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# 1 DNA stable isotope probing reveals contrasted activity and phenanthrene- 2 degrading bacteria identity in a gradient of anthropized soils

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10

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12

## 13 ABSTRACT

14 Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous soil organic pollutants. Although PAH-  
15 degrading bacteria are present in almost all soils, their selection and enrichment have been shown in  
16 historically high PAH contaminated soils. We can wonder if the effectiveness of PAH biodegradation  
17 and the PAH-degrading bacterial diversity differ among soils. The stable isotope probing (SIP)  
18 technique with <sup>13</sup>C-phenanthrene (PHE) as a model PAH was used to: i) compare for the first time a  
19 range of ten soils with various PAH contamination levels, ii) determine their PHE-degradation  
20 efficiency, and iii) identify the active PHE-degraders using 16S rRNA gene amplicon sequencing from  
21 <sup>13</sup>C-labelled DNA. Surprisingly, the PHE degradation rate was not directly correlated to the initial level  
22 of total PAHs and phenanthrene in the soils, but was mostly explained by the initial abundance and  
23 richness of soil bacterial communities. A large diversity of PAH-degrading bacteria was identified for  
24 seven of the soils, with differences among soils. In the soils where the PHE degradation activities  
25 were the higher, *Mycobacterium* species were always the dominant active PHE degraders. A positive  
26 correlation between PHE-degradation level and the diversity of active PHE-degraders (Shannon  
27 index) supported the hypothesis that cooperation between strains led to a more efficient PAH  
28 degradation.

29

## 30 INTRODUCTION

31 Polycyclic aromatic hydrocarbons (PAHs) are organic contaminants that represent a major source of  
32 environmental pollution, especially in soils. Anthropogenic activities are the main source of these  
33 contaminants due to the incomplete combustion of organic materials such as coke and petroleum

34 products during industrial processing. In industrial brownfields, although pollution stopped several  
35 decades ago, soil contamination can still reach very high levels (Biache *et al.* 2017). PAHs can  
36 accumulate in soils and be a serious threat for human health and ecosystem functioning with regard  
37 to their carcinogenicity and toxicity (Eom *et al.* 2007; Abdel-Shafy and Mansour 2016). However, the  
38 stress from high PAH concentrations leads to adaptation and selection of soil microbial communities  
39 (Bourceret *et al.* 2016). In these soils, a selection of microorganisms able to tolerate PAHs or degrade  
40 them has been shown (Cébron *et al.* 2008; Sawulski, Clipson and Doyle 2014), and involves  
41 detoxification (Sutherland 1992) or metabolism (PAHs used as a carbon source; Ghosal *et al.* 2016)  
42 mechanisms. Therefore, high levels of PAH contamination could impair soil functioning and lead to  
43 loss of functions (i.e. functions involved in C and N cycling; Liang *et al.* 2009). But it also leads to gain  
44 of functions (i.e. functions involved in PAH biodegradation; Cébron *et al.* 2008). Nevertheless,  
45 communities from weakly PAH-contaminated soils (soil background level) can also harbour the PAH  
46 degradation function (Crampon *et al.* 2017). Therefore, we can wonder if the effectiveness (i.e. the  
47 rate) of PAH biodegradation is similar between non- or low-contaminated soils on the one hand and  
48 soils with high and historical PAH contamination on the other hand. This aspect needs to be further  
49 explored to better understand the factors controlling the soil PAH biodegradation efficiency, which  
50 sometimes limits soil bioremediation (Chauhan *et al.* 2008; Ghosal *et al.* 2016).

51 In order to study the PAH biodegradation function, most studies have only sought to detect the  
52 presence of certain functional genes. PAH-RHD $\alpha$  genes, encoding the alpha subunit of PAH-ring  
53 hydroxylating dioxygenase enzymes involved in the first step of the bacterial biodegradation  
54 pathway, are choice targets indicating the functional potential of the microbial community (Cébron  
55 *et al.* 2008; Sawulski, Clipson and Doyle 2014), but this approach does not identify the actors of  
56 biodegradation. Stable isotope probing (SIP) is a culture-independent technique that links microbial  
57 identity with functions in complex systems such as soils (Dumont and Murrell 2005). This technique  
58 uses  $^{13}\text{C}$ -labelled compounds and allows for the monitoring of  $^{13}\text{C}$  during biodegradation and the  
59 labelling of cell components (e.g. DNA) of microbes using the  $^{13}\text{C}$  labelled substrate as a carbon  
60 source for their growth. SIP has until now been extensively used to study the degradation of single  
61 carbon compounds (Dumont *et al.* 2006; Mosbaek *et al.* 2016; He *et al.* 2019), but more and more  
62 studies use SIP to decipher the pathways and actors involved in degradation of natural and anthropic  
63 complex compounds, such as cellulose/lignin (Wilhelm *et al.* 2019) and various organic pollutants (Li  
64 *et al.* 2015; Khawand *et al.* 2016), among which the PAHs, i.e. naphthalene, phenanthrene, pyrene  
65 (Cébron *et al.* 2011; Wald *et al.* 2015; Song *et al.* 2016; Guo *et al.* 2017; Thomas *et al.* 2019) in order  
66 to identify microbial degraders. However, only few investigations have been carried out on PAH  
67 degraders in weakly contaminated soils (Song *et al.* 2016; Chen *et al.* 2018), and we do not know  
68 whether their PAH-degrading bacterial diversity is similar to historically and highly PAH-contaminated

69 soils. Most studies have used SIP to compare the PAH degradation yield and degrader identity in one  
70 soil under various conditions. For example, certain authors sought to understand the impact of  
71 surfactants (Crampon *et al.* 2017; Guo *et al.* 2017), root exudates (Cébron *et al.* 2011; Lv *et al.* 2018;  
72 Thomas, Corre and Cébron 2019), or temperature (Wald *et al.* 2015). On the other hand, SIP was  
73 never used to study a wide range of soils and compare their communities of PAH degraders in order  
74 to see i) whether some taxa are more efficient at PAH degradation than others, and ii) whether  
75 historically contaminated soils harbour a different diversity of active microorganisms than weakly  
76 contaminated soils.

77 In this context, the aim of this study was to compare a range of ten soils displaying a gradient of  
78 anthropization, determine their PAH-degradation efficiency, and identify the active PAH degraders.  
79 Our hypothesis was to test whether i) the intensity of PAH degradation was correlated to the level of  
80 PAH contamination of the soils, ii) the microbial communities from historically and highly PAH-  
81 contaminated soils were more efficient than those from weakly polluted soils, and iii) PAH degrader  
82 identity could explain the differences in PAH degradation rates, involving more or less efficient taxa.  
83 Phenanthrene (PHE), which is widely distributed in the environment and in contaminated soils, was  
84 used as a model PAH in SIP experiments. PHE-degrading bacteria were identified using high-  
85 throughput 16S rRNA gene amplicon sequencing from <sup>13</sup>C-labelled DNA.

86

## 87 **MATERIALS AND METHODS**

### 88 **Soil sampling and characteristics**

89 The soils used in this study were collected in November 2015 from various woodland ecosystems  
90 located on industrial wastelands, natural forest or ancient gravel pits, located in the “Grand Est”  
91 region (north-east of France); all sites are located within a 50-km radius (**Figure S1**). At each of the 10  
92 sites, samples were collected from three independent sub-sites 1 m apart. The three sub-site soil  
93 blocks were mixed to get one composite sample per site. Back to the laboratory, the soil samples  
94 were air-dried at room temperature for 1 week, and then sieved at 2 mm. All the dried and sieved  
95 soils were stored similarly at room temperature in the dark (6 months) before stable isotope probing  
96 experiment setup. Three soils were considered as weakly anthropized soils: two natural forest soils  
97 collected at Hémilly (He; Moselle) and Montiers-sur-Saulx (Mo; Meuse), and one anthropized but  
98 unpolluted ancient gravel pit soil collected at Dieulouard (Di; Meurthe-et-Moselle). Seven  
99 anthropized soils known to be polluted by metals and/or PAHs were collected from i) former slag  
100 heaps at Homécourt (Ho; Meurthe et-Moselle), Terville (Ter; Moselle), Uckange (Uc; Moselle), and  
101 Neuves-Maisons (NM; Meurthe-et-Moselle), and ii) former settling ponds at Pompey (Po; Meurthe-  
102 et-Moselle), Mont-St-Martin (MsM; Meurthe-et- Moselle) and Russange-Micheville (RM; Moselle).

103 This soil collection was chosen to cover a wide range of anthropization situations described in  
104 Lemmel *et al.* (2019). Briefly, a gradient of polycyclic aromatic hydrocarbon contamination was  
105 shown based on the 16 US-EPA PAHs ranging from 0.03 to 1,095.90 mg kg<sup>-1</sup> dw soil (**Table 1**). The  
106 soils presented variable 16S rRNA gene abundances ranging from 4.4 to 41.5 x 10<sup>10</sup> copies g<sup>-1</sup>. The  
107 lowest and highest 16S rRNA gene abundances were found in the Uc and NM soils and the Po and Te  
108 soils, respectively, while the other soils presented intermediate abundance values. The soil bacterial  
109 communities were characterised by Illumina MiSeq sequencing, and indices describing alpha-  
110 diversity such as the Chao1 richness index were calculated (**Table S1**; Lemmel *et al.* 2019). The soils  
111 presented variable Chao1 richness indices ranging from 2,541 to 4,966, and the lowest and highest  
112 values were found in the RM and NM soils and Di and Ho soils, respectively. A gradient of metal  
113 contamination, mainly by Zn (60 to 119,000 mg kg<sup>-1</sup>), Pb (23 to 39,500 mg kg<sup>-1</sup>), and Cd (0.09 to 152  
114 mg kg<sup>-1</sup>), was also shown (**Table S1**). The pH, known to be one of the main drivers of microbial  
115 communities, was similar (from 7.2 to 8.0) among soils except for soil He (pH = 5.4). Other soil  
116 characteristics (Laboratoire d'analyse des sols, INRA, Arras, France), namely total organic carbon (24  
117 to 159 g kg<sup>-1</sup>), total nitrogen (0.56 to 7.19 g kg<sup>-1</sup>), the C/N ratio (14 to 54), and texture (silty to sandy)  
118 varied among soils (**Table S1**; Lemmel *et al.* 2019).

119

## 120 **Soil respiration**

121 Basal respiration (BR) and substrate-induced respiration after PHE addition (PHE-SIR) were assessed,  
122 using two sets (i.e. unspiked and PHE-spiked) of soil samples. The two sets were prepared in  
123 triplicates, using 2.0 g dw of unspiked soil and of PHE-spiked soil, respectively. The PHE-spiked soil  
124 was prepared by mixing 1.8 g of soil with 0.2 g of spiked soil with PHE (Fluka, purity > 97.0 %)   
125 dissolved in n-hexane and left under a fume hood until complete evaporation of the solvent. The  
126 final PHE concentration was 200 mg kg<sup>-1</sup>. Our experimental design included 60 samples (10 unspiked  
127 soils and PHE-spiked soils, in triplicates).

128 The two-gram soil samples were placed in 125-ml Plasma glass flasks and rewetted using sterilised  
129 water to reach 80 % water retention capacity (WRC). Then they were hermetically sealed with  
130 rubber-butyl corks and incubated in the dark at 24 °C (optimal temperature and humidity conditions)  
131 for 17 days for soil samples Di, MsM, Mo, NM, Te, and Uc and incubations were continued until 23  
132 days for soil samples Ho, He, RM, and Po for which limited difference of respiration between BR and  
133 PHE-SIR could be observed. Respiration levels were monitored by measuring CO<sub>2</sub> contents using an  
134 infrared spectrophotometer (Binos, absorption at 2,325.6 cm<sup>-1</sup>) on 4-ml fractions of the flask  
135 atmosphere sampled after 1, 2, 4, 7, 10, 14, 17, and 23 days of incubation using a syringe. Produced  
136 CO<sub>2</sub> was expressed as the carbon mass produced by one gram of dw soil per hour (mg of C g<sup>-1</sup> h<sup>-1</sup>).

137 The flasks were left open under the fume hood for 15 min to renew the atmosphere after each  
138 sampling.

139

#### 140 **Stable isotope probing incubations**

141 For stable isotope probing (SIP) experiments, two sets of soil samples were prepared in triplicates by  
142 spiking all samples with PHE as described above. The first set was spiked with non-labelled PHE (i.e.  
143  $^{12}\text{C}$ -PHE; Fluka, > 97.0 % purity) and the second set was spiked with uniformly  $^{13}\text{C}$ -labelled PHE (Sigma  
144 Aldrich, 99 atom %  $^{13}\text{C}$ ). Both sets were prepared twice: one was harvested at the beginning of the  
145 experiment (T0) and the other was incubated and harvested after 12 days (Tf). The SIP incubation  
146 experimental design included 120 samples (10 soils in triplicates, spiked with  $^{12}\text{C}$ - or  $^{13}\text{C}$ -PHE, and  
147 analysed at 2 time points T0 and Tf).

148 Each soil sample was placed in 125-ml Plasma glass flasks and rewetted using sterilised water to  
149 reach 80 % WRC. Then, the T0 samples were directly harvested by freezing at  $-80\text{ }^{\circ}\text{C}$ . A  $\text{CO}_2$  trap (2 ml  
150 of 1M NaOH in a 5-ml vial) was added in the Tf flasks before being hermetically sealed and incubated  
151 for 12 days in the dark at  $24\text{ }^{\circ}\text{C}$ , to avoid  $^{13}\text{C}$ - $\text{CO}_2$  fixation (autotrophic and heterotrophic) during  
152 incubation. The atmosphere of the flasks was renewed once after 6 days, as described above. Tf  
153 incubations were harvested by freezing at  $-80\text{ }^{\circ}\text{C}$ .

154

#### 155 **$^{13}\text{C}$ -Phenanthrene degradation and $^{13}\text{C}$ -dissipation measurements**

156 The soil samples (T0 and Tf) from SIP incubation stored at  $-80\text{ }^{\circ}\text{C}$  were freeze-dried and ground to  
157  $500\text{ }\mu\text{m}$  with a grinder (Mixed Mill MM 400, Retsch) before PAH extraction, PHE-measurements and  
158  $\delta^{13}\text{C}$  estimation.

159 Total PHE PAH were extracted from one aliquot of ca. 500 mg of each dw soil with dichloromethane  
160 (DCM) at a high temperature ( $130\text{ }^{\circ}\text{C}$ ) and a high pressure (100 bars) using accelerated solvent  
161 extraction (DIONEX<sup>®</sup> 200 ASE), as described in Cennerazzo *et al.* (2017). Solvent extracts were  
162 evaporated under a nitrogen flow and dissolved in acetonitrile for PHE analysis. PHE was analysed by  
163 UV detection (254 nm) using a reverse-phase chromatography UHPLC DIONEX<sup>®</sup> Ultimate 3000  
164 system equipped with a DAD (Diode Array Detector) and a Zorbax Eclipse PAH column ( $2.1 \times 100\text{ mm}$ ,  
165  $1.8\text{ }\mu\text{m}$ , Agilent). As some soils were already contaminated with aged PHE, the  $^{13}\text{C}$ -PHE  
166 concentrations ( $^{13}\text{C}$ -PHE<sub>t</sub>) at T0 or after 12 days were calculated as follow:  $^{13}\text{C}$ -PHE<sub>t</sub> = PHE<sub>t</sub> - PHE<sub>i</sub>,  
167 where PHE<sub>t</sub> is the total PHE concentration measured and PHE<sub>i</sub> is the initial PHE concentration of the  
168 soils before  $^{13}\text{C}$ -PHE spiking.

169 Delta<sup>13</sup>C measurements were performed at the PTEF platform (INRA, Champenoux, France). Briefly,  
170 δ<sup>13</sup>C was measured from one aliquot of ca. 5 mg dw of soil using an elemental analyser (vario  
171 ISOTOPE cube, Elementar, Hanau, Germany) interfaced in line with a gas isotope ratio mass  
172 spectrometer (IsoPrime 100, Isoprime Ltd, Cheadle, UK; 0.2 ‰ sensitivity). δ<sup>13</sup>C values were then  
173 transformed into R<sup>13</sup>C/<sup>12</sup>C using the following formula:  $R^{13}C/^{12}C = R_{VPDB} \times (1 + \delta^{13}C/1000)$ , with  $R_{VPDB}$   
174 equal to 0.0112375, value of the reference standard (Vienna Pee Dee Belemnite). From these  
175 calculated ratios, we determined the <sup>13</sup>C-dissipation corresponding to the percentage of <sup>13</sup>C content  
176 (coming from the <sup>13</sup>C-PHE) after the 12 days of incubation relative to the T0 <sup>13</sup>C content just after the  
177 spiking (representing 100%), taking also into account the natural <sup>13</sup>C/<sup>12</sup>C ratio of the unspiked soils.

178 Available PAH content of unspiked soils was measured using cyclodextrin-based extraction and  
179 analysed by HPLC and UV-detection as described in Lemmel *et al.* (2019).

180

### 181 **DNA extraction, isopycnic ultracentrifugation, and gradient fractionation**

182 Total genomic DNA was extracted from ca. 300 mg of freeze-dried and ground ~~dw~~ soil (60 Tf samples  
183 from both <sup>12</sup>C and <sup>13</sup>C incubations) using a Fast DNA Spin Kit for Soil (MP Biomedicals, France),  
184 following the manufacturer's instructions. Then DNA was resuspended in 100 µl of DNase and  
185 pyrogen-free water. Concentration and purity ( $A_{260}/A_{280}$  ratio) were measured using a  
186 spectrophotometer (UV1800, Shimadzu) equipped with a TrayCell™ adapter (Hellma®). Heavy (<sup>13</sup>C-  
187 labelled) DNA was separated from light (<sup>12</sup>C) DNA according to the protocol described by Neufeld *et*  
188 *al.* (2007). Briefly, approximately 3,000 ng of DNA (except NM and Uc, for which 650 ng of DNA were  
189 used because lower genomic DNA quantities were recovered from these soils) were added to Quick-  
190 Seal polyallomer tubes (13 x 51 mm, 5.1 ml, Beckman Coulter), along with a gradient buffer (0.1M  
191 Tris-HCl, 0.1 M KCl, 1 mM EDTA) mixed with a CsCl solution, to a final buoyant density (BD) of ≈ 1.725  
192 g ml<sup>-1</sup>. The tubes were centrifuged at 42,400 rpm (i.e. 176,985 x g; VTI 65.2 rotor, Beckman) at 20 °C  
193 for 40 h. Following ultracentrifugation, 13 fractions of ca. 400 µl were collected from each tube using  
194 a fraction recovery system (Beckman). Then the BD value of each fraction was measured, and CsCl  
195 was removed by glycogen-assisted polyethylene glycol precipitation. DNA fractions were finally  
196 resuspended in 30 µl of TE buffer (pH 8.0).

197

### 198 **Real-time quantitative PCR**

199 In order to determine the distribution of heavy and light DNA and compare samples from <sup>12</sup>C and <sup>13</sup>C  
200 incubations, the abundance of 16S rRNA genes in each fraction was quantified using real-time  
201 quantitative PCR. The qPCR assay was performed as described in Cébron *et al.* (2008) by using the  
202 primer sets 968F/1401R (Felske, Akkermans and Vos 1998). Briefly, the reaction mixture (20 µl) was

203 composed of 10  $\mu\text{l}$  of iQ SYBR green SuperMix (Bio-Rad), 0.8  $\mu\text{l}$  of each primer (10  $\mu\text{M}$ ), 0.4  $\mu\text{l}$  of  
204 bovine serum albumin (3%), 0.2  $\mu\text{l}$  of dimethyl sulphoxide, 0.08  $\mu\text{l}$  of T4gp32 (MP Biomedicals,  
205 France), and 1  $\mu\text{l}$  of DNA as a template (DNA fraction samples or 10-fold dilution series from  $10^8$  to  
206  $10^1$  copies  $\mu\text{l}^{-1}$  of the standard plasmid). Quantifications were performed using a CFX96 Real-Time  
207 PCR detection system (Bio-Rad), under the following conditions: an initial denaturation step at 95  $^\circ\text{C}$   
208 for 5 min, and then 39 cycles of denaturation at 95  $^\circ\text{C}$  for 30 s, annealing at 56  $^\circ\text{C}$  for 20 s, extension  
209 at 72  $^\circ\text{C}$  for 30 s, and measurement of SYBR Green signal intensities at 82  $^\circ\text{C}$  for 5 s. Similarly, the  
210 abundance of 18S rRNA genes was quantified using the primer sets Fung5F/FF390R (Lueders *et al.*  
211 2004) as previously described in Thion *et al.* (2012a).

212 Additionally, PAH-RHD $\alpha$  genes were also quantified in the genomic DNA from unspiked soils (before  
213 incubation) and in each DNA fraction (from the SIP assay) using the primer pairs PAH-RHD $\alpha$  GP-F/R  
214 for Gram-positive bacteria (Actinobacteria) and PAH-RHD $\alpha$  GN-F/R for Gram-negative bacteria  
215 (Proteobacteria) (Cébron *et al.* 2008). The reaction mixtures and quantification conditions were as  
216 described above except annealing temperatures that were 57  $^\circ\text{C}$  and 54  $^\circ\text{C}$  for PAH-RHD $\alpha$  GN and GP,  
217 respectively as described in Cébron *et al.* (2008).

218 Based on qPCR quantifications, the relative abundances of PAH-RHD $\alpha$  GP and GN genes (related to  
219 16S rRNA genes) were calculated in unspiked soils. Similarly, the abundances of PAH-RHD $\alpha$  GP and  
220 GN genes in the soils after incubation and in the sequenced DNA fractions were calculated based on  
221 their average relative abundance in the light  $^{12}\text{C}$ -DNA fraction and in the heavy  $^{13}\text{C}$ -DNA fraction,  
222 respectively. All these gene relative abundance values are reported in **Table 1**.

223

## 224 **Identification of phenanthrene-degrading bacteria**

225 Based on the results of the distribution of heavy and light DNA in the recovered fractions, we  
226 observed an enrichment of the 16S rRNA gene in the heavy fraction (numbers 4-5-6; i.e. BD from  
227 1.725 to 1.717  $\text{g ml}^{-1}$ ) of the soils incubated with  $^{13}\text{C}$ -PHE, indicating a significant use of  $^{13}\text{C}$  for  
228 bacterial growth. In order to identify these phenanthrene-degrading bacteria, 5  $\mu\text{l}$  of the three heavy  
229 fractions were pooled from samples of both  $^{12}\text{C}$  and  $^{13}\text{C}$  incubations. These pooled fractions were  
230 used as templates to perform 16S rRNA gene amplicon libraries for Illumina MiSeq sequencing. To do  
231 so, the V3/V4 region of bacterial 16S rRNA genes (ca. 550 bp) was amplified using primers S-D-Bact-  
232 0341-a-S-17 and S-D-Bact-0787-b-A-20 (Muyzer, de Waal and Uitterlinden 1993; Caporaso *et al.*  
233 2011), and following a previously described dual-index strategy (Kozich *et al.* 2013).

234 PCR reactions were performed on 1  $\mu\text{l}$  of the pooled DNA fractions in a final volume of 50  $\mu\text{l}$ ,  
235 containing 10  $\mu\text{l}$  of 5X Phusion HF buffer, 1.5  $\mu\text{l}$  of 50 mM  $\text{MgCl}_2$ , 0.25  $\mu\text{l}$  of DMSO, 0.1  $\mu\text{l}$  of T4gp32  
236 (MPBiomedicals, France), 0.1  $\mu\text{l}$  of Phusion high-fidelity polymerase (Thermo Scientific), and 1  $\mu\text{l}$  of  
237 each primer at 10  $\mu\text{M}$ . PCR reactions were heated at 94  $^\circ\text{C}$  for 5 min, followed by 33 cycles of 30 s at



238 94 °C, 30 s at the annealing temperature (18 cycles starting at 63 °C, with a subsequent decrease of  
239 0.5 °C at each cycle, and 15 cycles at 54 °C), 30 s at 72 °C, and a final extension step of 7 min at 72 °C.  
240 Amplification products were checked by 1% agarose gel electrophoresis and purified using the  
241 UltraClean-htp 96 Well PCR Clean-Up kit (Qiagen) following the manufacturer's instructions. After  
242 quantification using a Quant-iT Picogreen ds-DNA assay Kit (Invitrogen), an amplicon library was  
243 prepared as an equimolar pool of amplicons (10 nM), purified on a QIAquick PCR purification kit  
244 column (Qiagen), and sent for sequencing to Genewiz platform (South Plainfield, NJ, USA) using an  
245 Illumina MiSeq V2 Kit for 2 x 250 bp paired-end sequencing. Illumina MiSeq paired-end reads have  
246 been deposited in the SRA database under BioProject accession number PRJNA507956.  
247 Sequence data were analysed following the MiSeq SOP procedure available in March 2017 and  
248 described in Kozich *et al.* (2013), using Mothur v.1.36.0 (Schloss *et al.* 2009). Due to the altered  
249 sequence quality of soil Uc as compared to the other 9 soils, two separate analyses were performed,  
250 with the Uc read treated separately from all the others. Paired-end reads were trimmed to a  
251 minimum QScore of 20 and joined using the following criteria: 404 bp < length < 480 bp, and a  
252 maximum of 6 ambiguous bases or no ambiguous base for paired-end reads from soil Uc or the other  
253 9 soils, respectively. Alignment of unique sequences was performed against the Silva database.  
254 Chimeras were detected using Uchime (Edgar *et al.* 2011) and removed. Taxonomy was assigned  
255 using the Silva 132 bacteria database (released in Dec. 2017) using a cutoff of 80. Sequences  
256 affiliated to archaea, eukaryota, unknown, mitochondria, and chloroplasts were removed for further  
257 analysis. Singletons (sequences appearing only once among all samples) were removed. Sequences  
258 were clustered in Operational Taxonomic Units (OTUs) at 97% similarity. Finally, datasets were  
259 rarefied to the lowest number of sequences per sample (1,096 or 20,764 reads/sample for sequences  
260 from Uc or the other 9 soils, respectively).

261 Using a method describe by Thomas *et al.* (2019), active PHE-degraders were determined as OTUs  
262 present in the pooled heavy fractions of the <sup>13</sup>C-PHE incubation at a significantly higher abundance  
263 than in the pooled heavy fractions of the <sup>12</sup>C-PHE control incubation. We first selected major OTUs  
264 (i) represented by at least 5 sequences in each <sup>13</sup>C-PHE triplicate and (ii) with a higher average  
265 abundance in <sup>13</sup>C-PHE samples than in <sup>12</sup>C-PHE controls. After log-transformation, Welch's test  
266 followed by Benjamini-Hochberg correction of the p-values allowed determining the active PHE-  
267 degrading OTUs among major OTUs, separately for each soil. Statistically selected OTUs with exactly  
268 the same taxonomic affiliation were grouped and their sequence numbers were summed. For each  
269 soil, active PHE-degraders were reported with (i) their relative abundance related to the whole pool  
270 of PHE-degraders, (ii) their enrichment in <sup>13</sup>C-PHE heavy fractions compared to <sup>12</sup>C-PHE controls, and  
271 (iii) the corresponding number of OTUs in the groups.

272

## 273 **Statistical analyses**

274 All statistical analyses were performed using RStudio v1.1.442. Significant differences in PHE  
275 degradation, <sup>13</sup>C dissipation, and 16S rRNA gene copy numbers among soils were assessed by using  
276 Kruskal-Wallis rank sum test and the multiple comparison test included in the agricolae R package  
277 (Mendiburu 2017). Significant differences between PHE degradation and <sup>13</sup>C dissipation in a given soil  
278 were assessed using Welch's test. Linear correlations between all data were explored using the *rcorr*  
279 function included in the Hmisc package in R (Harell, Dupont and others 2018). Linear multiple  
280 regressions and factor interactions were explored using the *lm* function. Diversity indices were  
281 calculated using the *specnumber* and *diversity* functions included in the vegan package in R (Oksanen  
282 *et al.* 2017).

283

## 284 **RESULTS**

### 285 **PAH contamination and PAH-dioxygenase genes of initial soils**

286 The initial PAH and phenanthrene contents are presented in **Table 1**, together with PAH-RHD $\alpha$  gene  
287 relative abundances in the ten initial unspiked soils. Soil collection showed a gradient of total PAH  
288 contents ranging from 0.03 (He) to 1,095.90 mg kg<sup>-1</sup> (NM) with ca. 1 to 10% of available PAHs  
289 (Lemmel *et al.* 2019). The initial unspiked soil PHE content followed a similar trend and ranged from  
290 below detection limit (He) to 199.24 mg kg<sup>-1</sup> (Ho). We can note that highest initial available PAH and  
291 PHE contents were measured in Ho (54.14 and 18.10 mg kg<sup>-1</sup>, respectively) and NM (82.98 and 15.77  
292 mg kg<sup>-1</sup>, respectively) soils. Based on the PAH content, we considered three groups of soil displaying  
293 low (< 20 mg kg<sup>-1</sup>), medium (20 - 200 mg kg<sup>-1</sup>), or high (> 200 mg kg<sup>-1</sup>) PAH contamination.  
294 Interestingly, the relative abundance of PAH-RHD $\alpha$  genes in initial unspiked soils showed linear  
295 Pearson correlation with total soil PAH contents ( $p = 2.62 \times 10^{-4}$ ,  $r = 0.91$ , and  $p = 3.38 \times 10^{-8}$ ,  $r = 0.99$   
296 for the PAH-RHD $\alpha$  -GN and -GP genes, respectively) and available ones ( $p = 5.77 \times 10^{-5}$ ,  $r = 0.96$ , and  
297  $p = 3.70 \times 10^{-6}$ ,  $r = 0.98$  for the PAH-RHD $\alpha$  -GN and -GP genes, respectively).

298

### 299 **Initial soil respiration**

300 Depending on the soil activity, basal respiration (BR) and respiration induced after phenanthrene  
301 addition (PHE-SIR) were monitored for 17 or 23 days (**Figure S2**). Soils Mo, Te, and Po had the highest  
302 basal respiration activity. A globally higher CO<sub>2</sub> production was observed in the PHE-SIR flasks than in  
303 the BR flasks, except for soil He and Po. Soils Te, Di and Mo had the highest PHE-SIR level as  
304 compared to their BR, with the difference in CO<sub>2</sub> production between BR and PHE-SIR representing at  
305 the end more than 200  $\mu$ gC per g of soil possibly indicating the complete mineralisation of the PHE  
306 (**Figure S2**). The Uc, MSM, NM, RM and Ho soils have lower CO<sub>2</sub> production representing between

307 90% (Uc) to 18% (Ho) of the corresponding C added through PHE spiking. Globally, after 10-12 days of  
308 incubation, most of the soils showed already a high difference in CO<sub>2</sub> production between the two  
309 conditions, indicating that most of the spiked phenanthrene had been degraded. Based on these  
310 results, we decided to stop SIP incubation after 12 days.

311

### 312 **<sup>13</sup>C-Phenanthrene degradation in the soils**

313 We measured PHE concentrations and δ<sup>13</sup>C levels to estimate <sup>13</sup>C-PHE degradation and <sup>13</sup>C dissipation  
314 in the soils at the end of SIP incubation (**Figure 1**). The soils presented variable percentages of <sup>13</sup>C-  
315 PHE degradation, ranging from 17.9% (RM) to 91.2% (Di) of the initial quantity of spiked <sup>13</sup>C-PHE.  
316 Soils Di and Po presented the highest PHE degradation, while soils RM and NM presented the lowest  
317 (Kruskal Wallis tests, p = 0.002). There was no correlation (Pearson) between the level of <sup>13</sup>C-PHE  
318 degradation and the soil characteristics (**Table S2**) and no correlation between <sup>13</sup>C-PHE degradation  
319 and the initial PAH and PHE contents (p-value of 0.21 and 0.59, respectively). Besides, medium PAH-  
320 polluted soils (Po, MsM, Te) presented higher <sup>13</sup>C-PHE degradation than low contaminated soils (He,  
321 Uc, Mo, RM, except Di) (Kruskal Wallis test, p = 0.002). Based on the soil δ<sup>13</sup>C levels, we calculated <sup>13</sup>C  
322 dissipation in the soils; it ranged from 18.8% (NM) to 59.5% (Di) of the initial <sup>13</sup>C quantity, with  
323 significant differences (Kruskal Wallis test, p = 0.002). No correlation was found between <sup>13</sup>C  
324 dissipation and the soil characteristics, including the initial PAH and PHE contents (p-value of 0.16  
325 and 0.52, respectively). Interestingly, the percentage of degraded PHE showed a positive linear  
326 correlation with both initial soil bacterial abundance (16S gene copy number; P = 0.0001, R = 0.65)  
327 and initial soil bacterial richness (Chao1 index; P = 0.0002, R = 0.62) (**Figure 2A and 2B**). A strong  
328 linear relationship (p = 0.0004; R = 0.90) was also found between the percentage of dissipated <sup>13</sup>C  
329 and degraded <sup>13</sup>C-PHE. The difference between the percentages of dissipated <sup>13</sup>C and degraded <sup>13</sup>C-  
330 PHE (**Figure 1**) indicate the amount of <sup>13</sup>C still present in the soil but not attributed to the remaining  
331 PHE (i.e. mainly <sup>13</sup>C-labeled microbial biomass) at the end of the incubations. Five soils (Di, Po, MsM,  
332 Te, and Ho) presented significantly higher PHE degradation than <sup>13</sup>C dissipation (Welch's test,  
333 p < 0.05), and this difference was positively correlated to the initial soil bacterial abundance (16S  
334 rRNA gene copy number) in the soils (Pearson; p = 0.01; R = 0.76).

335

### 336 **Evidence of <sup>13</sup>C-labelled DNA**

337 Bacterial 16S rRNA gene abundance was quantified from the DNA fractions obtained after  
338 ultracentrifugation of gDNA from the <sup>12</sup>C-PHE (control) and <sup>13</sup>C-PHE microcosms (**Figure 3**). The  
339 "heavy" DNA was in the fractions with a buoyant density ranging from 1.717 to 1.725 g ml<sup>-1</sup>, while  
340 most of the "light" DNA corresponded to the fractions with a buoyant density ranging from 1.699 to  
341 1.704 g ml<sup>-1</sup>. The numbers of 16S rRNA gene copies were higher in the heavy DNA fractions collected

342 from the <sup>13</sup>C microcosms than in the <sup>12</sup>C controls, highlighting the presence of <sup>13</sup>C-labelled DNA  
343 belonging to the active <sup>13</sup>C-PHE-degrading bacteria. This <sup>13</sup>C-DNA enrichment was observed for all  
344 soils but Mo, and only a weak signal was observed for soil RM. 18S rRNA gene copies were also  
345 quantified in all fractions (**Figure S3**), but no fungal <sup>13</sup>C-DNA enrichment was detected, indicating that  
346 fungi were not significantly involved in PHE degradation or did not use carbon from PHE for their  
347 growth, as they did not incorporate <sup>13</sup>C into their DNA.

348 In addition, PAH-RHD $\alpha$ -GN and -GP genes were also quantified in the DNA recovered from the CsCl  
349 gradient fractions to calculate their relative abundance in the soils after incubation and in the  
350 sequenced heavy <sup>13</sup>C-DNA fractions (**Table 1**). By comparing PAH-RHD $\alpha$ -GP and -GN abundances in  
351 the soils before and after SIP incubation, we calculated a value of enrichment during incubation  
352 (**Table 1**), ranging from 4 (NM) to 221 (Di) and from 3 (NM) to 126 (Te) for PAH-RHD $\alpha$ -GP and -GN,  
353 respectively. Interestingly, PAH-RHD $\alpha$ -GP gene abundance and PAH-RHD $\alpha$ -GP gene enrichment in the  
354 soils after SIP incubation were positively correlated to both the percentages of degraded PHE and  
355 dissipated <sup>13</sup>C during SIP incubation (Pearson; p = 0.03, R = 0.68, and p = 0.01, R = 0.75, respectively,  
356 for abundance; and p = 0.005, R = 0.81, and p = 0.0006, R = 0.89, respectively, for enrichment).  
357 Additionally, PAH-RHD $\alpha$ -GP gene abundance in the heavy <sup>13</sup>C-DNA fractions was positively correlated  
358 to the percentage of <sup>13</sup>C dissipated during SIP incubation (Pearson; p = 0.02, R = 0.72). No correlation  
359 was found for PAH-RHD $\alpha$ -GN gene abundance in the soils after SIP incubation or in the <sup>13</sup>C-heavy  
360 DNA fractions, or for PAH-RHD $\alpha$ -GN gene enrichment.

361

### 362 **Identity of PHE degraders**

363 Based on the results of the quantification of bacterial 16S rRNA genes, the pooled heavy DNA  
364 fractions (**Figure 3**) were used as a template to prepare a 16S rRNA gene amplicon library for Illumina  
365 sequencing. Due to the altered sequence quality of soil Uc as compared to the other 9 soils, two  
366 separate analyses were performed, with Uc reads treated separately from all the other soils. Totals  
367 of 7,150,234 and 405,380 reads were obtained after sequencing, and 3,485,709 and 22,402 reads  
368 were kept after the trimming steps for the 9 soils and soil Uc (in triplicates and from <sup>12</sup>C and <sup>13</sup>C  
369 incubations), respectively. Finally, data were rarefied to 20,764 and 1,096 reads/sample for the 9  
370 soils and soil Uc, respectively.

371 Active PHE-degraders were determined as OTU present in the pooled heavy fractions of the <sup>13</sup>C-PHE  
372 incubation at a significantly higher abundance than in the pooled heavy fractions of the  
373 corresponding <sup>12</sup>C-PHE controls. Then, OTU corresponding to active PHE-degraders and sharing the  
374 same taxonomic affiliation were gathered in groups. Relative abundance (relative to the whole pool

375 of PHE degraders) and enrichment (during  $^{13}\text{C}$ -SIP incubation) are reported in **Figure 4** for each PHE-  
376 degrader group in the corresponding soil. Only 7 soils were reported in **Figure 4** as no OTU was  
377 detected with a significantly higher abundance in  $^{13}\text{C}$ - than  $^{12}\text{C}$ - heavy DNA fractions in He, Mo and  
378 RM soils, confirming the fact that we could not detect 16S rRNA gene copies number enrichment in  
379 heavy fractions of the  $^{13}\text{C}$ -PHE incubations compared to the  $^{12}\text{C}$ -PHE controls (**Figure 3**). A total of 2  
380 (NM) to 12 (Di and Te) OTU groups (representing 3 to 30 OTUs) were highlighted as active PHE  
381 degraders. Most of these OTU groups were detected in  $^{13}\text{C}$ -DNA fractions with enrichment higher  
382 than 10 times compared to the  $^{12}\text{C}$ -controls. Active PHE degraders were relatively diverse and  
383 belonged to the Actinobacteria, Proteobacteria ( $\alpha$  and  $\beta$ ), Firmicutes, Patescibacteria and  
384 Cyanobacteria phyla; the last three were in minority compared to other phyla. Depending of the soil,  
385 dominant PHE degraders were mainly affiliated to *Micrococcaceae*, *Sphingomonadaceae* and  
386 *Burkholderiaceae* family, *Mycobacterium*, *Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium*  
387 (*ANPR*) and *Massilia* genera, and *Arthobacter crystallopoietes*, *Rhizobium rhizogenes* species.  
388 Altogether, those 8 taxonomic groups represented 78% (Te) to 100% (MsM) on average of the whole  
389 active PHE degraders in the corresponding soil. Among Actinobacteria, OTUs affiliated to  
390 *Mycobacterium* were the major PHE degraders in soils Di (55%), Po (98%), and MsM (57%), while  
391 *Arthobacter crystallopoietes* and *Micrococcaceae* family were the major PHE degraders in soil Te  
392 (44%) and NM (>99%), respectively. Besides, *Mycobacterium* affiliated OTUs were in the minority  
393 among the PHE degraders in both soils Te (20%) and Ho (4%). Concerning  $\alpha$ -Proteobacteria, the  
394 *ANPR*, *Rhizobium rhizogenes* and *Sphingomonadaceae* family represented the second major PHE  
395 degrader groups in soil Di (34%), Uc (44%) and MsM (43%), respectively. Finally, among  $\beta$ -  
396 Proteobacteria, *Burkholderiaceae* family members were the major PHE degraders in soil Uc (51%),  
397 and more specifically the *Massilia* genus in soil Ho (87%).

398 The percentage of PHE degraded was positively correlated to the relative abundance of the globally  
399 most abundant PHE degrader, namely *Mycobacterium* ( $p = 6.1 \times 10^{-5}$ ,  $R = 0.76$ ; **Figure 2D**). For each  
400 soil replicate, we calculated diversity indices (i.e. species richness, Shannon diversity, and Pielou's  
401 evenness; **Table S3**) to describe the PHE-degrader community. Shannon diversity index of PHE-  
402 degraders was positively correlated to the percentage of degraded PHE ( $p = 0.02$ ,  $R = 0.48$ ; **Figure**  
403 **2C**). The percentage of degraded PHE was not linearly correlated with the specific richness of PHE-  
404 degraders (**Figure 2E**). However, this relationship could be modeled by a saturation curve, suggesting  
405 a functional redundancy in the PHE-degrader community (beyond a certain species richness, several  
406 species have the same role in PHE degradation).

407

## 408 **DISCUSSION**

409 We evaluated the level of phenanthrene degradation and tried to identify the phenanthrene  
410 degraders in ten soils presenting a PAH pollution gradient. We hypothesised that the microbial  
411 communities of soils with a high and historical PAH contamination would be better adapted to PAH  
412 degradation than the ones from low- or non-contaminated soils. Consequently, the phenanthrene  
413 degradation rate would be positively correlated to the level of soil PAH contamination. Previous  
414 works indeed showed that greater PHE or PAH mineralisation appeared in soils pre-exposed to PAHs  
415 or with the highest PAH contamination level as compared to low- or non-contaminated soils (Johnsen  
416 and Karlson 2005; Carmichael and Pfaender 2009).

417 In the present study, we did not show any correlation between phenanthrene degradation rates and  
418 initial PAH or PHE contamination levels. Pre-exposure to PAH does not seem to be essential for  
419 efficient phenanthrene degradation. Nevertheless, this hypothesis was partly confirmed by the  
420 positive linear correlation between PAH-dioxygenase (both PAH-RHD $\alpha$ -GP and -GN) gene abundances  
421 and the total PAH contamination levels of the 10 soils. Our finding suggests that the selective  
422 pressure exerted by PAH pollution led to an enrichment in bacteria capable of PAH degradation. PAH-  
423 RHD $\alpha$  gene abundance is indeed a good indicator of the PAH contamination level in environmental  
424 samples (Cébron *et al.* 2008). However, although PHE degradation was surprisingly detected in all the  
425 tested soils, the PHE degradation rates were highly contrasted among soils and did not correlate with  
426 the PAH concentration or the PAH-dioxygenase gene abundance of the unspiked soils. Yet, although  
427 the moderately contaminated soil Di displayed higher PHE degradation than the non-contaminated  
428 ones, the highly contaminated soil NM did not display the highest PHE degradation rate. These  
429 results show that detecting the potential function (i.e. PAH-RHD $\alpha$  gene abundance) is not the sole  
430 predictor of the actual measured activity (i.e. PHE degradation) in the ten soils.

431 These differences in PHE biodegradation activity among soils could be explained by a myriad of  
432 abiotic and biotic factors specific to each soil, such as texture (Haghollahi *et al.* 2016), nutrient  
433 content (Breedveld and Sparrevik 2000), pH (Kästner *et al.* 1998), bioavailability of pollutants (Biache  
434 *et al.* 2017), multi-contamination (i.e. metal pollution; Sandrin and Maier 2003), and microbial  
435 diversity (Cébron *et al.* 2008).

436 Surprisingly, no correlation was found between the percentage of degraded PHE and any of the soil  
437 characteristics. No impact of the nitrogen or phosphorus contents or of the C:N ratio on the PHE  
438 degradation level was highlighted, even if it is well established that low nutrient levels limit microbial  
439 growth and activity (Wardle 1992) and hydrocarbon degradation (Leahy and Colwell 1990). C:N:P  
440 ratios could indeed drive PHE degradation (Smith, Graham and Cleland 1998) and the selection of  
441 active microorganisms in SIP experiments (Cébron *et al.* 2007); besides, adding N or P can increase  
442 the soil respiration (Breedveld and Sparrevik 2000) and improve the biodegradation of crude oil or  
443 gasoline (Leahy and Colwell 1990) and of PAHs (Joner *et al.* 2002; Jones *et al.* 2008) in soils.

444 Moreover, one of the main drivers of soil PAH degradation is PAH availability, mostly impacted by the  
445 soil texture (Carmichael and Pfaender 2009), organic matter type and content (Dictor *et al.* 2003),  
446 and the presence of aged pollution (Biache *et al.* 2008). Dictor *et al.* (2003) showed that even after a  
447 short period (i.e. few hours), a significant part of the spiked PHE became unavailable for soil  
448 microorganisms, resulting in reduced degradation. We can thus hypothesise that spiked PHE  
449 availability differed depending on the soils. The PHE degradation rate was well predicted from the  
450 mineralization data of soil spiked with PHE compared to the basal mineralization, except for the soil  
451 Mo where we overestimated it from mineralization pre-experiments. For Ho and NM soil, where the  
452 initial PAH contamination was the highest, the difference in mineralisation was low compared to the  
453 PHE degradation rates. This phenomenon could be due to the adaptation of bacterial communities in  
454 these aged PAH contaminated soils, leading to a shift in mineralization from the aged carbon (mostly  
455 PAHs) to the fresh added PHE by the same bacteria.

456 We also hypothesised that differences in PHE degradation rates between soils may be explained by  
457 the diversity of PHE degraders and/or by the presence of specific taxa with a higher PHE-degrading  
458 activity. In addition to the analysis of the diversity of the whole bacterial community in unspiked soils  
459 (Lemmel *et al.* 2019), the use of stable isotope probing allowed us to directly link the <sup>13</sup>C-PHE  
460 degradation function with the diversity of <sup>13</sup>C-labeled PHE degraders.

461 We get a very original result showing that the PHE degradation level was positively correlated to  
462 both bacterial abundance and bacterial richness of the initial soils, previously measured on unspiked  
463 soils (Lemmel *et al.* 2019). This relationship could be explained by i) the fact that soils harbouring a  
464 more abundant and diversified microbial community might contain more PHE-degrading bacteria  
465 (the sampling effect hypothesis, e.g. Hector *et al.* 2002) resulting in higher PHE degradation rates, or  
466 ii) facilitation between species improving PHE degradation. As broadly described in plant community  
467 ecology (Hooper and Vitousek 1998; Caldeira *et al.* 2001), facilitation between species enhances an  
468 ecosystem function and may be the mechanism responsible for the positive relationship between  
469 taxonomic diversity and ecosystem processes. We can consider that some bacterial species indirectly  
470 contributed to increase PHE degradation by facilitating the PHE degrader activity in the soil (for  
471 example by increasing their access to essential elements or by reducing the toxicity due to a high soil  
472 metal content). Our finding is in agreement with previous finding in microbial ecology. Jung *et al.*  
473 (2016) showed that a large proportion of functional gene categories were significantly altered by a  
474 reduction in microbial biodiversity, even if these authors also found that the efficiency of diesel  
475 biodegradation was increased in the low-diversity community suggesting that the relationship  
476 between microbial diversity and ecological function involves trade-offs among ecological processes.  
477 PHE degradation activity was also positively correlated with the active PHE-degrader Shannon  
478 diversity index calculated from SIP data, but no correlation was found with PHE-degrader richness.

479 Complementarity between species for the use of PHE should be considered here. When the diversity  
480 of PHE-degrading species increases, the use of PHE as a carbon substrate is optimized and a greater  
481 amount of PHE is degraded. For example, PHE degradation results from a sum of reactions (Ghosal *et*  
482 *al.* 2016), and when the diversity of PHE degraders increases, the joint functioning of the different  
483 species ensures a greater number of its reactions and thus maximises PHE degradation. Thomas,  
484 Corre and Cébron (2019) recently demonstrated, using SIP combined to metagenomic analysis, that  
485 active PHE-degraders act in a consortium, whereby complete PHE mineralization is achieved through  
486 the combined activity of taxonomically diverse co-occurring bacteria performing successive  
487 metabolic steps. Similarly, positive relationships between species diversity and soil respiration (Bell  
488 *et al.* 2005) or the efficiency of hydrocarbon biodegradation in marine sediment (Dell'Anno *et al.*  
489 2012) have been observed. All these observations refer to well-known ecological concept where  
490 increased microbial diversity corresponds to increased catabolic potential and, hence, to better  
491 removal of metabolites and pollutants as explained by Dejonghe *et al.* (2001). The positive  
492 correlation found with Shannon diversity index, and not with PHE-degrader species richness, tends to  
493 show that PHE degradation efficiency depends of the presence of keystone species, that if they are  
494 absent, greatly decreases the biodegradation rate. Indeed, below 10 active PHE-degrading OTUs, the  
495 PHE degradation rate is low, but when at least 10 OTUs are active in even proportion the PHE  
496 degradation is higher, leading to a more efficient PHE removal. This phenomenon was previously  
497 observed for other organic pollutants such as atrazine (Monard *et al.* 2011).

498 Through labelling of microbes metabolizing PHE and their degradation by-products, DNA-SIP allowed  
499 us identifying the active PHE-degraders in the studied soils. For three soils (He, Mo and RM),  
500 apparently, the <sup>13</sup>C-PHE degradation was too low and PHE-degraders growth too weak to generate a  
501 sufficient <sup>13</sup>C-labeling of the microbial biomass. Indeed, we could not detect significant enrichment of  
502 <sup>13</sup>C-DNA in heavy fractions of the CsCl gradients and, even after sequencing of the heavy DNA  
503 fractions, no OTUs could be detected as significantly enriched in the <sup>13</sup>C-PHE condition compared to  
504 the <sup>12</sup>C controls. When looking at the 7 other soils, a large number of bacterial OTUs was identified as  
505 PHE degraders (from 3 to 30 OTUs per soil representing 31 taxonomic groups). We found that the  
506 presence of some taxa seems to maximise the PHE degradation rate. Among them, the  
507 *Mycobacterium*, *Massilia*, *Arthrobacter*, *ANPR* and *Rhizobium* genera, and unclassified OTUs  
508 belonging to Sphingomonadaceae, Burkholderiaceae and Micrococcaceae seemed to be the main  
509 PHE degraders best explaining the PHE degradation rates in the 7 soils. PHE-degrading *Massilia* were  
510 isolated from soils after PHE exposure (Bodour *et al.* 2003; Zhang *et al.* 2010), and from PAH-polluted  
511 soils (Baquiran *et al.* 2012; Wang *et al.* 2016), or were detected in an oilfield by metagenomics (Zhou  
512 *et al.* 2017). Although no detailed information is available about the role and potential of *Massilia sp.*  
513 in PAH degradation in environmental samples, one study showed that *Massilia sp.* strain WF1 had



514 high degradation ability and tolerance to PHE (Gu *et al.* 2016). Additionally, *Massilia* seems to also  
515 tolerate metal contamination (Zhang *et al.* 2016). Micrococcaceae and among which *Arthrobacter*  
516 genus has been shown for its PAH degradation capacity both in pure culture (Aryal and Liakopoulou-  
517 Kyriakides 2013) and in the environment (Thion *et al.* 2012b). Similarly to our result, a recent study of  
518 Storey *et al.* (2018) showed also a strong increase of one unclassified OTU affiliated to  
519 Micrococcaceae family, being dominant in a soil after PHE amendment. *ANPR* genera, which relate to  
520 N-fixing bacteria through symbiosis with legumes, contain various *Rhizobium* species described for  
521 their capacity to tolerate and degrade PAHs (Poonthrigpun *et al.* 2006; González-Paredes *et al.* 2013),  
522 and have been used to enhance PAH phytoremediation (Johnson, Anderson and McGrath 2005; Teng  
523 *et al.* 2011). Members of the Sphingomonadaceae family are also well-known PAH degraders  
524 (Thomas, Corre and Cébron 2019) and many isolates belonging to Sphingomonadaceae were  
525 frequently isolated from PAH-contaminated environments (Johnsen, Wick and Harms 2005).  
526 Similarly, Burkholderiaceae family is known to contain various bacteria able to grow on PAHs or  
527 having PAH degradation genes, such as members of *Burkholderia* and *Ralstonia* genera (Fuenmayor  
528 *et al.* 1998; Cébron *et al.* 2008; Andreolli *et al.* 2011), and recently members of *Cupriavidus* genus  
529 (Kuppusamy *et al.* 2016; Oyehan and Al-Thukair 2017).

530 OTUs affiliated to the *Mycobacterium* (Actinobacteria) genus were the dominant PHE degraders in  
531 the soils displaying the higher PHE degradation rates. The PHE degradation rate was positively  
532 correlated to the relative abundance of OTUs affiliated to *Mycobacterium*. This relationship  
533 confirmed that *Mycobacterium* strains were essential drivers of PHE degradation in our soils and  
534 could be defined as a key stone species for efficient PHE-degradation. Due to their prevalence in  
535 many PAH-contaminated soils, *Mycobacterium* species have been suggested to potentially play a  
536 major role in the natural attenuation of PAHs (Cheung and Kinkle 2001; Chen, Peng and Duan 2016;  
537 Chen *et al.* 2018). Numerous *Mycobacterium* species have indeed been described for their ability to  
538 degrade various low- and high-molecular-weight PAHs, in PAH-contaminated soils (Chen *et al.* 2018;  
539 Li *et al.* 2018). Previous studies suggest that PAH-degrading *Mycobacterium* are well adapted to  
540 oligotrophic and low PAH availability conditions, usually found in PAH-contaminated soils.  
541 *Mycobacterium* can degrade PAHs under optimal conditions (C:N:P ratio of 100:10:1) as well as under  
542 N and P excess or deficiency (Leys *et al.* 2005). Additionally, Miyata *et al.* (2004) suggested that both  
543 passive diffusion and high-affinity transport systems contributed to the PHE uptake by  
544 *Mycobacterium* strain RJGII-135, and enabled the bacterium to use aqueous-phase PHE in high and  
545 low concentrations. Moreover, *Mycobacterium* strains can attach on the PAH source and form  
546 biofilms which increase PAH bioavailability (Bastiaens *et al.* 2000; Wick *et al.* 2002). Given that  
547 *Mycobacterium* were highlighted as main active PHE degraders in our soils presenting high PHE  
548 degradation rate, and that numerous studies describe *Mycobacterium* strains with PAH degradation

549 capacity, their presence could constitute a relevant indicator of PAH degradation. Further, they might  
550 be of interest for bioaugmentation of PAH degradation in contaminated environments.

551

552

## 553 **CONCLUSION**

554 For the first time we studied in parallel and through stable isotope probing the degradation of <sup>13</sup>C-  
555 phenanthrene in a collection of 10 anthropized soils presenting a PAH pollution gradient and  
556 identified major active phenanthrene degraders for 7 soils of the collection. Surprisingly, the  
557 phenanthrene degradation rate was neither correlated to the initial soil PAH contamination level nor  
558 to the functional PAH-degradation potential (occurrence of PAH-RHD $\alpha$  genes) but was best explained  
559 by the initial soil bacterial community richness and abundance. No specific taxa, representative of  
560 aged and highly contaminated soils, were identified. In the studied soils, the phenanthrene  
561 degradation efficiency depended on two parameters: i) to have a wide diversity of active  
562 phenanthrene degraders (correlation between PHE degradation and Shannon diversity index of  
563 active taxa) and ii) to have representatives of the *Mycobacterium* genus as dominant phenanthrene  
564 degrading taxa (correlation between PHE degradation and *Mycobacterium* occurrence). To conclude,  
565 our study shows that microbial community characteristics and the presence of specific key-stone  
566 species (*Mycobacterium*), but also species complementarity are more important for PAH degradation  
567 efficiency than initial levels of soil PAH contamination and the initial PAH-degradation potential.

568

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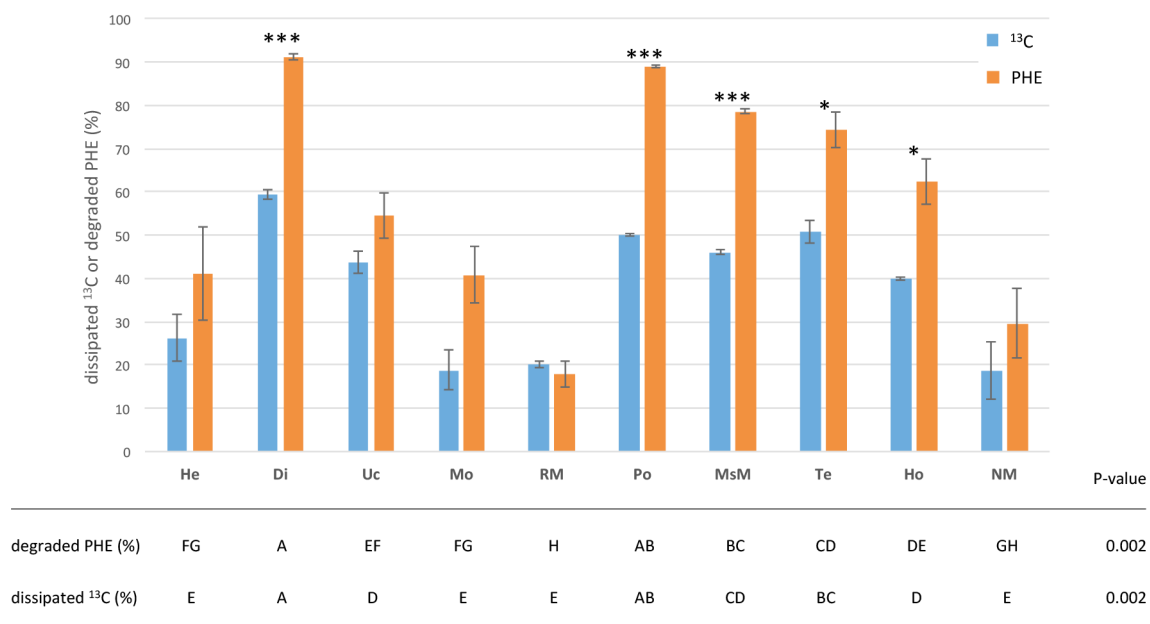
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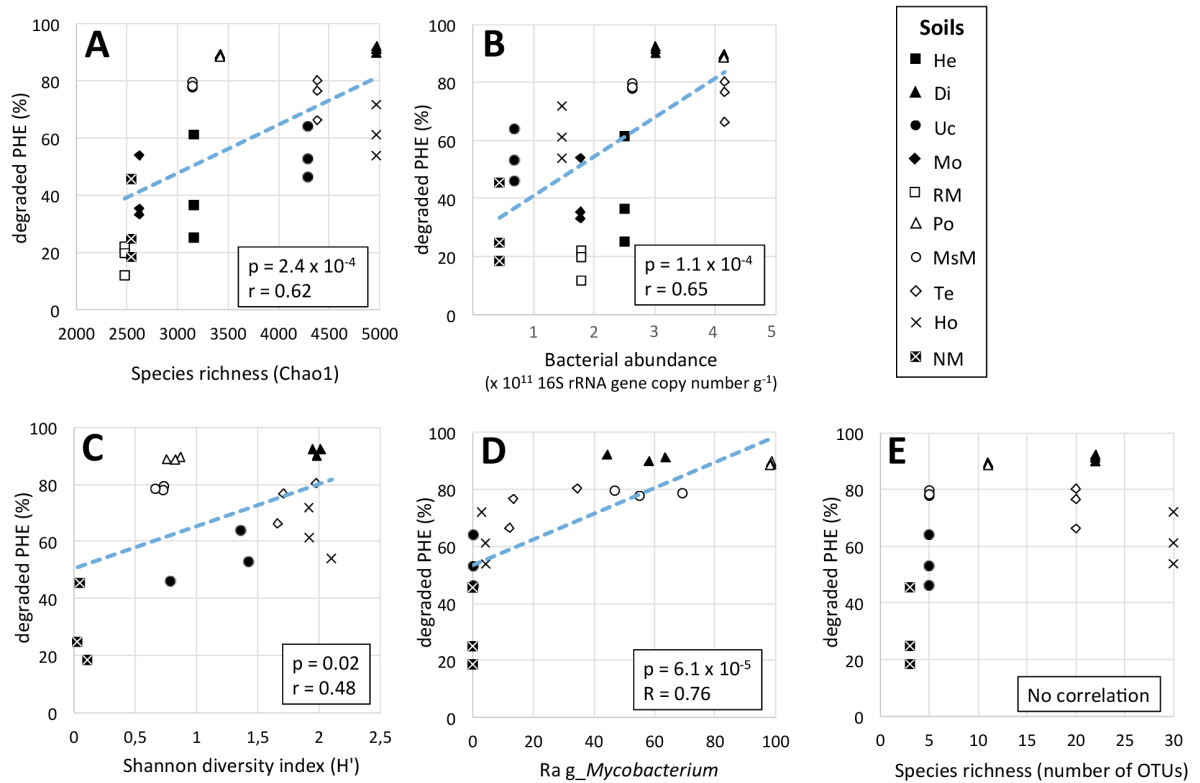


791 **Figure 1.** Percentages of dissipated  $^{13}\text{C}$  and degraded phenanthrene (PHE) at the end of SIP  
 792 incubations (after 12 days).  
 793 Error bars represent standard errors of the mean (n=3). Statistical differences (Kruskal-Wallis test,  $p <$   
 794 0.05) of dissipated  $^{13}\text{C}$  and degraded PHE among the soils are indicated by capital letters (different  
 795 letters indicate significant differences). Significant difference (Welch's test) between degraded PHE  
 796 and dissipated  $^{13}\text{C}$  for one soil are indicated by stars, with \*, \*\* and \*\*\* corresponding to  $p <$ 0.05,  $p <$   
 797 0.01 and  $p <$  0.001, respectively. Soils were ranked from left to right according to their total PAH  
 798 concentration.  
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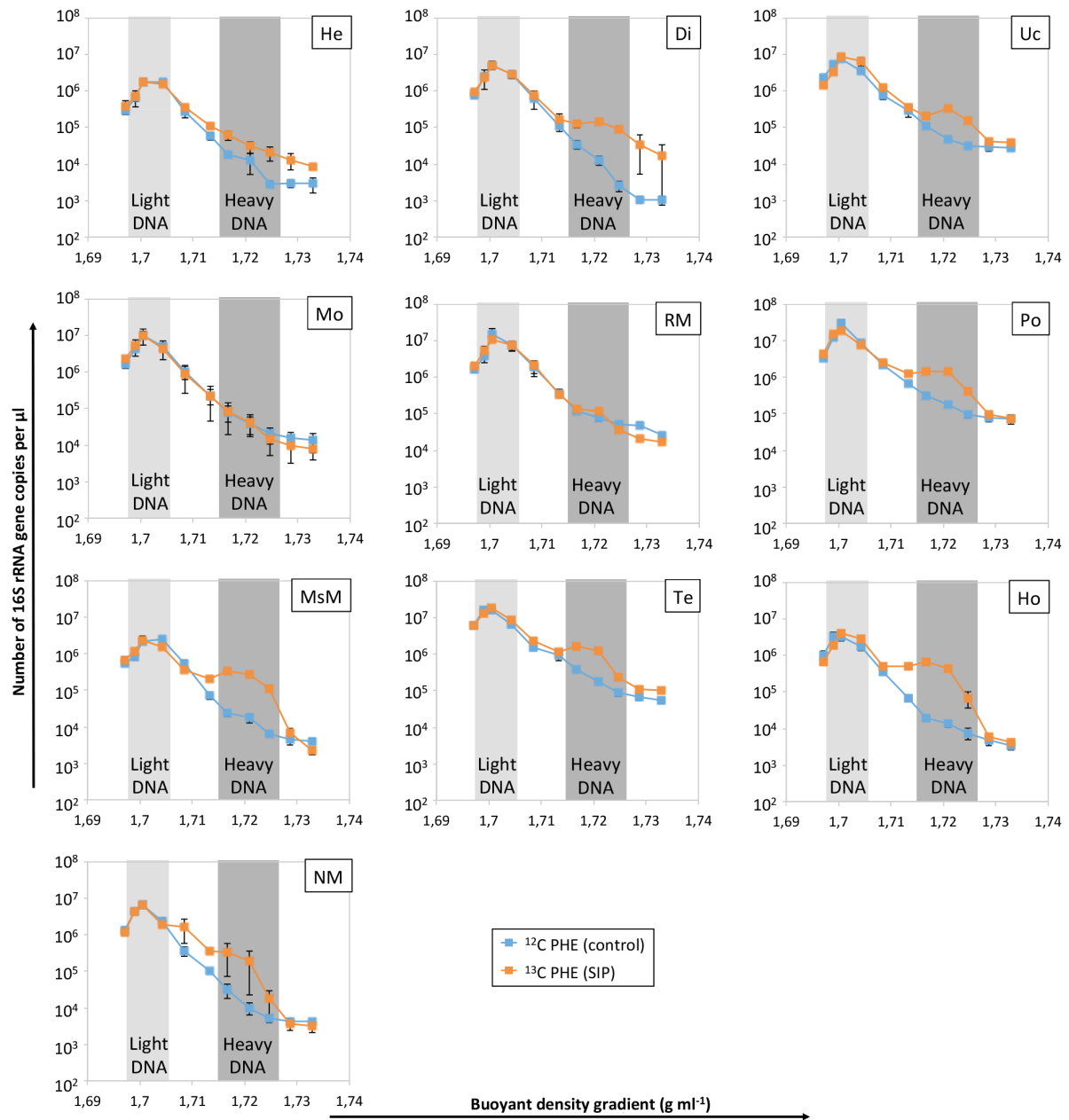
801 **Figure 2.** Relations between the percentage of degraded PHE and biotic factors for unspiked soils or  
 802 PHE-degrader characteristics found using SIP.  
 803 Correlation with total bacterial community Chao1 index (A) and bacterial abundance (B) of unspiked  
 804 soils, PHE-degrader Shannon diversity index (C), and relative abundance of *Mycobacterium* taxa  
 805 among PHE-degraders (D). No significant linear correlation was detected with PHE-degraders  
 806 richness (E).



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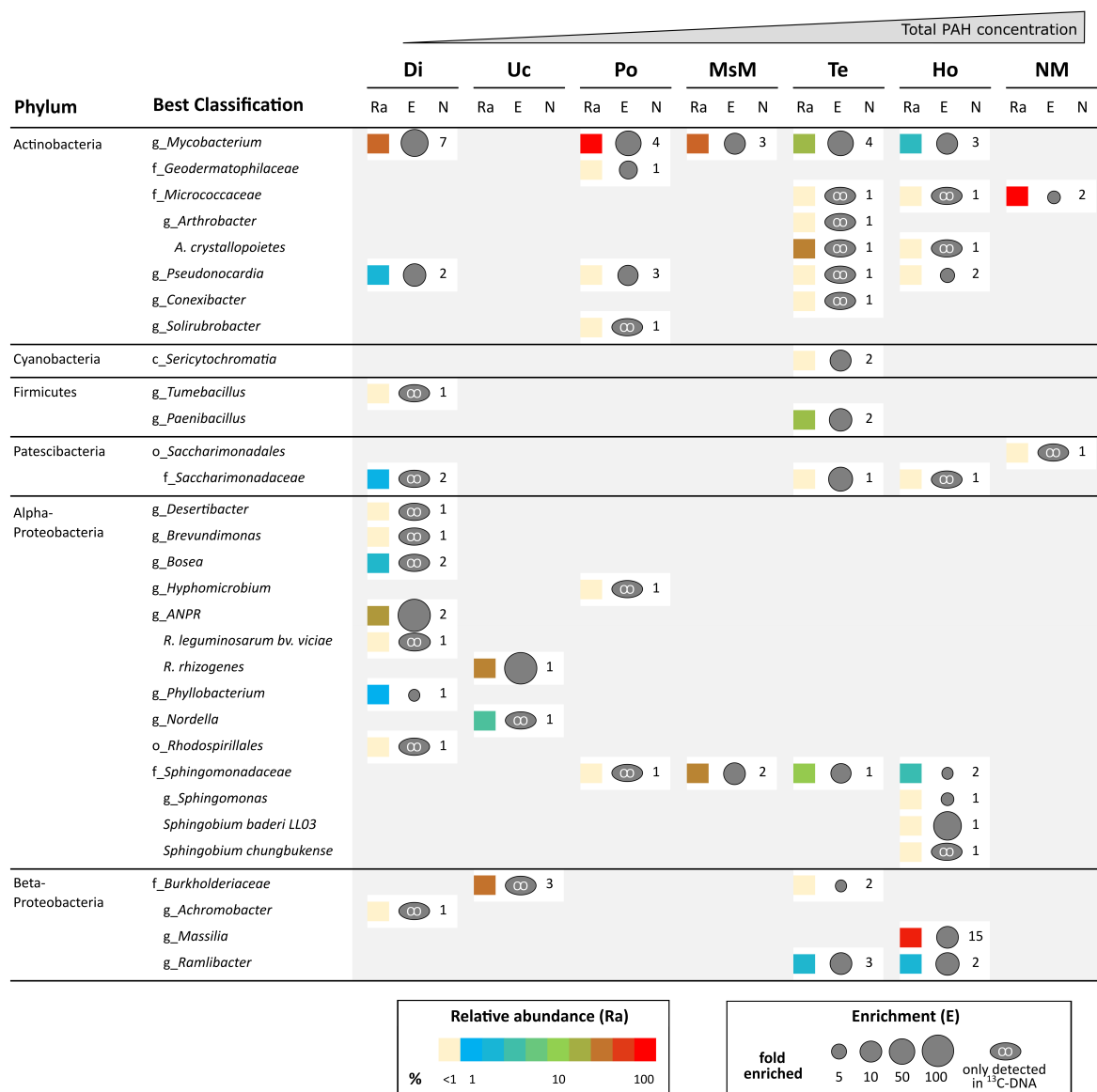
809 **Figure 3.** Quantification of bacterial 16S rRNA gene copy numbers in the 11 fractions (fractions n°1  
810 and 13 were discarded) separated by CsCl gradients using real-time quantitative PCR in the <sup>12</sup>C (blue)  
811 and <sup>13</sup>C (orange) microscosms. Data are presented for the 10 soils of the collection. Error bars  
812 represent standard errors of the mean (n=3). Light and dark grey bars represent the light and heavy  
813 fractions used for PAH RDHA $\alpha$  gene quantification and enrichment calculations, respectively. Heavy  
814 DNAs corresponding to fractions 4 to 6 (highlighted by a dark grey bar) were selected for DNA  
815 sequencing.



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818 **Figure 4.** Active <sup>13</sup>C-labeled PHE degrading taxa identified in each soil, based on differential OTU  
819 abundance in heavy-DNA fractions between <sup>13</sup>C and <sup>12</sup>C-PHE incubations.  
820 OTU having exactly the same taxonomic affiliation were summed as taxa groups. Their lowest  
821 possible classifications are given with the prefix corresponding to taxonomic rank (c = class, o = order,  
822 f = family, g = genus, no prefix = species). For each active PHE degrading taxa in one corresponding  
823 soil, Ra indicate the abundance relative to the whole pool of active PHE degraders identified through  
824 colour gradient, E indicates the enrichment factor of each taxa in <sup>13</sup>C-PHE compared to <sup>12</sup>C-PHE  
825 control through circle size (with the infinity symbol meaning that OTUs were only detected in <sup>13</sup>C-  
826 and not in <sup>12</sup>C-heavy DNA fractions), and N indicates the corresponding number of OTUs in the taxa  
827 groups. OTU groups originally classified as the Betaproteobacteriales order in the γ-Proteobacteria  
828 class (in Silva V132 database) are indicated here as β-Proteobacteria. Soils were ranked from left to  
829 right according to their total initial PAH concentration.



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834 **Table 1.** PAH contents (sum of the 16 regulatory PAHs and of phenanthrene concentration) and PAH-  
835 degradation genes (PAH-RHD $\alpha$  GN and GP) copy numbers and abundances in the soils and in the  
836 sequenced  $^{13}\text{C}$ -heavy DNA fraction, allowing to calculate functional gene enrichment in the  $^{13}\text{C}$ -heavy  
837 DNA fraction consequently to the Stable Isotope Probing (SIP) incubations.

	Sum of 16 regulatory PAH (mg kg $^{-1}$ )		Phenanthrene (mg kg $^{-1}$ )		Percentage of functional gene copy relative to 16S rRNA genes						Gene enrichment during SIP incubation	
					In soils (before SIP incubation)		In soils (after SIP incubation)		In sequenced $^{13}\text{C}$ -heavy DNA fractions			
	Total	Available	Total	Available	PAH-RHD $\alpha$ GP (x 10 $^{-3}$ )	PAH-RHD $\alpha$ GN (x 10 $^{-5}$ )	PAH-RHD $\alpha$ GP (x 10 $^{-2}$ )	PAH-RHD $\alpha$ GN (x 10 $^{-3}$ )	PAH-RHD $\alpha$ GP	PAH-RHD $\alpha$ GN (x 10 $^{-3}$ )	PAH-RHD $\alpha$ GP	PAH-RHD $\alpha$ GN
<b>He</b>	0.03 $\pm$ 0.01	<dl	<dl	<dl	7.0 $\pm$ 0.3	15.7 $\pm$ 1.0	5.7 $\pm$ 1.6	5.1 $\pm$ 2.6	0.3 $\pm$ 0.2	73.7 $\pm$ 10.5	8 $\pm$ 2	32 $\pm$ 16
<b>Di</b>	0.87 $\pm$ 0.06	<dl	0.11 $\pm$ 0.01	<dl	8.3 $\pm$ 0.4	11.5 $\pm$ 1.4	182.6 $\pm$ 23.0	N/A	39.4 $\pm$ 9.8	22.8 $^+$	221 $\pm$ 28	N/A
<b>Uc</b>	0.90 $\pm$ 0.09	<dl	0.26 $\pm$ 0.04	<dl	26.9 $\pm$ 3.6	14.0 $\pm$ 1.4	446.2 $\pm$ 27.6	1.0 $\pm$ 0.1	113.4 $\pm$ 28.9	18.3 $\pm$ 7.7	166 $\pm$ 10	7 $\pm$ 1
<b>Mo</b>	3.47 $\pm$ 1.35	<dl	1.34 $\pm$ 1.34	<dl	2.2 $\pm$ 0.2	4.9 $\pm$ 0.5	4.3 $\pm$ 0.6	0.5 $\pm$ 0.2	0.2 $\pm$ 0.1	18.7 $^+$	19 $\pm$ 3	11 $\pm$ 4
<b>RM</b>	6.59 $\pm$ 0.31	<dl	1.33 $\pm$ 0.03	N/A	11.2 $\pm$ 0.3	109.0 $\pm$ 9.3	26.6 $\pm$ 2.4	10.1 $\pm$ 1.4	1.9 $\pm$ 0.9	33.1 $\pm$ 16.7	24 $\pm$ 2	9 $\pm$ 1
<b>Po</b>	21.17 $\pm$ 2.33	0.18 $\pm$ 0.01	5.27 $\pm$ 2.79	<dl	22.1 $\pm$ 1.4	12.4 $\pm$ 0.8	462.7 $\pm$ 20.4	1.1 $\pm$ 0.4	81.1 $\pm$ 5.9	2.7 $\pm$ 0.8	209 $\pm$ 9	9 $\pm$ 3
<b>MsM</b>	49.65 $\pm$ 1.01	4.65 $\pm$ 0.62	4.40 $\pm$ 0.07	0.83 $\pm$ 0.06	31.4 $\pm$ 4.3	16.4 $\pm$ 4.7	505.1 $\pm$ 39.2	9.0 $\pm$ 0.8	24.5 $\pm$ 2.3	66.0 $^+$	161 $\pm$ 12	55 $\pm$ 5
<b>Te</b>	114.74 $\pm$ 0.50	6.31 $\pm$ 1.00	15.51 $\pm$ 0.09	0.88 $\pm$ 0.13	28.3 $\pm$ 3.0	6.1 $\pm$ 0.5	197.2 $\pm$ 22.4	7.7 $\pm$ 3.5	29.0 $\pm$ 6.4	2.2 $\pm$ 0.6	70 $\pm$ 8	126 $\pm$ 57
<b>Ho</b>	937.69 $\pm$ 14.31	54.14 $\pm$ 9.17	199.24 $\pm$ 3.25	18.10 $\pm$ 1.43	143.7 $\pm$ 12.2	329.0 $\pm$ 77.5	317.3 $\pm$ 29.3	159.2 $\pm$ 22.4	12.1 $\pm$ 3.1	274.9 $\pm$ 39.9	22 $\pm$ 2	48 $\pm$ 7
<b>NM</b>	1,095.90 $\pm$ 32.44	82.98 $\pm$ 8.47	104.08 $\pm$ 2.74	15.77 $\pm$ 0.56	160.5 $\pm$ 25.1	888.5 $\pm$ 475.6	69.9 $\pm$ 18.5	24.7 $\pm$ 8.0	0.9 $\pm$ 0.2	376.0 $\pm$ 197.6	4 $\pm$ 1	3 $\pm$ 1

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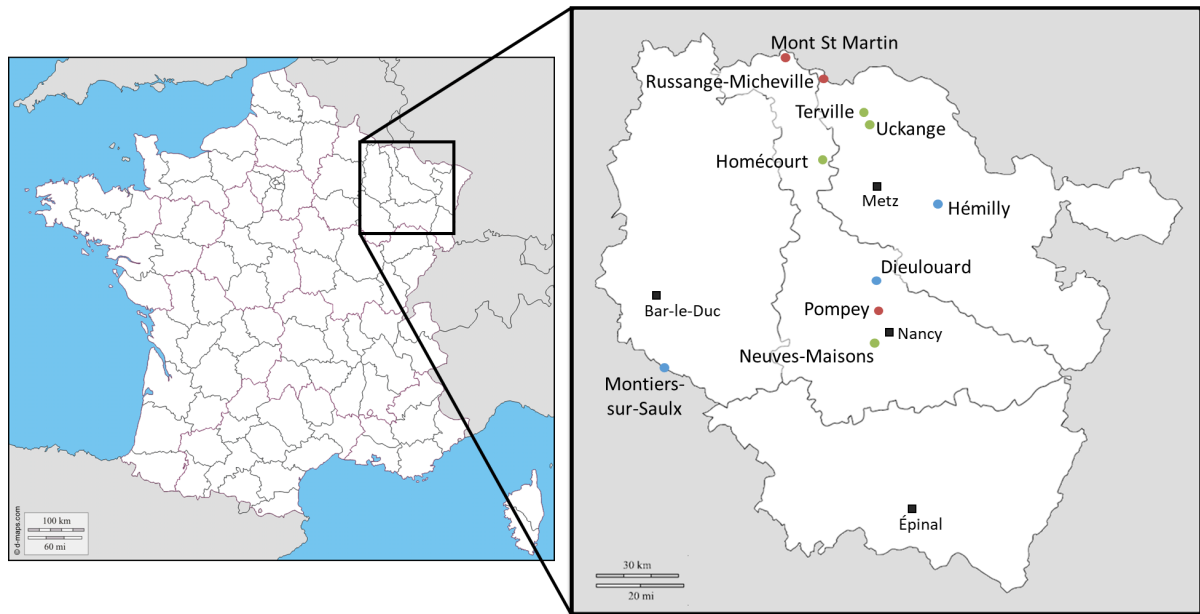
839 *Values are means (n = 3)  $\pm$  standard errors of the mean (SEM). For values indicated by +, SEMs were*  
840 *not calculated because only one replicate showed results for gene quantification. <dl means lower*  
841 *than the detection limit.*

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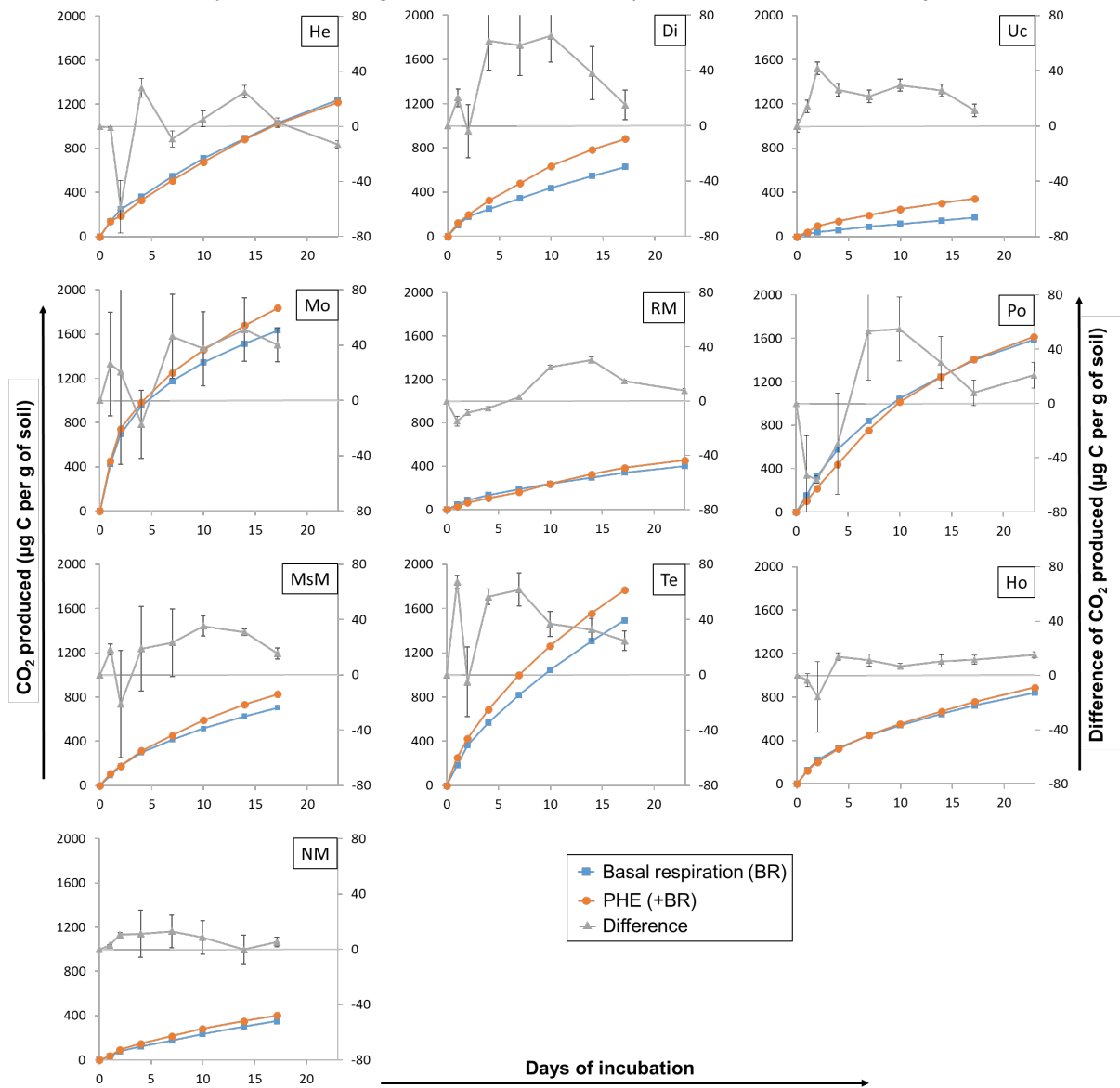
845 **Figure S1.** Geographic localisation of the sites. *Blue, green, and red points correspond to weakly*  
846 *anthropised, slag heap, and settling pond soils, respectively.*



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849 **Figure S2.** Soil respirations in the absence of a substrate (basal respiration, blue) or after  
 850 phenanthrene spiking (orange), and differences in produced CO<sub>2</sub> (grey). *Basal respiration and*  
 851 *respiration after PHE addition are expressed as cumulative curves and their values correspond to the*  
 852 *left axis, whereas each difference in CO<sub>2</sub> production is represented at each time of measurement and*  
 853 *their values correspond to the right axis. Error bars represent standard errors of the mean (n=3).*



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856 **Table S1.** Microbial and physico-chemical characteristics of unspiked soils. *16S rRNA gene copy*  
 857 *numbers and Chao1 indices are represented by means (n = 3) ± standard errors of the mean (SEM).*  
 858 *Zn, Pb, and Cd values correspond to total concentrations. From Lemmel et al. (2019).*

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	16S rRNA Gene copy number per g dw soil (x 10 <sup>10</sup> )	Chao1 richness index	pH (H <sub>2</sub> O)	Total organic carbon (g kg <sup>-1</sup> )	Nitrogen (g kg <sup>-1</sup> )	C/N	Clay (%)	Silt (%)	Sand (%)	Zn (mg kg <sup>-1</sup> )	Pb (mg kg <sup>-1</sup> )	Cd (mg kg <sup>-1</sup> )
He	25.0 ± 2.2	3,162 ± 441	5.4	72.1	2.85	25.3	25.5	69.0	5.5	248	93	0.27
Di	30.1 ± 1.1	4,966 ± 56	7.3	24.3	1.74	14.0	11.5	14.3	74.2	60	31	0.15
Uc	6.8 ± 0.5	4,293 ± 505	8.0	29.9	0.56	53.6	5.5	17.6	76.9	74	23	0.09
Mo	17.8 ± 2.4	2,621 ± 356	7.0	81.5	4.56	17.9	48.9	46.0	5.1	144	44	0.71
RM	17.9 ± 0.4	2,478 ± 26	7.6	149.0	3.83	38.8	10.9	74.6	14.5	119,000	39,500	22.00
Po	41.5 ± 0.4	3,422 ± 386	7.8	109.0	7.19	15.2	17.5	53.3	29.2	29,600	34,700	152.00
MsM	26.4 ± 1.2	3,151 ± 4	7.2	119.0	4.18	28.5	10.9	72.7	16.4	55,400	14,400	17.60
Te	41.5 ± 1.2	4,381 ± 66	7.7	88.2	4.35	20.3	10.0	20.7	69.3	1,650	475	4.55
Ho	14.8 ± 0.7	4,966 ± 173	7.5	159.0	2.92	54.6	7.0	12.0	81.0	314	303	1.10
NM	4.4 ± 0.3	2,541 ± 174	7.4	75.2	2.35	32.0	13.3	27.9	58.8	2,540	653	3.27

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863 **Table S2.** P-value and adjusted R-squared of the soil physicochemical characteristics tested in order  
 864 to explain PHE degradation among soils. Based on simple linear correlations, the three soil  
 865 characteristics showing the lowest P-values were selected to search for multiple interactions that  
 866 could explain different PHE degradation rates among soils.

Explanatory variable	P-value	Adjust R-squared
P_Olsen	0.256	0.052
Cd_tot	0.265	0.046
C/N	0.281	0.036
P_Olsen * Cd_tot	0.185	0.292
P_Olsen * C/N	0.295	0.156
Cd_tot * C/N	0.396	0.054
P_Olsen * Cd_tot * C/N	0.568	0.041

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869 **Table S3.** Richness, Shannon diversity and Pielou's evenness indices describing the active PHE-  
 870 degrader's community diversity based on OTU identified through <sup>13</sup>C-DNA sequencing from Stable  
 871 Isotope Probing incubations for the 7 soils studied.

	Richness index	Shannon diversity index	Pielou's evenness index
Di	22	1.98 ± 0.02	0.64 ± 0.01
Ho	30	1.98 ± 0.06	0.58 ± 0.02
MsM	5	0.71 ± 0.02	0.44 ± 0.01
NM	3	0.06 ± 0.02	0.06 ± 0.02

<b>Po</b>	11	$0.82 \pm 0.03$	$0.34 \pm 0.01$
<b>Te</b>	20	$1.78 \pm 0.10$	$0.59 \pm 0.03$
<b>Uc</b>	5	$1.19 \pm 0.20$	$0.74 \pm 0.13$

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