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1 **High PAH degradation and activity of degrading bacteria during alfalfa growth where a**  
2 **contrasted active community developed in comparison to unplanted soil**

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15 **Key words: historically contaminated soil, alfalfa, rhizosphere, RNA, bacterial diversity,**  
16 **PAH-degraders, functional community**

17 **Abstract**

18 PAH biodegradation in plant rhizosphere has been investigated in many studies, but the  
19 timescale of degradation and degrading bacteria activity was rarely considered. We explored  
20 the impact of plants on the temporal variability of PAH degradation, microbial abundance,  
21 activity and bacterial community structure in a rhizotron experiment. A historically  
22 contaminated soil was spiked with PAHs, planted or not with alfalfa, over 22 days with  
23 sampling once a week. In both conditions, most of the spiked PAHs were dissipated during  
24 the first week, conducting to polar polycyclic aromatic compound production and to  
25 decreased richness and diversity of bacterial communities. We showed a rapid impact of the  
26 rhizosphere on PAH degradation via the increased activity of PAH-degrading bacteria. After  
27 12 days, PAH degradation was significantly higher in the planted (100% degradation) than in  
28 unplanted (70%) soil. Gram-negative (*Proteobacteria*) PAH-dioxygenase genes and  
29 transcripts were higher in planted than unplanted soil and were correlated to the spiked PAH  
30 degradation. Conversely, Gram-positive (*Actinobacteria*) PAH-dioxygenase gene  
31 transcription was constant over time in both conditions. At 12 days, plant growth favoured the

32 activity of many *Gamma-Proteobacteria* (*Pseudomonadaceae*, *Stenotrophomonas* and  
33 *Acinetobacter*) while in unplanted soil *Alpha-Proteobacteria* (*Sphingomonadaceae*,  
34 *Sphingobium* and *Magnetospirillum*) and *Actinobacteria* (*Iamia*, *Geodermatophilaceae*, and  
35 *Solirubrobacterales*) were more active.

36

## 37 **Introduction**

38 Polycyclic Aromatic Hydrocarbons (PAHs) are organic pollutants resulting from coal-related  
39 activities (coking plants, gas plants) and derived activities (iron- and steel-production plants).  
40 These toxic compounds are persistent in the environment. Their high hydrophobicity, which  
41 leads to adsorption to the soil matrix, reduces their bioavailability to microorganisms (Bogan  
42 and Sullivan 2003). This aging process decreases PAH bioavailability in soils over time, and  
43 slows down the rate of PAH biodegradation (Allard et al. 2000). Microbial diversity also  
44 evolves over time, suggesting an adaptation to contamination (Bourceret et al. 2016). Thus, it  
45 is essential to better understand the temporal variations in microbial activity, to monitor it,  
46 and to relate it to the dynamics of PAHs in soils. To address this, many studies have used soils  
47 spiked with model PAH compounds (Allard et al. 2000; Thomas and Cébron 2016), but this  
48 makes them more bioavailable and therefore more degradable than aged PAHs in historically  
49 contaminated soils. Therefore it is appeared to us more realistic to study the biodegradation of  
50 a complex mixture of PAHs so as to investigate the activity of autochthonous microbial  
51 populations from aged contaminated soils and involved in biodegradation (Cébron et al.  
52 2013).

53 Rhizodegradation is a cost-effective and environment-friendly process using microorganisms  
54 associated to roots to dissipate soil organic pollutants, such as PAHs (Haritash and Kaushik  
55 2009). Plants release root exudates composed of sugars, amino and organic acids, among  
56 other compounds (Walker et al. 2003; Gao et al. 2015). They provide carbon growth sources  
57 for microorganisms, and biosurfactant compounds that increase PAH bioavailability and  
58 dissipation (Joner et al. 2002; Zhou et al. 2011). In PAH-contaminated soils, root exudates  
59 can increase microbial abundance (Kirk et al. 2005) and activity (Cébron et al. 2011). Several  
60 studies showed that plants enhanced PAH dissipation by modifying microbial diversity  
61 (Kawasaki et al. 2012) and activity (Yergeau et al. 2014; Peng et al. 2015), and selecting PAH  
62 degraders (Storey et al. 2014; Siciliano et al. 2003) in the rhizosphere. Consequently, most  
63 studies indicated that the combination of all these processes increased PAH degradation in the  
64 presence of plants (Liste and Alexander 2000; Tejeda-Agredano et al. 2013) and more

65 precisely in the rhizosphere. However, other experiments also evidenced no effect (Gartler et  
66 al. 2014), or slower PAH degradation in planted soil as compared to unplanted soil (Thomas  
67 and Cébron 2016). These contrasting results could be explained by spatio-temporal variations  
68 that occur within the rhizosphere and activate various processes during plant development.  
69 This hypothesis justifies further research on the time course of PAH-degradation processes  
70 and on the activity of the functional microbial community in soil-plant systems.

71 The temporal dynamics of rhizospheric processes could explain the variable efficiency of  
72 PAH degradation observed with plants. In uncontaminated soil, it has been extensively shown  
73 that the composition and quantity of root exudates can vary depending on the plant  
74 developmental stage and on root location (Hinsinger et al. 2005; Walker et al. 2003), and can  
75 thus modify the microbial community (Yang and Crowley 2000; Hannula et al. 2010;  
76 Baudoin et al. 2002), functional diversity (Haichar et al. 2008), and microbial activity  
77 (Baudoin et al. 2002). On the other hand, these rhizosphere processes have received little  
78 attention in contaminated environments where pollution is an additional factor impacting  
79 microorganism activity. It is therefore essential to assess the impact of both PAH pollution  
80 and root development on overall microbial activity and on the PAH-biodegrading function to  
81 know which factors are the most determining ones in a rhizoremediation context.

82 Among plant species tested for PAH rhizodegradation, the efficiency of legumes – e.g. alfalfa  
83 - is relevant, because they have extensive root systems that allow soil colonization and  
84 nutrient uptake (Muratova et al. 2003; D’Orazio et al. 2013). Alfalfa is a plant commonly  
85 used *in situ* during land-farming treatment of organic pollutants (Trapp and Karlson 2001). It  
86 is also commonly used in microcosm studies to evaluate rhizodegradation efficiency, and  
87 more precisely study the processes involved (Bourceret et al. 2015). A legume such as alfalfa  
88 is thus a good and realistic model plant to evaluate the time course of PAH rhizodegradation  
89 in aged contaminated soil.

90 This study aimed to evaluate 1) the dynamics of PAH dissipation in alfalfa rhizosphere as  
91 compared to unplanted soil, and 2) the variability of microbial community activity and PAH-  
92 degrading bacterial abundance and activity over three weeks of plant growth. We developed a  
93 specific 2-compartment rhizotron device to rapidly establish an active root system and put it  
94 in contact with PAH spiked soil so as to avoid the time gap between root growth from seeds  
95 germination and the degradation of bioavailable pollutants. Rhizotrons were filled with an  
96 aged PAH-contaminated soil, initially spiked with a complex mixture of PAHs (i.e. a soil



97 organic extract). Rhizotrons were sampled once a week to monitor concentrations of PAHs  
98 and potential metabolites (polar polycyclic aromatic compounds, pPAC). Dissolved organic  
99 carbon (DOC), pH, and humidity were measured to characterize the rhizosphere and soil  
100 conditions. Working on extracted DNA and RNA fractions, we also assessed total bacterial  
101 and fungal abundance, PAH-degrading bacterial abundance and transcriptional activity levels.  
102 Finally, we assessed active bacterial community structures by pyrosequencing of 16S rRNA  
103 transcripts.

## 104 **Materials and Methods**

### 105 **Experimental device**

106 We used an aged PAH contaminated soil, from a former coking plant (Neuves-Maisons (NM),  
107 Lorraine, France) to perform rhizotron experiments. Soil characteristics and spiking procedure  
108 were described previously by Bourceret et al. (2015). Briefly, the NM soil ( $1033 \pm 148$  mg  
109 per kg of dw soil of  $\Sigma 13$  PAHs (fluorene, phenanthrene, anthracene, fluoranthene, pyrene,  
110 benzo(a)anthracene, chrysene, benzo(b)fluoranthene, benzo(k)fluoranthene, benzo(a)pyrene,  
111 dibenzo(a,h)anthracene, benzo(g,h,i)perylene, indeno(1,2,3-cd)pyrene) was air-dried, sieved  
112 at 2 mm and spiked with a complex organic pollutant extract (**Table S1**), rich in PAHs and  
113 obtained from the same soil using chloroform extraction (Cébron et al. 2013). After solvent  
114 evaporation, the spiked soil was mixed with NM soil not exposed to chloroform, in a one-  
115 tenth ratio. Experimental device was divided in two parts (**Fig. 1**): the top part (mini-  
116 rhizotron) was a poly-methyl methacrylate box (7 cm high x 29.8 cm long x 2.3 cm wide)  
117 without bottom, that fits into the second part: a rhizotron (30 cm high x 30 cm long x 2.5 cm  
118 wide) with a removable front face allowing spatially localized depth sampling as described by  
119 Bourceret et al. (2015). First, 8 mini-rhizotrons were filled with NM soil, closed at the bottom  
120 by bolting cloth (5  $\mu\text{m}$  mesh), and adjusted to 80% of the soil water holding capacity (WHC)  
121 (corresponding to 203 ml deionized water per kg of dw soil). Alfalfa (*Medicago sativa* var.  
122 Europe) was planted on 4 of these devices by seeding 2.4 g of seeds (c.a. 26 seeds per  $\text{cm}^2$ )  
123 and the other 4 devices were left unplanted. After germination, the 8 devices were placed in a  
124 plant growth chamber under controlled conditions (22°C/18°C day/night, 80% relative  
125 humidity, c.a. 250  $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ , 16 hours photoperiod) and were maintained at 80%  
126 of the WHC by weighing the devices every two days. The previous growth of plant in NM  
127 soil allowed to avoid the delay between time needed for root development and the PAH  
128 degradation, that was already shown to be fast in spiked soil with a previous study (Bourceret  
129 et al. 2015). After 15 days, when roots reached the bottom of the mini-rhizotron devices, eight

130 rhizotrons were filled with spiked NM soil ( $1418 \pm 193$  mg of  $\Sigma 13$  PAHs per kg of dw soil),  
131 bolting cloths of the mini-rhizotrons were removed and devices were fitted in their  
132 corresponding rhizotron to allow root penetration and growth in spiked soil. This time  
133 corresponded to T0. Initial spiked NM soil was sampled: six aliquots from each of the eight  
134 rhizotrons corresponding to 48 T0 samples were stored at  $-80^{\circ}\text{C}$  for further analyses. Then the  
135 4 planted and 4 unplanted devices were incubated in plant growth chamber in conditions  
136 described above. After 6 (T1), 12 (T2), 16 (T3) and 22 (T4) days of plant growth, two  
137 rhizotrons (one planted and one unplanted) were harvested and opened to allow core sampling  
138 using a stainless steel cylinder equipped with a PTFE piston (1.6 cm diameter, 2.5 cm long,  
139 thus enabling to collect soil cores of around  $5\text{ cm}^3$ ). At 3 cm from the interface of the two  
140 compartments, 6 samples with 3 cm between the core centers were collected per device (**Fig.**  
141 **1**). Soil samples were prepared as previously described by Bourceret et al. (2015). Briefly,  
142 after 3 rinsing by sterile water, roots were spread on a black paper and photographed to  
143 estimate the size of each root fragment using ImageJ software (<http://rsbweb.nih.gov/ij/>).  
144 Roots were then air-dried at  $60^{\circ}\text{C}$  and weighed to quantify dry root biomass. Rhizosphere  
145 soil, collected from root rinsing water by centrifugation at 4400 rpm for 10 minutes, and bulk  
146 soil were weighed to calculate the ratio between the two fractions and were mixed together to  
147 obtain a unique soil sample stored at  $-80^{\circ}\text{C}$  before analyses: soil characteristics (pH, DOC,  
148 organic acids, and sugar concentrations), PAH and pPAC concentrations, and DNA/RNA  
149 extractions. Soil humidity was measured by collecting 3 other samples on the side of the  
150 rhizotrons and by weighing the soil before and after drying at  $60^{\circ}\text{C}$  for 48 hours.

### 151 **Soil characteristics**

152 Soil solutions were prepared with 1 g of soil in distilled water (1:5 w/v ratio), and pH was  
153 measured using a pH-meter electrode (BioBlock scientific, pHM210 Radiometer analytical).  
154 After centrifugation (10 min, 4000 rpm,  $20^{\circ}\text{C}$ ) supernatant was filtered ( $0.45\ \mu\text{m}$ ) and diluted  
155 20 times to quantify dissolved organic carbon (DOC) with a total organic carbon analyzer  
156 (TOC-V CSH, Shimadzu). Sugars (inositol, trehalose, sucrose, glucose, fructose) and organic  
157 acids (formate, butyrate, oxalate) were quantified from non-diluted extract with an ion-  
158 exchange chromatograph ICS 5000 (CarboPac SA10 column,  $40^{\circ}\text{C}$ ) and ICS 3000 (AS 11-  
159 HC column,  $35^{\circ}\text{C}$ ), respectively.

### 160 **Extraction and quantification of total PAHs, pPACs and bioavailable PAHs**

161 An aqueous solution of hydroxypropyl- $\beta$ -cyclodextrin (20 ml, 50 mM) (Acros organics, New  
162 Jersey, USA) was used to extract available PAH fraction from 1 g of fresh soil from 5 NM

163 soil samples before spiking, 6 T0 samples, and 48 rhizotron samples (6 samples from both  
164 planted and bare rhizotrons at each sampling time) in Oak Ridge Centrifuge tubes (FEP,  
165 Nalgene, USA) (Reid et al. 2000). After stirring (100 rpm) for 16 hours at 24 °C and 2  
166 centrifugation steps (10000 rpm, 20 min), dichloromethane was added (24 ml) to the aqueous  
167 supernatant (c.a. 30 ml) to extract available PAHs. An Accelerated Solvent Extractor  
168 (ASE350 Dionex ®) was used to extract total PAHs and pPACs with dichloromethane  
169 (130°C, 100 bars) from 0.25 g of soil (stored at -80°C) from 12 NM soil samples before  
170 spiking, 48 T0 samples, and 48 rhizotron samples previously lyophilized and crushed (<500  
171 µm). Dichloromethane extracts were split in two fractions to quantify PAHs using HPLC/UV-  
172 Fluo (High Pressure Liquid Chromatography) and pPACs using GC-MS (gas chromatography  
173 coupled to mass spectrometry). Firstly, the two dichloromethane extracts containing available  
174 and total PAHs were evaporated under nitrogen flow and diluted in 5 ml and 20 ml of  
175 acetonitrile, respectively. HPLC (Dionex Ultimate 3000) system, with a 100-mm long and  
176 4.6-mm internal diameter separation column (SupelcoAscendis Express C10), 2.6-mm  
177 granulometry allowed quantifying PAHs from 10 µl of extract. From total and available  
178 extracts, 13 PAHs (16 US-EPA PAHs excluding the three more volatile molecules:  
179 naphthalene, acenaphthylene and acenaphthene) were quantified by using a UV (254 nm) and a  
180 fluorescence detector, respectively. Secondly, from dichloromethane extracts pPACs were  
181 quantified on a GC-6890 (Agilent Technologies) equipped with a DB5- MS column (60 ×  
182 0.25 mm id × 0.25µm film thickness) coupled to a MS-5973 Inert (Agilent Technologies)  
183 operating SIM mode (Single Ion Monitoring). The oven temperature program was as follows:  
184 2 minutes at 70 °C, then from 70 °C to 130 °C at 15 °C min<sup>-1</sup>, then from 130 °C to 315 °C at 4  
185 °C min<sup>-1</sup> and then a 25 min hold at 315 °C. The carrier gas was helium at 1.4 ml min<sup>-1</sup>  
186 constant flow. Fifteen pPACs including 11 oxygenated pPACs (O-PACs) and 4 nitrogenated  
187 pPACs (heterocyclic PACs containing nitrogen N-PACs) were quantified by an internal  
188 calibration using deuterated internal standards (the list of the 15 quantified polar PAC is given  
189 in Hanser et al. (2015)). During a sequence, calibration controls were injected every 12  
190 samples to check the overall calibration.

### 191 **DNA/RNA extraction**

192 Nucleic acids were extracted from 0.5 g of soil using a FastDNA SPIN Kit for soil (MP  
193 Biomedicals, France). DNA and RNA were co-extracted from the same aliquots of samples  
194 stored at -80°C. DNA was resuspended in 100 µl of DES (Dnase-free water) and quantified at  
195 480 nm (Xenius, SAFAS) by using Quant-iTPicoGreenasDNA Assay kit (Invitrogen), and

196 then extracts were diluted to 2 ng/μl before amplification. RNA was collected from the  
197 filtrates at the DNA binding to the column step. After sodium acetate (0.1Vol, 3M, pH 5.2)  
198 and isopropanol precipitation for one night at 4°C and centrifugation, RNA was resuspended  
199 in 45 μl of Rnase-free water. Two steps of DNase (Thermo scientific) treatment (addition of  
200 10 μl of DNase buffer (10X), 1 μl of DNase (1u/μl) and incubated 1 hour at 37°C) and  
201 purification using RNeasyMinElute Cleanup kit (Qiagen) allowed obtaining 30 μl of pure  
202 extract (presence of DNA contamination was PCR checked). After quantification at 480 nm  
203 (Xenius, SAFAS) by Quant-iTRiboGreen RNA Assay kit (Invitrogen), extracts were  
204 converted in cDNA using SuperScript III First-Strand kit (Invitrogen). Reverse transcription  
205 reaction (20 μl) was performed using 1 μl of random hexamer (50 ng/μl), 1 μl of hybridization  
206 buffer and 22.27 ng of RNA (5 min at 65°C and 1 min on ice), then 10 μl of reaction buffer  
207 (2X) and 2 μl of enzyme mix were added and incubated 10 min at 25°C, 50 min at 50°C and 5  
208 min at 85°C.

### 209 **Real-time PCR quantification**

210 The primer sets Fung5F/FF390R (Lueders et al. 2004; Thion et al. 2013), 968F/1401R (Felske  
211 et al. 1998; Cébron et al. 2008) and PAH-RHD<sub>α</sub>GN F/R, PAH-RHD<sub>α</sub> GP F/R (Cébron et al.  
212 2008), targeting the fungal 18SrRNA genes, the bacterial 16S rRNA genes, and the PAH-ring  
213 hydroxylating dioxygenase genes from Gram-negative and Gram-positive bacteria,  
214 respectively, were used to quantify by real-time PCR the abundance of active and total fungal,  
215 bacterial and PAH-degrading bacterial populations. Amplification reactions were performed  
216 on 20 μl of reaction mix containing 10 μl of iQ SYBR green SuperMix (Bio-Rad), 0.8 μl of  
217 each primer (10 μM), 0.4 μl of 3% BSA (bovine serum albumin) solution, 0.2 μl of DMSO  
218 (dimethyl sulfoxide), 0.08 μl of T4 bacteriophage gene 32 product (MP Biomedicals, France)  
219 and 1 μl of diluted gDNA or pure cDNA. Amplification and quantification were achieved  
220 using a CFX96 Real-Time PCR detection system (Bio-Rad) according to standard plasmid  
221 dilution series and program described by Cébron et al. (2008) and Thion et al. (2012). After  
222 soil humidity correction, data were expressed in number of gene copies per gram of dry  
223 weight soil, in percentage of transcripts relative to gene copies, and in percentages of 18S  
224 rRNA or PAH-dioxygenase relative to 16S rRNA gene or transcript copies.

### 225 **Pyrosequencing and sequence analysis**

226 The primer set 515F ((5'-GTG CCA GCM GCC GCG GTA A-3') / 907R (5'-CCG TCA ATT  
227 CMT TTR AGT TT-3')) (Turner et al. 1999) was used to amplify the V4-V5 region of  
228 bacterial 16S rDNA from gDNA and cDNA. Samples were identified with a MID (Multiplex

229 Identifier, Roche). PCR reactions were achieved as described in Bourceret et al. (2016).  
230 Beckman Coulter Genomics (Danvers, MA, USA) sequenced libraries using a 454/Roche GS-  
231 FLX Titanium system. Data were deposited in the NCBI Sequence Read Archive (SRA)  
232 under the BioProject ID: PRJNA324193. Denoised 16S rDNA sequenced were analysed in  
233 QIIME 1.8.0 pipeline (Caporaso et al. 2010) as previously described by Bourceret et al.  
234 (2017). Briefly, after filtering and reverse complement non-sense sequences, data from gDNA  
235 and cDNA were gathered and clustered in Operational Taxonomic Units (OTUs) at 97% of  
236 similarity and their representative sequences were affiliated at different taxonomic levels  
237 (from phyla to genus) using RDP classifier (Wang et al. 2007) and the Greengenes 13\_5  
238 database (McDonald et al. 2012). Then subsampling was performed to the lowest number of  
239 sequences counted in one of the samples (4,739 sequences per sample) and alpha-diversity  
240 indexes (richness and diversity estimates) were calculated using QIIME 1.8.0 pipeline.

#### 241 **Statistical analyses**

242 Statistical analyses were performed using XLStat 2013 software (Addinsoft). One-way  
243 analysis of variance (ANOVA, with  $p < 0.05$ ) followed by a Newman-Keuls multiple  
244 comparison test was performed to measure the time effect in each condition (planted and  
245 unplanted) between 0 and 22 days. Two-way analysis of variance (ANOVA, with  $p < 0.05$ )  
246 followed by a Newman-Keuls multiple comparison tests were performed to measure the time  
247 and plant effect between T1 and T4. Box plots were drawn using PAH data to compare their  
248 distribution and variability. Significant differences in relative abundance of dominant OTUs  
249 ( $\geq 1\%$  in at least one sample) over time, were identified in cDNA samples with STAMP  
250 version 2.0.8 (Parks and Beiko 2010) by a Tukey Kramer ANOVA, with a Benjamini  
251 Hochberg correction of p value ( $q < 0.05$  and Effect size (ES)  $> 0.26$ ). Significant differences in  
252 the ratio cDNA/gDNA OTU abundance, according to the presence of plant was studied by  
253 Student-t test with a Benjamini Hochberg correction of p value with the differential  
254 expression function in XLStatOmics package. Canonical correspondence analysis (CCA)  
255 followed by a Monte Carlo permutation test (1000 permutations) was performed by using  
256 XLStat-ADA 2015 software (Addinsoft) to study, considering both conditions (alfalfa and  
257 unplanted soil) and 4 sampling times (0, 6, 12, 16 and 22 days), relationship between the  
258 relative abundance of the 41 most abundant OTUs ( $\geq 1\%$  in at least one sample) and  
259 environmental variables (root biomass and length, pH, percentage of Gram-negative and  
260 Gram-positive PAH dioxygenase transcripts and genes, sugars, organic acids, and PAH  
261 content).

## 262 **Results**

### 263 **Rhizosphere parameters**

264 Humidity stayed constant (around 80% of the WRC) over time in both planted and unplanted  
265 soil rhizotrons but was globally significantly higher in unplanted soil ( $78.36 \pm 20.91$  and  
266  $70.85 \pm 9.98$  %, in unplanted and alfalfa planted soil, respectively). In alfalfa rhizotron, root  
267 biomass was significantly ( $p < 0.05$ ) higher after 22 days of plant development than after 6  
268 days, whereas root length significantly increased over time and was significantly different  
269 between 6 and 12 days and then between 16 and 22 days (**Fig. 2A**). Related to the plant  
270 growth, several parameters, highlighting the effect of root exudation on soil properties,  
271 increased over time. Globally sugar concentration and DOC increased significantly over time  
272 in the planted compared to unplanted soil. Indeed sugar concentration was significantly higher  
273 after 12 days in planted than in unplanted soil where no sugar was detected (**Fig. 2B**) and  
274 DOC stayed constant over time in both rhizotrons until the 16<sup>th</sup> day, and then increased  
275 significantly in planted soil on the 22<sup>nd</sup> day (**Fig. 2C**). At this time point, organic acids were  
276 detected in alfalfa planted rhizotron ( $0.21 \pm 0.13$  mg.kg<sup>-1</sup>) but none in unplanted soil.  
277 Surprisingly soil pH increased significantly over time in both conditions and was stable after  
278 12 days. However it increased more rapidly in the planted than in the unplanted soil rhizotron  
279 with a significant difference at 6 days (**Fig. 2D**).

### 280 **PAHs and pPACs contents**

281 The total aged PAH concentration ( $\Sigma 13$  PAHs) of the NM soil ( $1033 \pm 148$  mg.kg<sup>-1</sup>dw soil)  
282 was significantly increased ( $p < 0.05$ ), i.e 27 %, after soil spiking with the organic pollutant  
283 extract and reached  $1418 \pm 193$  mg.kg.dw soil<sup>-1</sup> at the beginning (T0) of the experiment (**Fig.**  
284 **3**). The bioavailable PAH fraction was also increased by 41 % and reached  $63.7 \pm 18.0$  mg.kg<sup>-1</sup>  
285 dw soil, while it represented  $37.3 \pm 15.6$  mg.kg<sup>-1</sup>dw soil before spiking (data not shown).  
286 Spiking increased the concentration in total and bioavailable fractions of most of the 13 PAH  
287 compounds (**Fig. S1**). The total PAH concentration decreased significantly between 0 and 6  
288 days and stayed constant until the 22<sup>nd</sup> day in both planted and unplanted rhizotrons (**Fig. 3**).  
289 From the 6<sup>th</sup> days, the total PAH concentration dropped down to  $1200 \pm 210$  and  $1024 \pm 190$   
290 mg.kg.dw soil<sup>-1</sup> in unplanted and planted soil, respectively, corresponding to a decrease in 19  
291 and 24% relative to the initial concentration, and to 70 and 100% of the spiked PAH fraction,  
292 respectively. The concentration in total PAHs was significantly lower in alfalfa than in  
293 unplanted soil when considering all times together (from 6 to 22 days, two-way ANOVA) and  
294 was mostly explained by a significant difference after 12 days (Student t-test) of incubation

295 **(Fig. 3)**. The concentrations of several compounds decreased significantly from the 6<sup>th</sup> day  
296 (fluorene, phenanthrene, fluoranthene, pyrene, benzo(k)fluoranthene, benzo(a)pyrene and  
297 benzo(g,h,i)perylene only in planted condition) whereas the concentration of other PAHs  
298 remained constant over the time course (anthracene, benzo(a)anthracene, chrysene,  
299 benzo(b)fluoranthene, dibenzo(a,h)anthracene, indeno(1,2,3-cd)pyrene) or even increased  
300 significantly (benzo(g,h,i)perylene) from the 22<sup>nd</sup> day, in presence of plant **(Fig. S2)**.  
301 Bioavailable PAH fraction decreased also significantly between T0 and the 6<sup>th</sup> day in both  
302 conditions, reaching  $0.05 \pm 0.01$  and  $0.08 \pm 0.03$  mg.kg<sup>-1</sup>dw soil, corresponding to ca.100 %  
303 decreases, for both planted and unplanted soil rhizotron. After one week, the bioavailable  
304 PAH fraction was significantly lower than in the soil before spiking. Ten oxygenated and one  
305 nitrogenous pPAC compounds were detected in initial soil (T0) and in both conditions after 6  
306 days **(Table 1)**. After 6 days the concentration of 4 oxygenated pPAC (methylanthracene-  
307 9,10-dione, benzoanthracenedione, naphacene-5,12-dione) was significantly higher than at  
308 T0, without significant differences between planted and unplanted soils. Benzo(cd)pyrenone  
309 was only significantly higher at 6 days than at T0 in the planted rhizotron. The concentration  
310 of the other pPAC stayed constant over the first week for both planted and unplanted soils.

311

## 312 **Microbial communities**

### 313 *Abundance of active and total microbial communities*

314 Bacterial abundance was higher than fungal, and bacteria were also more active over time in  
315 both planted and unplanted soil (mean value of  $7.84 \times 10^8 \pm 6.64 \times 10^8$  16S rRNA gene copies  
316 and  $1.12 \times 10^{10} \pm 1.25 \times 10^{10}$  transcript copies g dw soil<sup>-1</sup>) while fungi were 100 times less  
317 represented (mean value of  $9.94 \times 10^6 \pm 7.04 \times 10^6$  18S rRNA gene copies and  $2.05 \times 10^8 \pm$   
318  $1.48 \times 10^8$  transcript copies g dw soil<sup>-1</sup>) **(Fig. 4A, B)**. Abundance of 16S rRNA genes tended  
319 to increase over time in alfalfa and stayed constant in unplanted rhizotron whereas number of  
320 16S rRNA transcripts increased significantly between T0 and the 6<sup>th</sup> day for both rhizotrons  
321 and then stayed constant. Abundance of 16S rRNA genes and transcripts was significantly  
322 higher in planted than unplanted rhizotron and plant effect was mainly detected at 12 (T2) and  
323 22 days (T4). Abundance of 18S rRNA genes and transcripts tended to be constant in both  
324 unplanted and planted rhizotrons and no plant effect was observed.

325 Abundance of Gram-negative PAH-dioxygenase genes and transcripts increased significantly  
326 between T0 and the 6<sup>th</sup> day in both rhizotron types, and were higher in planted than in  
327 unplanted soils, with a significant plant effect after 12 days **(Fig. 4A and B)**. Abundance of  
328 Gram-positive PAH-dioxygenase genes slightly increased over time whereas transcripts

329 largely increased between T0 and 6 days in both soil and further increased in planted soil till  
330 12 days, resulting in a higher content in planted than in unplanted rhizotron, significant at  
331 each sampling time. The level of PAH-dioxygenase gene transcription was expressed by the  
332 ratio of the number of transcripts relative to corresponding gene copies (**Fig. 4C**). The level of  
333 PAH-dioxygenase gene transcription was significantly higher in alfalfa than in unplanted soil  
334 specifically after 12 days, and then decreased (for Gram-negative bacteria) and stayed  
335 constant (for Gram-positive bacteria) over time without significant differences with unplanted  
336 soil (**Fig. 4C**).

337

#### 338 *Richness, evenness and diversity of the active community*

339 Globally, bacterial richness (Chao1) and diversity (Inverse of Simpson's) indices were  
340 significantly higher in unplanted than in alfalfa planted rhizotron (**Table 2**). This difference  
341 was significant after 16 days for richness and 12 days for diversity index (**Table 2**). No  
342 significant impact of plant was detected on the bacterial community evenness. For both  
343 studied conditions, bacterial richness, diversity and evenness indices decreased significantly  
344 during the 6 first days and after this time point increased significantly over time.

345 To explore the potential bacterial population involved into the rapid PAH degradation  
346 appearing at the beginning of the incubation period, we looked for bacterial community  
347 structure modification over time (**Fig. 5**). Among the active community, 12 phyla were  
348 identified and their proportion changed over time. In both rhizotrons, *Proteobacteria* was the  
349 more active phylum all over the incubation to the detriment of *Chloroflexi*, *Actinobacteria*  
350 and *Nitrospirae* that represented a lower proportion of the active community along the time  
351 course compared to the T0. Activity of *Beta-* and *Gamma-Proteobacteria* increased between 0  
352 and 6 days and stayed constant or decreased until the end of incubation while the proportion  
353 of active *Alpha*-subdivision members further increased. An opposite trend was observed for  
354 the activity of *Planctomycetes*, *Acidobacteria* and *Delta-Proteobacteria* that was first  
355 repressed between 0 and 12 days and then favored at the end of the incubation (**Fig. 5**). The  
356 relative abundance of some active phyla evolved also differently over time according to the  
357 presence of alfalfa. In alfalfa planted rhizotron, *Bacteroidetes* and *TM6* were more and less  
358 active over time, respectively, while the activity of *Unclassified Proteobacteria* was  
359 transiently high and then low over the 22 days. In planted soil, 3 OTUs affiliated to *Beta-*  
360 *Proteobacteria* (*Thiobacillus*, and *Un.Oxalobacteraceae*), 1 OTU affiliated to *Alpha-*  
361 *Proteobacteria* (*Mycoplana*) and 1 OTU affiliated to *Firmicutes* (*Bacillus selenatarsenatis*)  
362 were significantly more abundant among the active community after 6 days of incubation than



363 at all other times, contrary to the unplanted rhizotron where no OTU was favored after 6 days  
364 (**Fig. S3**). After 12 days of incubation, three other OTUs belonging to *Alpha-* (*Sphingobium*),  
365 *Beta-* (*Janthinobacterium*) and *Gamma-* (PYR10d3) *Proteobacteria* were favored in planted  
366 soil. In unplanted rhizotron, 5 OTUs affiliated to *Alpha-* (*Mycoplana*, *Phenylobacterium*) and  
367 *Beta-* (*Thiobacillus*, *unclassified. Comamonadaceae*) *Proteobacteria* were significantly more  
368 abundant among the active community after 12 days of incubation.

369 Significant positive or negative impact of plants on shaping active bacterial community was  
370 observed on 7 and 18 OTUs after 6 and 12 days of incubation, respectively (**Fig. 6**). After 6  
371 days, the activity ratio between 16S rRNA and rDNA for 3 OTUs belonging to *Gamma-*  
372 (*Pseudomonadaceae*), *Beta-*(*Achromobacter*) *Proteobacteria* and *Firmicutes* (*Paenibacillus*)  
373 was significantly higher in alfalfa planted than in unplanted soil. Four other OTUs, affiliated  
374 to *Alpha-* (*Un. Sphingomonadaceae*), *Beta-* (*Un. Burkholderiales*) *Proteobacteria* and  
375 *Actinobacteria* (*Un. Micrococcaceae*) were more actives in unplanted soil. After 12 days,  
376 plant growth favored significantly the activity of 11 OTUs belonging to *Alpha-* (*Devosia*),  
377 *Beta-* (*Methylophilaceae*), *Gamma-* (*Pseudomonadaceae*, *Stenotrophomonas*, *Acinetobacter*)  
378 *Proteobacteria*, *Bacteroidetes* (*Dyadobacter*) and *Actinobacteria* (*Un. Micrococcaceae*). In  
379 unplanted soil the activity of 6 OTUs affiliated to *Alpha-Proteobacteria* (*Un.*  
380 *Sphingomonadaceae*, *Sphingobium*, *Magnetospirillum*) and *Actinobacteria* (*Un.*  
381 *Solirubrobacterales*, *Un. Geodermatophilaceae*) was significantly higher than in alfalfa  
382 planted soil. After 16 and 22 days of incubation, 56% and 76% of the OTUs whose activity is  
383 significantly impacted by the presence of plant, were more active in alfalfa planted rhizotron  
384 (**Fig. S4**).

385

### 386 **Multivariate analysis**

387 The two first axes of the canonical correspondence analysis (CCA) explained 62.5 % of  
388 bacterial diversity variability among samples (**Fig. 7A and B**). The F1 axis (explaining 41.8%  
389 of the variability) separates samples from the T0 (located on the right) to all other sampling  
390 times. The F2 axis (explaining 20.8% of the variability) separates samples according to the  
391 presence of plant and time, with 6to 22 day samples from the bottom to the top. Alfalfa  
392 planted samples were separated from unplanted soil samples according to sugar and DOC  
393 contents, root development (biomass and length), abundance of Gram-negative PAH-  
394 dioxygenase genes and transcripts, and abundance of Gram-positive PAH-dioxygenase  
395 transcripts (**Fig. 7A**). These parameters are correlated to the higher activity of 11 bacterial  
396 OTUs, most belonging to *Proteobacteria* except one *Bacteriodetes* (*Dyalobacter*), in the

397 alfalfa planted rhizotron (**Fig. 7B**). These OTUs gathered bacteria affiliated to  
398 *Pseudomonadales* (*Acinetobacter* and *Pseudomonas*), *Sphingomonadales* (*Sphingobium*),  
399 *Burkholderiales* (*Janthinobacterium* and *Achromobacter*) and *Rhizobiales* (*Un.*  
400 *Methylobacteriaceae*) orders. Unplanted soil samples were mostly discriminated according to  
401 the higher PAH content ( $\Sigma$ 13 PAHs and 2-3, 4 and 5-6 cycles). According to the Pearson  
402 correlation matrix (**Table S2**), pH and the root biomass and length were positively and  
403 significantly correlated to the percentage of Gram-positive PAH-dioxygenase transcripts  
404 contrary to the abundance of 18S transcripts, showing a negative correlation. Moreover  
405 concentrations of 2-3, 4- rings and  $\Sigma$ 13PAHs were significantly and negatively correlated to  
406 the percentage of Gram-negative PAH-dioxygenase transcripts, shown as the most active  
407 degrading community at the beginning of the monitoring.

408

## 409 **Discussion**

### 410 **Rapid PAH dissipation in a spiked aged-contaminated soil**

411 After 6 days of incubation 100 and 70% of the spiked PAH fraction was dissipated in the  
412 planted and unplanted rhizotrons, respectively. A similar rapid dissipation, of bioavailable  
413 PAHs after a few days was previously observed in simplified sand or soil microcosms spiked  
414 with phenanthrene (Louvel et al. 2011; Cébron et al. 2011), but never observed with such a  
415 complex PAH contaminant mixture. We evidenced the degradation of almost all spiked PAHs  
416 including fluorene, phenanthrene, fluoranthene, pyrene, benzo(k)fluoranthene and  
417 benzo(a)pyrene (which have from 2 to 5 aromatic cycles) in both conditions. In the same soil  
418 (NM) incubated in bioslurry, 30 to 55% of the aged PAH compounds (fluorene, phenanthrene,  
419 fluoranthene, pyrene, benzo(a)anthracene and chrysene) were degraded after 1.5 months of  
420 incubation (Biache et al. 2017), indicating the presence of a very effective microbial  
421 community for the degradation of a wide range of PAH compounds. Degradation of PAHs by  
422 microbial consortia and co-metabolism are often described as playing crucial roles in the  
423 degradation of PAH mixtures in the environment (Ghosal et al. 2016) because the presence of  
424 low-molecular-weight PAHs could have a synergistic effect on the degradation of more  
425 recalcitrant compounds (Van Herwijnen R et al. 2003; Roy et al. 2013). During the first 6  
426 days of the experiment, spiked PAHs were probably the main available carbon sources in both  
427 the planted and unplanted rhizotrons, as no increase in DOC or sugar content was observed in  
428 either condition. As the microbial community of the aged-contaminated NM coking plant soil  
429 was well adapted to PAH pollutants (Cébron et al. 2009; Bourceret et al. 2016), the bacterial

430 community was rapidly activated, and the spiked PAH fraction was in turn rapidly  
431 biodegraded. The main parameter that limited the biodegradation of aged PAHs in this soil  
432 was their low bioavailability (Cébron et al. 2013) and spiking transiently led to more easily  
433 biodegradable PAHs. In our experiment, before being irreversibly adsorbed to soil mineral or  
434 organic materials, such as clay, black carbon or coal-tar substances (Ghosal et al. 2016),  
435 PAHs were rapidly degraded, proving that their availability is crucial for biodegradation.  
436 Besides PAH degradation, oxygenated polar compounds (pPAC) were produced during the  
437 first week of the experiment. This kind of bioavailable compounds, previously described in  
438 water from industrial wasteland (Rollin et al. 2005; Lundstedt et al. 2006) and in coal tar  
439 (Northcott and Jones 2001; Benhabib et al. 2010), could result from biotic PAH degradation.  
440 Lundstedt et al. (2007) indeed described the production of oxygenated compounds during  
441 microbial biodegradation, with apparent concentrations resulting from the balance between  
442 pPAC degradation and pPAC production during PAH biodegradation (Biache et al. 2017).  
443 The production of oxygenated pPAC possibly more available and more toxic than parent  
444 molecules could contribute to shape the bacterial community structure.

445 Together with PAH biodegradation, we observed that bacterial community abundance and  
446 activity rapidly increased during the first 6 days of incubation. Moreover the community  
447 composition was drastically modified in both planted and unplanted conditions. Most of  
448 bacterial OTUs with increasing activity were affiliated to *Beta-* and *Gamma-Proteobacteria*,  
449 at the expense of *Actinobacteria* whose proportions fell sharply. PAH amendment may have  
450 caused a shift of bacterial communities from oligotroph, K-strategist, Gram-positive bacteria  
451 (such as *Actinobacteria*) to copiotroph, r-strategist, Gram-negative bacteria (such as  
452 *Proteobacteria*) (Bevivino and Dalmastri 2017). These *Proteobacteria* are potentially Gram-  
453 negative PAH-degraders. We did find a predominance of bacteria known to be involved in  
454 PAH degradation, such as OTUs affiliated to the *Achromobacter*, *Janthinobacterium*,  
455 *Pyr10d3*, *Sphingobium*, and *Thiobacillus* genera and *Sphingomonadaceae*, *Micrococcaceae*,  
456 and *Comamonadaceae* families (Fernández-Luqueño et al. 2011; Kunihiro et al. 2013). The  
457 abundance and transcription level of PAH-dioxygenase genes increased significantly during  
458 the first 6 days in both conditions, confirming the higher potential of PAH bacterial  
459 degradation. Greater activity of Gram-negative than Gram-positive (*Actinobacteria*) PAH  
460 degraders was evidenced during these early days, indicating that *Proteobacteria* were most  
461 probably the main phylum involved in the biodegradation of the spiked PAHs. This finding  
462 was also supported by a higher activity of *Proteobacteria* as compared to other taxa in both  
463 planted and unplanted soils, and by a negative correlation between the activity of Gram-

464 negative PAH-degrading bacteria and PAH contents. Several studies indicated that PAH  
465 contamination induces a selection of and an enrichment in PAH degraders (Cébron et al.  
466 2008; Zhang et al. 2013; Sawulski et al. 2014), and also up-regulates the transcription of the  
467 PAH-dioxygenase gene in pure culture (Kim et al. 2006) or complex communities (Louvel et  
468 al. 2011). A negative correlation between the transcription of PAH-dioxygenase genes of  
469 Gram-negative bacteria and PAH contents would confirm the major involvement of this  
470 population in early pollutant degradation, although we cannot rule out the involvement of  
471 Gram-positive *Actinobacteria* that could be the main active PAH-degraders after PAH spiking  
472 (de Menezes et al. 2012). Furthermore, fungi could also be involved in PAH degradation  
473 (Cerniglia and Sutherland 2010), but we did not detect any increase of fungal 18S rRNA gene  
474 or transcript copies during incubation in either condition, suggesting a fairly limited  
475 involvement of these microorganisms in our soil.

476

#### 477 **Plant effect**

478 The presence of plants induced a shift in the active bacterial community structure and the  
479 PAH-degrading population. The timescale of the experiment (22 days) allowed us to monitor  
480 the root development of alfalfa, and compare processes between planted and unplanted soils.  
481 In addition to increasing the PAH biodegradation capacity, alfalfa plants globally increased  
482 the abundance and activity of bacteria, the abundance of Gram-negative PAH-degrading  
483 bacteria and the expression of both Gram-negative and Gram-positive PAH-dioxygenase  
484 genes. However, the presence of plants led to a decrease of the bacterial richness and diversity  
485 indexes. This finding contrasts with previous observation from the same soil (NM) spiked  
486 with phenanthrene and planted with ray-grass. The authors found that plants indeed induced  
487 an increase of bacterial species richness and slightly inhibited phenanthrene degradation, even  
488 if the abundance of PAH-dioxygenase genes increased, yet without modification of the  
489 transcription level as compared to unplanted soil (Thomas and Cébron 2016). These  
490 contrasting results can be explained by two different types of contamination, i.e. a PAH  
491 mixture *vs.* phenanthrene, and two different plant species with contrasted root systems and  
492 root exudation (Bourceret et al. 2015). Root exudates probably partly inhibit the degradation  
493 of one sole PAH compound, while they might help in co-metabolism processes when PAH  
494 contamination is complex and contains high-molecular-weight compounds (Rentz et al. 2005)  
495 as shown for PCB degradation (Vrchotová et al. 2013).

496 After 6 days, even if root biomass and length were low, the presence of alfalfa had already  
497 modified a few parameters. The pH, the abundance and proportion of active Gram-negative

498 PAH-degrading bacteria were significantly higher in the planted rhizotron than in the  
499 unplanted one. Similar findings about PAH-degrading bacterial activity were reported in  
500 batch experiments with sand spiked with phenanthrene (Louvel et al. 2011). After 6 days,  
501 plants had already shaped the bacterial community structure: several OTUs belonging to *Beta-*  
502 *and Gamma-Proteobacteria* were significantly more active in the planted rhizotron, while  
503 other OTUs belonging to *Alpha-, Beta-Proteobacteria* and *Actinobacteria* were favoured in  
504 the unplanted rhizotron. In our experiment, after 12 days, root length and sugar content were  
505 significantly higher than after 6 days, and root biomass and DOC tended to increase,  
506 suggesting a stronger rhizosphere effect. At this sampling time, significantly higher PAH-  
507 degradation was observed in the planted rhizotron than in the unplanted one. This could be  
508 linked to an increasing level of PAH-dioxygenase transcription in both Gram-negative and  
509 Gram-positive PAH-degrading bacterial populations that was significantly higher in the  
510 planted soil than in the unplanted one. Our results clearly show a positive and rapid impact of  
511 the rhizosphere on the PAH degradation potential *via* the increased activity of a wider  
512 diversity (both GN and GP) of PAH-degrading bacteria. Even if *Actinobacteria* were enriched  
513 in the unplanted soil, the activity of the PAH degraders belonging to this phylum was favored  
514 in the rhizosphere. The higher activity of Gram-positive PAH degraders could help in the  
515 degradation of more recalcitrant PAH compounds (Kanaly and Harayama 2000). We  
516 highlighted a time-dependent effect in the course of the process dynamics, which could  
517 explain why some authors did not observe an increase in the activity of PAH-degrading  
518 populations in certain rhizospheres (Kawasaki et al. 2015). The addition of artificial root  
519 exudates can increase the density of soil PAH degraders, with an increase (Joner et al. 2002)  
520 or no difference (Cébron et al. 2011) in the degradation of spiked PAHs. We showed the  
521 activity of growing roots in a more natural rhizosphere system. A rhizotron device is probably  
522 the most adapted laboratory system to study the dynamics of rhizosphere processes because it  
523 makes it possible to perform analyses on precisely located samples with specific  
524 characteristics (depth, root density, roots age...) instead of mean rhizosphere samples usually  
525 obtained from pot experiments. Rhizotrons can allow studying the root turnover (Leigh et al.  
526 2002), the isothiocyanate degradation rate, microbial communities (Rumberger and  
527 Marschner 2003) or spatial heterogeneity of PAH degradation and microbial communities  
528 (Bourceret et al. 2015).

529 In our study, the carbon released by actively growing roots and also leached from the top part  
530 of the device presenting a higher root density may have activated PAH degradation through  
531 co-metabolism (Ambrosoli et al. 2005). This phenomenon could also be attributed to a

532 priming effect, whereby the release of labile carbon stimulates microbial density and activity  
533 (Haichar et al. 2014), promoting PAH degradation or biotransformation (Rentz et al. 2005).  
534 As shown by the canonical analysis the higher differentiation between the planted and  
535 unplanted conditions was observed after 6 and 12 days of incubation. Root growth seemed to  
536 activate bacterial population mostly belonging to *Proteobacteria*, such as OTUs affiliated to  
537 the *Gamma-* (*Pseudomonadaceae*, *Stenotrophomonas* and *Acinetobacter*), *Alpha-*  
538 (*Sphingobium*, *Devosia*), and *Beta-*(*Methylophilaceae*, *Janthinobacterium* and  
539 *Achromobacter*) subdivisions. In addition to their ability to degrade PAHs (Fernández-  
540 Luqueño et al. 2011; Tauler et al. 2015; Gupta et al. 2015), OTUs affiliated to *Proteobacteria*  
541 can be favoured in the presence of plants as compared to unplanted soil (Thomas and Cébron  
542 2016; Thomas et al. 2016). On the contrary, in unplanted soil other members of the *Alpha*  
543 (*Sphingomonadaceae*, *Sphingobium*, *Sphingomonas*, *Mycoplana*, *Magnetospirillum*) and *Beta*  
544 (*Ralstonia*, *Oxalobacteraceae*, *Comamonadaceae*, *Thiobacillus*) subdivisions were favoured,  
545 as well as *Actinobacteria* (*Iamia*, *Geodermatophilaceae*, *Georgenia*, and *Micrococcaceae*).  
546 After 16 and 22 days of root development, when no further PAH dissipation was observed,  
547 the transcriptional level of the PAH-dioxygenase gene from Gram-negative and Gram-  
548 positive bacteria decreased or remained constant over time in both planted and unplanted  
549 soils. At these time points, root development seemed to mainly influence the structure of the  
550 bacterial community. The proportion of many OTUs indeed differed between the planted and  
551 unplanted soils, but organic pollutant degradation no longer did.

552

## 553 **Conclusion**

554 Our results suggest that the dissipation of spiked PAHs is very fast in the soil when these  
555 pollutants represent the main available carbon sources for microbial communities and PAH-  
556 degrading bacteria are present. The presence of plants, which release root exudates, could  
557 favour microbial degradation by co-metabolism and increase the PAH degradation yield. The  
558 transcription of the PAH-dioxygenase gene was higher in the presence of plants than in  
559 unplanted soil, and transcription from Gram-negative bacteria (*Proteobacteria*) was high  
560 during the first 12 days when most of the PAHs were degraded. In the planted condition,  
561 Gram-positive PAH-degrading bacteria (*Actinobacteria*) were also highly active, and  
562 probably contributed to the higher level of PAH degradation in the rhizosphere. The  
563 characterization of the bacterial community structure and activity over time indicated different  
564 dynamics between the two conditions, with *Beta-* and *Gamma-Proteobacteria* mostly active

565 in planted soil while *Alpha-Proteobacteria* and *Actinobacteria* were more active in unplanted  
566 soil.

567

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575

576

### 577 **Figure captions**

578 **Table 1.** Nitrogenous and oxygenated pPAC concentration ( $\text{mg.kg}^{-1}$ ) over time (at T0 and 6  
579 days) in planted (Alf) and unplanted (BS) soils (Means  $\pm$  SD, n=6). Letters indicate  
580 significant differences in pPAC concentrations between T0 and 6 days, and depending on  
581 presence of plant (one-way ANOVA).

582 **Table 2.** Bacterial richness (Chao1) and diversity estimators (Inverse of Simpson's index and  
583 Evenness) in unplanted soil (BS) and soil planted with alfalfa (Alf) at the beginning (T0) and  
584 after 6 , 12 , 16 and 22 days of incubation from cDNA data (Means  $\pm$  SD, n=4). The plant  
585 effect was tested (from 6 to 22 days) using a two-way analysis of variance (ANOVA)  
586 followed by Newman-Keuls multiple comparison test (letters represents groups with  
587 significant different values ( $p<0.05$ )). One-way ANOVA was used to test time effect (from  
588 T0 to 22 days), separately for each condition.

589 **Figure 1.** Experimental devices and sampling points. (A) The device was composed of a  
590 mini-rhizotron (in orange) fitting in a corresponding rhizotron (in black). According to the  
591 sampling map (in white) at the depth 3 cm, six samples (in green) were collected, 3 samples  
592 (in gray) were used for soil humidity measurements, and 4 supplemental samples collected  
593 between green locations allowed quantification of dissolved organic carbon (DOC). (B)  
594 Pictures of alfalfa rhizotron devices after 6 , 12 , 16 and 22 days of plant growth.

595

596 **Figure 2.** Soil and root colonization parameters over time. (A) Dry root biomass and length,  
597 (B) sugar content, (C) dissolved organic carbon (DOC) and (D) pH after 6, 12, 16 and 22  
598 days in alfalfa planted (green) and unplanted (brown) soils. Means (n=6 for root biomass and  
599 length, and n=4 for pH and DOC) and standard errors. Letters indicate significant (p<0.05)  
600 combined effect of time and presence of plant evaluated by using two-way ANOVA followed  
601 by Neuman-Keuls multiple comparison test.

602

603 **Figure 3.** PAH concentration (sum of 13 PAH, i.e. the 16 US-EPA PAHs, except  
604 naphthalene, acenaphthylene and acenaphthene) after 6, 12, 16 and 22 days in planted (green)  
605 and unplanted (brown) soils. Box plots show data variability and means (black cross), n=24  
606 (for T0 samples) and n=6 (from 6 to 22 day samples). Dashed line indicate mean of PAH  
607 concentration in the NM soil before spiking. Black asterisks indicate significant differences  
608 between planted and unplanted soil (Student t test, p<0.05) and letters indicate significant  
609 differences depending on time (one-way ANOVA).

610

611 **Figure 4.** Abundance values of genes (A) and transcripts (B) and ratio of transcripts relative  
612 to their corresponding genes (C) for fungal 18S rRNA, bacterial 16S rRNA and PAH-  
613 degrading bacterial communities after 6, 12, 16 and 22 days in alfalfa planted (green) and  
614 unplanted (brown) soils. Values are means and standard deviations of 3 replicates (T0) and 6  
615 replicates for 6 to 22 days. The effect of time was evaluated for each condition (planted,  
616 unplanted) by using one-way ANOVA (p<0.05) and presence of plant was evaluated between  
617 6 and 22 days by using two-way ANOVA (p<0.05) followed by Neuman Keuls multiple  
618 comparison test. In A and B, different letters indicate significant changes in microbial  
619 community abundance depending on time for each condition, black asterisks indicate plant  
620 effect. In C, letters indicate cross effect of plant and time on the ratio of transcript relative to  
621 gene copies.

622

623 **Figure 5.** Relative proportion (mean, n=4) of dominant active bacterial phyla ( $\geq 1\%$  in at least  
624 one sample) in alfalfa planted (Alf) and unplanted soil (BS) at 0, 6, 12, 16 and 22 days of  
625 incubation. The twelve significant phyla were identified by a Tukey Kramer ANOVA with a  
626 Benjamini Hochberg correction of p-value (q<0.05, and effect size >0.26).

627 **Figure 6.** Ratio of the 16S rRNA relative to 16S rDNA for OTU ( $\geq 10$  sequences in one  
628 sample) significantly discriminating plant effect, after 6 and 12 days of incubation. Both



629 conditions were compared using Student t-tests with Benjamini-Hochberg correction of p-  
630 value. Asterisks denote degree of significance (green and brown for higher ratio with alfalfa  
631 and unplanted soils, respectively; \*  $q < 0.05$ ; \*\*  $q < 0.01$ ; \*\*\*  $q < 0.001$ ). OTUs were affiliated at  
632 97% of similarities to the deeper taxonomic level, leading to affiliations at different levels.

633 **Figure 7.** Canonical correspondence analysis (CCA) ordination including abiotic (A) and  
634 (B) biotic variables. (A) environmental variables (in red) (DOC, sugar and organic acid  
635 contents, pH, percentage of 18S rRNA, PAH-RHD<sub>α</sub> GN and GP genes and transcripts relative  
636 to 16S rRNA), root biomass (RootB) and length (RootL), sum of 13 PAH concentrations, 2-3-  
637 ring PAHs (fluorene, phenanthrene, anthracene), 4-ring PAHs (fluoranthene, pyrene,  
638 benzo(a)anthracene, chryzene), 5-6-ring PAHs (benzo(b)fluoranthene, benzo(k)fluoranthene,  
639 benzo(a)pyrene, dibenzo(a,h)anthracene, benzo(g,h,i)perylene, indeno(1,2,3-cd)pyrene), and  
640 (B) the relative abundance of dominant active bacterial OTUs ( $\geq 1\%$  in at least one sample,  
641 taxonomic affiliation at 97% of similarities to the deeper taxonomic level, leading to  
642 affiliations at different levels) (in black) were used to discriminate the 24 samples (4  
643 replicates for each sampling time corresponding to 0, 6, 12, 16 and 22 days) collected for  
644 alfalfa planted (green) and unplanted (brown) rhizotrons. A Monte Carlo permutation test  
645 (1000 permutations) was performed and showed a significant ( $p < 0.0001$ ) effect of  
646 environmental variables on the relative abundance of dominant OTUs. Un. Coma.: Un.  
647 Comamonadaceae; Un. Pseudo.: Un. Pseudomonadaceae; Un. Sphingo.: Un.  
648 Sphingomonadaceae; Un. Geo.: Un. Geodermatophilaceae; Un. Meth.: Un.  
649 Methylobacteriaceae; Phen.: Phenylobacterium; Phaeos. Fulvum: PhaeospirillumFulvum;  
650 Un. Xantho.: Un. Xanthomonadaceae; Un. Oxalo.: Un. Oxalobacteraceae.

651

## 652 Supplemental data

653 **Table S1.** PAH concentration ( $\text{mg.L}^{-1}$ ) in the organic extract used to spike NM soil. Values  
654 are means ( $n=3$ ) and standard errors.

655 **Table S2.** Pearson's correlation matrix between soil properties and the percentage of PAH-  
656 dioxygenase transcripts and genes. Values in bold correspond to significant correlation  
657 between variables ( $p < 0.05$ ).

658

659 **Figure S1.** Bioavailable (A) and total (B) PAH concentration in NM soil before (grey) and  
660 after (blue) spiking. Black asterisks indicate significant differences between both conditions

661 (Student t test,  $p < 0,05$ ). Values are means and standard deviation of 12 and 48 samples (total  
662 fraction) and 8 and 6 samples (bioavailable fraction), before and after spiking respectively.

663 **Figure S2.** Total PAH concentration of 13 individual compounds (the 16 US-EPA PAHs  
664 except naphthalene, acenaphthylene and acenaphthene) after 6, 12, 16 and 22 days in alfalfa  
665 planted (green) and unplanted (brown) soils. Values are means and standard deviations of 24  
666 replicates (0 day) and 6 replicates from 6 to 22 days. The effect of time was evaluated for  
667 each condition (planted, unplanted) independently by using one-way ANOVA ( $p < 0.05$ ) and  
668 effect of presence of plant was evaluated between T1 and T4 by using two-way ANOVA  
669 ( $p < 0.05$ ) followed by Neuman Keuls multiple comparison test. Different letters indicate  
670 significant differences in PAH concentration depending on time for each condition, black  
671 asterisks indicate plant effect.

672 **Figure S3.** Evolution over time of the relative proportion of dominant OTUs ( $\geq 1\%$  in at least  
673 one sample), in alfalfa planted and unplanted soil, reported to the transcript 16S rRNA copy  
674 number based on cDNA analysis. Forty-seven significant OTUs were identified by a Tukey  
675 Kramer ANOVA with a Benjamini Hochberg correction of p-value ( $q < 0.05$ , and effect size  
676  $> 0.26$ ) and non-significant OTUs correspond to population presenting constant relative  
677 proportion over time. Asterisks denote OTUs with significant differences in relative  
678 abundance over time specifically in planted (green) or in unplanted soil (brown). OTUs were  
679 affiliated at the most precise level.

680 **Figure S4.** Ratio of the 16S rRNA relative to 16S rDNA for OTU (with at least 10 sequences  
681 in one sample) significantly discriminating plant effect, after 16 and 22 days of incubation.  
682 Both conditions were compared using Student t-tests with Benjamini-Hochberg correction of  
683 p-value. Asterisks denote degree of significance (green and brown for higher ratio with alfalfa  
684 planted and unplanted soils, respectively; \*  $q < 0.05$ ; \*\*  $q < 0.01$ ; \*\*\*  $q < 0.001$ ).

685

686

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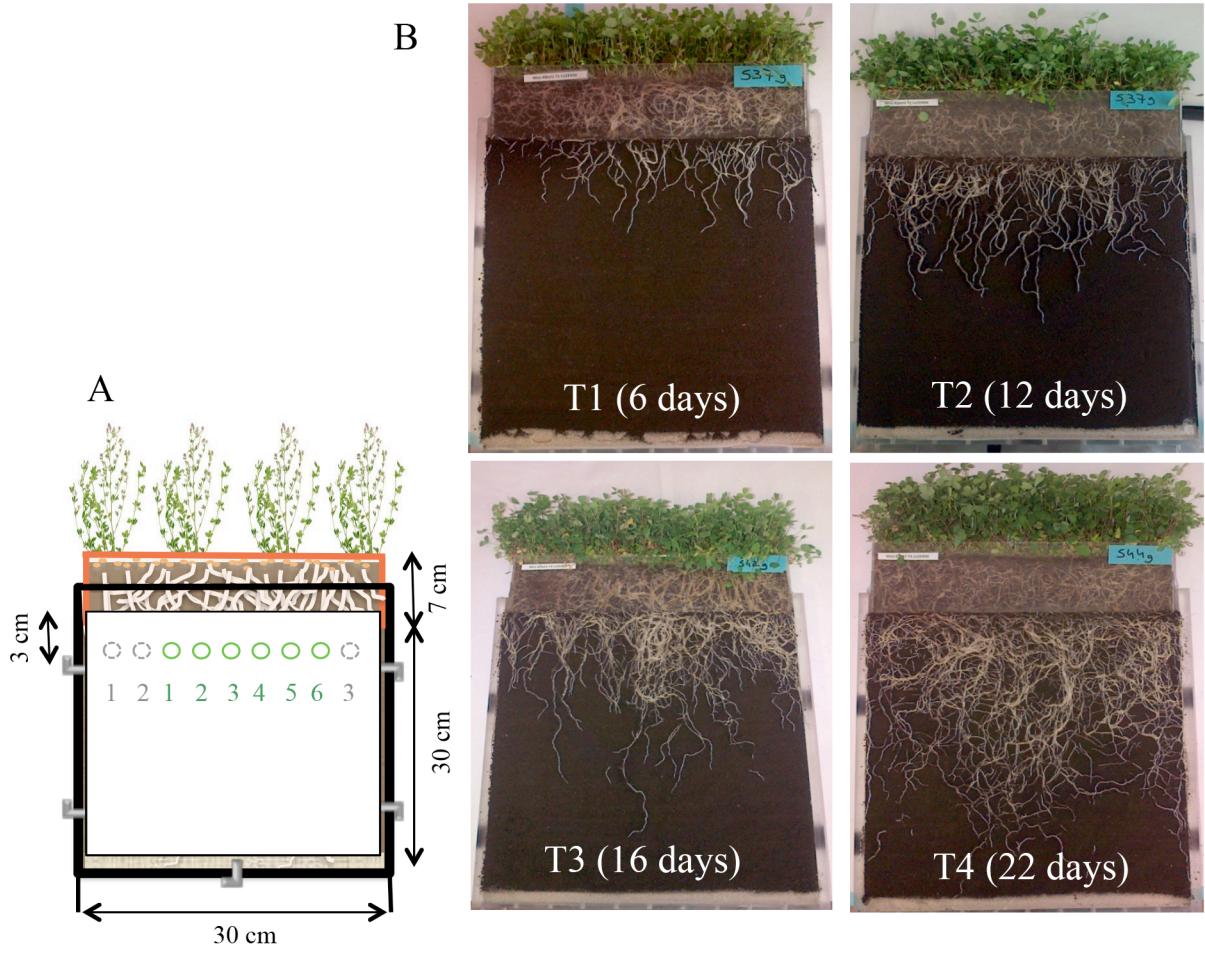
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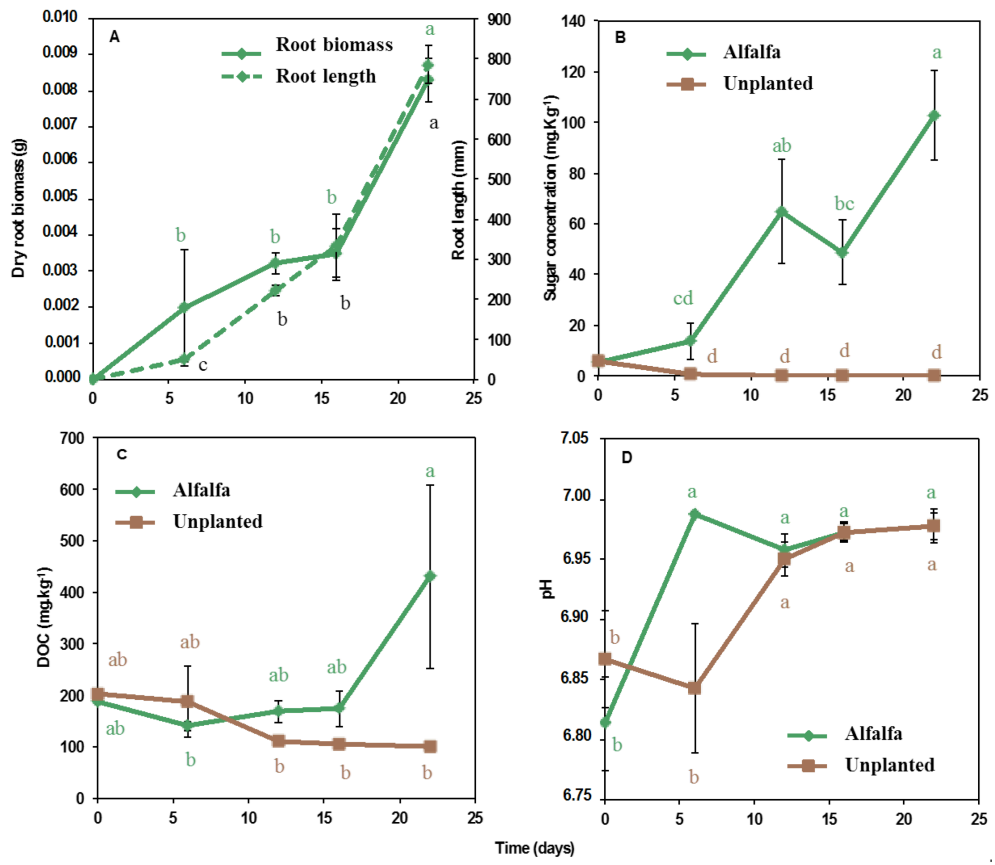
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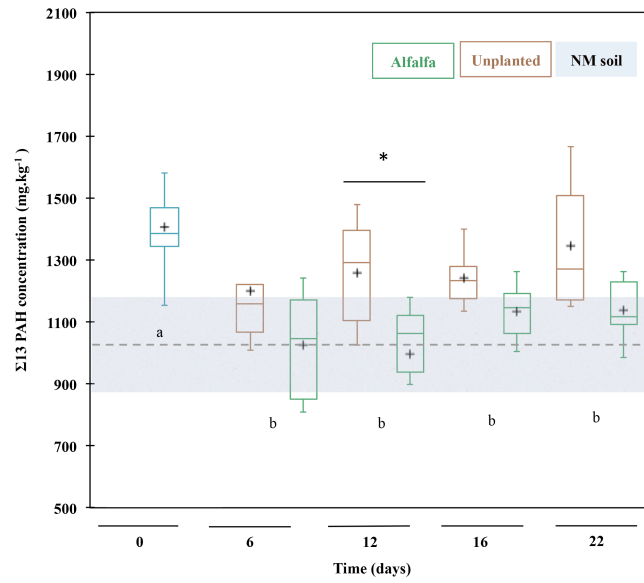
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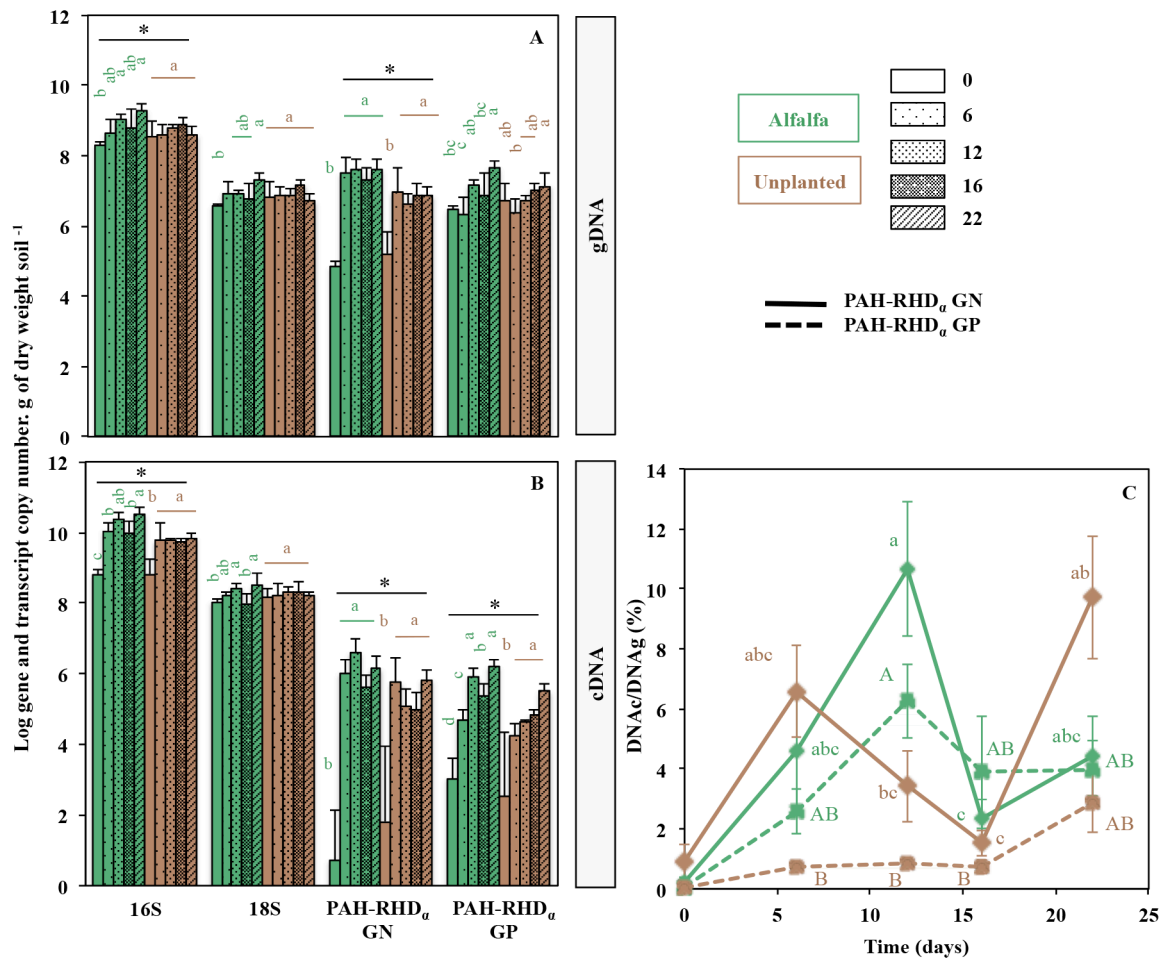
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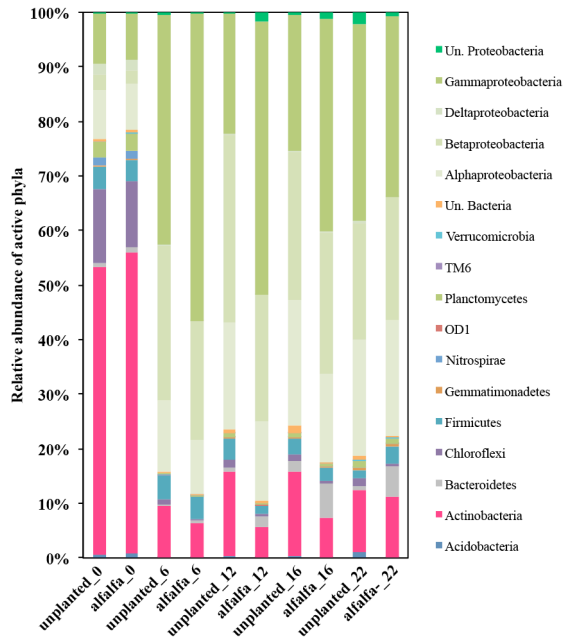
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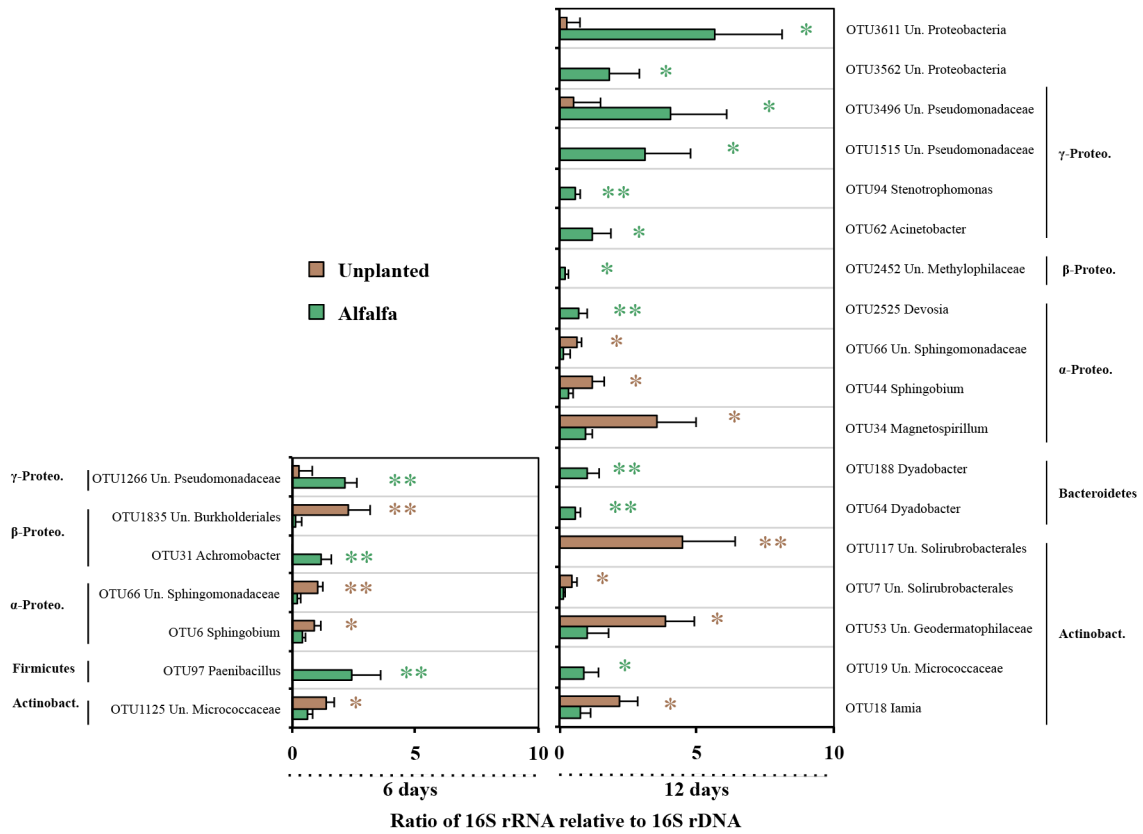
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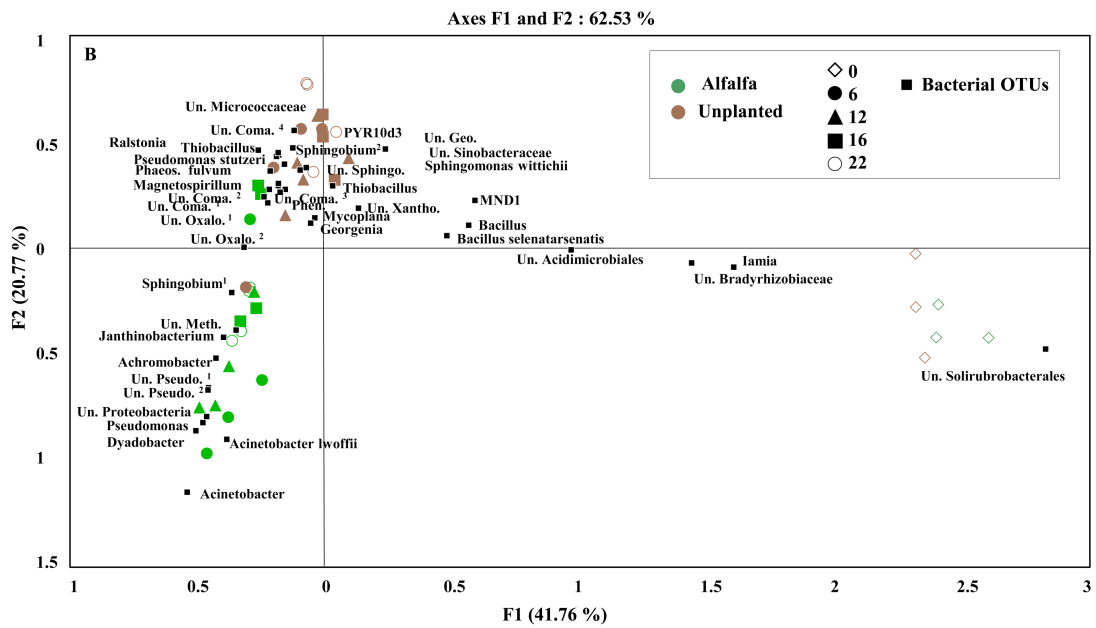
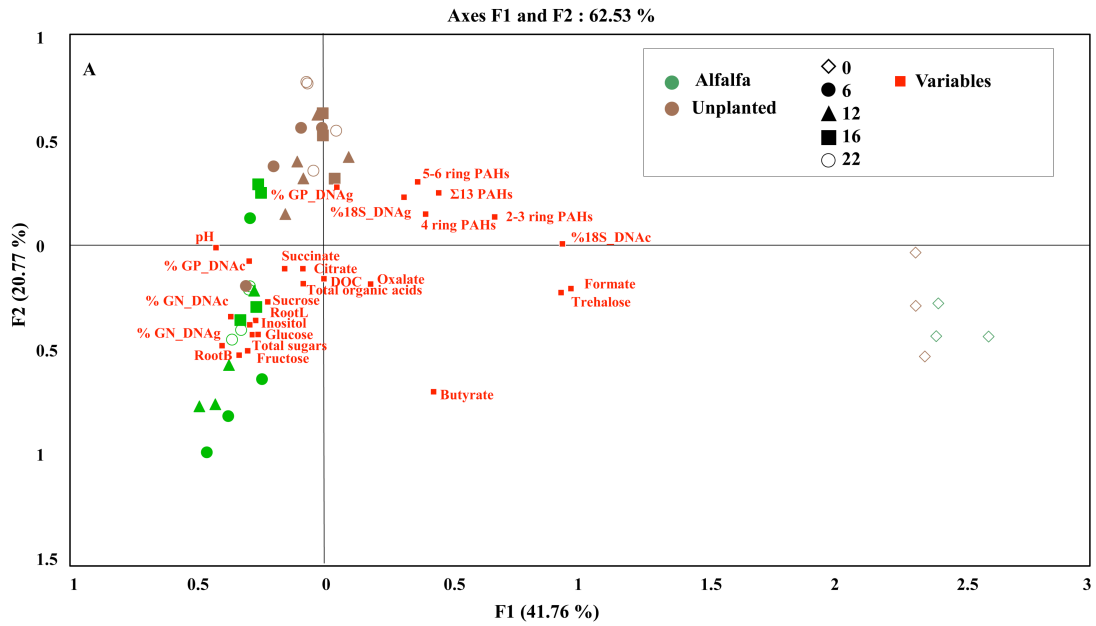


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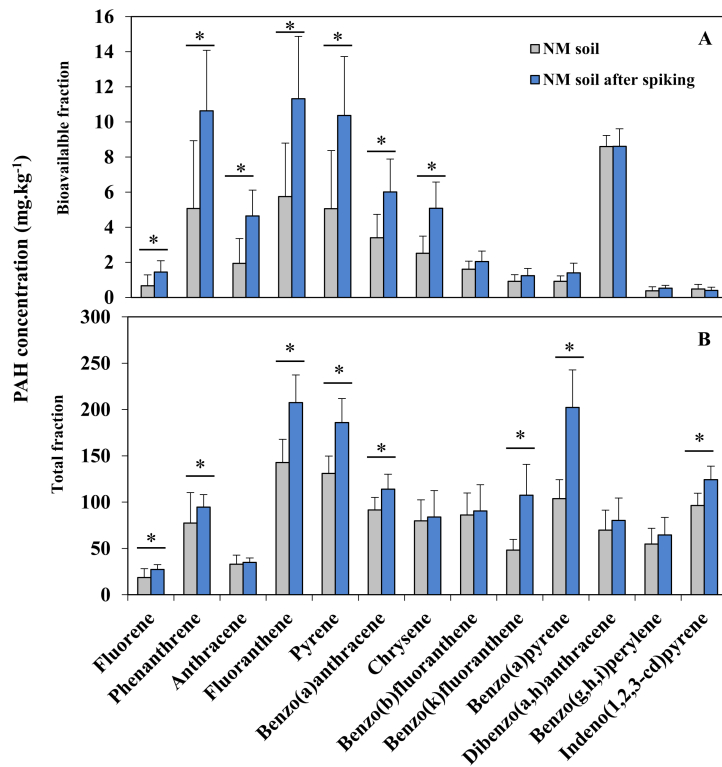
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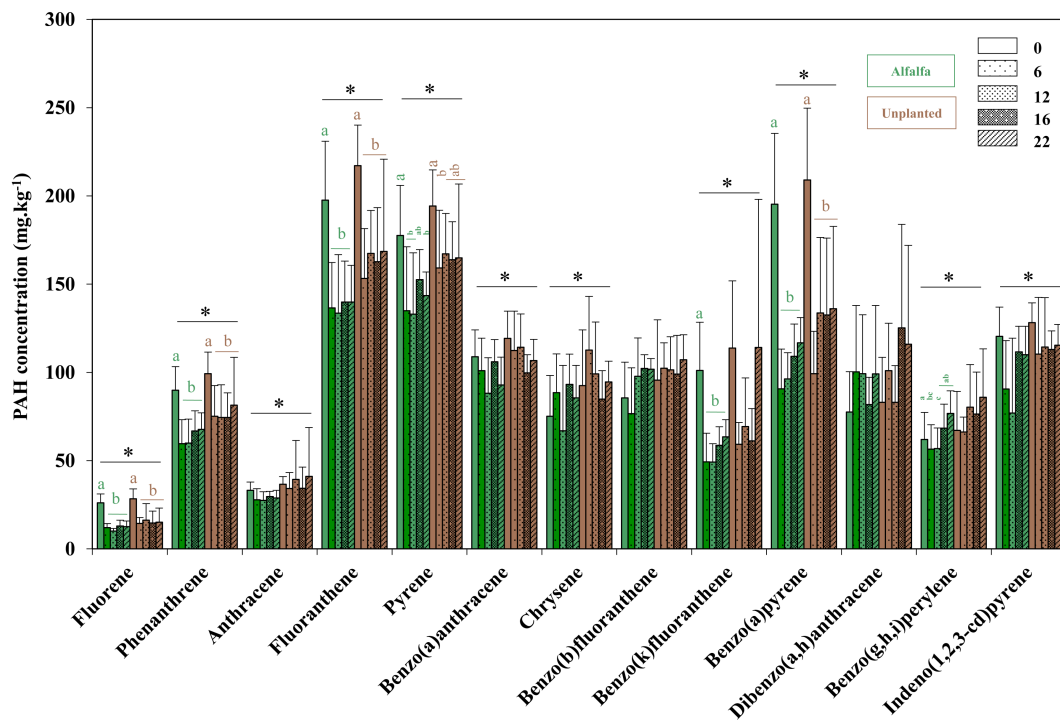


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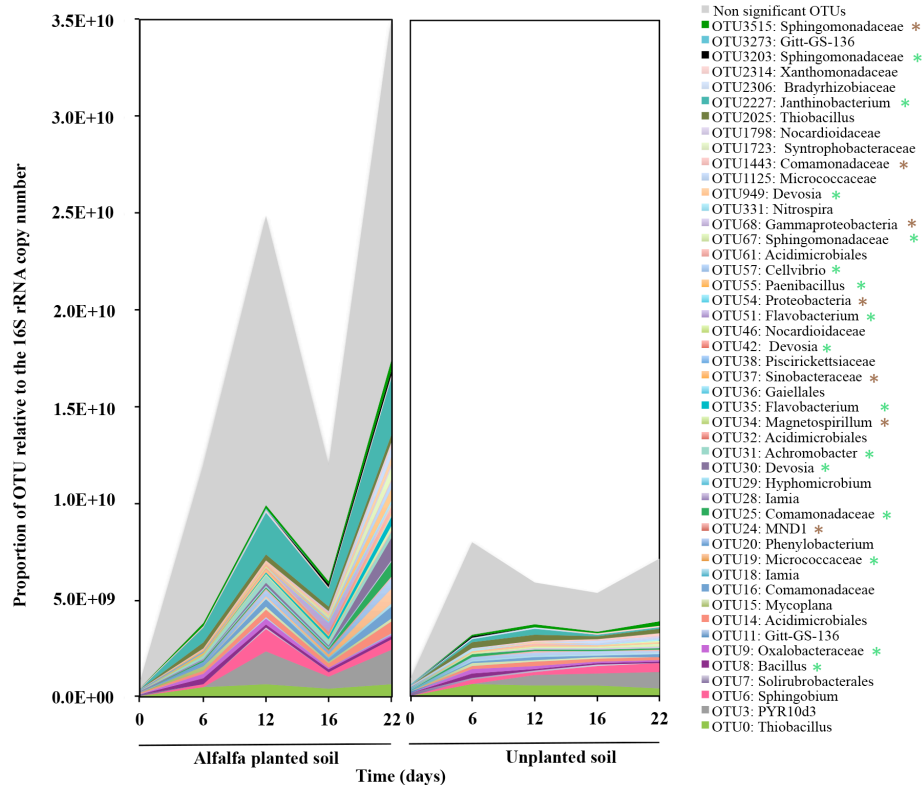


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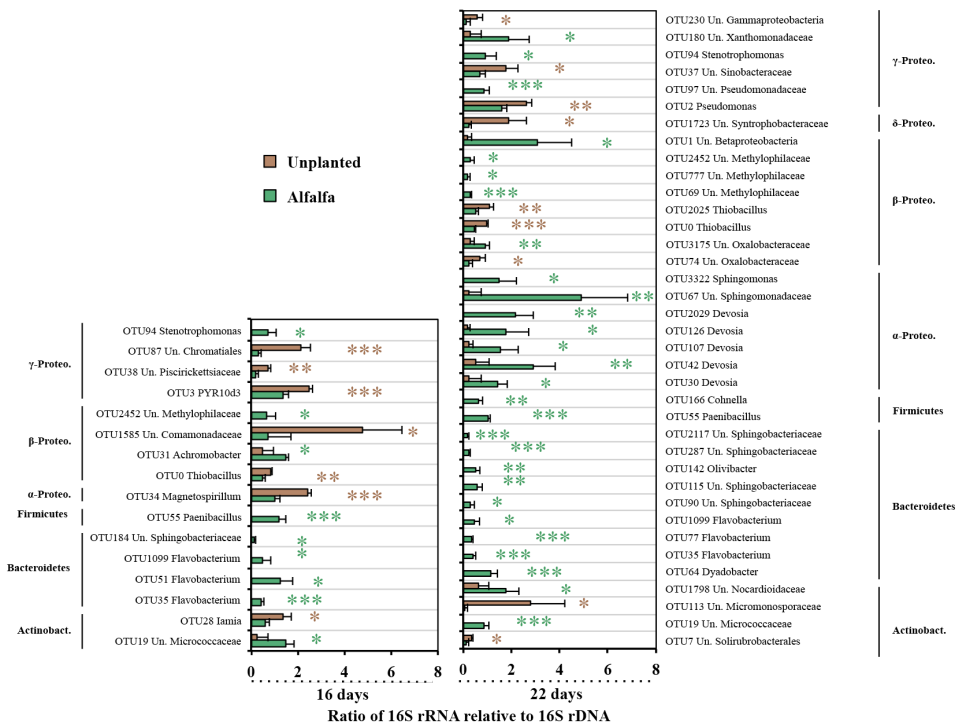


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939 **Table S2.** PAH concentration (mg.L<sup>-1</sup>) in the organic extract used to spike NM soil. Values are means (n=3) and standard errors.

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PAHs concentration (mg/L)												
Fluorene	Phenanthrene	Anthracene	Fluoranthene	Pyrene	Benzo(a) anthracene	Chrysene	Benzo(b) fluoranthene	Benzo(k) fluoranthene	Benzo(a) pyrene	Dibenzo(a,h) anthracene	Benzo(g,h,i) perylene	Indeno(1,2,3-cd) pyrene
24.8 ± 2.3	98.7 ± 4.2	35.9 ± 1.6	221.8 ± 16.7	200.9 ± 11.7	126.3 ± 12.2	101.1 ± 6.8	115.7 ± 10.8	70.7 ± 10.3	28.2 ± 7.6	70.3 ± 7.0	77.2 ± 7.8	101.7 ± 10.3

943

Variables	Time	2-3 ring PAH	4 ring PAH	5-6 ring PAH	Σ13 PAHs	pH	DOC	RootB	RootL
%18S_DNAc	<b>-0.646</b>	<b>0.686</b>	<b>0.451</b>	<b>0.361</b>	<b>0.474</b>	<b>-0.603</b>	-0.028	<b>-0.360</b>	<b>-0.338</b>
% PAH-RHDα GN_DNAc	0.106	<b>-0.434</b>	<b>-0.391</b>	-0.220	<b>-0.339</b>	0.059	-0.094	0.062	0.010
% PAH-RHDα GP_DNAc	<b>0.733</b>	-0.204	-0.253	0.137	-0.053	<b>0.409</b>	0.154	<b>0.452</b>	<b>0.538</b>
%18S_DNAg	<b>-0.502</b>	0.283	0.199	0.064	0.154	<b>-0.386</b>	-0.151	<b>-0.363</b>	<b>-0.485</b>
% PAH-RHDα GN_DNAg	-0.032	<b>-0.512</b>	<b>-0.448</b>	<b>-0.530</b>	<b>-0.547</b>	0.213	-0.029	<b>0.349</b>	0.052
% PAH-RHDα GP_DNAg	<b>0.497</b>	0.163	0.094	<b>0.388</b>	0.277	0.170	0.092	0.049	0.120

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949 Table S1. Pearson's correlation matrix between soil properties and the percentage of PAH-dioxygenase transcript and genes. Values in bold correspond to  
950 significant correlation between variables ( $p < 0.05$ ).

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