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

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17 Abstract

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

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

36

37 Introduction

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

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

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

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

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

This study aimed to evaluate 1) the dynamics of PAH dissipation in alfalfa rhizosphere as compared to unplanted soil, and 2) the variability of microbial community activity and PAHdegrading bacterial abundance and activity over three weeks of plant growth. We developed a specific 2-compartment rhizotron device to rapidly establish an active root system and put it in contact with PAH spiked soil so as to avoid the time gap between root growth from seeds germination and the degradation of bioavailable pollutants. Rhizotrons were filled with an aged PAH-contaminated soil, initially spiked with a complex mixture of PAHs (i.e. a soil organic extract). Rhizotrons were sampled once a week to monitor concentrations of PAHs
and potential metabolites (polar polycyclic aromatic compounds, pPAC). Dissolved organic
carbon (DOC), pH, and humidity were measured to characterize the rhizosphere and soil
conditions. Working on extracted DNA and RNA fractions, we also assessed total bacterial
and fungal abundance, PAH-degrading bacterial abundance and transcriptional activity levels.
Finally, we assessed active bacterial community structures by pyrosequencing of 16S rRNA
transcripts.

104 Materials and Methods

105 **Experimental device**

We used an aged PAH contaminated soil, from a former coking plant (Neuves-Maisons (NM), 106 Lorraine, France) to perform rhizotron experiments. Soil characteristics and spiking procedure 107 were described previously by Bourceret et al. (2015). Briefly, the NM soil (1033 \pm 148 mg 108 per kg of dw soil of Σ 13 PAHs (fluorene, phenanthrene, anthracene, fluoranthene, pyrene, 109 benzo(a)anthracene, chrysene, benzo(b)fluoranthene, benzo(k)fluoranthene, benzo(a)pyrene, 110 dibenzo(a,h)anthracene, benzo(g,h,i)perylene, indeno(1,2,3-cd)pyrene) was air-dried, sieved 111 112 at 2 mm and spiked with a complex organic pollutant extract (Table S1), rich in PAHs and obtained from the same soil using chloroform extraction (Cébron et al. 2013). After solvent 113 evaporation, the spiked soil was mixed with NM soil not exposed to chloroform, in a one-114 tenth ratio. Experimental device was divided in two parts (Fig. 1): the top part (mini-115 rhizotron) was a poly-methyl methacrylate box (7 cm high x 29.8 cm long x 2.3 cm wide) 116 without bottom, that fits into the second part: a rhizotron (30 cm high x 30 cm long x 2.5 cm 117 wide) with a removable front face allowing spatially localized depth sampling as described by 118 Bourceret et al. (2015). First, 8 mini-rhizotrons were filled with NM soil, closed at the bottom 119 by bolting cloth (5 µm mesh), and adjusted to 80% of the soil water holding capacity (WHC) 120 (corresponding to 203 ml deionized water per kg of dw soil). Alfalfa (Medicago sativa var. 121 Europe) was planted on 4 of these devices by seeding 2.4 g of seeds (c.a. 26 seeds per cm²) 122 and the other 4 devices were left unplanted. After germination, the 8 devices were placed in a 123 plant growth chamber under controlled conditions (22°C/18°C day/night, 80% relative 124 humidity, c.a. 250 μ mol photons m⁻² s⁻¹, 16 hours photoperiod) and were maintained at 80% 125 of the WHC by weighing the devices every two days. The previous growth of plant in NM 126 soil allowed to avoid the delay between time needed for root development and the PAH 127 degradation, that was already shown to be fast in spiked soil with a previous study (Bourceret 128 et al. 2015). After 15 days, when roots reached the bottom of the mini-rhizotron devices, eight 129

rhizotrons were filled with spiked NM soil (1418 \pm 193 mg of Σ 13 PAHs per kg of dw soil), 130 bolting cloths of the mini-rhizotrons were removed and devices were fitted in their 131 corresponding rhizotron to allow root penetration and growth in spiked soil. This time 132 corresponded to T0. Initial spiked NM soil was sampled: six aliquots from each of the eight 133 rhizotrons corresponding to 48 T0 samples were stored at -80°C for further analyses. Then the 134 4 planted and 4 unplanted devices were incubated in plant growth chamber in conditions 135 described above. After 6 (T1), 12 (T2), 16 (T3) and 22 (T4) days of plant growth, two 136 137 rhizotrons (one planted and one unplanted) were harvested and opened to allow core sampling using a stainless steel cylinder equipped with a PTFE piston (1.6 cm diameter, 2.5 cm long, 138 thus enabling to collect soil cores of around 5 cm³). At 3 cm from the interface of the two 139 compartments, 6 samples with 3 cm between the core centers were collected per device (Fig. 140 1). Soil samples were prepared as previously described by Bourceret et al. (2015). Briefly, 141 after 3 rinsing by sterile water, roots were spread on a black paper and photographed to 142 estimate the size of each root fragment using ImageJ software (http://rsbweb.nih.gov/ij/). 143 144 Roots were then air-dried at 60°C and weighed to quantify dry root biomass. Rhizosphere soil, collected from root rinsing water by centrifugation at 4400 rpm for 10 minutes, and bulk 145 soil were weighed to calculate the ratio between the two fractions and were mixed together to 146 obtain a unique soil sample stored at -80°C before analyses: soil characteristics (pH, DOC, 147 organic acids, and sugar concentrations), PAH and pPAC concentrations, and DNA/RNA 148 extractions. Soil humidity was measured by collecting 3 other samples on the side of the 149 rhizotrons and by weighing the soil before and after drying at 60°C for 48 hours. 150

151 Soil characteristics

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

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

161 An aqueous solution of hydroxypropyl-β-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

soil samples before spiking, 6 T0 samples, and 48 rhizotron samples (6 samples from both 163 planted and bare rhizotrons at each sampling time) in Oak Ridge Centrifuge tubes (FEP, 164 Nalgene, USA) (Reid et al. 2000). After stirring (100 rpm) for 16 hours at 24 °C and 2 165 centrifugation steps (10000 rpm, 20 min), dichloromethane was added (24 ml) to the aqueous 166 supernatant (c.a. 30 ml) to extract available PAHs. An Accelerated Solvent Extractor 167 (ASE350 Dionex ®) was used to extract total PAHs and pPACs with dichloromethane 168 (130°C, 100 bars) from 0.25 g of soil (stored at -80°C) from 12 NM soil samples before 169 spiking, 48 T0 samples, and 48 rhizotron samples previously lyophilized and crushed (<500 170 µm). Dichloromethane extracts were split in two fractions to quantify PAHs using HPLC/UV-171 Fluo (High Pressure Liquid Chromatography) and pPACs using GC-MS (gas chromatography 172 173 coupled to mass spectrometry). Firstly, the two dichloromethane extracts containing available and total PAHs were evaporated under nitrogen flow and diluted in 5 ml and 20 ml of 174 175 acetonitrile, respectively. HPLC (Dionex Ultimate 3000) system, with a 100-mm long and 4.6-mm internal diameter separation column (SupelcoAscendis Express C10), 2.6-mm 176 granulometry allowed quantifying PAHs from 10 µl of extract. From total and available 177 extracts, 13 PAHs (16 US-EPA PAHs excluding the three more volatile molecules: 178 naphtalene, acenaphtylene and acenaphtene) were quantified by using a UV (254 nm) and a 179 fluorescence detector, respectively. Secondly, from dichloromethane extracts pPACs were 180 quantified on a GC-6890 (Agilent Technologies) equipped with a DB5- MS column (60 \times 181 0.25 mm id \times 0.25µm film thickness) coupled to a MS-5973 Inert (Agilent Technologies) 182 operating SIM mode (Single Ion Monitoring). The oven temperature program was as follows: 183 2 minutes at 70 °C, then from 70 °C to 130 °C at 15 °C min⁻¹, then from 130 °C to 315 °C at 4 184 °C min⁻¹ and then a 25 min hold at 315 °C. The carrier gas was helium at 1.4 ml min⁻¹ 185 constant flow. Fifteen pPACs including 11 oxygenated pPACs (O-PACs) and 4 nitrogenated 186 pPACs (heterocyclic PACs containing nitrogen N-PACs) were quantified by an internal 187 calibration using deuterated internal standards (the list of the 15 quantified polar PAC is given 188 in Hanser et al. (2015). During a sequence, calibration controls were injected every 12 189 190 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 μ l of DES (Dnase-free water) and quantified at ¹⁹⁵ 480 nm (Xenius, SAFAS) by using Quant-iTPicoGreenasDNA Assay kit (Invitrogen), and

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

209 Real-time PCR quantification

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

225 Pyrosequencing and sequence analysis

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

IDentifier, Roche). PCR reactions were achieved as described in Bourceret et al. (2016). 229 Beckman Coulter Genomics (Danvers, MA, USA) sequenced libraries using a 454/Roche GS-230 FLX Titanium system. Data were deposited in the NCBI Sequence Read Archive (SRA) 231 under the BioProject ID: PRJNA324193. Denoised 16S rDNA sequenced were analysed in 232 QIIME 1.8.0 pipeline (Caporaso et al. 2010) as previously described by Bourceret et al. 233 (2017). Briefly, after filtering and reverse complement non-sense sequences, data from gDNA 234 and cDNA were gathered and clustered in Operational Taxonomic Units (OTUs) at 97% of 235 similarity and their representative sequences were affiliated at different taxonomic levels 236 (from phyla to genus) using RDP classifier (Wang et al. 2007) and the Greengenes 13 5 237 database (McDonald et al. 2012). Then subsampling was performed to the lowest number of 238 239 sequences counted in one of the samples (4,739 sequences per sample) and alpha-diversity indexes (richness and diversity estimates) were calculated using QIIME 1.8.0 pipeline. 240

241 Statistical analyses

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

262 **Results**

263 Rhizosphere parameters

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

280 PAHs and pPACs contents

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

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

311

312 Microbial communities

313 Abundance of active and total microbial communities

Bacterial abundance was higher than fungal, and bacteria were also more active over time in 314 both planted and unplanted soil (mean value of 7.84 x $10^8 \pm 6.64$ x 10^8 16S rRNA gene copies 315 and $1.12 \times 10^{10} \pm 1.25 \times 10^{10}$ transcript copies g dw soil⁻¹) while fungi were 100 times less 316 represented (mean value of 9.94 x $10^6 \pm 7.04$ x 10^618 S rRNA gene copies and 2.05 x $10^8 \pm$ 317 1.48 x 10⁸ transcript copies g dw soil⁻¹) (Fig. 4A, B). Abundance of 16S rRNA genes tended 318 to increase over time in alfalfa and stayed constant in unplanted rhizotron whereas number of 319 16S rRNA transcripts increased significantly between T0 and the 6thday for both rhizotrons 320 and then stayed constant. Abundance of 16S rRNA genes and transcripts was significantly 321 322 higher in planted than unplanted rhizotron and plant effect was mainly detected at 12 (T2) and 22 days (T4). Abundance of 18S rRNA genes and transcripts tended to be constant in both 323 unplanted and planted rhizotrons and no plant effect was observed. 324

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

largely increased between T0 and 6 days in both soil and further increased in planted soil till 329 12 days, resulting in a higher content in planted than in unplanted rhizotron, significant at 330 each sampling time. The level of PAH-dioxygenase gene transcription was expressed by the 331 ratio of the number of transcripts relative to corresponding gene copies (Fig. 4C). The level of 332 PAH-dioxygenase gene transcription was significantly higher in alfalfa than in unplanted soil 333 specifically after 12 days, and then decreased (for Gram-negative bacteria) and stayed 334 335 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*

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

To explore the potential bacterial population involved into the rapid PAH degradation 345 appearing at the beginning of the incubation period, we looked for bacterial community 346 structure modification over time (Fig. 5). Among the active community, 12 phyla were 347 identified and their proportion changed over time. In both rhizotrons, Proteobacteria was the 348 more active phylum all over the incubation to the detriment of Chloroflexi, Actinobacteria 349 and Nitrospirae that represented a lower proportion of the active community along the time 350 course compared to the T0. Activity of Beta- and Gamma-Proteobacteria increased between 0 351 and 6 days and stayed constant or decreased until the end of incubation while the proportion 352 of active Alpha-subdivision members further increased. An opposite trend was observed for 353 the activity of Planctomycetes, Acidobacteria and Delta-Proteobacteria that was first 354 repressed between 0 and 12 days and then favored at the end of the incubation (Fig. 5). The 355 356 relative abundance of some active phyla evolved also differently over time according to the presence of alfalfa. In alfalfa planted rhizotron, Bacteroidetes and TM6 were more and less 357 active over time, respectively, while the activity of Unclassified Proteobacteria was 358 transiently high and then low over the 22 days. In planted soil, 3 OTUs affiliated to Beta-359 360 Proteobacteria (Thiobacillus, and Un.Oxalobacteraceae), 1 OTU affiliated to Alpha-Proteobacteria (Mycoplana) and 1 OTU affiliated to Firmicutes (Bacillus selenatarsenatis) 361 362 were significantly more abundant among the active community after 6 days of incubation than at all other times, contrary to the unplanted rhizotron where no OTU was favored after 6 days
(Fig. S3). After 12 days of incubation, three other OTUs belonging to *Alpha- (Sphingobium)*, *Beta- (Janthinobacterium)* and *Gamma-* (PYR10d3) *Proteobacteria* were favored in planted
soil. In unplanted rhizotron, 5 OTUs affiliated to *Alpha- (Mycoplana, Phenylobacterium)* and *Beta- (Thiobacillus, unclassified. Comamonadaceae) Proteobacteria* were significantly more
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 days, the activity ratio between 16S rRNA and rDNA for 3 OTUs belonging to Gamma-371 (Pseudomonadaceae), Beta-(Achromobacter) Proteobacteria and Firmicutes (Paenibacillus) 372 was significantly higher in alfalfa planted than in unplanted soil. Four other OTUs, affiliated 373 to Alpha- (Un. Sphingomonadaceae), Beta- (Un. Burkolderiales) Protebacteria and 374 Actinobacteria (Un. Micrococcaceae) were more actives in unplanted soil. After 12 days, 375 plant growth favored significantly the activity of 11 OTUs belonging to Alpha- (Devosia), 376 Beta- (Methylophilaceae), Gamma- (Pseudomonadaceae, Stenotrophomonas, Acinetobacter) 377 Proteobacteria, Bacteroidetes (Dyadobacter) and Actinobacteria (Un. Micrococcaceae). In 378 unplanted soil the activity of 6 OTUs affiliated to Alpha-Proteobacteria (Un. 379 Sphingomonadaceae, Sphingobium, Magnetospirillum) and Actinobacteria (*Un*. 380 Solirubrobacterales, Un. Geodermatophilaceae) was significantly higher than in alfalfa 381 planted soil. After 16 and 22 days of incubation, 56% and 76% of the OTUs whose activity is 382 significantly impacted by the presence of plant, were more active in alfalfa planted rhizotron 383 384 (Fig. S4).

385

386 Multivariate analysis

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

alfalfa planted rhizotron (Fig. 7B). These OTUs gathered bacteria affiliated to 397 Pseudomonadales (Acinetobacter and Pseudomonas), Sphingomonadales (Sphingobium), 398 and 399 Burkolderiales (Janthinobacterium *Achromobacter*) and *Rhizobiales* (Un. Methylobacteriaceae) orders. Unplanted soil samples were mostly discriminated according to 400 the higher PAH content (Σ 13 PAHs and 2-3, 4 and 5-6 cycles). According to the Pearson 401 correlation matrix (Table S2), pH and the root biomass and length were positively and 402 significantly correlated to the percentage of Gram-positive PAH-dioxygenase transcripts 403 404 contrary to the abundance of 18S transcripts, showing a negative correlation. Moreover concentrations of 2-3, 4- rings and Σ 13PAHs were significantly and negatively correlated to 405 the percentage of Gram-negative PAH-dioxygenase transcripts, shown as the most active 406 407 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 planted and unplanted rhizotrons, respectively. A similar rapid dissipation, of bioavailable 412 413 PAHs after a few days was previously observed in simplified sand or soil microcosms spiked with phenanthrene (Louvel et al. 2011; Cébron et al. 2011), but never observed with such a 414 complex PAH contaminant mixture. We evidenced the degradation of almost all spiked PAHs 415 416 including fluorene, phenanthrene, fluoranthene, pyrene, benzo(k)fluoranthene and benzo(a)pyrene (which have from 2 to 5 aromatic cycles) in both conditions. In the same soil 417 (NM) incubated in bioslurry, 30 to 55% of the aged PAH compounds (fluorene, phenanthrene, 418 fluoranthene, pyrene, benzo(a)anthracene and chrysene) were degraded after 1.5 months of 419 incubation (Biache et al. 2017), indicating the presence of a very effective microbial 420 community for the degradation of a wide range of PAH compounds. Degradation of PAHs by 421 microbial consortia and co-metabolism are often described as playing crucial roles the in 422 degradation of PAH mixtures in the environment (Ghosal et al. 2016) because the presence of 423 low-molecular-weight PAHs could have a synergistic effect on the degradation of more 424 recalcitrant compounds (Van Herwijnen R et al. 2003; Roy et al. 2013). During the first 6 425 426 days of the experiment, spiked PAHs were probably the main available carbon sources in both the planted and unplanted rhizotrons, as no increase in DOC or sugar content was observed in 427 428 either condition. As the microbial community of the aged-contaminated NM coking plant soil was well adapted to PAH pollutants (Cébron et al. 2009; Bourceret et al. 2016), the bacterial 429

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

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

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

476

477 Plant effect

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

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

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

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

priming effect, whereby the release of labile carbon stimulates microbial density and activity 532 (Haichar et al. 2014), promoting PAH degradation or biotransformation (Rentz et al. 2005). 533 As shown by the canonical analysis the higher differentiation between the planted and 534 unplanted conditions was observed after 6 and 12 days of incubation. Root growth seemed to 535 activate bacterial population mostly belonging to Proteobacteria, such as OTUs affiliated to 536 (Pseudomonadaceae, Stenotrophomonas and Acinetobacter), Alpha-537 the Gamma-538 (Sphingobium, Devosia), and *Beta-(Methylophilaceae,* janthinobacterium and Achromobacter) subdivisions. In addition to their ability to degrade PAHs (Fernández-539 Luqueño et al. 2011; Tauler et al. 2015; Gupta et al. 2015), OTUs affiliated to Proteobacteria 540 can be favoured in the presence of plants as compared to unplanted soil (Thomas and Cébron 541 2016; Thomas et al. 2016). On the contrary, in unplanted soil other members of the Alpha 542 (Sphingomonadaceae, Sphingobium, Sphingomonas, Mycoplana, Magnetospirillum) and Beta 543 (Ralstonia, Oxalobacteraceae, Comamonadaceae, Thiobacillus) subdivisions were favoured, 544 as well as Actinobacteria (Iamia, Geodermatophilaceae, Georgenia, and Micrococcaceae). 545 546 After 16 and 22 days of root development, when no further PAH dissipation was observed,

the transcriptional level of the PAH-dioxygenase gene from Gram-negative and Grampositive bacteria decreased or remained constant over time in both planted and unplanted soils. At these time points, root development seemed to mainly influence the structure of the bacterial community. The proportion of many OTUs indeed differed between the planted and unplanted soils, but organic pollutant degradation no longer did.

552

553 **Conclusion**

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

in planted soil while *Alpha-Proteobacteria* and *Actinobacteria* were more active in unplantedsoil.

567

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575 576

577 Figure captions

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

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

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

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

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

610

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

622

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

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

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

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

651

652 Supplemental data

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

Table S2. Pearson's correlation matrix between soil properties and the percentage of PAHdioxygenase transcripts and genes. Values in bold correspond to significant correlation
between variables (p<0.05).

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Figure S1. Bioavailable (A) and total (B) PAH concentration in NM soil before (grey) and after (blue) spiking. Black asteriks indicates significant differences between both conditions (Student t test, p<0,05). Values are means and standard deviation of 12 and 48 samples (total
fraction) and 8 and 6 samples (bioavailable fraction), before and after spiking respectively.

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

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

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

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914 Figure 3.



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916 Figure 4.









922 Figure 6.





929 Figure S1.



930

931 Figure S2.





936 Figure S4



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939	Table S2. PAH	concentration (mg	L^{-1}) in th	e organic extra	ct used to spik	e NM soil.	Values are means (n=3) and standard errors.
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PAHs concentration (m	g/L)
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Fluorene	Phenanthrene	Anthracene	Fluoranthene	Pyrene	Benzo(a)	Chrysono	Benzo(b)	Benzo(k)	Benzo(a)	Dibenzo(a,h)	Benzo(g,h,i)	Indeno(1,2,3-cd)
			Fluorantinene		anthracene	Chrysene	fluoranthene	fluoranthene	pyrene	anthracene	perylene	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

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

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