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Rhizosphere effect is stronger than PAH concentration on shaping spatial bacterial assemblages along centimetre-scale depth gradients

Amélia Bourceret, Corinne Leyval, François Thomas, Aurélie Cebron

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1 **Rhizosphere effect is stronger than PAH concentration on shaping**
2 **spatial bacterial assemblages along centimeter-scale depth**
3 **gradients**

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6 Amélia BOURCERET^{1,2,†}, Corinne LEYVAL^{1,2}, François THOMAS^{1,2,§}, Aurélie
7 CÉBRON^{1,2,*}

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9
10 ¹ CNRS, LIEC UMR7360, Faculté des Sciences et Technologies, Bd des Aiguillettes, BP70239, 54506 Vandoeuvre-lès-
11 Nancy, France.

12 ² Université de Lorraine, LIEC UMR7360, Faculté des Sciences et Technologies, Bd des Aiguillettes, BP 70239, 54506
13 Vandoeuvre-lès-Nancy, France.

14 *Corresponding author: aurelie.cebron@univ-lorraine.fr ; phone: (+33) 383684296.

15 † Current address: Department of Plant Microbe Interactions, Max Planck Institute for Plant Breeding Research, Cologne,
16 Germany

17 § Current address: Sorbonne Université, UPMC, Centre National de la Recherche Scientifique, UMR 8227, Integrative
18 Biology of Marine Models, Station Biologique de Roscoff, Roscoff, France

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20

21 **Running-head:** Rhizosphere bacterial assemblage at centimeter-scale

22

23

24

25 Résumé

26

27 A l'échelle centimétrique, les paramètres biotiques et abiotiques (caractéristiques édaphiques et polluants)
28 modulent les assemblages bactériens. Dans une expérience en rhizotron sur un sol industriel contaminé en
29 hydrocarbures aromatiques polycycliques (HAP) et végétalisé, nous avons précédemment montré que la
30 pollution était distribuée spatialement de manière aléatoire et avec des points chauds. Nous avons donc étudié ici
31 l'effet de cette distribution hétérogène des HAP sur l'assemblage de la communauté bactérienne à l'échelle
32 centimétrique, et l'avons comparé à l'impact des gradients rhizosphériques formés en fonction de la profondeur,
33 en présence de luzerne ou ray-grass. Le séquençage d'amplicons d'ARNr 16S a permis de montrer une plus
34 grande diversité bactérienne dans la rhizosphère du ray-grass et un enrichissement en certains taxons selon le
35 type de plante. En effet, les *Bacteroidetes*, *Firmicutes* et *Gamma-Proteobacteria* ont été favorisés dans la
36 rhizosphère de la luzerne alors que le ray-grass a favorisé les *Acidimicrobiia*, *Chloroflexi*, *Alpha-* et *Beta-*
37 *Proteobacteria*. Dans la rhizosphère de la luzerne, les gradients de biomasse racinaire, de concentrations en
38 sucres et du pH en fonction de la profondeur, ont fortement modulé l'assemblage de la communauté
39 microbienne, en favorisant les *Actinobacteria* en surface, et les *Gemmatimonadetes* et *Proteobacteria* en
40 profondeur. Au contraire, la structure de la communauté bactérienne était homogène sur toutes les profondeurs
41 dans la rhizosphère du ray-grass. Pour les 2 plantes, ni la concentration ni la distribution spatiale des HAP n'a
42 d'effet significatif sur l'assemblage des communautés bactériennes. Globalement à l'échelle centimétrique,
43 l'assemblage des communautés bactériennes est principalement modulé par les gradients physiques et chimiques
44 induits par les systèmes racinaires mais pas par la pollution en HAP répartie de manière inégale.

45

46 Abstract

47 At centimeter scale, soil bacterial assemblages are shaped by both abiotic (edaphic characteristics and pollutants)
48 and biotic parameters. In a planted industrial soil, contaminated with polycyclic aromatic hydrocarbons (PAHs),
49 pollution was previously shown to be distributed randomly with hot and cold spots, using rhizoboxes. We
50 therefore investigated the effect of this patchy PAH distribution on the bacterial community assemblage, and
51 compared it to that of root depth gradients found in the rhizosphere of either alfalfa or ryegrass. Sequencing of
52 16S rRNA amplicons revealed a higher bacterial diversity in ryegrass rhizosphere and enrichment in specific
53 taxa by the two plant species. Indeed, *Bacteroidetes*, *Firmicutes* and *Gamma-Proteobacteria* were globally
54 favored in alfalfa while *Acidimicrobiia*, *Chloroflexi*, *Alpha-* and *Beta-Proteobacteria* were globally favored in

55 ryegrass rhizosphere. The presence of alfalfa that created depth gradients of root biomass, carbohydrate and pH,
56 actually shaped bacterial assemblage, favoring *Actinobacteria* near the surface, and *Gemmatimonadetes* and
57 *Proteobacteria* at greater depth, while it was spatially homogeneous all along the ryegrass root system. With
58 both plant species, the PAH content and random distribution had no significant effect on bacterial assemblage.
59 Globally, at cm-scale, bacterial community assemblages were mostly shaped by soil physical and chemical depth
60 gradients induced by root growth but not by PAH patchy content.

61

62 **Keywords:** Alfalfa, ryegrass, pyrosequencing, bacterial diversity, PAH-contaminated soil, rhizobox

63

Draft

64 **Introduction**

65

66 Identifying patterns and drivers of microbial heterogeneity in soil is one of the main current challenges in
67 microbial ecology. Spatial patterns of soil microbial communities are extremely variable across scales (Ettema
68 and Wardle, 2002; Tripathi et al. 2014; Fuka et al. 2009; Kuzyakov and Blagodatskaya 2015). At regional scale
69 (meter to kilometer) factors such as climate, topography and soil pedology explain microbial community
70 biogeography (Constancias et al. 2015a,b; Fierer and Jackson 2006; Dequiedt et al. 2011). At the field scale,
71 Naveed et al. (2016) showed that bacterial community structure was not random but strongly related with
72 edaphic properties, such as total organic carbon and clay content and soil water characteristics. At local scale
73 (millimeter to centimeter), heterogeneity is driven by the patchy distribution of physico-chemical characteristics,
74 carbon and nutrient resources and biotic and/or abiotic stressors, creating a mosaic of different habitats (Bahram
75 et al. 2015; Ettema and Wardle, 2002). At centimeter scale O'Brien et al. (2016) observed an extreme patchiness
76 with abrupt changes in relative abundance of bacterial phyla from sample to sample. At micrometer scale, spatial
77 distribution of soil microbes is determined by microscale soil heterogeneity caused by variation in soil
78 architecture, water fluxes and biotic interactions in aggregates and pore space (Nunan et al. 2002; Grundmann
79 2004; Stolpovsky et al. 2012; Raynaud and Nunan 2014). At all these scales, the presence of plants can further
80 increase the spatial heterogeneity of microbial communities. Indeed, the rhizosphere soil compartment is a
81 hotspot of microbial density and activity (Marschner et al. 2001; Garbeva et al. 2008). Plant roots create specific
82 microniches (eg. oxygen concentration, pH, available substrates, chemical signals) that can regulate microbial
83 community structure (Jaeger et al. 1999; Hinsinger et al. 2009). In planted soils, microbiome structure and
84 activity vary according to plant species (Berg and Smalla 2009), stages of plant development (Remenant et al.
85 2009; Philippot et al. 2013; Schmidt and Eickhorst 2014) and differences in quality and quantity of root exudates
86 released by young, mature or senescent roots (Yang and Crowley 2000; Marschner et al. 2002; DeAngelis et al.
87 2009; Schmidt and Eickhorst 2014). However, little information is currently available on the spatial assemblage
88 of soil microbial community in the rhizosphere of different plant species harboring contrasted root architectures
89 and releasing various exudates. Such an approach should be studied at the millimeter to centimeter scale
90 corresponding to the distance of rhizosphere influence.

91

92 The picture becomes even more complex when considering anthropogenized soils. Organic pollutants are
93 recognized as one major bacterial community stressor and induce shifts in bacterial community composition

94 (Yang et al. 2014). Polycyclic aromatic hydrocarbon (PAH) contamination also selects populations especially
95 adapted for degradation (Cébron et al. 2008; Yrjälä et al. 2010). The distribution of persistent organic pollutants
96 has been demonstrated to be patchy, therefore emphasizing the relevance of scale consideration for microbial
97 ecology studies in urban zones and industrial polluted soils. At regional scale, pollution presents gradients of
98 concentration decreasing spatially from the source points from urban and industrialized area to more rural zones
99 (Peng et al. 2016). At the field scale, spatial patchy distribution of pollution can be found, influencing the
100 relative proportion of some bacterial phyla (Mukherjee et al. 2014), as well as microbial diversity, evenness and
101 PAH catabolic genes (Bengtsson et al. 2013). At local centimeter-scale, organic matter and pollutants are
102 heterogeneously distributed and can form hotspots of concentration, conducting to a nugget effect (Bourceret et
103 al. 2015, Hybolt et al. 2011). Unfortunately, the link between PAH hotspots and variation of community
104 assemblage was never explored at this scale and needs to be specified. At smaller scale, diffusion mechanisms
105 control the heterogeneity, but knowledge is limited by methodological flows due to the minimum quantity of soil
106 necessary for organic pollutant analyses.

107

108 Interest is rising on the use of plants to treat PAH-polluted soils through rhizoremediation, by stimulating
109 microorganism capacity to degrade pollutants in the rhizosphere (Kuiper et al. 2004). However, reports on
110 rhizoremediation efficiencies vary, with sometime even inhibition of PAH-biodegradation in the rhizosphere of
111 certain plants. Indeed plants select specific microbial communities depending on species (Kowalchuk et al.
112 2002), and different plants could preferentially enrich PAH-degraders (Siciliano et al. 2003; Kirk et al. 2005;
113 Liste and Prust 2006; Cébron et al. 2009). Then, in polluted soils, the root zone could contribute to microbial
114 community heterogeneity. In the past, most studies on rhizosphere microbiome overlooked this small-scale
115 heterogeneity, comparing a composite sample of rhizosphere soil collected from a whole plant root system (e.g.
116 plants collected *in situ* or cultivated in pots) to another plant species or to bulk or unplanted soils (Smalla et al.
117 2001; Yergeau et al. 2014; Breidenbach and Conrad 2015). Unfortunately, this one-dimensional approach does
118 not inform on spatial heterogeneity and selection of specific microbial community assemblage depending on the
119 plant species, the depth along the root system, the stage of plant development, the root biomass and age, and
120 does not allow understanding rhizosphere soil biological functioning and processes. Based on these
121 considerations, the impact of contrasted root architectures, and root density on shaping the microbial community
122 structure should be investigated at the local centimeter scale to give insight into the rhizosphere influence, that
123 has been demonstrated to extend to 10-20 millimeters from roots (Schmidt and Eickhorst 2013; Uteau et al.

124 2015). As such specific patterns have not yet been analyzed or discussed for the spatial distributions of bacterial
125 taxa in the rhizosphere of PAH-polluted soils at fine cm-scale, we wanted to fill this gap. The development of
126 root window-based approaches, such as rhizoboxes, has opened new ways to reveal the distribution of microbes
127 in rhizosphere soil and have been used in the present study to sample numerous soil cores close to the roots and
128 spatially distributed at different depths.

129

130 The present study focuses on the bacterial community structure along two plant root systems (alfalfa and
131 ryegrass) in a PAH-contaminated soil in order to point out the principal environmental biotic and abiotic drivers
132 at centimeter scale using rhizobox experimental device. Spatial variability of bacterial community has been
133 rarely studied in the past, at small scale, and never in contaminated soil presenting spatial patchy distribution of
134 pollutants and depth rhizosphere gradients as found in Bourceret et al. (2015). Based on these previous findings,
135 we hypothesized that bacterial community could be influenced by two main forcing factors identified previously:
136 i) rhizosphere processes linked to plant species and root depth gradients of root biomass and carbon from
137 exudates, and/or ii) pollutant content that shows a random spatial distribution with cold and hot spots of PAHs.
138 To test these hypotheses, we used tag-encoded amplicon pyrosequencing of bacterial 16S rRNA genes to
139 investigate bacterial community assemblages in samples from rhizobox experiment.

140

141

142 **Materials and methods**

143

144 *Experimental device, sampling and soil characteristics*

145

146 Aged PAH-contaminated industrial soil, collected from a former coking plant site (Neuves-Maisons [NM],
147 Lorraine, France), was used for the same rhizobox experiment as described in Bourceret et al. (2015). The NM
148 industrial soil contains an historical and recalcitrant PAH contamination, ca 1224 mg.kg⁻¹ (Cébron et al. 2009;
149 Ouvrard et al. 2011) of the 16 US-EPA PAHs. Briefly, rhizoboxes were poly-methyl-methacrylate boxes (H: 30
150 cm x D: 30 cm x W: 2.5 cm) with a removable front face, and holes (0.4 cm in diameter) at the bottom to allow
151 water flow. Two rhizoboxes were filled with sieved NM soil, previously spiked with a complex organic pollutant
152 extract from the same soil that increased the PAH content to 1524 mg.kg⁻¹ of 16 US-EPA PAHs and increased
153 the bioavailability of PAHs and their biodegradation. Rhizoboxes were watered to 80% water retention capacity

154 (WRC). At the start of the experiment, soil properties were spatially homogeneous in the two rhizoboxes.
155 Rhizoboxes were used to grow two plant species with contrasted root systems. After sowing alfalfa (Alf;
156 *Medicago sativa* var. Europe) and ryegrass (Rye; *Lolium multiflorum* var. Podium) at 22.3 ± 2.0 seeds per cm^2 ,
157 plants were grown in a regulated growth chamber ($22^\circ\text{C} / 18^\circ\text{C}$ day/night, 80% relative humidity, ca $250 \mu\text{mol}$
158 photons $\text{m}^{-2} \text{s}^{-1}$, 16-hour photoperiod) (Bourceret et al. 2015). Rhizoboxes were opened after 37 days (T37) when
159 few roots reached the bottom of the rhizoboxes and root density gradients could easily be visualized for both
160 plants. At this time 16 soil cores (1.6 cm diameter, 2.5 cm long; ca 5.03 cm^3) were sampled every 6 cm at 2, 8,
161 14 and 20-cm depth (4 replicate cores per depth) from each rhizobox (*i.e.* 32 samples). Given the high root
162 density measured in each core sample (Table S1), we considered that the entire soil was influenced by roots and
163 regarded it as rhizosphere. Roots were rinsed 3 times with sterile water to collect the root-adhering soil, and
164 weighed to measure the dry root biomass of each sample. Centrifugation of the first root rinsing water allowed
165 collecting the adhering soil that was weighed (mass of root-adherent soil) before being mixed to the rest of soil
166 core sample. Considering the totality of rhizobox samples, root biomass was similar between the two plants
167 (mean of 13 and 12 mg ($n=28$) for alfalfa and rye-grass, respectively; Table S1). Although two depth gradients
168 were found, the one in alfalfa rhizosphere was more pronounced than the ryegrass one, due to higher root
169 biomass at 2 cm depth (Table S1). The ratio mass of root adherent to total soil sample was higher in ryegrass
170 than in alfalfa (Table S1). The 32 mixed soil samples were stored at -80°C until analysis.

171 Soil samples were previously characterized (Bourceret et al. 2015) for some biotic and abiotic characteristics that
172 are summarized in the Table S1. Bacterial abundance (16S rRNA gene copy number, measured through real-
173 time quantitative PCR) was higher in alfalfa than in ryegrass rhizosphere, without depth gradient. Dissolved
174 organic carbon (DOC) and carbohydrates were measured from soil aqueous extract (1/5 w/v ratio) prepared for
175 pH measurement, using TOC-meter and ion-exchange chromatography. pH was similar between the two plants,
176 with lower values at 2 cm depth. DOC was higher with alfalfa than with ryegrass, while carbohydrates such as
177 fructose and xylose were mostly detected with ryegrass. Although no DOC depth gradient was observed,
178 concentration of individual carbohydrates varied, *ie.* inositol was more concentrated at 2 cm depth in alfalfa than
179 in ryegrass rhizosphere, and mannitol was more concentrated at deeper depth in ryegrass than in alfalfa
180 rhizosphere. Total and bioavailable PAH concentration were measured using High-Pressure Liquid
181 Chromatography (HPLC) after Accelerated Solvent Extraction (ASE) using dichloromethane and
182 hydroxypropyl- β -cyclodextrin extraction, respectively. Although higher concentrations of the total and
183 bioavailable 16 US-EPA PAHs were observed in ryegrass than in alfalfa rhizosphere (Table S1), no depth

184 gradient was shown because PAH were distributed randomly with patches of hot and cold spots (Bourceret et al.
185 2015).

186

187

188 ***DNA Extraction***

189

190 Total genomic DNA (gDNA) was extracted from *ca* 0.5 g wet weight of the 32 soil samples (16 samples for
191 each plant after 37 days of growth: T37_alf and T37_rye), using the FastDNA SPIN Kit for Soil (MP
192 Biomedicals, Illkirch, France) and resuspended in 100 µl of Dnase-free water. A spectrophotometer (UV1800,
193 Shimadzu) equipped with a TrayCell unit (Hellma) allowed measuring gDNA concentration and evaluating its
194 quality (purity ratio A_{260}/A_{280}).

195

196 ***Library preparation and pyrosequencing***

197

198 The V4-V5 region of bacterial 16S rRNA gene was amplified using the primer set 515F (5'-GTG CCA GCM
199 GCC GCG GTA A-3') / 907R (5'-CCG TCA ATT CMT TTR AGT TT-3') (Turner et al. 1999), combined at the
200 5'-end with a MID (Multiplex Identifier, Roche) corresponding to a sample identification sequence of 10
201 nucleotides. PCR reactions were performed for each sample in five replicates, in a final volume of 50 µl
202 containing 5X Taq-&Go™ ready to use PCR Mix (MP Biomedicals, Illkirch, France), 10 pM of each primer,
203 and 2 µl of ten-fold diluted template gDNA. Amplicons from the five independent reactions were pooled and
204 purified using the QIAquick PCR Purification Kit (Qiagen, France). Amplicon concentration was measured on a
205 spectrophotometer as described above for gDNA. An equimolar mix of the thirty-two 16S rRNA gene amplicons
206 was prepared in a final volume of 50 µl, reaching a total concentration of 40 ng.µl⁻¹. Library was sequenced by
207 Beckman Coulter Genomics (Danvers, MA, USA) on a half run of 454/Roche GS-FLX Titanium system using
208 Lib-L kit. Sequence data from the 32 samples have been deposited in the NCBI Sequence Read Archive (SRA)
209 and are available under the BioProject ID: PRJNA305229.

210

211 ***Analysis of 16S rDNA sequences***

212

213 After a denoising step from fasta- and qual-files with Acacia 1.52-b0 program (Bragg et al. 2012) at the default

214 parameters, a total of 565,240 16S rDNA sequences were retained. Denoised data were further analyzed in the
215 QIIME 1.8.0 pipeline (Caporaso et al. 2010). First, sequences were distributed to the 32 samples according to
216 their MIDNs and filtered according to different criteria: a length between 300 and 500 bp, a minimum average
217 quality score allowed in sequence of 25, an absence of ambiguous bases and a maximum length of homopolymer
218 runs of 6 bp. Both sense and non-sense sequences were included in the analysis. Second, sequences were
219 clustered in Operational Taxonomic Units (OTUs) at 97% of similarity using USEARCH program including
220 chimera detection (Edgar 2010). Taxonomy was assigned to a representative sequence from each OTU,
221 identified as the most abundant read, using RDP classifier (Wang et al. 2007) and the Greengenes 13_5 database
222 (McDonald et al. 2012). Finally, subsampling was performed to 3,223 sequences per sample, corresponding to
223 the lowest number of sequences retrieved in one of the samples. Alpha-diversity indexes (OTU₉₇ and Chao1
224 richness estimates, Shannon H' and inverse Simpson diversity indexes and Evenness) were calculated from this
225 data set using QIIME 1.8.0 pipeline.

226

227 *Statistical analysis*

228

229 Differences in alpha-diversity estimates between groups were tested using Student t-test and one-way analyses of
230 variance (ANOVA) followed by Newman-Keuls Tukey HSD multiple comparison Post-Hoc test in XLstat
231 software version 2.01 (Addinsoft) with significance level set at $p < 0.05$. To evaluate the impact of soil
232 characteristics (PAH concentration, root density and soil properties, among others) on the bacterial community
233 structure, non-metric multidimensional scaling (nMDS) was performed in R (version 3.0.1) on a Bray-Curtis
234 dissimilarity matrix by using pyrosequencing data at the OTU level for the two-plant species. Fitting of the soil
235 characteristics was performed using the envfit function from the vegan package (Oksanen et al. 2013). After
236 calculation of Pearson correlation between soil characteristics and bacterial diversity (Table S2), only significant
237 factors ($p < 0.05$) were conserved for visualization. We took advantage of the soil biotic and abiotic
238 characteristics previously published in Bourceret et al. (2015) and summarized above (Table S1). To evaluate
239 differences in bacterial community structures between the two plant rhizospheres, subsequent statistical analyses
240 were performed: i) Differences in the relative abundance of taxa composing the bacterial community depending
241 on plant species were analyzed with STAMP version 2.0.8 (Parks and Beiko 2010) that allowed considering the
242 effect size to evaluate the magnitude of the observed differences; ii) Welch's t-tests with filtering of the results
243 based on effect size (difference between proportions > 1 or ratio of proportions > 4) were used to evaluate

244 changes in relative abundance between the two plant conditions at T37. Finally, to evaluate the impact of root
245 depth gradient on bacterial community structures subsequent statistical analyses were performed: Discriminant
246 taxa between the four studied depths (2, 8, 14 and 20 cm) inside alfalfa or ryegrass condition were identified
247 using an ANOVA followed by a Tukey-Kramer post-hoc comparison test, with filtering of the results based on
248 effect size $\eta^2 > 0.26$, that is considered as a large value (Nakagawa and Cuthill 2007). For Welch's tests and
249 ANOVA performed in STAMP, significance level was set at $q=0.05$ after Benjamini-Hochberg correction for
250 multiple testing, and only taxa with relative abundance representing at least 3 sequences in one sample were
251 considered.

252

253

254 **Results and Discussion**

255

256 *Bacterial community description*

257

258 After quality-filtering and subsampling, a total of 103,136 16S rDNA sequences were clustered in 1,848 OTUs.
259 OTUs represented by more than 3 sequences belonged to 12 known phyla (Fig. 1). In aged-contaminated
260 environments, selection and adaptation processes lead to the development of a specific bacterial community
261 tolerant to the pressure exerted by the pollution (Yang et al. 2014b, Golebiewski et al. 2014). This is in contrast
262 with more recent pollution, which induces toxicity mechanisms (Yang et al. 2014a). Accordingly, the NM soil
263 bacterial community is well adapted to contamination due to its long-term (*ca* 100 years) PAH exposure (Cébron
264 et al. 2009; Bourceret et al. 2016), but we could expect that PAH-spiking and biodegradation activity could have
265 exerted a new pressure of selection combined to the plant growth, during the 37 days in rhizoboxes. As
266 previously shown on *in situ* experimental plots planted with alfalfa, the NM soil is dominated by members of the
267 *Proteobacteria*, *Actinobacteria* and *Bacteroidetes* phyla (Bourceret et al. 2016), followed by *Chloroflexi* and
268 *Planctomycetes*. By gathering the twenty most abundant OTUs in both alfalfa and ryegrass conditions, we found
269 that the bacterial community was dominated by 26 OTUs (accounted for $51.9 \pm 3.4\%$ of the bacterial 16S rDNA
270 sequences), of which 70% belongs to *Proteobacteria*. *Proteobacteria* often dominates most soil bacterial
271 communities (Fierer et al. 2007), but we notice here that *Actinobacteria* is the second dominant phylum ~~group~~
272 while usually it is in lower abundance in most soils (Janssen et al. 2006, Fierer et al. 2007). High proportion of
273 spore forming bacterial group such as *Actinobacteria*, likely indicates an adaptation of the community to

274 environmental stress imposed by multi-contamination in NM soil (Ouvrard et al. 2011). It has been described
275 that *Actinobacteria* dominate in soils with <10% organic matter whereas *Proteobacteria* prevailed in higher-
276 organic matter soils (Bell et al., 2013). Even if NM soil has a high organic carbon content due to PAH
277 contamination, it mainly consists of highly recalcitrant carbon (Biache et al. 2014) with low bioavailability.
278 Furthermore, previous studies showed that *Actinobacteria* dominated in hydrocarbon and diesel-polluted soils
279 (Yergeau et al. 2012; Bell et al. 2013) as some isolates are capable of hydrocarbon biodegradation (Kanaly and
280 Harayama 2010). In NM soil, members of the *Bacteroidetes* were the third dominating group, while Janssen
281 (2006) reported that they are generally in low abundance in soils and most of the time belong to the rare-
282 biosphere (Nacke et al. 2011). *Bacteroidetes* were previously shown to be: i) negatively correlated with total soil
283 carbon content, ii) more abundant in relatively high soil pH, and iii) rapidly activated by easily degradable plant
284 derived bio-available organic matter (Acosta-Martinez et al. 2010). These traits could explain the relatively high
285 proportion of *Bacteroidetes* in alfalfa and ryegrass planted NM soil. The relative abundance of *Acidobacteria* (<
286 0.71%) was low in comparison with other soil types where it ranges from 2 to 78% (Jones et al. 2009). This
287 could be explained by a mean pH of 7.3 and recent modification and adaptation of the NM soil community to the
288 continual input of pollutants during the 20th century. Indeed *Acidobacteria* are better adapted to environments
289 with low pH (Fierer et al. 2007) and are scarce in young soils (Nemergut et al. 2007; Tarlera et al. 2008).

290

291 *Identification of parameters influencing the bacterial community assemblage*

292

293 Although the bacterial community composition of the NM soil was globally similar in both planted rhizoboxes,
294 we asked whether it is specifically shaped by plant species and spatially structured according to rhizosphere
295 depth gradient or heterogeneous patchy PAH distribution. Non-metric multidimensional scaling (nMDS, Fig. 2)
296 based on relative abundances of OTUs revealed a clear distinction of bacterial community assemblage after 37
297 days of plant growth depending on plant species, along the first axis (dim1) that also discriminated samples
298 according to sampling depth for alfalfa. The second axis (dim2) discriminated samples according to sampling
299 depth for both alfalfa and ryegrass condition. Fitting of soil parameters to the ordination further showed that
300 depth, bacterial abundance, root biomass, mass of root adherent soil (collected after washing the roots), pH,
301 dissolved organic carbon and carbohydrate contents (inositol, mannitol, xylose and fructose) were significantly
302 correlated with bacterial community structure (see Pearson correlations in Table S2). Among these correlated
303 parameters, the four most impacting were the depth, the mass of root-adherent soil, pH and bacterial abundance

304 (Pearson correlation $R^2 = 0.78, 0.66, 0.59$ and 0.56 , respectively; Table S2). DOC and bacterial abundance,
305 significantly higher in alfalfa rhizosphere, and carbohydrate quality (*i.e.* inositol concentration significantly
306 higher in alfalfa contrary to mannitol, fructose and xylose concentrations that were higher in ryegrass), were
307 linked to differences in bacterial diversity between the two plant species. pH, depth and root biomass were
308 correlated together and influenced similarly both rhizospheres.

309 On the contrary, the total and bioavailable PAH concentrations were not significantly correlated with bacterial
310 community structure (Fig. 2; Table S2). Although PAHs are randomly distributed, their concentrations are
311 spatially variable in both rhizoboxes with local hot and cold spots, as well as higher variability and lower mean
312 concentration in alfalfa than in ryegrass rhizosphere (1074 ± 359 and 1297 ± 340 mg/kg of total PAHs,
313 respectively; Table S1). The PAH contamination was homogeneous at the beginning of the experiment with a
314 low standard deviation (*i.e.* 1524 ± 174 mg/kg of total PAHs, coefficient of variation $CV=11\%$), while after 37
315 days of PAH biodegradation a high heterogeneity appeared in both rhizoboxes ($CV= 33\%$ and 26% for alfalfa
316 and ryegrass, respectively). However the present data, supported by absence of Pearson correlation (Table S2),
317 suggests that local variations in PAH content do not influence the NM soil bacterial community assemblage
318 when comparing for each soil core sample, the PAH content and the bacterial diversity. Since the bacterial
319 community has adapted to the pollution over decades (Cébron et al. 2009), a large fraction of the soil
320 microbiome could be tolerant to high PAH concentration level, as shown in soils regularly exposed to PAHs
321 (Macleod and Semple 2002; Haritash and Kaushik 2009). Therefore, the fate of PAH probably depends mainly
322 on bacterial activity level (mRNA) that could locally be higher while diversity does not change (Johnsen and
323 Karlson 2007; Thomas and Cébron 2016). Our data suggests that at centimeter-scale, rhizosphere effect was
324 stronger than PAH concentration effect on spatially shaping bacterial assemblages. The pre-eminence of
325 rhizosphere effect against PAH concentration contrasts with what was previously found at larger meter-scale
326 where microbial diversity, evenness and the distribution of PAH catabolic genes matched the patchy PAH
327 distribution (Bengtsson et al. 2013) and where abundance of some phyla (*i.e.* increase in *Proteobacteria* and
328 decrease in *Actinobacteria*, TM7 and *Planctomycetes*) was linked to hot spots of PAH pollution (Mukherjee et
329 al. 2014). Working at meter-scale, in the field, might underestimate the effect of plants due to limitation of
330 sampling. A study at a smaller scale can be useful to consider the impact of rhizosphere that is spatially limited
331 by short-range diffusion of rhizodeposits in vegetated soils. Our finding highlights the urgent need of
332 considering relevant, fine-scale effects when evaluating rhizosphere impact on pollutant biodegradation.
333 Moreover, the effect of plant rhizosphere could be more intense during the early stage of plant growth than when

334 they are installed for longer.

335

336

337 ***Plant species effect on the bacterial community***

338

339 We characterized the effect of plant species on shaping the rhizosphere bacterial communities by analyzing
340 results from both rhizoboxes using the variability among the 16 sample points per condition after 37 days of
341 plant growth. Technical constraints allowed investigating only one rhizobox per plant species, thus it is difficult
342 to fully account for unexpected variations due to the rhizobox system itself. However, the two rhizoboxes were
343 prepared simultaneously with the same batch of soil, previously sieved at 2 mm, and kept under the same
344 conditions. Furthermore at the finely sampled centimeter scale, we can consider that we compared two
345 rhizosphere systems (containing each dozens of plants), one planted with alfalfa and the other with ryegrass
346 (Kuske et al. 2002). All estimates of bacterial richness ($p=0.017$ and 0.001 for Chao1 and OTU₉₇, respectively),
347 taxon evenness ($p=0.003$) and diversity ($p=0.001$ and 0.005 for H' and $1/D$, respectively) were significantly
348 higher in soil planted with ryegrass compared to alfalfa (Table 1). As previously shown in Bourceret *et al.*
349 (2015), root biomass was globally similar in both rhizobox (mean of 13 mg dry roots, $n=16$) but ryegrass roots
350 were more homogeneously distributed all over the rhizobox than for alfalfa that had a higher root biomass at
351 shallow depth (2 cm) in comparison to the three other greater depths (26 ± 17 and 9 ± 5 mg, respectively). Thus,
352 the commonly described rhizosphere impact (i.e. reduction of the alpha-diversity indices; Kowalchuk et al. 2002;
353 Marilley and Aragno 1999) was less pronounced in ryegrass than in alfalfa rhizobox because alfalfa root biomass
354 was higher. Although a higher ratio of root-adherent soil was detected for ryegrass than for alfalfa (0.11 ± 0.03
355 and 0.04 ± 0.02 , respectively), a higher dissolved organic carbon concentration (190 ± 65 and 121 ± 37 mg/kg)
356 and a higher bacterial abundance ($3.44 \times 10^9 \pm 9.57 \times 10^8$ and $2.37 \times 10^9 \pm 5.60 \times 10^8$ 16S rDNA copies, respectively)
357 were detected in alfalfa than in ryegrass rhizosphere, respectively. Root structure, and concentration,
358 composition and production rate of exudates, as well as nature of root symbiosis are the main factors driving
359 bacterial colonization of the rhizosphere and bacterial community structure (Marschner et al. 2001; Berg and
360 Smalla 2009). Indeed, the two studied plants possess a very contrasted root system (Bourceret et al. 2015).
361 Alfalfa develops a tap root system composed of a central root from which few lateral roots emerge (Aprill and
362 Sims 1990). Meanwhile ryegrass features an extensively branched and fibrous root system with fine and profuse
363 roots, that leads to high root length and higher total rhizoplane surface than alfalfa (Soleimani et al. 2010).

364 The effect of plant species was highlighted when taking relative abundance into account. At different taxonomic
365 levels (Table 2), members of the *Bacteroidetes* (*Flavobacteriia* class and *Flavobacteriales* order), *Firmicutes*
366 (*Bacilli* class, *Bacillales* order and *Paenibacillus*), *Alpha-Proteobacteria* (unclassified *Caulobacteraceae*,
367 *Devosia*, unclassified *Sphingomonadaceae*), *Gamma-Proteobacteria* (*Pseudomonadales* order and
368 *Pseudomonas*) and *Actinobacteria* (unclassified *Micrococcaceae*) were significantly more abundant in alfalfa
369 than in ryegrass rhizosphere. Reversely, members of the *Chloroflexi*, *Alpha-Proteobacteria* (*Sphingomonadales*
370 and *Caulobacterales* orders, and *Mycoplana* and unclassified *Sphingomonadaceae*), *Beta-Proteobacteria*
371 (*Burkholderiales* and *Hydrogenophilales* orders, and *Thiobacillus*) and *Actinobacteria* (*Acidimicrobiales* order)
372 were significantly more abundant with ryegrass than with alfalfa (Table 2). Similarly, several studies showed
373 such species-dependent effect of plants on the assemblage of rhizosphere bacterial communities (Kirk et al.
374 2005; Kawasaki et al. 2012; Oliveira et al. 2014). Although additional unmeasured parameters might be of
375 importance (e.g. nitrogen content), we managed to establish relationships between bacterial community
376 assemblage and a number of soil characteristics (Fig. 2). Higher content of dissolved organic carbon in alfalfa
377 rhizosphere could explain the higher bacterial abundance compared to ryegrass. A positive correlation between
378 microbial biomass and water-soluble organic carbon was previously described and variability between plant
379 species was observed (Chantigny et al. 1996). Although carbohydrates are not the main components of root
380 exudates (ca 5% of DOC), their analysis provides information about differences in the nature of compounds
381 released by the two plant species. The higher inositol content in alfalfa rhizosphere (Bourceret et al. 2015) might
382 select specific bacteria compared to ryegrass rhizosphere, which was richer in mannitol, xylose and fructose.

383

384 *Spatial structuration of bacterial community assemblage with depth*

385

386 We demonstrated here that the physical and chemical depth gradients, observed at centimeter-scale, shaped
387 differentially the bacterial community under alfalfa and ryegrass systems. Some studies have shown that
388 bacterial community structure is modified with stage of plant development (Chiarini et al. 1998; BauDoin et al.
389 2002) and position along the root (Baudoin et al. 2001; Cavaglieri et al. 2009), which both affect the quality of
390 root exudates. So far, these studies focused mainly on the maize rhizosphere and on non-contaminated soil.
391 Working on the same dataset as the present experiment, Bourceret *et al.* (2015) previously indicated that the pH
392 was positively correlated and the root biomass was negatively correlated with depth in rhizoboxes, and that
393 carbohydrate concentrations varied both with plant species and depth.

394 Ryegrass root biomass was relatively homogeneous along the depth profile. Accordingly we did not evidence
395 spatial gradient of bacterial diversity (Table 1), nor did we identify OTUs impacted significantly with depth in
396 ryegrass rhizosphere despite the presence of sugar gradients and a small trend of depth structuration in
397 multivariate analysis (Fig. 2). These results contrast with previous observations on functional population,
398 showing increased abundance of PAH-degrading *Actinobacteria* dioxygenase genes, with depth in ryegrass
399 rhizosphere (Bourceret et al. 2015).

400 On the contrary, all diversity estimators increased significantly with depth in the soil planted with alfalfa (Table
401 1; $p=0.003$, 0.001 , 0.017 , 0.029 and 0.052 for OTU₉₇, Chao1, H' , $1/D$ and E , respectively). Furthermore, they
402 were negatively correlated to alfalfa root biomass that was more abundant at shallow depth (Pearson's correlation
403 coefficient $R^2 = -0.60$, -0.61 , -0.64 , -0.54 and -0.62 for OTU₉₇, Chao1, H' , $1/D$ and E , respectively). Exudation is
404 known to be greater at the root apex, and hence expected to be higher at greater depth in alfalfa rhizobox due to
405 its root structure. The quality of exudate compounds varies depending on the age of plant roots (Walker et al.
406 2003; Yang and Crowley 2000), thus creating depth gradient due to the presence of older roots at shallow depths.
407 This phenomenon could explain the highest bacterial diversity at greater depth potentially due to differences in
408 exudate quality. These results contrast with previous studies in non-contaminated soil, where root exudation
409 leads to nutrient selection for fast-growing species and to a lower diversity (Kowalchuk et al. 2002; Marilley and
410 Aragno 1999). Moreover, the relative abundance of some phyla and OTUs varied also significantly with depth in
411 the soil from the alfalfa rhizobox (Fig. 1 and 3). Few authors suggested that spatial variability across the
412 rhizosphere induces spatial gradient of bacteria, due to heterogeneity of nutrient release. For instance Corgié et
413 al. (2006) observed in a compartmented device that bacterial community structure depended on the distance from
414 roots. In alfalfa rhizobox, at phylum level, the relative abundance of *Actinobacteria* was significantly higher at
415 shallow depth where root biomass and inositol content were higher and pH more acidic (pH was 6.91 ± 0.22 and
416 7.32 ± 0.08 at 2 cm depth and mean at the three deeper depths, respectively), while *Gemmatimonadetes* and
417 *Proteobacteria* phyla were significantly more abundant at greater depth (Fig. 1). Five OTUs affiliated to
418 *Actinobacteria* (unclassified *Micrococcaceae*), *Bacteroidetes* (unclassified *Chitinophagaceae*), *Firmicutes*
419 (unclassified *Bacillales*), *Planctomycetes* (*Planctomyces*) and *Alpha-Proteobacteria* (unclassified
420 *Methylobacteriaceae*) were significantly favored at shallow depth (Fig. 3). The slight acidification linked to the
421 higher root biomass at shallow depth (Hinsinger et al. 2003) could select some taxa, as the pH was previously
422 described as a main driver of bacterial community (Rousk et al. 2010). Five OTUs affiliated to *Actinobacteria*
423 (unclassified *Nocardioideae*), *Firmicutes* (*Bacillus selenatarsenatis*), *Alpha-Proteobacteria* (unclassified

424 *Phyllobacteriaceae*) and *Gamma-Proteobacteria* (unclassified *Xanthomonadaceae*) were significantly favored at
425 greater depth (Fig. 3).

426

427

428 **Conclusion**

429

430 Overall, our results showed that rhizosphere effect is stronger than PAH concentrations on shaping bacterial
431 communities at the spatial centimeter-scale in heterogeneous contaminated soil. This finding could lead to a
432 better management of polluted sites by choosing the best plants capable of shaping the microbial community
433 through their root structure and according to predefined criteria (higher bacterial abundance, higher proportion of
434 some specific taxa, etc...).

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437

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442

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449

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699 **Tables**

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701 **Table 1.** Bacterial richness and diversity estimators in soil planted with alfalfa (Alf) or ryegrass (Rye) after 37
 702 days (T37) of plant growth, at four depths (2, 8, 14 and 20 cm from the surface of the rhizobox). OTUs were
 703 created with 97% sequence similarity (OTU₉₇). Means \pm SD, n=4. The effect of plant species was evaluated
 704 using Student t-tests (p<0.05). The effect of depth was tested (different letters (a, b, c) denote groups with
 705 significantly different values (p<0.05)) separately for each plant species using one-way ANALYSES OF VARIANCE
 706 (ANOVA) followed by Tukey HSD multiple comparison tests.

707

Conditions	Richness estimator		Diversity estimator		
	Observed OTU ₉₇	Chao1	Shannon's index (H')	Inverse of Simpson's index (1/D)	Evenness (E)
Alf_T37-2	351 \pm 7 ^b	519 \pm 33 ^c	6.42 \pm 0.11 ^b	29.78 \pm 4.50 ^b	0.759 \pm 0.012 ^b
Alf_T37-8	375 \pm 22 ^b	581 \pm 57 ^{bc}	6.64 \pm 0.27 ^{ab}	42.02 \pm 13.88 ^{ab}	0.777 \pm 0.024 ^{ab}
Alf_T37-14	389 \pm 25 ^{ab}	629 \pm 57 ^{ab}	6.70 \pm 0.19 ^{ab}	42.08 \pm 7.47 ^{ab}	0.779 \pm 0.014 ^{ab}
Alf_T37-20	415 \pm 15 ^a	723 \pm 52 ^a	6.92 \pm 0.13 ^a	54.10 \pm 10.01 ^a	0.796 \pm 0.013 ^a
Rye_T37-2	415 \pm 10 ^{ab}	684 \pm 76	6.91 \pm 0.14	53.31 \pm 9.44	0.795 \pm 0.017
Rye_T37-8	415 \pm 7 ^{ab}	707 \pm 47	6.99 \pm 0.07	60.06 \pm 8.95	0.804 \pm 0.008
Rye_T37-14	396 \pm 5 ^b	619 \pm 70	6.85 \pm 0.14	50.65 \pm 8.66	0.793 \pm 0.016
Rye_T37-20	434 \pm 14 ^a	722 \pm 39	6.96 \pm 0.08	56.98 \pm 5.11	0.795 \pm 0.007
Plant effect	Rye > Alf	Rye > Alf	Rye > Alf	Rye > Alf	Rye > Alf

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711 **Table 2.** Bacterial taxa (at the phylum, class, order and OTU levels) for which significant effect of the plant
 712 species was detected on their relative abundance after 37 days of plant growth. Mean \pm SD (n=16) over the entire
 713 depth profile. The effect of plant was evaluated using Welch's t-tests followed by Benjamini-Hochberg
 714 correction of the p-values. Significant taxa were defined based on stringent criteria: abundance (at least 3
 715 sequences in one sample), corrected p-value ($q < 0.05$) and effect size (difference between proportions > 1 or ratio
 716 of proportions > 4). Based on these statistical analyses, values in boldface denote the group with significantly
 717 highest relative abundance for each taxon.

718

Bacterial taxa		Relative abundance (%)	
		Alf T37	Rye T37
Phylum level			
<i>Bacteroidetes</i>		6.6 \pm 2.5	2.5 \pm 0.8
<i>Chloroflexi</i>		3.1 \pm 0.8	4.4 \pm 0.6
<i>Firmicutes</i>		2.3 \pm 1.0	1.3 \pm 0.3
<i>Proteobacteria</i>		60.1 \pm 5.4	64.7 \pm 4.2
Class level			
<i>Acidimicrobiia</i>	(<i>Actinobacteria</i>)	3.0 \pm 1.0	4.5 \pm 0.7
<i>Flavobacteriia</i>	(<i>Bacteroidetes</i>)	3.6 \pm 2.2	0.4 \pm 0.3
<i>Bacilli</i>	(<i>Firmicutes</i>)	2.1 \pm 1.0	0.9 \pm 0.2
<i>Alphaproteobacteria</i>	(<i>Proteobacteria</i>)	25.7 \pm 2.0	30.5 \pm 2.5
<i>Betaproteobacteria</i>		11.0 \pm 2.7	15.9 \pm 3.4
<i>Gammaproteobacteria</i>		22.6 \pm 4.2	17.7 \pm 3.2
Order level			
<i>Acidimicrobiales</i>	(<i>Actinobacteria</i>)	3.0 \pm 1.0	4.5 \pm 0.7
<i>Flavobacteriales</i>	(<i>Bacteroidetes</i>)	3.6 \pm 2.2	0.4 \pm 0.3
<i>Bacillales</i>	(<i>Firmicutes</i>)	2.1 \pm 1.0	0.9 \pm 0.2
<i>Sphingomonadales</i>	(<i>Alphaproteobacteria</i>)	11.6 \pm 1.7	16.0 \pm 2.6
<i>Caulobacterales</i>		1.9 \pm 0.3	3.1 \pm 0.6
<i>Burkholderiales</i>	(<i>Betaproteobacteria</i>)	4.3 \pm 0.9	6.1 \pm 1.4
<i>Hydrogenophilales</i>		5.8 \pm 2.0	8.8 \pm 2.2
<i>Pseudomonadales</i>	(<i>Gammaproteobacteria</i>)	7.4 \pm 2.8	3.1 \pm 1.3
OTU level			
Unclass. <i>Micrococcaceae</i> _1	(<i>Actinobacteria</i>)	6.1 \pm 4.6	0.6 \pm 0.6
Unclass. <i>Micrococcaceae</i> _2		0.1 \pm 0.1	0.01 \pm 0.02
<i>Paenibacillus</i>	(<i>Firmicutes</i>)	0.4 \pm 0.3	0.01 \pm 0.01
Unclass. <i>Caulobacteraceae</i> _1	(<i>Alphaproteobacteria</i>)	0.2 \pm 0.1	0.01 \pm 0.02
Unclass. <i>Caulobacteraceae</i> _2		0.17 \pm 0.1	0.002 \pm 0.01
<i>Mycoplana</i>		0.7 \pm 0.2	1.9 \pm 0.5
<i>Devosia</i>		2.6 \pm 1.3	0.4 \pm 0.3
Unclass. <i>Sphingomonadaceae</i> _1		0.6 \pm 0.5	0.03 \pm 0.04
Unclass. <i>Sphingomonadaceae</i> _2		1.1 \pm 0.4	3.0 \pm 0.3
<i>Thiobacillus</i>	(<i>Betaproteobacteria</i>)	3.6 \pm 1.1	5.7 \pm 1.8
<i>Pseudomonas</i>	(<i>Gammaproteobacteria</i>)	6.7 \pm 2.9	1.7 \pm 0.7

719

720 **Figure caption**

721

722 **Figure 1.** Relative abundance of the bacterial phyla (represented by at least 3 sequences) after 37 days (T37) of
723 alfalfa (Alf) and ryegrass (Rye) growth for samples (means, n=4) located at four depths (2, 8, 14 and 20 cm from
724 the surface of the rhizobox). *Proteobacteria* were represented at the class level for better taxonomic resolution.
725 The group named other phyla includes: MVP-21, OD1, SBR1093, TM6, and TM7.

726

727 **Figure 2.** Non-metric multidimensional scaling ordination (nMDS) representing the impact of environmental
728 factors (arrows: edaphic parameters in black, sugar content in green, rhizospheric parameters in green and
729 bacterial abundance in red) on bacterial community assemblage in alfalfa (circle) and ryegrass (triangle)
730 rhizospheres at four depths (color gradients) after 37 days of plant growth. Each arrow represents one
731 environmental parameter significantly correlated to the bacterial diversity ($p < 0.05$ and $R^2 > 0.2$), excepted for
732 PAHs ($p = 0.441$ and $R^2 = 0.0511$) and bioavailable PAHs ($p = 0.477$ and $R^2 = 0.0514$). The nMDS 2D-stress was
733 0.161.

734

735 **Figure 3.** OTUs for which significant effect of depth was detected on their relative abundance in alfalfa
736 rhizosphere. A Tukey-Kramer ANOVA with Benjamini-Hochberg correction of p-value (q-value) allowed
737 identifying OTUs with significant (considering $q < 0.05$, and effect size > 0.26) differences with depth (2, 8, 14
738 and 20 cm from the surface of the rhizobox). Means \pm SD (n=4). For each OTU, letters denote groups with
739 significant differences in relative abundance.

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