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

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

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

Abstract

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

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

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

Materials and methods

Experimental device, sampling and soil characteristics

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

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

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

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

DNA Extraction

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

Library preparation and pyrosequencing

The V4-V5 region of bacterial 16S rRNA gene was amplified using 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), combined at the 5'-end with a MID (Multiplex Identifier, Roche) corresponding to a sample identification sequence of 10 nucleotides. PCR reactions were performed for each sample in five replicates, in a final volume of 50 µl containing 5X Taq-&Go™ ready to use PCR Mix (MP Biomedicals, Illkirch, France), 10 pM of each primer, and 2 µl of ten-fold diluted template gDNA. Amplicons from the five independent reactions were pooled and purified using the QIAquick PCR Purification Kit (Qiagen, France). Amplicon concentration was measured on a spectrophotometer as described above for gDNA. An equimolar mix of the thirty-two 16S rRNA gene amplicons was prepared in a final volume of 50 µl, reaching a total concentration of 40 ng.µl⁻¹. Library was sequenced by Beckman Coulter Genomics (Danvers, MA, USA) on a half run of 454/Roche GS-FLX Titanium system using Lib-L kit. Sequence data from the 32 samples have been deposited in the NCBI Sequence Read Archive (SRA) and are available under the BioProject ID: PRJNA305229.

Analysis of 16S rDNA sequences

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

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

226

227 *Statistical analysis*

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

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

Results and Discussion

Bacterial community description

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

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

Identification of parameters influencing the bacterial community assemblage

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

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

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

they are installed for longer.

Plant species effect on the bacterial community

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

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

383

Spatial structuration of bacterial community assemblage with depth

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

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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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Tables

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

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

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

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>Caulobacteriales</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

Figure caption

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

Figure 2. Non-metric multidimensional scaling ordination (nMDS) representing the impact of environmental factors (arrows: edaphic parameters in black, sugar content in green, rhizospheric parameters in green and bacterial abundance in red) on bacterial community assemblage in alfalfa (circle) and ryegrass (triangle) rhizospheres at four depths (color gradients) after 37 days of plant growth. Each arrow represents one environmental parameter significantly correlated to the bacterial diversity ($p < 0.05$ and $R^2 > 0.2$), excepted for 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 0.161.

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