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The bacterial and fungal diversity of an aged PAH- and heavy metal-contaminated soil is affected by plant cover and edaphic parameters

BOURCERET Amélie^{1,2}, CÉBRON Aurélie^{1,2,†}, TISSERANT Emilie³, POUPIN Pascal^{4,5},
BAUDA Pascale^{4,5}, BEGUIRISTAIN Thierry^{1,2}, LEYVAL Corinne^{1,2}

¹ CNRS, LIEC UMR7360, Faculté des Sciences et Technologies, BP70239, 54506 Vandoeuvre-lès-Nancy, France.

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

³ INRA, IAM UMR1136, Centre INRA de Nancy, 54280 Champenoux, France.

⁴ Université de Lorraine, LIEC UMR7360, Campus Bridoux - Rue du Général Delestraint, 57070 Metz Borny, France.

⁵ CNRS, LIEC UMR7360, Campus Bridoux - Rue du Général Delestraint, 57070 Metz Borny, France.

† Corresponding author: aurelie.cebron@univ-lorraine.fr; Phone: (+33) 3 83 68 42 96

ABSTRACT

Industrial wasteland soils with aged PAH and heavy metal contaminations are environments where pollutant toxicity has been maintained for decades. Although the communities may be well adapted to the presence of stressors, knowledge about microbial diversity in such soils is scarce. Soil microbial community dynamics can be driven by the presence of plants, but the impact of plant development on selection or diversification of microorganisms in these soils has not been established yet. To test these hypotheses, aged-contaminated soil samples from a field trial were collected. Plots planted with alfalfa were compared to bare soil plots, and bacterial and fungal diversity and abundance were assessed after 2 and 6 years. Using pyrosequencing of 16S rRNA gene and ITS amplicons, we showed that the bacterial community was dominated by *Proteobacteria*, *Actinobacteria* and *Bacteroidetes* and was characterized by low *Acidobacteria* abundance, while the fungal community was mainly represented by members of the *Ascomycota*. The short-term toxic impact of pollutants usually reduces the microbial diversity, yet in our samples bacterial and fungal species richness and diversity was high suggesting that the community structure and diversity adapted to the contaminated soil over decades. The presence of plants induced higher bacterial and fungal diversity than in bare soil. It also increased the relative abundance of bacterial members of the

36 *Actinomycetales*, *Rhizobiales* and *Xanthomonadales* orders and of most fungal orders.
37 Multivariate analysis showed correlations between microbial community structure and heavy
38 metal and PAH concentrations over time, but also with edaphic parameters (C/N, pH,
39 phosphorus and nitrogen concentrations).

40
41 **Key words: bacteria, fungi, PAH, heavy metals, rhizosphere, 454-pyrosequencing**

42 43 **1. Introduction**

44 The decline of coal-mining activities has left behind large areas of industrial wasteland soils
45 that display high levels of multi-contamination by polycyclic aromatic hydrocarbons (PAHs)
46 and heavy metals (HMs) [1]. During the 20th century, industrial facilities such as gas or
47 coking plants caused chronic pollution of soils. After an ageing period, both PAHs and HMs
48 persisted in the environment due to their low availability/mobility and low
49 degradability/reactivity. Thus the high concentrations of pollutants and their toxicity have
50 been exerting a selection pressure on microorganisms for decades. These particular
51 environmental conditions potentially enabled the microbial community to adapt, and favored
52 microorganisms able of using pollutant as carbon source or tolerate pollutant by detoxification
53 mechanisms [2]; [3]. This long-term process should lead to the development of a new
54 microbial community diversity after a few decades [4]; [5]. Yet, little is known about the
55 impact of ancient contamination on microbial diversity in such aged multi-polluted soils.
56 Most studies focus on the short-term modifications of microbial diversity following recent
57 contamination events, mainly in laboratory experiments (*i.e.* spiking of unpolluted soils)
58 [8];[9];[10]. Environmental conditions and edaphic parameters are components that shape the
59 soil microbial communities [11]. Similarly, chronic pollution could also be an important
60 parameter that can select soil microbial diversity. While the short-term impact of
61 contamination tends to decrease microbial abundance, richness and diversity, we
62 hypothesized that in an aged multi-contaminated soil the microbial community would adapt
63 over time toward a unique and rich diversity pattern [4]; [5].

64 Some studies show that vegetation can help to restore contaminated environments.
65 Phytoremediation strategies have been developed based on plants' contribution to the
66 stabilization, extraction and degradation of pollutants [12]. While shaping microbial diversity
67 by stimulating some microbial populations, plant root exudates could favor PAH degradation

68 through rhizodegradation processes [13];[14];[15] and modify HM speciation, mobility and
69 availability [12] in the rhizosphere. Among different plant species used in rhizoremediation
70 assays, alfalfa is described as tolerant to pollution and interesting for PAH remediation
71 [16];[17], similarly to other legumes such as red lentil [18]. A contribution of mycorrhizal
72 colonization of plant roots to PAH dissipation [19] and HM tolerance [20] has also been
73 shown. There is an effect of plants on soil microbial biomass and diversity because vegetal
74 litter or residues bring complex organic matter into soils [21] and also pose the question of
75 selection or diversification of microorganisms. As most studies focus on one particular
76 microbial function or population, little is known about the impact of plants and the effect of
77 the rhizosphere on bacterial and fungal richness and diversity in multi-contaminated soils. A
78 dramatic shift in the bacterial community structure in the rhizosphere of sunflower in a
79 creosote-contaminated soil was demonstrated in a pot experiment after 90 days in the
80 greenhouse during PAH-degradation [22]. Although fungal community dynamic is mainly
81 driven by plants [23], the diversity of this microbial community needs to be explored. Overall,
82 as the rhizosphere effect could modify soil parameters and fate of pollutants, there is still a
83 need to study the long-term impact of plants in comparison to bare soil, on shaping the
84 microbial communities during natural attenuation processes of aged multi-contaminated soils.
85 It is well established that environmental parameters impact microbial community composition
86 [11]. Although bacteria and fungi are in constant interaction in soils [24], these two
87 components of the microbial community have a different ecology (e.g. nutrient and carbon
88 source requirement, growth rate...) and respond differently to environmental conditions and
89 stressors. To study the system as a whole, bacterial and fungal diversity must be studied
90 together. To our knowledge, no study has yet evaluated how plants and edaphic parameters
91 drive both fungal and bacterial diversity of a multi-contaminated soil on the long term.

92 In this context, the aims of our study were to (1) describe the diversity of bacterial and fungal
93 communities in an aged multi-contaminated soil from a former coking plant site (Neuves-
94 Maisons, France), (2) determine the impact of plant development on microbial richness and
95 diversity, (3) characterize the temporal modifications of microbial diversity according to
96 evolution of edaphic parameters over 6 years. To address this issue, we collected soil samples
97 from a field trial where a long-term plant-assisted natural attenuation experiment was being
98 conducted [25]. We studied three replicate plots of planted and non-planted soil, sampled at
99 three time-points (0, after 2 and 6 years). We analyzed bacterial and fungal community
100 abundance using real-time quantitative PCR, and diversity using tag-encoded pyrosequencing

101 of the 16S rRNA gene and ITS amplicons, respectively.

102

103 **2. Materials and methods**

104 **2.1. Site description and sample collection**

105 The field trial, previously described by Ouvrard et al. [25], was set up in September 2005.

106 Among the 24 plots filled with an aged PAH- and HM-contaminated soil (NM) from a coking

107 plant wasteland (Neuves-Maisons, Meurthe et Moselle, France), only three replicates of two

108 treatments were studied. The bare soil plots (NM-BS) were kept clear of vegetation by hand

109 weeding and the planted plots (NM-Msm) were sown with alfalfa (40 g of seeds of *Medicago*

110 *sativa* var. Europe *per* plot) in September 2005 and co-inoculated with two mycorrhizal

111 fungal strains (*Glomus mosseae* and *Glomus intra-radices*, recently renamed *Funneliformis*

112 *mosseae* and *Rhizophagus intraradices* [26], commercial inoculum from the Institut für

113 Pflanzkultur, Germany). The plots were monitored twice a year, in May and September, for

114 6 years. In September, the alfalfa biomass was cut and dry weight was measured (Table 1).

115 Mycorrhizal colonization of roots was determined by Trypan blue staining as described by

116 [27] and notation was performed using the method described in [28]. Six soil subsamples

117 were collected *per* plot and mixed to obtain one composite soil sample *per* plot. After sieving

118 to 5 mm, 500 g were dried and dedicated to soil characterization, and 50 g were stored at -

119 80°C until DNA extraction. Three sampling dates were studied: samples T0 (September

120 2005), T4 (September 2007) and T12 (September 2011), i.e. 18 soil samples altogether.

121 Sample characteristics (humidity level, physico-chemical properties, total and extractable

122 heavy metals, and PAH concentrations) were measured at the LAS-INRA laboratory (Arras,

123 France) and are listed in Table 1.

125 **2.2. DNA extraction**

126 Total genomic DNA was extracted from 0.5 g wet weight of each of the 18 soil samples with

127 the FastDNA SPIN Kit for Soil (MP Biomedicals, Illkirch, France), and resuspended in 100

128 µl of DES (Dnase-Free water). DNA concentrations and quality (purity ratio A_{260}/A_{280}) were

129 measured using a spectrophotometer (UV1800, Shimadzu) equipped with a TrayCell unit

130 (Hellma).

132 **2.3. Assessment of fungal and bacterial abundance by real-time quantitative PCR**

133 Real-time quantitative PCR was performed as previously described in Cébron et al. [29] and

134 Thion et al. [23]. Primer sets FF390R (5'-GAGGTCTCGTTCGTTATCG-3') / Fung5F (5'-
135 GGGAACCAGGACTTTTAC-3') [30] and 968F (5'-AACGCGAAGAACCTTAC-3') /
136 1401R (5'-CGGTGTGTACAAGACCC-3') [31] were used to quantify fungi and bacteria by
137 targeting the 18S rRNA and 16S rRNA genes, respectively. Amplification reactions (20 µl)
138 were carried out using 1 µl of ten-fold diluted DNA (corresponding to 2.8 to 6.5 ng), 10 µl of
139 iQ SYBR Green SuperMix (Bio-Rad), 0.8 µl of each primer (10 µM), 0.4 µl of Bovine Serum
140 Albumin (3%), 0.2 µl of Dimethylsulfoxide, and 0.08 µl of T4gp32 (500 µg/ml) (MP
141 Biomedicals). Quantification was performed using a CFX96 Real-Time PCR detection system
142 (Bio-Rad) and standard plasmid dilution series from 10⁸ to 10¹ copies µl⁻¹. Data were then
143 expressed as gene copy numbers *per* gram of dry weight soil after data correction using soil
144 sample humidity values.

2.4. PCR and pyrosequencing

147 The primer set 515F (5'-GTG CCA GCM GCC GCG GTA A-3') / 907R (5'-CCG TCA ATT
148 CMT TTR AGT TT-3') [32] was used to amplify the V4-V5 region of bacterial 16S rRNA
149 genes and the primer set ITS1F (5'-CTTGGTCATTTAGAGGAAGTAA-3') [33] / ITS2 (5'-
150 GCTGCGTTCTTCATCGATGC-3') [34] was used to amplify the ITS1 (Internal Transcribed
151 Spacer) region of fungal rRNA genes. Primers were combined at the 5' end with a MID
152 (Multiplex Identifier, Roche) corresponding to a sequence of 10 sample-specific nucleotides.
153 The PCR mixture (50 µl) contained 5X Taq-&Go™ ready to use PCR Mix (MP Biomedicals),
154 10 pM of each primer, and 2 µl of ten-fold diluted template DNA (or 2 µl of sterile water for
155 the negative control). Five independent PCR reactions were performed for each sample. The
156 PCR conditions were 94°C for 5 min; 30 cycles of 94°C for 30 s, 55°C or 50°C for 1 min,
157 72°C for 1 min, followed by 72°C for 10 min for primers 515F/907R or ITS1F/ITS2,
158 respectively. PCR efficiency was checked by agarose gel electrophoresis (1%) and visualized
159 after ethidium bromide staining under UV light with a GelDoc XR transilluminator (Bio-
160 Rad).

161 The five independent PCR products were pooled, purified using the QIAquick PCR
162 Purification Kit (Qiagen, France) and DNA was quantified by using a spectrophotometer
163 (UV1800, Shimadzu) equipped with a TrayCell unit (Hellma) and by visualization and
164 comparison with a mass ladder (Low DNA Mass Ladder, Invitrogen) on 1% agarose gels as
165 described above. Two equimolar mixes (multiplexed samples) from the 18 samples, one for
166 16S rRNA genes and one for ITS amplicons, were prepared in 50 µl to reach a total
167 concentration of 40 ng/µl. Pyrosequencing was performed by Beckman Coulter Genomics

168 (Danvers, MA, USA) using a 454/Roche GS-FLX Titanium system.

169

170 **2.5. Analysis of 16S rRNA genes and ITS pyrosequencing data**

171 **2.5.1. Bacterial community analysis**

172 Ribosomal Database Project (RDP, [35]) pyrosequencing pipeline was used for filtering and
173 trimming of 16S rRNA gene reads based on sff-files. The presence of both forward and
174 reverse primers (max. edit distance of 2) was checked to recover sense and anti-sense
175 sequences. At the end, 482,163 reads passed the length (longer than 300 bp) and quality
176 criteria (exclusion a reads with unresolved nucleotides and a low average quality score below
177 20) before being distributed into 18 samples according to their MIDs, the 16S rRNA gene
178 reads were filtered and trimmed according to different criteria. Mothur 1.29.2 program [36]
179 was used following previously described analyses steps [37]. Alignment of bacterial
180 sequences used the SILVA database as a template. Based on distance matrix generation, the
181 sequences were clustered and OTU (Operational Taxonomic Unit) were formed to 97%
182 sequence similarity. To compare all sample conditions, a sub-sampling to 18,100 sequences
183 per sample was performed. Finally, the taxonomic affiliation was obtained using the RDP
184 database as a template.

185

186 **2.5.2. Fungal community analysis**

187 For the fungal community, 360,477 quality reads of ITS were obtained from the Mothur-
188 based pipeline previously described by Counce et al. [38]. The fungal ITS sequences were
189 distributed into 18 samples according to their MIDs, the reads were cleaned in Mothur 1.20.1
190 with the default parameters [36]. ITS1 was extracted by using Fungal ITS extractor version 2
191 [39] and a filtration step by length (> 100 bp) was performed. Quality ITS reads were aligned
192 and clustered into OTUs (97% similarity) and consensus sequences were generated by using
193 Uclust version 3.0 (parameter iddef = 2.97% similarity). Then sub-sampling to 9,100
194 sequences per sample was performed. Consensus sequences were assigned against the UNITE
195 database by using the Basic Local Alignment search Tool algorithm Blastn version 2.2.2.3
196 [40].

197

198 **2.5.3. Diversity analysis**

199 Rarefaction curves were obtained using the Analytic Rarefaction 2.0 tool (Hunt Mountain
200 Software); richness and diversity indices were calculated using EstimateS 9.1.0 [41]. The
201 sequence data generated in this study were deposited in the NCBI Sequence Read Archive

202

203

204

205

206

202 (SRA) and are available under the BioProject ID: PRJNA269565 and PRJNA269566 for
203 bacteria and fungi, respectively

205 **2.6. Statistical analyses**

206 Statistical analyses were performed using XLStat2012 software (Addinsoft). A two-way
207 analysis of variance (ANOVA) followed by a Newman-Keuls post-hoc test was performed to
208 determine statistical differences ($p < 0.05$) in the microbial communities among the six
209 conditions, depending on time and the presence of a plant cover. Canonical correspondence
210 analysis (CCA) followed by a Monte Carlo permutation test (1000 permutations) in XLStat-
211 ADA 2015 (Addinsoft) was used to evaluate the relationship between environmental factors
212 (plant biomass, Log_{10} of bacterial and fungal abundances obtained through qPCR and edaphic
213 characteristics that were significantly different (ANOVA, $p < 0.05$) between samples). Two-
214 dimensional hierarchical clustering was performed on standardized data set (z-score) of the
215 most abundant ($> 2\%$ in at least one sample) bacterial and fungal OTUs (affiliation at 97%
216 similarity; i.e. 24 bacterial and 37 fungal OTUs) in the three replicates of the six conditions.
217 This was performed within the XLStat2011-Omics package (Addinsoft). Both samples and
218 OTUs were clustered with Ascending Hierarchical Classification based on distance metric
219 (euclidian), generating dendrograms. Heat maps were reordered by dendrogram clustering to
220 relocate covarying OTUs and samples.

222 **3. Results**

223 **3.1. Evolution of soil characteristics with time and plant colonisation**

224 The edaphic parameters of the NM soil evolved over time as described in Table 1. Over the
225 six years, pH values, C/N, available phosphorous, and humidity increased (or were higher at
226 T12), while PAH concentrations, extractable Mn and Zn decreased with time, and total metal
227 concentration remained unchanged.

229 **3.2. Bacterial and fungal abundance**

230 Over time, bacterial abundance (mean value of $1.69 \times 10^9 \pm 0.58 \times 10^9$ 16S rRNA gene copies
231 g dw soil^{-1}) was about ten-fold higher than fungal abundance (mean value of $2.14 \times 10^8 \pm 0.15$
232 $\times 10^8$ 18S rRNA gene copies g dw soil^{-1} ; Figure 1). Bacterial abundance was significantly
233 higher ($p < 0.05$) at T0 and then decreased with time. Conversely, fungal abundance remained
234 constant over time. In the Msm plots, no colonization of roots by mycorrhizal fungi was
235 observed at T4, while the frequency of mycorrhizal roots reached 24% at T12 and arbuscules

236 were observed (Table 1). The presence of vegetation had no impact on bacterial abundance,
237 while it enhanced fungal abundance: it was significantly higher ($p < 0.05$) in MsmT4 samples
238 when the biomass of alfalfa colonizing the Msm plots reached its highest value (1693 ± 156 g
239 plot^{-1} ; Figure 1). Consequently, the ratio fungi to bacteria was higher in MsmT4 soil
240 compared to the other samples.

3.3. Bacterial and fungal richness and diversity

241
242 After filtering steps, 88% and 82% of 16S rRNA genes and ITS reads were kept for further
243 analyses (Table 2). The rarefaction analysis revealed that plateau levels were not reached in
244 any of the samples (Supplementary Figure S1). Based on complete data, we found ten times
245 more OTUs for the bacterial (1,963 to 3,057 OTUs g dw soil^{-1}) than for the fungal community
246 (284 to 592 OTUs g dw soil^{-1} ; Table 2). Similarly, the Chao1 estimator of OTU richness
247 predicted a ten-fold higher richness for the bacterial community (mean of 6,350 estimated
248 species at T0) as compared to the fungal community (mean of 463 estimated species at T0;
249 Table 2). Bacterial richness was neither influenced by the presence of a plant cover or by time
250 (Table 2 and Supplementary Figure S1). By contrast, fungal richness increased significantly
251 ($p < 0.05$) with time and was significantly higher ($p < 0.05$) under the MsmT4 condition (Table
252 2). The Shannon and inverse of Simpson indices of bacterial and fungal diversity increased
253 significantly ($p < 0.05$) with time and with the presence of plants at MsmT4, as well as at T12
254 for fungi (Table 2).
255

256
257 Taxonomic assignment of OTUs allowed us to identify at least 17 bacterial phyla and 5 fungal
258 phyla (Figure 2). The bacterial community was mainly composed of *Proteobacteria* ($57.4 \pm$
259 13.4%), *Actinobacteria* ($13.2 \pm 7.7\%$), *Bacteroidetes* ($6.0 \pm 1.9\%$) and unclassified bacteria
260 ($2.4 \pm 1.0\%$). *Proteobacteria* were dominated by members of the *Gamma-* ($21.3 \pm 8.8\%$),
261 *Alpha-* ($19.0 \pm 1.5\%$) and *Beta-Proteobacteria* ($15.0 \pm 6.4\%$) classes. The fungal community
262 was mainly composed of *Ascomycota* ($53.6 \pm 9.4\%$), unclassified fungi ($41.3 \pm 8.2\%$) and
263 *Basidiomycota* ($4.2 \pm 5.2\%$).

264 We found 24 and 37 dominant bacterial and fungal OTUs, represented by at least 2% of the
265 sequences for at least one sample (Figure 3). They accounted for $28.6 \pm 7.5\%$ and $67.9 \pm$
266 3.3% of the bacterial 16S rRNA genes and fungal ITS sequences, respectively. At T0, the
267 bacterial community was dominated by OTU11 affiliated to the *Thiobacillus* genus (*Beta-*
268 *Proteobacteria*) and the fungal community was dominated by two OTUs affiliated to the

269 *Talaromyces* and *Scedosporium* genera (*Ascomycota*) and by a third OTU affiliated to
1 270 unclassified fungi.

3 271

5 272 **3.4. Modification of bacterial and fungal diversity over time**

7 273 The relative abundance of some bacterial phyla significantly ($p < 0.05$) increased
8
9 274 (*Bacteroidetes*, *Firmicutes*, *Nitrospirae*, *OD1*, *Gemmatimonadetes*, *Verrucomicrobia*) or
10
11 275 decreased (*Gamma-Proteobacteria*, *Beta-Proteobacteria*) over time, while others were
12
13 276 specifically increased at T4 (*Actinobacteria*, *Acidobacteria*, *Alpha-Proteobacteria*) (Figure
14
15 277 2). The relative abundance of *Glomeromycota* phylum increased significantly ($p = 0.007$) at
16
17 278 T12 (Figure 2). Moreover, the relative abundance of two orders belonging to the *Ascomycota*
18
19 279 (*Hypocreales* and *Pleosporales*) increased significantly ($p = 0.062$ and $p = 0.009$) over time. On
20
21 280 the contrary, the relative abundance of *Eurotiales* decreased significantly ($p < 0.001$) over time.

22 281

23 282 Clustering of samples according to the dominant OTUs (horizontal dendrogram, Figure 3)
24
25 283 showed higher similarity between samples at T0 than between samples at the two others
26
27 284 times. This clustering of T0 samples is mainly due to 19 OTUs, mostly belonging to bacteria
28
29 285 (Figure 3). The relative abundance of 12 bacterial and 10 fungal OTUs increased significantly
30
31 286 ($p < 0.05$) over time (Figure 3 and Table S1). The 12 bacterial OTUs were affiliated to one
32
33 287 *Actinobacteria* (*Arthrobacter*), one *Bacteroidetes* (*Ohtaekwangia*), one unclassified
34
35 288 *Firmicute*, one *Gemmatimonadetes* (*Gemmatimonas*), one *Nitrospirae* (*Nitrospira*), two
36
37 289 *Alpha-Proteobacteria* (unclassified *Erythrobacteraceae* and unclassified *Bradyrhizobiaceae*),
38
39 290 three *Beta-Proteobacteria* (*Rhodocyclaceae*, *Methyloversatilis* and unclassified
40
41 291 *Burkholderiales*), and two *Gamma-Proteobacteria* (unclassified and *Thiohalophilus*). The 10
42
43 292 fungal OTUs were affiliated to 5 different genera: 3 were affiliated to the *Ascomycota*
44
45 293 phylum, the *Pezizomycotina* subphylum and the *Sordariomycetes* class (*Acremonium*,
46
47 294 *Bionectria*, *Fusarium*), one belonged to unclassified *Basidiomycota*, and one was affiliated to
48
49 295 unclassified fungi. The relative abundance of 2 bacterial OTUs affiliated to the *Acidobacteria*
50
51 296 *Gp7* and *Ohtaekwangia* genera (*Bacteroidetes*) and of 7 fungal OTUs affiliated to *Phoma*,
52
53 297 unclassified *Plectosphaerellaceae* and unclassified fungi, increased significantly at T4 (Figure
54
55 298 3 and Table S1). The relative abundance of 9 bacterial and 3 fungal OTUs decreased
56
57 299 significantly ($p < 0.05$) over time (Table S1). Seven of these bacterial OTUs were affiliated to
58
59 300 *Gamma-Proteobacteria* and belonged to the orders *Acidithiobacillales* (*Thermithiobacillus*),
60
61 301 *Chromatiales* (*Thiohalophilus*, *Halothiobacillus*), *Xanthomonadales* (unclassified
62
63 302 *Xanthomonadaceae*, *Dyella*, *Rhodanobacter*), *Methylohalomonas* or unclassified *Gamma-*

303 *Proteobacteria*, one was affiliated to *Chloroflexi* (*Sphaerobacter*) and one to *Beta-*
304 *Proteobacteria* (*Thiobacillus*). The 3 fungal OTUs belonged to the *Ascomycota* phylum,
305 *Pezizomycotina* subphylum and *Eurotiomycetes* (*Talaromyces*) or *Sordariomycetes*
306 (*Pseudallescheria*) class, and one was affiliated to unclassified fungi (Figure 3).

308 **3.5. Modification of bacterial and fungal diversity with the presence of plant**

309 Clustering of samples according to the dominant OTUs (horizontal dendrogram, Figure 3)
310 showed high similarities in planted soils and between bare soils at T4 and T12. Clustering of
311 the dominant OTUs (vertical dendrogram, Figure 3) showed that plant development increased
312 the relative abundance of 15 and 11 OTUs, at T4 and T12 respectively, mainly belonging to
313 fungi. The relative abundance of other bacterial OTUs was increased in bare soil (Figure 3
314 and Table S1). The relative abundance of some bacterial phyla increased significantly
315 ($p < 0.05$) (*Actinobacteria*) or decreased (*Bacteroidetes*, *Nitrospira*, *Planctomycetes*, *Gamma-*
316 *Proteobacteria*, *OD1*, *Gemmatimonadetes*) with the presence of plants. Concerning fungal
317 phyla, the relative abundance of *Zygomycota* and *Glomeromycota* increased significantly in
318 alfalfa-planted plots ($p < 0.05$). Moreover, the fungal order *Microascales* was significantly
319 ($p < 0.001$) less represented in planted soils (Figure 3).

320
321 The relative abundance of 5 bacterial and 10 fungal OTUs increased significantly ($p < 0.05$)
322 with the presence of plants (Figure 3 and Table S1). These bacterial OTUs were affiliated to
323 one *Actinobacteria* (*Arthrobacter*), one unclassified *Firmicute*, one *Alpha-Proteobacteria*
324 (unclassified *Bradyrhizobiaceae*) and two *Gamma-Proteobacteria* belonging to the
325 *Xanthomonadaceae* family (unclassified and *Dyella*). Among the fungal OTUs, four belonged
326 to the *Sordariomycetes* class among the *Pezizomycotina* subphylum (*Acremonium*, *Bionectria*,
327 and *Fusarium* and unclassified *Plectosphaerellaceae*), and the other six were unclassified.
328 The relative abundance of 6 bacterial and 5 fungal OTUs decreased significantly ($p < 0.05$)
329 with the presence of plants (Table S1). These bacterial OTUs were affiliated to one
330 *Bacteroidetes* (*Ohtaekwangia*), one *Gemmatimonadetes* (*Gemmatimonas*), one *Nitrospiraea*
331 (*Nitrospira*), one *Alpha-Proteobacteria* (unclassified *Erythrobacteraceae*), and two *Beta-*
332 *Proteobacteria* (unclassified *Rhodocyclaceae* and *Burkholderiales*). Three fungal OTUs
333 belonged to the *Sordariomycetes* (*Phoma*, *Pseudallescheria*, *Scedosporium*) and two to
334 unclassified fungi (Figure 3).

336 **3.6. Multivariate analysis**

337 A Canonical correspondence analysis (CCA) was used to evaluate which variables best
1 338 explained the microbial community compositions (Figure 4). CCA ordination of bacterial and
2 339 fungal taxons (relative abundance at the order level) and environmental variables (plant
3 340 biomass, microbial abundance and edaphic characteristics of the samples) depict relationships
4 341 between the different plots and sampling dates. The first 2 CCA axes explained 69.63% of the
5 342 total variance in the microbial diversity data (Figure 4). The six T0 samples grouped together
6 343 and were separated from the other samples on the 1st axis (F1) discriminating the samples
7 344 according to the effect of time. These T0 samples were discriminated according to higher
8 345 bacterial abundance (16S), higher total calcium carbonate concentration, and higher
9 346 extractable zinc concentration. At T4, the three samples from the planted plots MsmT4 were
10 347 clearly separated from the BST4 samples on the 2nd axis (F2) discriminating the samples
11 348 according to the presence of plants, higher total N content and fungal abundance (18S). At
12 349 T12, although the samples were still discriminated, the difference between the Msm and BS
13 350 plots was thinner. T12 samples were discriminated according to higher total metal contents,
14 351 extractable Fe and Cu, higher pH, C/N ratio and available phosphorous.
15 352 At T0, four bacterial orders belonging to *Gamma-proteobacteria* (*Chromatiales*), *Beta-*
16 353 *proteobacteria* (*Acidithiobacillales* and *Hydrogenophilales*) and *Elusimicrobia* phyla
17 354 (*Elusimicrobiales*) and one fungal order (*Sporidiobolales*) belonging to *Basidiomycota* were
18 355 the most correlated with high extractable Zinc concentration, high CaCO₃ and high bacterial
19 356 abundance. Over 6 years part of the microbial community was modified and more affected by
20 357 changes in edaphic parameters. At T12, five bacterial orders belonging to five different phyla:
21 358 *Nitrospirae* (*Nitrospirales*), *Verrucomicrobia* (*Puniceicoccales*), *Actinobacteria* (*Euzebyales*),
22 359 *Acidobacteria* (Unclassified *Acidobacteria_GPI6*), and *Chloroflexi* (Unclassified
23 360 *Thermomicrobia*), and two fungal orders: Unclassified *Glomeromycota*, and Unclassified
24 361 *Basidiomycota*, were highly correlated with higher C/N ratio, higher pH, higher P₂O₅, higher
25 362 extractable iron and copper content and higher zinc and chrome concentrations. Moreover,
26 363 seven bacterial orders belonging to five different phyla: *Actinobacteria* (*Solirubrobacterales*
27 364 *Thermoleophilales*), *Chloroflexi* (Unclassified *Chloroflexi*), *Acidobacteria* (Unclassified
28 365 *Acidobacteria_GPI0*), *Planctomycetes* (Unclassified), and *Firmicutes* (Unclassified), were
29 366 highly correlated with higher copper and lead concentrations. At T4, most fungal orders were
30 367 positively correlated to alfalfa biomass and to the higher fungal abundance. Among these
31 368 seven orders, six belong to the Ascomycota phylum and *Pezizomycotina* sub-phylum and
32 369 belong to four different classes: *Dothideomycetes* (*Capnodiales*), *Eurotiomycetes*
33 370 (*Onygenales*), *Leotiomycetes* (*Helotiales*, *Thelebolales*), and *Sordariomycetes*

371 (*Chaetosphaeriales*, and Unclassified *Hypocreomycetidae*), and one to the *Basidiomycota*
372 phylum (*Tremellales*).

374 4. Discussion

375 4.1. Microbial diversity of the aged multi-contaminated NM soil

376 The NM soil had a high carbon concentration, neutral pH, low nutrient concentrations and
377 high concentrations of PAHs and heavy metals that explain its low fertility and high toxicity
378 [25]. This soil represented a unique ecosystem: contamination dated back to ca. 100 years, so
379 selection pressure on soil microorganisms had lasted for decades. While the short-term impact
380 of contamination tends to decrease microbial abundance, richness and diversity, we
381 hypothesized that in such aged multi-contaminated soil the microbial community would adapt
382 over time toward a unique diversity as compared to unpolluted soil.

383 In NM soil, bacteria were approximately ten-fold more abundant than fungi regardless of time
384 or of the presence of vegetation. The same trend was previously observed in the same soil
385 [42], and in many uncontaminated soils but with a higher difference between bacterial and
386 fungal abundance levels. In fact, the environments harboring such high fungal abundance and
387 a high fungal to bacterial ratio are usually rich in carbon, like the litter horizon in forest soils
388 [43]. The Chao1 index evidenced higher bacterial than fungal species richness in our NM soil
389 samples, as previously shown in other soils [44]. Freshly contaminated soils usually harbor
390 low bacterial richness, evenness and diversity after spiking [8]. Yet bacterial richness and
391 diversity in our NM soil was relatively high and comparable to the levels found in a diesel-
392 contaminated soil in Poland [45] and in an aged PAH-contaminated soil from a coking plant
393 wasteland in Beijing, China [46], using a similar sequencing effort. The result from our NM
394 soil suggests a long-term adaptation of the microbial community toward a diversified and
395 pollutant-resistant community. In addition, fungal richness was close to the richness found in
396 forest soils [38], but fungal diversity was relatively low and close to the level found in a soil
397 highly contaminated by hydrocarbons [6].

398 The dominance of 3 bacterial phyla (*Proteobacteria*, *Actinobacteria*, and *Bacteroidetes*) and
399 *Ascomycota* and unclassified fungi with poor representation of *Basidiomycota* is in
400 accordance with fingerprinting analyses of dominant bacterial and fungal species inhabiting
401 the NM soil [47];[23]. Overall, at the phylum level, the bacterial and fungal community
402 diversities were close to other soil types. However the relative proportion of each bacterial
403 phylum seems to be specific to polluted environments, contrary to fungal ones.
404 *Proteobacteria* are usually the dominant phylum in contaminated soils [7]. The co-occurrence

405 of *Alpha*-, *Beta*- and *Gamma-Proteobacteria* in similar relative proportions (15 - 21%) seems
406 typical of polluted soils: *Alpha*- are usually in higher proportions in non-contaminated soils,
407 while *Beta*- and *Gamma*- subdivisions are favored in hydrocarbon-contaminated soils [48].
408 Furthermore, *Actinobacteria* was the second most abundant phylum in our NM soil as found
409 in other heavy-metal contaminated soils [49];[7]. *Actinobacteria* were previously described in
410 extremely arid environments [50], with similar properties (low nutrient and low carbon
411 availability) to aged multi-contaminated soil. Finally, some genera belonging to the
412 *Actinobacteria* are able to degrade PAH compounds [51]. Members of *Acidobacteria* were
413 found scarce in our NM soil as found in other PAH-contaminated soil [46], but they were
414 largely less represented than in non-polluted soils [52];[53]. Overall, similar fungal phylum
415 diversity was also found in non-polluted soils [54].

4.2. Impact of edaphic parameters on soil microbial diversity over time

418 Although this result could be specific to this particular site under specific climatic condition,
419 the NM soil monitoring over 6 years revealed interesting relationship between bacterial and
420 fungal diversity and edaphic parameters. Our canonical correspondence analysis showed that
421 a temporal shift occurred in the composition of the soil microbial community, with some taxa
422 correlated with modifications in edaphic parameters.

423 Among the different soil parameters that increased simultaneously over time (C/N ratio,
424 available phosphorous, extractable iron, copper, chrome, zinc concentrations), the pH can be
425 an important driver of the bacterial community but not of the fungal one [55]. Most orders
426 belonging to *Acidobacteria* were favored over time, with subgroup-16 positively correlated
427 with pH values. Unlike the other subgroups the relative abundance of *Acidobacteria* subgroup
428 1 was greater at T0 and was negatively correlated with the pH level, as previously found [55].
429 Despite a low availability and mobility of PAH and HM, and significant decrease of these
430 fractions over time [25], we observed a significant decrease in total PAH (c.a. 40%
431 dissipation) and extractable Zn concentrations over the 6 years of monitoring that could also
432 contribute to explain changes in microbial diversity. Xu et al. [46] suggested that a decrease
433 in pollutant concentration allowed for the development of members of the *Acidobacteria*
434 phylum in a PAH-contaminated soil. The PAH decrease could also explain the emergence of
435 PAH-degrading microorganisms that benefited from the biodegradation of the pollutant. As
436 previously observed [47];[23], OTUs affiliated to the *Arthrobacter* and *Fusarium* genera
437 increased over time in NM soil samples. Members of the *Arthrobacter* and *Fusarium* genera
438 have been found in hydrocarbon-contaminated environments [56];[57] and could be potential

PAH degraders [51];[58]. The relative abundance of the *Nitrospirae* phylum, one *Nitrospira* OTU and the *Nitrospirales* order increased over time. On CCA it was positively correlated to the C/N ratio, and negatively correlated to total nitrogen content. This bacterial group is more competitive with other nitrite-oxidizing bacteria (i.e. *Nitrobacter*) in soils with low N availability [59]. Relative abundance of the *Gemmatimonadetes* phylum and one OTU affiliated to the *Gemmatimonas* genus also increased over time. Although members of this phylum are frequently detected in soils and more prevalent in dry soils with poor nutrient availability and low microbial activity [60], their ecological properties are still unclear. On the contrary, the relative abundance of other microbial taxa decreased over time. The fungal *Eurotiales* order and one OTU affiliated to *Talaromyces* (*Ascomycota*; *Eurotiales*) were more abundant at T0. This fungal taxon seems to be well adapted to high PAH contamination levels, as similar strains were isolated from an oil-contaminated soil and were able to grow at high PAH concentrations [61]. Within the bacterial community, the relative abundance of the *Gamma-Proteobacteria* phylum and of seven OTUs affiliated to *Gamma-Proteobacteria* decreased over time. Among these, *Thiohalophilus*, *Halothiobacillus*, *Methylohalomonas*, known as halophilic bacteria, could be repressed by the leaching of extractable metals and sulfate salts over time. The relative abundance of *Thiobacillus* (*Beta-proteobacteria*; *Hydrogenophilales*) was the most abundant bacterial OTU at T0 (ca. 15%), but also decreased over time. *Thiobacillus* was previously detected in heavy metal-contaminated soils [7]. The implication of the *Thiobacillus* genus in PAH degradation has also been described [62] although our results indicate a drastic decrease over time.

4.3. Impact of vegetation on soil microbial abundance and diversity

Our results show that the plant cover influenced microbial abundance in the NM soil, especially at T4 when the plant cover was the thickest. Although the role of root exudates in regulating soil fungal community composition and diversity is well known [63], the impact of the rhizosphere on increasing soil fungal abundance, as found here, is less documented as compared to bacteria [63].

The plant cover had an unexpected impact on fungal and bacterial community structure in the NM soil. At T4, PAH dissipation was significantly lower in presence of plants than in bare soil, probably explained by the release of more easily degradable carbon compounds in root exudates [64]. At T4, the community diversity is contrasted between bare and alfalfa planted soils, and the multivariate analysis showed that plant biomass is the dominant explicative factor compared to PAH content. Bacterial and fungal diversity indices increased with the

472 presence of vegetation, as previously shown [47];[23]. Although the impact of plants varies
1 473 depending on seasons, usually fungal and bacterial diversity usually decrease in the
2 474 rhizospheric or planted soils as compared to the bulk or unplanted controls [65];[66]. Lower
3 475 microbial diversity in the rhizosphere could be due to selective pressure from root exudates.
4 476 In aged hydrocarbon- and heavy metal-contaminated soils that are extremely oligotrophic
5 477 environments, microbial activity is limited by nutrient availability [67]. Most studies describe
6 478 the impact of plants on microbial diversity in non-contaminated and/or nutrient-rich soils (e.g.
7 479 agricultural or forest soils). The presence of plants on a nutrient-poor soil could increase
8 480 diversity due to the input of diversified carbon and nutrient sources, such as carbohydrates
9 481 from exudates and plant debris preferentially degraded by bacteria and fungi, respectively.
10 482 Moreover, the development of plants and roots creates numerous novel niches favoring the
11 483 establishment of more diverse microbial communities. As previously shown, the functional
12 484 community was also impacted by plant covers because enzymatic activity measured (FDase,
13 485 arylamidase, aryl sulfatase, cellulose, urease...) were higher in vegetalized NM soil than in
14 486 bare soil [42].

15 487 Our analyses allowed us to discriminate between planted soil and bare soil plots and to
16 488 identify microbial taxa whose relative abundance levels were differently impacted by the
17 489 presence of plants. *Actinomycetales* and *Arthrobacter*, the most represented order and most
18 490 abundant OTU of the bacterial community, were positively correlated to alfalfa biomass.
19 491 Increased numbers of *Actinobacteria* in the rhizosphere of different plant species such as
20 492 maize [66] and strawberry [68] are documented. In addition, the *Bradyrhizobiaceae* family
21 493 and the *Rhizobiales* order were among the dominant bacterial OTUs favored by the
22 494 development of alfalfa. These nitrogen-fixing bacteria are known to be abundant in the
23 495 rhizosphere of legumes due to their symbiotic association [69]. However we never observed
24 496 nodules throughout the monitoring period. Finally, the relative abundance of the *Nitrospirae*
25 497 phylum and *Nitrospira* OTUs, a well-known nitrite-oxidizing group [70], decreased in alfalfa-
26 498 planted plots. Competition for nitrogen between plants and nitrifying bacteria has already
27 499 been reported in unfertilized nutrient-poor soil [71].

30 500 Plants have a higher impact on fungal than on bacterial community structure. According to
31 501 our multivariate analysis, most fungal orders were positively correlated to the presence of
32 502 plants (alfalfa biomass). The relative abundance of *Ascomycota* belonging to the
33 503 *Pezizomycotina* sub-division and affiliated to various classes, *i.e.* *Sordariomycetes*,
34 504 *Leotiomycetes*, *Dothideomycetes*, and *Eurotiomycetes*, increased. Among the
35 505 *Sordariomycetes*, the *Chaetosphaeriales* and the unclassified *Hypocreales* were the most

506 important orders allowing for discrimination between treatments. Among these orders, the
1 507 relative abundance of some dominant fungal OTUs affiliated to the *Acremonium*, *Bionectria*,
2 508 and *Fusarium* genera significantly increased with the presence of alfalfa. While the
3 509 *Acremonium* genus is well-known as a fungal endophyte of plants that improves mineral
4 510 nutrition [72], *Bionectria* and *Fusarium* species are often plant pathogens [73];[74].
5 511 Conversely, in bare soil the *Microascales* order was the only one whose relative abundance
6 512 increased. Although alfalfa was co-inoculated with two mycorrhizal *Glomus* strains,
7 513 *Glomeromycota* was not a dominant phylum, but its relative abundance was higher in planted
8 514 plots. Our results show that unclassified *Glomeromycota* were more correlated with planted
9 515 soil after 6 years than after 2 years. Colonization of roots by mycorrhizal fungi was detected
10 516 only after 6 years, suggesting potential initial toxicity of the contaminated soil towards
11 517 mycorrhizal fungi, as suggested previously [25].
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23 518 **5. Conclusion**

24 519 To conclude, the high fungal and bacterial abundance levels in the NM soil probably reflect
25 520 its high carbon content due to contamination. Microbial diversity and richness in the aged-
26 521 contaminated soil were surprisingly high and closer to that found in an unstressed soil than in
27 522 freshly contaminated soil. A specific microbial community settled over time through
28 523 modifications of edaphic parameters, pollution content and due to the presence of plants. Over
29 524 time the C/N increased, due to a decrease of soil nitrogen content (no fertilization), favoring
30 525 *Nitrospirae* phylum, and nitrogen-fixing bacteria in planted soil. Over time the pH increased,
31 526 probably due to sulphate leaching that favoured some *Acidobacteria* groups and repressed
32 527 halophilic bacteria. In 6 years the decrease of PAH content by c.a. 40% favoured the
33 528 appearance of few taxa previously described as PAH degraders. The modification of pollution
34 529 content and bioavailability contributed probably to reduce environment toxicity and led to
35 530 adaptation in microbial community composition. The alfalfa cover structured the microbial
36 531 community, probably due to a rhizosphere effect while this partly inhibited the PAH
37 532 dissipation. Indeed vegetation (i) selected a specific community by releasing root exudates or
38 533 plant debris that input nutrient sources more easily degraded than PAHs in the oligotrophic
39 534 soil; (ii) increased bacterial diversity and (iii) favoured most fungal species compared to bare
40 535 soil.
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2
3 540 and Annie Buchwalter for English editing.
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6 541 **Tables**

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8
9 542 Table 1. Summary of the soil characteristics for the six conditions (mean and standard
10
11 543 deviation, n=3). Different letters indicate significant differences ($p<0.05$) among treatments
12
13 544 by two-way analysis of variance (ANOVA) followed by Newmans-Keuls multiple
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15 545 comparison test (nd: not determined).
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19 547 Table 2. Pyrosequencing data characteristics, and richness and diversity estimators (3%
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21 548 cutoff) of the bacterial and fungal communities in the six different conditions (means and
22
23 549 standard deviations, n=3): BS (Bare soil) and Msm (planted with *Medicago sativa* and
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25 550 inoculated with mycorrhizal fungi) at three time-points (T0: setting up, T4: after 2 years and
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27 551 T12: after 6 years). We created OTUs based on 97% sequence similarity (OTU₉₇) and
28
29 552 randomly subsampled 16S rRNA genes and ITS data to 18,100 and 9,100 reads *per* sample. A
30
31 553 two-way analysis of variance (ANOVA, $p<0.05$) followed by Newman-Keuls multiple
32
33 554 comparison test was performed on richness and diversity estimator data to determine
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35 555 statistical differences (lowercase) between treatments.
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37 556
38 557 Supplemental Table S1. Affiliation at 97% similarity of the most abundant (>2% in at least
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40 558 one sample) bacterial and fungal OTUs in the six conditions (mean and standard deviation,
41
42 559 n=3). Two-way analysis of variance (ANOVA) followed by Neuman-Keuls multiple
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44 560 comparison test to study the effect of plant cover and time on the most abundant bacterial
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46 561 (16S) and fungal (ITS) OTUs. Sections colored in dark grey / grey / light grey and white
47
48 562 mean respectively that OTUs were significantly ($p<0.05$) favored at all time-points / favored
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50 563 at T4 / inhibited or not influenced by the plant cover or by time.
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52 565 **Figure captions**

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55 566 Figure 1. Abundance of fungal and bacterial (18S and 16S rRNA gene copy numbers)
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57 567 communities and alfalfa biomass over time (T0: September 2005, T4: September 2007 and
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59 568 T12: September 2011) for the two BS (bare soil) and Msm (planted with *Medicago sativa* and
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569 inoculated with mycorrhizal fungi) treatments. Different letters indicate significant
1 570 differences among plots ($p < 0.05$) resulting from a two-way analysis of variance (ANOVA)
2 571 followed by Neuman-Keuls multiple comparison test ; abc, AB and A'B' refer respectively to
3 572 statistics on 16S rRNA gene copy numbers. g of dry weight soil⁻¹, 18S rRNA gene copy
4 573 numbers. g of dry weight soil⁻¹ and alfalfa biomass.
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10 574 Figure 2. Relative abundance of the dominant (represented by more than 10 sequences) fungal
11 and bacterial phyla in the six conditions (means of 3 replicates).
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14 576 Figure 3. Clustering analysis generating double dendrograms (vertically: OTUs dendrogram
15 and horizontally: sample conditions dendrogram) and heat map representation reflecting data
16 577 values of the most abundant (>2% in at least one sample) bacterial and fungal OTUs in the six
17 578 conditions (affiliation at 97% similarity). The relative abundance of 24 bacterial and 37 fungal
18 579 OTUs were normalised (z-score) to compare studied conditions (3 replicates per condition).
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25 581 Figure 4. Canonical correspondence analysis (CCA) ordination of bacterial (in green) and
26 fungal (in blue) taxons (relative abundance of the dominant orders represented by more than
27 582 10 sequences), and environmental variables (abundance of fungal 18S and bacterial 16S
28 583 rRNA gene copy numbers, biomass of dry alfalfa and edaphic characteristics (in red),
29 584 showing the relationships between the different plots and sampling dates (3 replicates of the 6
30 585 conditions, in black bold type). Numbers in *Italic* correspond to the quantity of OTUs that
31 belong to the taxon. Fungal and bacterial orders: *Acidi.*: *Acidimicrobiales*, *Acidithio.*:
32 *Acidithiobacillales*, *Actinomy.*: *Actinomycetales*, *Altero.*: *Alteromonadales*, *Bacill.*:
33 *Bacillales*, *Bdello.*: *Bdellovibrionales*, *Burkhol.*: *Burkholderiales*, *Caldi.*: *Caldilineale*,
34 586 *Capno.*: *Capnodiales*, *Caulo.*: *Caulobacterales*, *Chaetos.*: *Chaetosphaeriales*, *Chlamy.*:
35 *Chlamydiales*, *Chroma.*: *Chromatiales*, *Clostri.*: *Clostridiales*, *Dehalo.*: *Dehalococcoidales*,
36 587 *Desulfur.*: *Desulfuromonadales*, *Elusi.*: *Elusimicrobiales*, *Euro.*: *Eurotiales*, *Euzeb.*:
37 *Euzebyales*, *Flavo.*: *Flavobacteriales*, *Gemma.*: *Gemmatimonadales*, *Helot.*: *Helotiales*,
38 588 *Hydrogeno.*: *Hydrogenophilales*, *Hypo.*: *Hypocreales*, *Lactob.*: *Lactobacillales*, *Legion.*:
39 *Legionellales*, *Methylo.*: *Methylococcales*, *Microasc.*: *Microascales*, *Mortier.*: *Mortierellales*,
40 590 *Myxoc.*: *Myxococcales*, *Nitro.*: *Nitrospirales*, *Nitroso.*: *Nitrosomonadales*, *Oceano.*:
41 *Oceanospirillales*, *Onyg.*: *Onygenales*, *Opitu.*: *Opitutales*, *Parvu.*: *Parvularculales*, *Pseudo.*:
42 *Pseudomonadales*, *Punicei.*: *Puniceicoccales*, *Rhizo.*: *Rhizobiales*, *Rhodo.*: *Rhodobacterales*,
43 591 *Rhodocyc.*: *Rhodocyclales*, *Rhodospiri.*: *Rhodospirillales*, *Rickett.*: *Rickettsiales*, *Soliru.*:
44 *Solirubrobacterales*, *Sphaero.*: *Sphaerobacterales*, *Sphingo.*: *Sphingomonadales*,
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601 *Sphingobacter*.: *Sphingobacteriales*, *Sporidio*.: *Sporidiobolales*, *Syntro*.:
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2 602 *Syntrophobacterales*, *Theleb*.: *Thelebolales*, *Thermo*.: *Thermoanaerobacterales*, *Thermoleo*.:
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4 603 *Thermoleophilales*, *Thiotric*.: *Thiotrichales*, *Treme*.: *Tremellales*, *Un. Acido_Gp1*:
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6 604 *Unclassified Acidobacteria_Gp1*, *Un. Acido_Gp10*: *Unclassified Acidobacteria_Gp10*, *Un.*
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8 605 *Acido_Gp16*: *Unclassified Acidobacteria_Gp16*, *Un. Acido_Gp17*: *Unclassified*
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10 606 *Acidobacteria_Gp17*, *Un. Acido_Gp3*: *Unclassified Acidobacteria_GP3*, *Un. Acido_Gp6*:
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12 607 *Unclassified Acidobacteria GP6*, *Un. Acido_Gp7*: *Unclassified Acidobacteria_GP7*, *Un.*
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14 608 *Actino*.: *Unclassified Actinobacteria*, *Un. Bacteria*.: *Unclassified Bacteria*, *Un. Bacteroid*.:
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16 609 *Unclassified Bacteroidetes*, *Un. Basidio*.: *Unclassified Basidiomycota*, *Un. Chloro*.:
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18 610 *Unclassified Chloroflexi*, *Un. Firmi. Unclassified Firmicutes*, *Un. Fungi*: *Unclassified Fungi*,
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20 611 *Un. Glomero*.: *Unclassified Glomeromycota*, *Un. Hypocre*.: *Unclassified*
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22 612 *Hypocreomycetidae*, *Un. ODI*: *Unclassified ODI*, *Un. Plancto*.: *Unclassified*
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24 613 *Planctomycetes*, *Un. Strepto*.: *Unclassified Streptophyta*, *Un. Thermo*.: *Unclassified*
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26 614 *Thermomicrobia*, *Un. α -proteo*.: *Unclassified Alphaproteobacteria*, *Un. γ -proteo*.:
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28 615 *Unclassified Gammaproteobacteria*, *Un. δ -proteo*.: *Unclassified Deltaproteobacteria*,
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30 616 *Xantho*.: *Xanthomonadales*. Edaphic characteristics: extractable copper, iron, and zinc (Cu
ext, Fe ext, and Zn ext, respectively), 16 PAHs (mg/Kg of dry weight soil).

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32 618 Supplementary Figure S1. Rarefaction curve representing the number of operational
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34 619 taxonomic units (OTUs) depending on the number of 16S rRNA genes (A) and ITS (B)
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36 620 sequences amplified from the six conditions. After OTU formation to 97% similarity, sub-
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38 621 sampling was performed to 18,100 and 9,100 sequences *per* sample for bacterial and fungal
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40 622 data, respectively.

41 42 623 **References**

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Figure1
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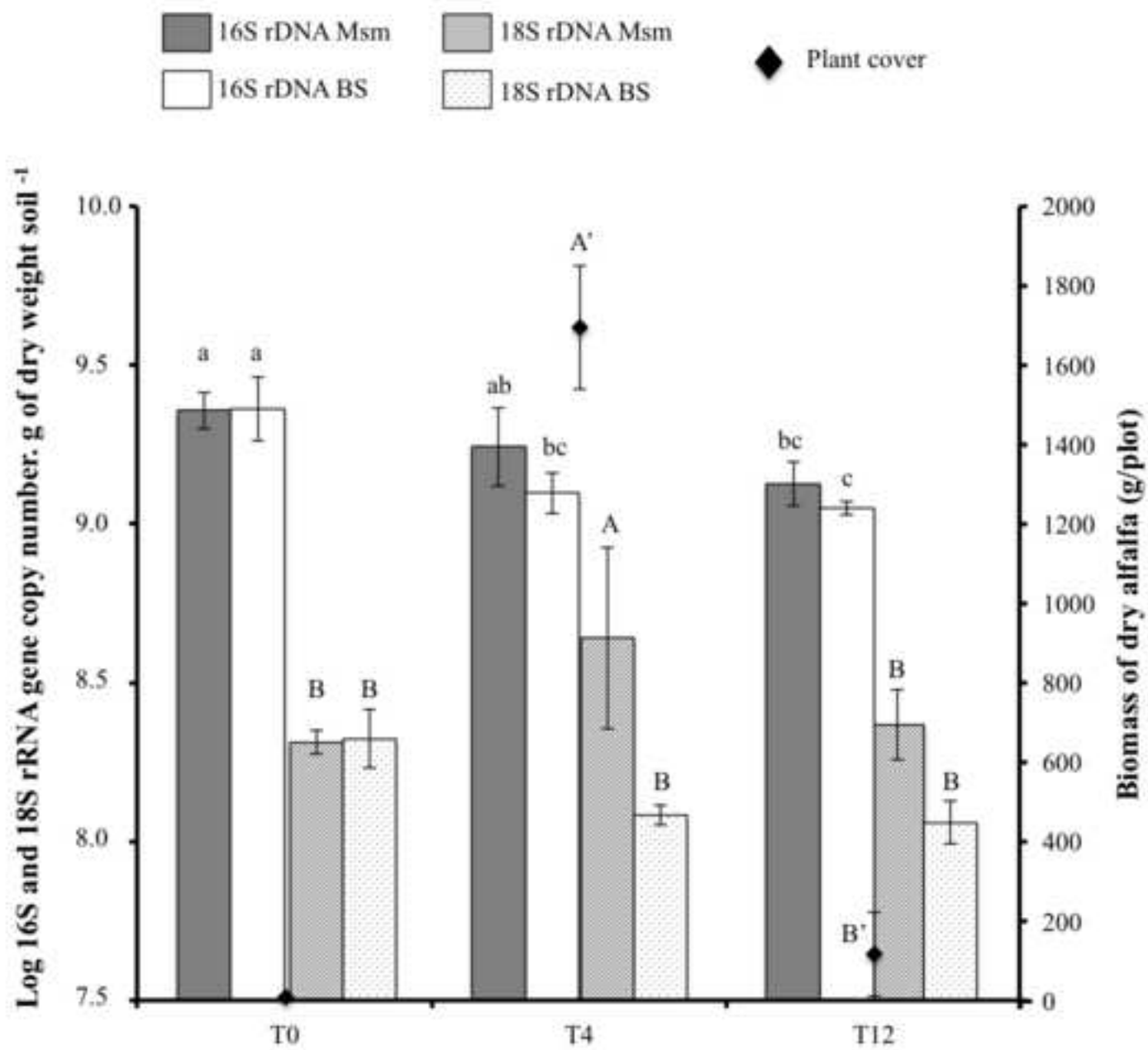


Figure2

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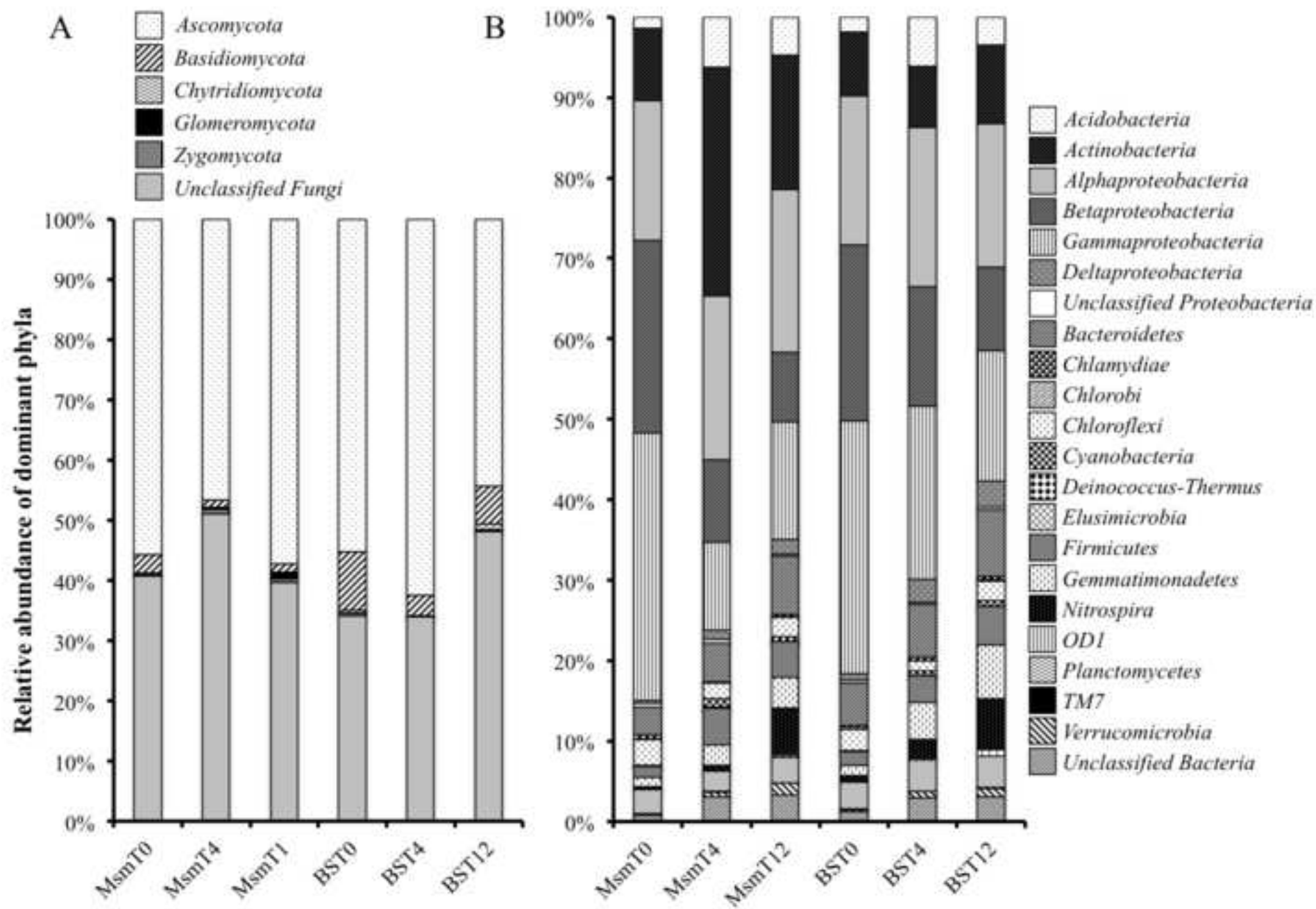


Figure4
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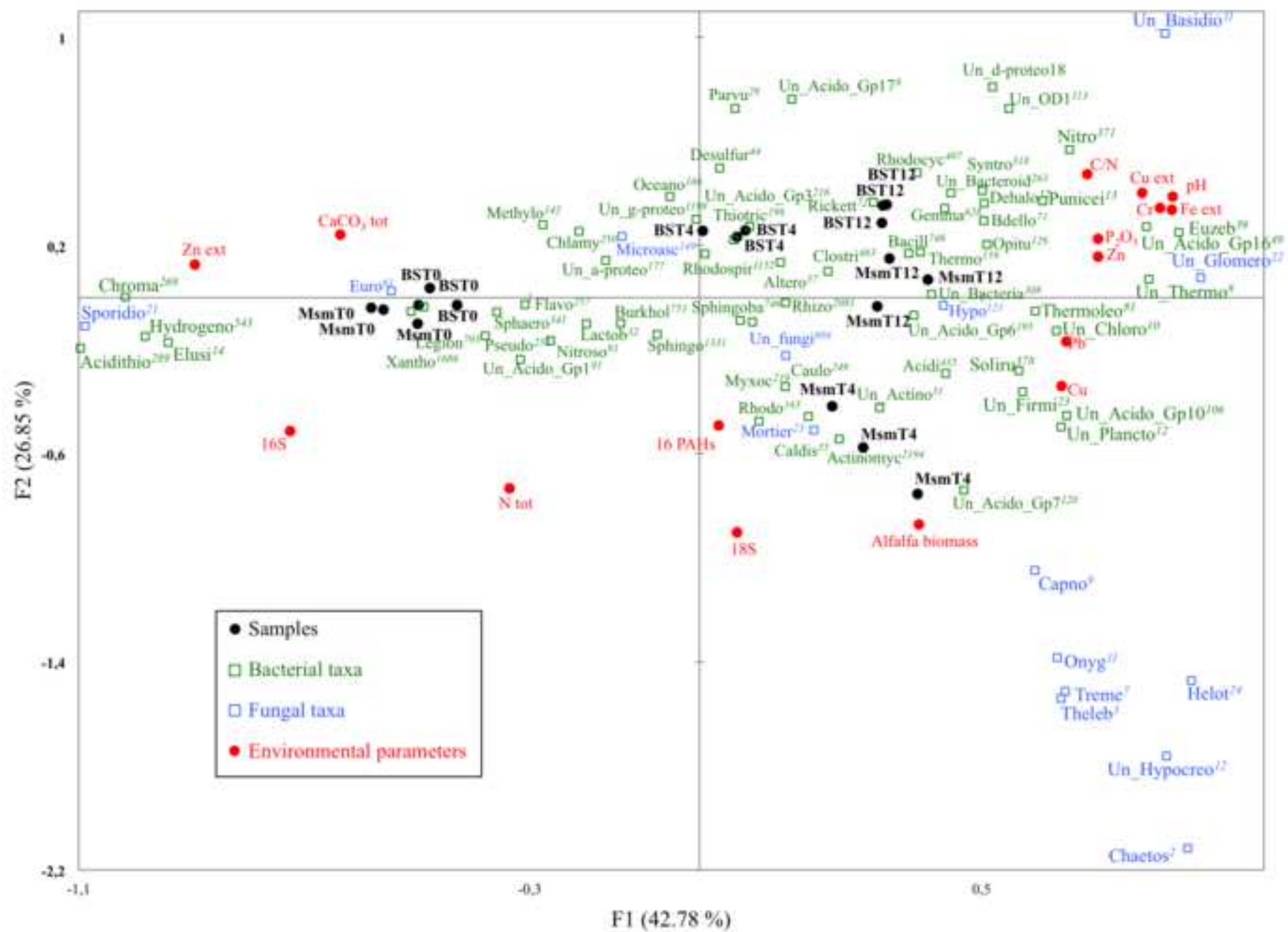


Table1

Condition	MsmT0	BST0	MsmT4	BST4	MsmT12	BST12
Sampling date	September 2005		September 2007		September 2011	
Humidity (%)	48.85 ± 7.83 ^b	44.74 ± 10.12 ^b	47.40 ± 5.81 ^b	58.65 ± 6.12 ^{ab}	64.37 ± 5.18 ^a	67.91 ± 3.89 ^a
Biomass of alfalfa (g)	0	0	1693.3 ± 156.3	0	109.0 ± 106.1	0
Mycorrhizal colonization						
Frequency of colonized roots (%)	nd	nd	0	nd	24.6 ± 10.1	nd
Frequency of arbuscules (%)	nd	nd	0	nd	15.6 ± 20.3	nd
N (g/Kg)	2.76 ± 0.14 ^a	2.73 ± 0.17 ^a	2.85 ± 0.07 ^a	2.51 ± 0.09 ^{bc}	2.66 ± 0.04 ^{ab}	2.40 ± 0.02 ^c
C/N	21.5 ± 0.7 ^b	22.2 ± 0.2 ^b	22.5 ± 0.8 ^b	23.5 ± 1.1 ^{ab}	25.4 ± 1.1 ^a	25.5 ± 1.7 ^a
TOC (g/Kg)	59.23 ± 1.45	60.67 ± 4.02	64.13 ± 1.88	59.07 ± 4.68	67.47 ± 2.68	61.27 ± 3.58
Organic matter (g/Kg)	102.33 ± 2.31	105.00 ± 6.93	111.00 ± 3.46	102.17 ± 8.28	117.00 ± 4.58	106.00 ± 6.08
pH	6.72 ± 0.16 ^c	6.96 ± 0.14 ^c	7.15 ± 0.07 ^{bc}	7.26 ± 0.15 ^b	7.47 ± 0.04 ^a	7.59 ± 0.01 ^a
CaCO ₃ (g/Kg)	14.83 ± 1.35 ^{ab}	18.40 ± 1.85 ^a	10.92 ± 1.29 ^b	13.93 ± 2.46 ^{ab}	10.10 ± 0.53 ^b	14.47 ± 3.86 ^{ab}
Available P ₂ O ₅ (Olsen) (g/Kg)	0.040 ± 0.004 ^b	0.048 ± 0.003 ^{ab}	0.055 ± 0.006 ^a	0.058 ± 0.009 ^a	0.055 ± 0.006 ^a	0.061 ± 0.007 ^a
Extractable metal (mg/Kg)						
Cu	10.28 ± 1.78 ^c	9.85 ± 0.21 ^b	12.03 ± 1.10 ^b	12.70 ± 0.50 ^b	13.03 ± 0.06 ^b	15.47 ± 0.23 ^a
Fe	82.00 ± 3.76 ^b	82.30 ± 4.71 ^b	97.00 ± 8.72 ^a	101.10 ± 4.25 ^a	99.83 ± 0.29 ^a	107.00 ± 3.00 ^a
Mn	435.33 ± 19.40 ^a	359.00 ± 73.75 ^b	126.00 ± 9.54 ^c	72.20 ± 6.39 ^c	64.40 ± 2.01 ^c	73.37 ± 2.97 ^c
Zn	242.67 ± 3.79 ^a	242.67 ± 9.07 ^a	152.67 ± 16.01 ^c	170.67 ± 7.64 ^{bc}	154.33 ± 8.50 ^c	188.67 ± 16.20 ^b
Metal (mg/Kg)						
B	9.2 ± 0.2 ^a	8.5 ± 0.3 ^b	5.4 ± 0.0 ^d	6.0 ± 0.2 ^c	3.8 ± 0.3 ^e	3.6 ± 0.1 ^e
Cr	658.7 ± 51.9 ^b	648.3 ± 23.5 ^b	783.7 ± 40.5 ^b	767.7 ± 84.1 ^b	1012.3 ± 61.9 ^a	1033.3 ± 75.1 ^a
Cu	140.7 ± 14.2 ^{ab}	131.0 ± 5.6 ^b	157.3 ± 4.2 ^a	147.0 ± 2.0 ^{ab}	146.7 ± 3.5 ^{ab}	146.3 ± 6.7 ^{ab}
Zn	1926.7 ± 55.1 ^b	2000.0 ± 187.3 ^b	2306.7 ± 20.8 ^{ab}	2300.0 ± 226.1 ^{ab}	2196.7 ± 40.4 ^{ab}	2416.7 ± 223.7 ^a
Pb	441.0 ± 5.6 ^c	451.0 ± 35.3 ^c	610.3 ± 19.5 ^a	596.0 ± 32.1 ^a	491.7 ± 6.4 ^{bc}	545.3 ± 56.0 ^{ab}
Σ16 PAH (US-EPA) (mg/kg)	1870.6 ± 146.5 ^a	1839.1 ± 262.3 ^a	2002.0 ± 100.5 ^a	1281.8 ± 314.2 ^b	1333.3 ± 57.7 ^b	1133.3 ± 152.8 ^b

Table2

	Conditions	Numbers of reads		Richness estimator		Diversity estimator	
		Raw data	Filtered data	Observed OTU ₉₇	Chao1	Shannon's index (H')	Inverse of Simpson's index (1/D)
16S	MsmT0	28922 ± 549	25784 ± 637	2658 ± 735	8173 ± 3599	5.46 ± 0.25 ^b	31.13 ± 8.04 ^c
	MsmT4	25205 ± 4287	21958 ± 3832	3057 ± 435	8433 ± 2061	6.38 ± 0.19 ^a	180.74 ± 24.73 ^a
	MsmT12	32767 ± 2910	28822 ± 2354	2754 ± 760	7301 ± 3622	6.22 ± 0.23 ^a	128.61 ± 11.77 ^b
	BST0	31608 ± 5355	27725 ± 4406	1963 ± 528	4527 ± 1931	5.45 ± 0.24 ^b	38.92 ± 10.08 ^c
	BST4	30640 ± 1828	26994 ± 1301	2402 ± 803	5610 ± 3373	6.06 ± 0.26 ^a	123.89 ± 6.03 ^b
	BST12	32736 ± 4867	29439 ± 4449	2762 ± 222	7136 ± 1228	6.10 ± 0.11 ^a	104.52 ± 14.24 ^b
	<i>Total of sequences</i>	<i>545631</i>	<i>482163</i>				
ITS	MsmT0	23887 ± 3613	19837 ± 2666	284 ± 26 ^c	452 ± 89 ^b	2.90 ± 0.18 ^c	7.20 ± 1.23 ^b
	MsmT4	26700 ± 13721	21722 ± 10983	592 ± 69 ^a	995 ± 157 ^a	4.20 ± 0.25 ^a	35.91 ± 19.50 ^a
	MsmT12	27495 ± 3256	22867 ± 2716	563 ± 80 ^a	977 ± 115 ^a	4.01 ± 0.27 ^a	16.39 ± 3.96 ^{ab}
	BST0	21020 ± 1517	17336 ± 868	294 ± 27 ^c	474 ± 117 ^b	2.98 ± 0.05 ^c	7.79 ± 0.28 ^b
	BST4	20951 ± 6250	16889 ± 4907	441 ± 11 ^b	728 ± 9 ^a	3.22 ± 0.10 ^c	7.21 ± 0.32 ^b
	BST12	26111 ± 3932	21508 ± 3276	519 ± 28 ^{ab}	847 ± 138 ^a	3.57 ± 0.10 ^b	18.39 ± 14.15 ^{ab}
	<i>Total of sequences</i>	<i>438492</i>	<i>360477</i>				

FigureS1

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TableS1

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