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► To cite this version:

Vanessa Alvarez-Lopez, Cyril Zappellini, Alexis Durand, Michel Chalot. Pioneer trees of *Betula pendula* at a red gypsum landfill harbour specific structure and composition of root-associated microbial communities. *Science of the Total Environment*, 2020, 726, pp.138530. 10.1016/j.scitotenv.2020.138530 . hal-02795934

HAL Id: hal-02795934

<https://hal.science/hal-02795934>

Submitted on 5 Jan 2022

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1 **Pioneer tress of *Betula pendula* at a red gypsum landfill harbour specific**
2 **structure and composition of root-associated microbial communities**

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11

12 **Keywords**

13 *Betula pendula*, *Populus tremula*, *Populus nigra*, *Salix purpurea*, native trees, Illumina
14 MiSeq,

15

16 **Abstract**

17 The study of root-associated microbial communities is important to understand the natural
18 processes involved in plant colonisation at degraded areas. Root associated bacterial and
19 fungal communities of woody species colonising a red gypsum landfill (a metal-enriched
20 environment) were characterised through metabarcoding. Among trees naturally growing
21 on the landfill, *Betula pendula* is the only tree species in the centre of the area, whereas

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22 companion tree species such as *Populus nigra*, *P. tremula* and *Salix purpurea* were
23 present on the edges. The bacterial community was dominated by *Proteobacteria* (38%),
24 *Actinobacteria* (35%) and *Bacteroidetes* (20%) and the most abundant bacterial OTU
25 belonged to the family *Streptomycetaceae*. The fungal community was dominated by
26 *Ascomycota* (60%) and *Basidiomycota* (30%) and the most abundant family was
27 *Pyronemataceae*. Analysis of similarities, heatmap and hierarchical cluster analysis
28 showed that *B. pendula* grown in the centre of the landfill harboured a specific microbial
29 community, which was unique and different, not only from other tree species (*Populus* or
30 *Salix* spp.), but also from other *B. pendula* growing at the edges. Our findings on relevant
31 indicator OTUs associated to the birches located in the centre of the landfill (such as
32 Otu00716 *Catellatospora* sp (family *Micromonosporaceae*, phylum *Actinobacteria*) or
33 Otu4_35502 *Russula* sp. (family *Russulaceae*, phylum *Basidiomycota*)) may have
34 important implications for the successful revegetation of these harsh environments using
35 microbial-based phytostabilisation approaches.

36

37

38 **1. Introduction**

39 Gentle Remediation Options (GRO) focus on the use of plants in combination with
40 associated microorganisms for the remediation and revitalisation of contaminated sites
41 (Kidd *et al.*, 2015). Metal(loid) toxicity can severely limit the performance and
42 establishment of plants in polluted areas and therefore, the selection of metal-tolerant
43 plant species able to successfully establish and grow at each specific site is of vital
44 importance for the success of GRO. Sites contaminated with potentially toxic elements
45 (PTE); such as mine tailings, spoils or landfills (made-up of by-products of metal
46 extraction) are important sources of metal-tolerant plant genotypes. Red gypsum landfills
47 are made of by-products of titanium refining; mainly used for the TiO₂ pigment industry
48 and enriched in S, Fe and Mn (Zappelini *et al.*, 2018, Zapata-Carbonell *et al.*, 2019).
49 Despite the unfavourable growth conditions at these sites, several metallophyte plants
50 have evolved mechanisms to tolerate these conditions and are able to colonise these types
51 of substrates (Whiting *et al.*, 2004; Batty, 2005; Mendez and Maier, 2008). Fast-growing
52 trees are ideal plants for the revegetation of these sites due to their extensive root systems,

53 rapid growth, large biomass production and easy harvesting with subsequent re-sprouting
54 (Peuke and Rennenberg, 2005).

55 In the last years, an increasing number of studies including the use of native
56 pioneer trees for the revegetation of degraded lands is found in the (Fernandez *et al.*,
57 2017; Mleczek *et al.*, 2017; Asension *et al.*, 2018) A greater understanding of the
58 mechanisms, which influence the response of plants to the local conditions frequently
59 found at these sites, could help to improve the efficiency of GRO techniques. Although
60 an increasing number of studies have addressed the influence of the associated microbial
61 community in plant metal tolerance, its role in harsh environments remains unravelled.
62 Studies approaching the description of plant-associated microbial communities are useful
63 as a first approximation to understand these interactions. Plants interact closely with
64 microbes, and these can enhance plant growth and health by increasing nutrient uptake
65 and improving plant resistance to pathogens and stress (Weyens *et al.*, 2009; Kidd *et al.*,
66 2017). Plant growth-promoting microorganisms include nitrogen-fixing bacteria,
67 biocontrol microorganisms or mycorrhizal fungi, among others (Coninx *et al.*, 2017; Kidd
68 *et al.*, 2017). Plant associated microorganisms can also improve nutrient availability
69 through the production of organic acids and siderophores or phosphate solubilisation.
70 Bacteria can also produce phytohormones, such as auxins, cytokinins and gibberellins,
71 which stimulate plant growth and development (Tanimoto, 2005; Taghavi *et al.*, 2009).
72 Plant associated bacteria can also reduce plant stress through the synthesis of 1-
73 aminocyclopropane-1-carboxylic acid (ACC) deaminase which reduces the level of
74 ethylene in stressed plants (Glick, 2014). Other well-known mechanisms of PGPBs
75 include increasing plant water uptake, alteration of root morphology, production of
76 antibiotics and the induction of plant defence mechanisms (van Loon and Bakker, 2003;
77 Kidd *et al.*, 2009).

78 Ectomycorrhizal fungal (ECM) are the dominant group of root symbionts of
79 pioneer trees that attempt to colonise heavily contaminated sites (Colpaert, 2008). Fungal
80 endophytes also play a key role on primary colonisation and subsequently on
81 phytoremediation. It has been observed on contaminated sites that pioneer populations of
82 mycorrhizal birches, willows, poplars or pines are able to successfully establish (Vrålstad
83 *et al.*, 2002; Adriaensen *et al.*, 2004; Colpaert *et al.*, 2004). Root-associated arbuscular
84 mycorrhizal fungi (AMF) usually improve the growth of plants in non-fertile soils by
85 increasing the exchange surface between plant and soil (Smith and Read, 1996). AMF
86 have been mostly studied for their role in plant growth, mainly due to their role in plant

87 P uptake from soils (Smith and Read, 2002). Moreover, AMF are able to alleviate metal
88 toxicity in plants (Hildebrandt *et al.*, 2007). Other key soil microorganisms such as
89 saprotrophic fungi are central in soils due to their capacity to degrade recalcitrant organic
90 compounds and may strongly interact with soil bacteria (Baldrian, 2008). Saprotrophic
91 fungi may also have a potential in mobilising metals from contaminated soils, due to their
92 ability to exudate chelating agents (Arwidsson *et al.*, 2009).

93 Further interactions between bacteria, fungi and plants have been shown to
94 contribute to microbial community stability (Bonfante and Anca, 2009; Bell *et al.*, 2014)
95 and many bacterial strains have indeed been reported to promote mycorrhizal symbioses
96 (Frey-Klett *et al.*, 2007; Hrynkiewicz *et al.*, 2010).

97 Here, we characterised the root microbial communities associated to *Betula*
98 *pendula*, which is the pioneer colonising vegetation at a red gypsum landfill in the region
99 of Thann in NE France (Zapata-Carbonell *et al.*, 2019). At this site, *B. pendula* is the only
100 woody species that had successfully established across the whole landfill while other
101 woody species showed only sparse growth at some specific spots (Zapata-Carbonell *et*
102 *al.*, 2019). Moreover, in previous studies *B. pendula* trees growing at this site have been
103 shown to efficiently accumulate Mn and therefore this species is a promising candidate
104 to be used as biocatalyst. The present study hypothesised that *B. pendula* hosts a specific
105 root-associated microbial community responsible for its high adaptability to the soil
106 conditions at this site. Therefore, the objective of this research was to analyse the structure
107 of these communities using high-throughput sequencing technologies (Illumina next-
108 generation sequencing).

109

110 **2. Material and methods**

111 2.1. Study site

112 The study site is located in the southern part of the Alsace region (47°47'47.7"N
113 7°08'18.5"E) near the city of Thann (NE of France). Here, Cristal France produces TiO₂
114 (an important pigment) and TiCl₄ (an excellent Lewis acid catalyst). The Thann plant
115 produces TiO₂ through a sulphate-based technology and TiCl₄ through a chlorine process
116 leading to the production of acidic effluents with a high load in trace metals. The
117 extractive industry led to the production of by-products, such as red gypsum that are
118 stored on large and open landfills. Red gypsum is produced by the neutralisation of waste-
119 waters with limestone and is characterised by high Mn and Fe concentrations and elevated

120 pH (Asad *et al.*, 2017). *Betula pendula* was found to be the dominant (> 80%) plant
121 species that had naturally recolonised the D1 landfill area after the deposit of red gypsum
122 ceased in 2006 (Zapata-Carbonell *et al.*, 2019). According to the “*National methodology*
123 *for the management of polluted sites and soils*”, (2017) developed by the French Ministry
124 of Environment, Energy and Sea, and based on the data recorded by Zapata-Carbonell *et*
125 *al.*, (2019), the D1 area presents values higher than those found in natural soils for Cd,
126 Co, Cr, Cu, Pb, Zn and (especially) for Ti.

127

128 2.2. Sampling procedure

129 All soil and plant samplings were performed in October 2016 in the D1 landfill area
130 (described previously by Zapata-Carbonell *et al.*, 2019). Roots and shoots of *B. pendula*
131 were collected across the D1 area. Ten individual plants of *B. pendula* were sampled at
132 the centre of the landfill where *B. pendula* is the only tree growing. One soil sample was
133 taken from the root system of each plant. For comparative purposes, 10 individual trees
134 of other woody species growing at the edges (*Populus tremula*, *P. nigra*, *Salix purpurea*)
135 were sampled at three specific spots. Moreover, 10 individual plants of *B. pendula*, which
136 were found growing in close proximity to these species at the edges, were also sampled.
137 These plant combinations were always growing at the same point and only one soil
138 sample was taken for each pair of woody species. Soil samples (although not rhizospheric
139 soils) were collected in the close proximity of the roots. To clarify, we named *B. pendula*
140 associated to *P. tremula* as *B. pendula*-(Pt), those associated to *P. nigra* as *B. pendula*-
141 (Pn) and those associated to *S. purpurea* as *B. pendula*-(Sp). Birch plants sampled at the
142 centre were named as *B. pendula* (Fig. 1).

143

144 2.3. Soil physicochemical properties and elemental composition of leaves

145 Soil pH and extractable-element concentrations were determined in the < 2mm fraction
146 of the dried soils. Soil pH was measured in H₂O using a 1:2.5 soil:solution ratio after 30
147 min agitation. To determine the extractable fractions of soil macro- and micro-elements,
148 5 g of 2 mm sieved soil were incubated with 50 mL of 10 mM CaCl₂ under agitation for
149 3 h at room temperature. The mixture was filtered with ash-free filters and acidified at
150 2% (v/v) with HNO₃ before inductively coupled plasma atomic emission spectrometry
151 analysis (ICP-AES, Thermo Fischer Scientific, Inc., Pittsburgh, PA, United States).

152 Plants were thoroughly washed with pressurised tap water to remove any adhering
153 soil particles, and rinsed with deionised water. Leaves were dried for 48 h at 40 °C and
154 0.1 g of dry material was digested in a 4:1 HNO₃:H₂O₂ mixture. Shoot nutrients and trace
155 elements were measured also by ICP-AES.

156 2.4. Molecular methods

157 The roots were carefully washed with tap water to remove visible soil and rinsed with
158 sterile deionised water. The fine roots were then cut off and transferred to Eppendorf
159 tubes and frozen at -20 °C. DNA was extracted from roots of 10 single individual trees
160 from each plant species at each sampling point. To isolate DNA from the roots, plant cell
161 lysis was first performed using 50 mg of frozen root samples that were pulverised with 3-
162 mm tungsten carbide beads (Qiagen S.A.S., Courtaboeuf, France) in a Mixer Mill for 3
163 min at 30 Hz (modelMM400; Retsch Inc., Newtown, PA). Root DNA was then extracted
164 with a PowerSoil® DNA Isolation Kit (MO-BIO Laboratories, Inc., Carlsbad, CA USA).
165 To improve DNA extraction, an additional step of 10 min at 60°C was realised after
166 addition of C1 solution. The DNA quality and quantity were assessed by agarose gel
167 electrophoresis and with the Quant-iT™ PicoGreen® dsDNA Assay Kit (Invitrogen,
168 Carlsbad, CA, USA) using an FLX-Xenius spectrofluorometer (SAFAS, Monaco). Two-
169 step PCR amplifications were performed with the bacterial primers 341F (5'- CCT ACG
170 GGR SGC AGC AG -3') and 802R (5'- GAC TAC HVG GGT ATC TAA TCC -3') (Baker
171 *et al.*, 2003; Beckers *et al.*, 2016) and the fungal primers ITS1F (5'- CTT GGT CAT TTA
172 GAG GAA GTA A -3') and ITS2 (5'- GCT GCG TTC TTC ATC GAT GC -3') (White
173 *et al.*, 1990; Gardes, 1993). The cycling conditions for 16S rRNA gene amplification
174 involved an initial 3 min denaturing step at 94°C followed by 30 cycles of 25 s at 94°C,
175 25 s at 43.6°C, and 1:30 min s at 72°C. The cycling conditions for ITS genes included an
176 initial 3 min denaturing step at 94°C followed by 30 cycles of 25 s at 94°C, 25 s at 45.4°C,
177 and 1:30 min s at 72°C. The sequencing of the V3-V4 domains of 16S rRNA genes and
178 the fungal ITS1 was performed with an Illumina MiSeq platform (Microsynth AG,
179 Switzerland).

180 2.5. Data analysis

181 Bioinformatics processing of the bacterial datasets was carried out using the MOTHUR
182 pipeline (Schloss *et al.*, 2009). Raw reads were filtered by length and quality and 16S
183 rRNA gene reads were pre-clustered using sumaclust (Mercier *et al.*, 2013) at 0.99

184 identity. Sequences with less than 8 reads were discarded, then remaining sequences were
185 aligned with those present in the Silva database to remove non-16S rRNA gene
186 sequences, and plant DNA contamination was removed by suppressing reads identified
187 as k__Bacteria (100), p__Cyanobacteria (100), c__Chloroplast (100), o__Streptophyta
188 (100), and unclassified (100). Taxonomic assignments were made using a Bayesian
189 approach (Wang *et al.*, 2007) with the Greengenes database (DeSantis *et al.*, 2006).
190 Finally, OTUs classification was derived using the Needleman distance and average
191 neighbour clustering at a distance of 0.03.

192 Fungal Sequence de-multiplexing and bioinformatics processing of the datasets
193 were performed using the PIPITS pipeline (Gweon *et al.*, 2015). This pipeline uses the
194 most recent RDP Classifier to classify sequences against the curated UNITE fungal
195 dataset. As for bacteria, fungal sequences were filtered by length and quality and clustered
196 into OTUs, defined at 97% similarity. Singleton OTUs were excluded (Dickie, 2010;
197 Tedersoo *et al.*, 2010) and the taxonomic assignment of OTUs was performed using the
198 UNITE (Kõljalg *et al.*, 2013) database at a 97% similarity threshold.

199 2.6. Statistical analysis

200 All statistical analyses were performed using R software v. 3.0.2 (R Core Team, 2013).
201 The Shapiro test was used to verify the normality of the data. Analysis of variance
202 (ANOVA) was used to detect significant differences in shoot and soil measured
203 parameters.

204 Richness and diversity indices were calculated based on a data set in which the
205 numbers of reads per sample were rarefied to 6972 and 28129 reads per sample for
206 bacteria and fungi, respectively. Rarefaction curves were created with the “rarecurve”
207 function in the Vegan package. NMDS was calculated using the Bray Curtis method (k =
208 3) using the “metaMDS” function in the Vegan package. Results of relative abundance
209 of the most abundant OTUs were paired with a heatmap of Spearman’s correlations
210 created with the “heatmap.2” function using the gplot package. The numbers of OTUs
211 that were shared between plants were visualised using Venn diagrams implemented by
212 “VennDiagram” in the Vegan package. Indicator species analysis was performed using
213 the “multipat” function of the indicpecies package (De Caceres and Legendre, 2009). An
214 ANalysis Of SIMilarities (ANOSIM) was carried out to obtain P (significance levels) and
215 R (the strength of the factors on the samples) values using the “anosim” function with the
216 vegan package. A spearman correlation was carried out to determine the relationships

217 between soil/plant physicochemical characteristics and the abundant OTUs and between
218 fungal and bacterial OTUs. Differences in family and OTUs abundance between plants
219 were analysed using the Kruskal-Wallis test.

220 **3. Results**

221 **3.1. Soil physicochemical properties and elemental composition of leaves**

222 Supporting Information Table S1 and Supporting Information Table S2 present the main
223 soil properties as well as the leaf macro- and micro-nutrients used in subsequent statistical
224 analysis as environmental variables. In the case of trees growing on the edges, only one
225 soil sample for each pair of plant species has been studied. Briefly, soils present a strongly
226 alkaline pH (between 7.8 up to 10). The main CaCl₂-extractable elements found at the
227 landfill were Mg > K > Fe > Mn > P > Cu and a relation between soil pH and CaCl₂-
228 extractable Mn and Fe was clearly observed. Supporting Information Table S2 evidences
229 the Mn accumulation capacity of *B. pendula*, showing the highest levels of Mn leaf
230 concentration (up to 570 mg kg⁻¹). Moreover, this accumulation was not related to the site
231 of growth (neither to the pH nor to the CaCl₂-extractable fraction of Mn) (Table S1 and
232 Table S2). Plants of birch also tended to accumulate a higher P concentration in their
233 leaves than the other plant species growing at the same sampling points although this
234 difference was not significant (Table S2).

235 **3.2. Richness and diversity of root bacterial and fungal communities for the 4 woody** 236 **species**

237 Following total genomic DNA extraction from root samples, amplicons of the V3-V4 16S
238 rRNA gene region were generated and a total of 5,262,333 paired-end reads were obtained
239 through Illumina MiSeq sequencing (Supporting Information Table S3). After carrying
240 out a quality, chimera, singleton and contaminant filter, a total of 907,006 filtered and
241 non-chimeric bacterial sequences constituted our final processed dataset, spread among
242 1,325 non-singleton OTUs defined by representative DNA sequences with sizes of 231
243 to 257 bp (mean = 237 bp). After subsampling, the dataset was rarefied to 6,972 reads per
244 sample (among the samples from each tree, those exhibiting a low sequence count were
245 eliminated from the rest of the analysis), which were distributed among 1,322 non-
246 singletons OTUs. Amplicons of the ITS region were generated and a total of 5,581,697
247 paired-end reads were obtained (Supporting Information Table S4). A total of 5,148,296

248 filtered and non-chimeric fungal sequences constituted the final processed dataset, spread
249 among 613 non-singleton OTUs defined DNA sequences with sizes above to 100 bp.
250 After subsampling, the dataset contained 28,129 reads per sample, distributed among 577
251 non-singletons OTUs.

252 Rarefaction curve analysis showed that all samples approached an asymptote in
253 both the bacterial and fungal dataset, revealing that the overall diversity was well
254 represented (Supporting Information Fig. S1). Moreover, the measured Good's coverage
255 values (an estimator of completeness of sampling) were greater than 98% for each sample
256 type in the case of the bacterial dataset, and 100% for the fungal dataset (Supporting
257 Information Table S3 and S4 respectively).

258 Richness and diversity estimations were calculated for each dataset (Table 1 and
259 Table 2). Both bacterial richness (based on Chao1 estimator and number of observed
260 OTUs) and diversity indices (estimated through the Shannon Index, Inverse Simpson
261 Index and Evenness) were generally lower in the roots of the *B. pendula* plants collected
262 at the centre of the landfill than in the roots of all other trees collected at the edges. The
263 highest bacterial richness and diversity indices were found to be associated with *P.*
264 *tremula* (Table 1). Fungal richness and diversity (Table 2) followed the same trend as for
265 bacteria and were generally lower in the roots of the *B. pendula* growing at the centre of
266 the landfill. In this case, both richness indices (Chao1 and number of observed OTUs)
267 were highest in the roots of all birches growing at the edges.

268 **3.3. Effects of plant species and growth location in microbial structure and** 269 **composition**

270 The bacterial dataset comprised a total of 17 phyla, 151 families, and 246 genera (17, 79
271 and 100 taxonomically identified respectively). The bacterial community was dominated
272 by *Proteobacteria* (38%), *Actinobacteria* (35%) and *Bacteroidetes* (20%). Only 26 (20
273 identified) families and 31 (14 identified) genera presented an abundance of higher than
274 0.5%. The fungal dataset was composed of a total of 7 phyla, 138 families, and 211 genera
275 (5, 98 and 128 taxonomically identified respectively). The fungal community was
276 dominated by *Ascomycota* (60%) followed by *Basidiomycota* (30%). Only 18 (4
277 identified) families and 19 (5 identified) genera presented an abundance higher than 0.5%.

278 Figure 2 shows a barplot for the 20 most abundant bacterial (Fig. 2a) and fungal
279 (Fig. 2b) families for each studied tree. The three most abundant identified families were
280 *Cytophagaceae* (19%), *Streptomyetaceae* (16%) and *Hyphomicrobiaceae* (15%) (Fig.

281 2a) and the three most abundant identified genera were *Lentzea* (7.11%), *Devosia* (5.52%)
282 and *Rhodoplanes* (3.33%) (data not shown). The roots of *B. pendula* showed a slight
283 enrichment in members belonging to the genera *Kribella*, *Rhodoplanes* and *Methylibium*
284 compared to the other trees (data not shown). The most abundant identified fungal family
285 corresponded to *Pyronemataceae* (33%), followed by *Russulaceae* (15.5%). The next
286 identified family was *Thelephoraceae*, although present in a much lower abundance
287 (2.0%) (Fig. 2b). The most abundant genera were *Geopora* (33.1%), *Lactarius* (15.1%)
288 and *Tomentella* (2.0%). Roots of *B. pendula* showed a slight increase in members of the
289 genera *Oidiodendron* and *Geopora* (data not shown).

290 Figure 2 shows a clear pattern in the distribution of families between each pair of
291 neighbouring trees (*P. tremula* - *B. pendula* (Pt), *P. nigra* - *B. pendula* (Pn) and *S.*
292 *purpurea* - *B. pendula* (Sp)). However, due to the high data scattering, few of these results
293 were significant. In the case of the bacterial dataset, the statistical differences were mainly
294 associated to *P. nigra* and the neighbour birch (named as *B. pendula* (Pn)). These trees
295 harboured a higher number of members of the families *Actinosynnemataceae* (Kruskal
296 Wallis $\chi^2=37.9$, $p=1.2\times 10^{-6}$), *Glycomycetaceae* (Kruskal Wallis $\chi^2=34.61$, $p=5.2\times 10^{-6}$)
297 and *Comamonadaceae* (Kruskal Wallis $\chi^2=20.6$, $p=2.1\times 10^{-3}$) and lower members of the
298 families *Pseudonocardiaceae* (Kruskal Wallis $\chi^2=32.4$, $p=1.4\times 10^{-5}$) and
299 *Methylocystaceae* (Kruskal Wallis $\chi^2=39.2$, $p=6.6\times 10^{-7}$) than all the other trees. In the
300 case of the fungal dataset the statistical differences were observed only for two families
301 *Pyronemataceae* (Kruskal Wallis $\chi^2=24.4$, $p=4.4\times 10^{-4}$) and *Sebacinaceae* (Kruskal
302 Wallis $\chi^2=20.5$, $p=2.3\times 10^{-3}$), which showed respectively a lower and higher number in
303 roots associated to *P. tremula* and the nearby growing birch (*B. pendula* (Pt))

304 Table 3 shows the result of the ANOSIM analysis for the dissimilarity values of
305 microbial communities between samples. A significant dissimilarity ($p < 0.05$) was
306 observed when all the tree species growing at the landfill were analysed together.
307 Interestingly, when comparing *B. pendula* from the centre of the landfill with the other
308 tree species (*Salix* and *Populus*) or with the other *B. pendula* growing at the edges, a
309 strong and significant dissimilarity in their root-associated bacterial communities was
310 observed. Similarly, trees growing at the different sampling points at the edges showed a
311 significant dissimilarity amongst their associated microbial communities. However, trees
312 of *B. pendula* growing at the edges of the landfill (*B. pendula* (Pt), *B. pendula* (Pn) and
313 *B. pendula* (Sp)) did not show a significant dissimilarity between their root bacterial

314 communities and those of their neighbour trees (*P. tremula*, *P. nigra* and *S. purpurea*
315 respectively).

316 The dissimilarity analysis between different root fungal communities, (ANOSIM,
317 Table 3) showed similar patterns as for bacteria although less marked. The highest
318 dissimilarity was found between *B. pendula* in the centre with either *B. pendula* (Pt)
319 ($R^2=0.621$, $p=0.001$) or with *P. tremula* ($R^2=0.804$, $p=0.001$). A high and significant
320 dissimilarity was found also when comparing together (a) all the plants of *B. pendula*
321 growing at the different locations ($R^2=0.327$, $p=0.001$); (b) the *B. pendula* at the centre
322 and the other tree species ($R^2=0.471$ $p=0.001$), (c) the other tree species growing at the
323 edges (either when comparing only *B. pendula* ($R^2=0.327$, $p=0.001$) or (d) all species
324 ($R^2=0.472$ $p=0.001$)). No significant dissimilarity was observed when comparing *B.*
325 *pendula* either with *B. pendula* (Pn) or with *P. nigra*.

326 Supporting Information Table S5 shows the number of total reads of the most
327 abundant OTUs (>0.5%) found in the bacterial collection. Only 33 OTUs (from the 1,322)
328 presented abundances higher than 0.5%, highlighting the presence of very rare OTUs
329 dominating the community. The most abundant OTU belongs to the family
330 *Streptomycetaceae* (OTU00001, 14.9%). This OTU presented the highest abundance in
331 roots of *B. pendula* and *S. purpurea*, although it was also uniformly distributed in the
332 roots of all the plant species and locations. Within the other abundant OTUs,
333 OTU00005_f:*Micromonosporaceae*, OTU00022_g:*Kribella*,
334 OTU00030_f:*Methylocystaceae* and OTU00034_g:*Methylibium* tended to be more
335 abundant in roots of *B. pendula* growing at the centre of the landfill (although significant
336 differences were not observed).

337 Total n° of reads of the most abundant OTUs (>0.5%) in the fungal dataset are
338 shown in Supporting Information Table S6. Similar to what was observed for bacteria,
339 only few OTUs presented abundance >0.5% (24 from a total of 577). The fungal
340 community was clearly dominated by the OTU 25_92637 belonging to *Geopora*
341 *arenicola* species (31.8% of the total dataset). This OTU was present in high abundance
342 in roots of all the studied trees, however, its abundance was slightly higher in the roots of
343 *B. pendula* at the centre. An extreme variability between replicates of the same plant
344 species or growth location (which is reflected by the high standard errors in Supporting
345 Information Table S6) was observed, leading to the lack of important statistical
346 differences. The other most abundant OTUs belonged to the genera *Lactarius* (15.7%)
347 and the order *Pezizales* (7.2%), *Agaricales* (5.7%) and *Helotiales* (3.6%).

348 Supporting Information Figure S2 shows the distribution of unique and shared
349 bacterial (Supporting Information Fig. S2a and S2b) and fungal OTUs (Supporting
350 Information Fig. S2c and S2d) amongst *B. pendula* growing at the centre and the other
351 trees growing at the edge. *B. pendula* from the centre presented 44 specific OTUs
352 compared to other tree species and 60 specific OTUs when compared to other birch trees
353 growing at the edges. The number of specific OTUs in the *B. pendula* growing at the
354 edges (compared to the *B. pendula* at the centre) was similar to that found in their
355 neighbour trees. A similar pattern in the fungal dataset was found: *B. pendula* from the
356 centre presented 5 specific OTUs when compared to the other *B. pendula* but also when
357 compared to the other tree species.

358 A heatmap was carried out using the most abundant bacterial and fungal OTUs
359 (>0.5% abundance) (Fig. 3). *B. pendula* growing at the centre clustered separately from
360 all other trees. Trees of different species, which were growing together at the edges were
361 clustered in couples, depending on their growth location. The birches at the centre of the
362 landfill were more closely associated to trees growing at the locations of *P. nigra*. When
363 a heatmap was performed separately for either bacterial or fungal communities (data not
364 shown) the same pattern was found in the case of bacteria. Similarly, birch trees also
365 presented a specific fungal OTU distribution but in this case, plants of *B. pendula* were
366 associated to the cluster made up of *P. tremula*-*B. pendula* (Pt) and *S. purpurea*-*B. pendula*
367 (Sp) (data not shown).

368 An analysis of indicator OTUs for each tree species and growth locations was also
369 carried out. A summary for *B. pendula* indicator OTUs is presented in Table 4. Fourteen
370 indicator bacterial OTUs and 4 indicator fungal OTUs were observed for this tree species.
371 The bacterial OTUs include one belonging to the class *Gammaproteobacteria*, one to the
372 family *Cytophagales*, 3 to the family *Rhizobiales*, one to the family *Streptomycetaceae*
373 and one to the family *Hyphomicrobiaceae*, one to the genus *Rhodoplanes* and one to the
374 genus *Catellatospora*. In the case of fungi, OTUs belonged to the species *Oidiodendron*
375 *maius*, the genus *Russula*, and the order *Agaricales* and *Hypocreales*.

376 **3.4. Relationships between microbial communities, physicochemical soil properties** 377 **and elemental composition of leaves.**

378 Considering an interspecies analysis based on the bacterial and fungal community
379 composition using the most abundant OTUs (>0.5%), (NMDS plots (Fig. 4)) it was
380 observed that roots of *B. pendula*, *P. tremula*, *P. nigra* and *S. purpurea* were clustered

381 separately for each tree. The distribution of the samples of *B. pendula* was related to the
382 abundance of 5 specific bacterial OTUs (Otu00001 (f:*Streptomyetaceae*), Otu00017
383 (g:*Rhodoplanes*), Otu00009 (f:*Cytophagaceae*), Otu00030 (f:*Methylocystaceae*) and
384 Otu00014 (f:*Haliangiaceae*)) and to leaf Mn- concentration.

385 Supporting Information Table S7 shows the one-by-one correlations found
386 between the most abundant bacterial OTUs (>0.5%), soil physicochemical parameters
387 and leaf metal and nutrient concentrations. On the whole, correlations of several OTUs
388 with both, CaCl₂-extractable Fe and Mn concentrations were observed (either positive in
389 the case of OTU00003_g:*Lentzea*, OTU00006_g:*Devosia*, OTU00012_g:*Glycomyces*,
390 and OTU00029_g:*Catellatospora* or negative in OTU00001 and OTU00026 both
391 belonging to the *Streptomyetaceae* family). Also strong positive correlations were found
392 with some soil nutrients, such as CaCl₂-extractable P (OTU00002_f:*Cytophagaceae*,
393 OTU00026_f:*Streptomyetaceae* and OTU00028_g:*Arenimonas*) or CaCl₂-extractable K
394 (OTU00005_f:*Micromonosporaceae*, OTU00016_o:*Rhizobiales* or
395 OTU00037_g:*Dyadobacter*). However, correlations with the corresponding leaf nutrient
396 concentrations were not observed (except in the case of OTU00002_f:*Cytophagaceae*
397 where a correlation was observed with soil CaCl₂-extractable P and leaf P concentration).
398 A positive correlation with both leaf K and P concentrations was found with
399 OTU00003_g:*Lentzea* and OTU00010_f:*Cytophagaceae*. Finally,
400 OTU00012_g:*Glycomyces* showed a positive correlation with leaf P and OTU00029 with
401 leaf K concentrations. Similarly, in the case of fungal community, a strong correlation
402 with both, CaCl₂-extractable Fe and Mn was observed for the OTUs
403 24_66088_o:*Pezizales*, 23_37852_o:*Agaricales* and 51_62345_Unclassified. The OTU
404 24_66088 also showed a correlation with leaf K and P concentrations while the OTU
405 67_38353_o:*Hypocreales* was correlated with Mn leaf concentrations and OTU
406 51_62345_Unclassified with leaf P concentrations (Supporting Information Table S8).

407 Finally, a spearman correlation was carried out to study significant co-occurrence
408 in the community of the most abundant bacterial and fungal OTUs. A positive co-
409 occurrence may be considered as co-presence while a negative co-occurrence as mutual
410 exclusion (Supporting Information Table S9). The bacterial OTUs
411 OTU00003_g:*Lentzea*, OTU00004_f:*Hyphomicrobiaceae* and
412 OTU00021_f:*Methylocystaceae* presented the highest numbers of significant co-
413 occurrence with fungal OTUs (either positive or negative). The fungal OTUs
414 44_70433_g:*Lactarius*, 24_66088_o:*Pezizales* and 18_28512_p:Ascomycota showed the

415 highest number of significant co-occurrence with bacterial OTUs. On the contrary, there
416 were 4 bacterial OTUs (Otu00005_f:*Micromonosporaceae*, Otu00009_f:*Cytophagaceae*,
417 Otu00030_f:*Methylocystaceae* and Otu00033_g:*Rhodoplanes*) and 3 fungal OTUs
418 (13_15910_o:*Agaricales*, 25_11792_s:*Sphaerosporella brunnea* and
419 51_62345:Unclassified) which did not show any significant correlation with the
420 occurrence of either fungal or bacterial OTUs, respectively. The most abundant fungal
421 OTU 25_92637_s:*Geopora arenicola* presented a significant correlation with 5 bacterial
422 OTUs and 4 of these were negative. The unique positive correlation was found with
423 Otu00003_g:*Lentzea*. Similarly, the major bacterial OTU,
424 Otu00001_f:*Streptomycetaceae* was found to be significantly related to three fungal
425 OTUs (from those positively correlated with 37_4836_c:*Sordariomycetes* and
426 3_31227_o:*Agaricales*). Overall, the strongest correlations ($\rho > 0.5$) were found between
427 fungal OTU 24_66088_o:*Pezizales* and bacterial Otu00012_g:*Glycomyces* ($\rho = 0.55$),
428 the fungal OTU 10_6322_Unclassified and the bacterial Otu00034_g:*Methylibium*
429 ($\rho = 0.53$), the fungal OTU 37_4836_c:*Sordariomycetes* and the bacterial
430 Otu00039_f:*Cytophagaceae* ($\rho = 0.58$) and finally between the 12_355_s:*Tomentella*
431 *ellisii* and the bacterial Otu00015_c:TM7_1 ($\rho = 0.64$).

432 **4. Discussion**

433 This study aimed to describe the root-associated microbiota of *B. pendula* which naturally
434 colonises a red-gypsum landfill in the NW of France. It is well known that birch is a
435 pioneering plant species, able to establish in nutrient deficient and degraded soils
436 (Kopponen *et al.*, 2001). Part of this success is related to their well-developed root
437 system(Birch is also a high biomass producer and fast-growing tree which can provide
438 high economic return with end-use applications such as the production of biofuels, pulp,
439 and paper and other bio-based products such as biocatalysts (Kidd *et al.*, 2015; Asad *et*
440 *al.*, 2017). Furthermore, birch can be grown on marginal land thus evading the food versus
441 fuel debate (Searchinger *et al.*, 2008). On the other hand, plants harbour multiple
442 microbial taxa, which affect several plant traits such as biomass production, nutrient
443 availability and transport from the soil (Sugiyama *et al.*, 2012), metabolite production
444 (Badri *et al.*, 2013), drought tolerance (Lau and Lennon, 2012) and flowering time
445 (Wagner *et al.*, 2014). In the last years, several studies have also highlighted the influence
446 of mycorrhizas on the alleviation on metal toxicity to plants through extracellular
447 inactivation, metal binding in fungal wall, enhanced efflux pumps activity, intracellular

448 inactivation and adaptive response to oxidative stress (Amir *et al.* 2014). In addition, the
449 composition of the plant microbiota can enhance host resistance to pathogens (Mendes *et*
450 *al.*, 2011; Busby *et al.*, 2016).

451 Little information about root microbial communities associated with *B. pendula*
452 growing at contaminated sites using metabarcoding approaches is available in the
453 literature. Some studies such as Mesa *et al.* (2017) studied the rhizospheric and
454 endospheric bacterial communities associated with *Betula celtiberica* growing at a
455 contaminated abandoned fertiliser industrial site in NW Spain. Data of the number of
456 observed OTUs and Chao index found in roots of *B. celtiberica* by these authors are
457 comparable with those found in the present study.

458 Here, *B. pendula* growing at the centre of the landfill showed, generally, lower
459 richness and diversity (either in bacterial and fungal communities) than the other trees
460 growing at the edges. One possible explanation could be the presence of harsher
461 physicochemical soil properties at the centre of the landfill, which would negatively affect
462 root microbial communities. However, we did not find any clear difference in the
463 measured parameters (pH and CaCl₂-extractable elements) between the different studied
464 areas. Similarly, in a deeper study of the area, Zapata-Carbonell *et al.* (2019) did not
465 observe any pattern of distribution on measured soil nutritional parameters (based on
466 organic matter and CaCl₂-extractable elements) between the centre and the edges of the
467 landfill. However, it is also important to note that other factors (such as water, N content
468 or CEC) could also be affecting the distribution and composition of plant and microbial
469 communities. It has been reported by several authors that beneficial microorganisms,
470 increase plant tolerance to drought, (Timmusk *et al.*, 2014). Moreover, the incidence and
471 virulence of diseases or pests can be higher in plants growing in stressed environments
472 (such as degraded soils). Associated microorganisms can also protect plants against
473 diseases through for example the induction of plant defence mechanisms or the
474 production of antibiotics (Compant *et al.*, 2010). Related to that point, it is worth
475 mentioning the importance of members of the order *Actinomycetales* (notably the
476 *Streptomyces* genus), as the richest natural source of antibiotics or antimetabolites (Bérdy,
477 2005; Olano *et al.*, 2009; Lemanceau *et al.*, 2006).

478 In the present study, the bacterial community was dominated by *Proteobacteria* and
479 *Actinobacteria*. It is important to note that here, no distinction between endophytes and
480 epiphytes was made, and therefore only one habitat was considered. However, roots were
481 exhaustively washed and we can consider that only strongly root-associated

482 microorganisms are being discussed. Interestingly, *Proteobacteria* (families such as
483 *Pseudomonadaceae*, *Burkholderiaceae* or *Xanthomonadales*) and several OTUs
484 belonging to *Actinobacteria* have been shown to be the most dynamic taxa associated
485 with disease suppression in the rhizosphere of sugar beet plants in a study using DNA
486 metagenomics with Phylochip (Mendes *et al.*, 2011).

487 Mesa *et al.* (2017) also found that in the endosphere of *B. celtiberica*,
488 *Proteobacteria* was the most abundant phylum (accounting for 64% of the total reads),
489 followed by *Bacteroidetes* (17%), *Actinobacteria* (7.9%), *Firmicutes* (1.8%), and
490 *Chlamydiae* (1.3%). It is generally accepted that the *Alpha*- and *Betaproteobacteria* are
491 copiotrophic members (Fierer *et al.*, 2007; Zhao *et al.*, 2014), since they are usually
492 associated with habitats having relatively low soil C/N ratio, high NO₃-N (Nugroho *et*
493 *al.*, 2005), and enriched nutrients (Leff *et al.*, 2015; Li *et al.*, 2016). Roots constitute a
494 nutrient-rich niche since they receive high amounts of available C and N as exudates,
495 which could promote the growth of these bacterial inside the root and at the rhizoplane.
496 Mesa *et al.* (2017) observed in roots of *B. celtiberica*, that the phylum *Proteobacteria*
497 was dominated by taxa related to *Burkholderiales*, and *Pseudomonadales*. However, in
498 our study, the phylum *Proteobacteria* was mostly represented by the family
499 *Hyphomicrobiaceae* (order *Rhizobiales*) and the genera *Devosia* and *Rhodoplanes*,
500 whereas *Pseudomonas*, (which are frequently described in contaminated soils) were very
501 rare (only three OTUs belonging to the *Pseudomonas* genera were observed (Otu00824,
502 Otu01204 and Otu00214)) with an abundance ranging from 0.001% to 0.064%.

503 Moreover, OTUs belonging to the order *Rhizobiales* (including members of the
504 family *Hyphomicrobiaceae* and the genera *Rhodoplanes*) were found to be indicators of
505 the roots of *B. pendula*. There is little information about the role of *Rhodoplanes* in the
506 environment, and several studies related their preference to inhabiting rhizospheric soil
507 rather than endospheric habitats (Touceda-González *et al.*, 2015; Gkarmiri *et al.*, 2017).
508 These bacteria were also abundant in the root DNA-based community of *Arabidopsis*
509 *thaliana* (Haichar *et al.*, 2012) and *Panax notoginseng* (Tan *et al.*, 2017). A study which
510 used ¹⁵N-DNA-SIP to investigate soil microorganisms responsible for N fixation,
511 identified, among others, bacteria of the genus *Rhodoplanes* as being potential N fixers
512 (Buckley *et al.*, 2007). The abundance of microorganism related to N-cycling together
513 with the low values of soil N reported in previous studies (Zapellini *et al.* 2018) seem to
514 indicate that these bacteria could be important for the growth of birch in the landfill.
515 Several members of rhizobiales (such as *Ensifer*, *Rhizobium*, *Phyllobacterium*) have been

516 shown to reduce plant stress response and improve plant growth in different hosts and
517 soils (Larcher *et al.*, 2008; Guo and Chi, 2014; Lafuente *et al.*, 2015; Mesa *et al.*, 2017).
518 Mesa *et al.* (2017) have also observed members of the *Rhizobiales* present in both the
519 rhizosphere and endosphere of *B. celtiberica*. Moreover, in the same study, most of the
520 culturable strains from the endosphere belonged to the genus *Rhizobium* (11%).

521 *Actinobacteria* was the second most represented phylum and the main OTU
522 (Otu00001) of the dataset belonged to the *Streptomycetaceae* family. The predominance
523 of this phylum was also found in several other studies involving either soil or plant
524 compartments (Lopez *et al.*, 2017). For example, Ghosh and Das (2018) studied the soil
525 bacterial community in Mn-enriched mine tailings by using a shotgun-metagenomic
526 approach and found that *Proteobacteria*, and *Actinobacteria* dominated the soil
527 community (and specifically the *Streptomycetaceae* family). Several studies have
528 confirmed the high adaptability of *Actinobacteria* to toxic concentrations of metals
529 (DeGroot *et al.*, 2005; Schmidt *et al.*, 2005) and it has been shown that their strong
530 secondary metabolism enables them to cope with stress factors (So *et al.*, 2001). In one
531 of our recent study (Zappelini *et al.* 2018), we found that *Actinobacteria* (and specifically
532 the genera *Streptomyces*) was the predominant phylum in the soil culturable bacterial
533 collection isolated from this same soil. The high proportion of the *Actinobacteria* phylum,
534 which was found amongst all tree roots (independently of the species and growth
535 location), could suggest the importance of the soil bacterial community in determining
536 the structure and composition of root-associated communities. Similarly, Yang *et al.*
537 (2017) found that after growing barley seeds in soils, an enrichment in *Actinobacteria*
538 was observed in roots of plantlets compared to seeds growing in axenic substrate,
539 suggesting that the soil had a major impact driving the root bacterial communities.

540 The biological control activity of these endophytic actinobacteria is also of
541 interest given their ability to colonise healthy plant tissues and produce antibiotics *in situ*
542 (Shimizu, 2011). For example, the endophytic *Streptomyces* isolated from healthy
543 banana plants (*Musa* sp.) were studied for the ability to produce antifungal molecules that
544 inhibited the growth of the pathogen *Fusarium oxysporum* (Cao *et al.*, 2005). An
545 enrichment of *Streptomyces* in roots compared to rhizospheric soil is frequently described
546 in the literature (Foulon *et al.*, 2016; Durand *et al.*, 2018). Moreover, Gkarmiri *et al.*
547 (2017) suggested that within the active bacterial community that *Streptomyces* were
548 highly active in the root compartment but not in the rhizosphere.

549 In the present study, Otu00716 belonging to *Catellatospora* sp. (family
550 *Micromonosporaceae*, phylum *Actinobacteria*) was found to be an indicator species of
551 the birch trees at the central location. The genus *Catellatospora* is considered one of the
552 rarely occurring genera of actinomycetes: only a few papers mentioning these
553 microorganisms can be found and most of them concern descriptions of the few species
554 and their taxonomic position (Saracchi *et al.*, 2004). During a study of the endophytic
555 root actinomycete population from 252 leaf litter and 205 plant root samples (Petrolini,
556 1992; Sardi *et al.*, 1992) found that most of the 2950 isolates obtained belonged to
557 *Streptomyces* (Sardi *et al.*, 1992) and 215 to the genus *Micromonospora* (Williams *et al.*,
558 1993). However, representatives of the “rare” genera were also found (Petrolini, 1992;
559 Petrolini *et al.*, 1995), and among these, 53 strains of *Catellatospora* sp. However, in the
560 present study 6 OTUs belonging to this genus were observed. Moreover, one of these
561 (Otu00029) was one of the abundant OTU (abundance >0.5%), although this was not the
562 OTU with highest abundance in the birch roots.

563 Members of the plant microbiota can be transmitted either horizontally (acquired
564 from the surrounding environment) or vertically (acquired directly from the parent)
565 (Gundel *et al.*, 2011). The results showed that *B. pendula* harboured a specific microbial
566 community which is unique and different, not only from other plant species growing at
567 the red gypsum landfill (*Populus* or *Salix* spp.) but also from other *B. pendula* growing at
568 the edges (analysis of similarities ANOSIM, heatmap and hierarchical cluster analysis of
569 the relative abundance of bacterial and fungal OTUs), suggesting that horizontally
570 transmission is an important via of microbial acquisition in this case. Moreover, this plant-
571 location specific community was more noticeable in the case of the bacterial community.
572 However, concerning the distribution of unique and shared OTUs, it was found that, even
573 though a high and consistent number of OTUs were unique for each plant species, most
574 of them were shared among different plant species or sites of growth. These results also
575 suggest that a generalised plant colonisation by certain indigenous soil bacterial species
576 is occurring and that the specificity of microbial communities can be related mainly to
577 the differences between OTU abundances. Yang *et al.* (2017) found that the differences
578 in root active microbiomes associated with different barley cultivars were mainly
579 quantitative and not as much qualitative. For example, the variation between the
580 genotypes was manifested in the abundance of many OTUs from diverse taxa, rather than
581 by the presence/absence of single OTUs in the given genotypes.

582 Data obtained from fungal sequencing showed a much higher replicate variability
583 than those obtained in bacterial sequencing. The same pattern was found in several other
584 studies when comparing fungal communities from soil and root habitats (Beckers *et al.*,
585 2017; Durand *et al.*, 2017; Kolaříková *et al.*, 2017). This result suggests that fungal
586 endophytic colonisation and formation of stable communities appears to be a more
587 variable process than bacterial colonisation (Beckers *et al.*, 2017).

588 Although in our study most taxa (of both bacterial and fungal communities but
589 especially in the case of fungi sequencing) were not sequenced at a high enough read
590 depth to enable powerful comparisons, an enrichment of the genus *Geopora* (specifically
591 *G. arenicola*) in the roots of all tree species was observed and tended to be slightly higher
592 in roots of *B. pendula* at the centre. Kolaříková *et al.* (2017) investigated the root-
593 associated fungal communities of two dominant trees *Salix caprea* and *B. pendula* along
594 a primary successional chronosequence on a mine spoil bank in the Czech Republic and
595 found that *G. arenicola* was specifically associated to the youngest trees in the
596 chronosequence independently of the tree species. *Geopora arenicola* is an
597 ectomycorrhizal fungus, which, among others has the characteristic to promote plant
598 growth in stressful environments (Hryniewicz *et al.*, 2009). Within the fungal indicator
599 OTU, we found the genera *Oidiodendron* and *Russula* (OTU 3_5515
600 s:*Oidiodendron_maius*|SH217755.06FU and OTU 4_35502 g:*Russula*). *Russula* is an
601 ECM fungus frequently found in soils and also associated with birch cultures (Bent *et al.*,
602 2011; Parker *et al.*, 2017). Moreover, Parker *et al.* (2017) screened plant/soil fungal
603 communities associating with *Picea mariana*, *Populus tremuloides* and *Betula papyrifera*
604 after a wildfire and found that each plant species seemed to be affected by different groups
605 of fungi. A higher shoot biomass of birch was associated with those associated with
606 ribotype 732 to 734 bp belonging to *Russula* spp. It is interesting to note, however, that
607 the *Russulaceae* family was not abundant in roots of the central *B. pendula*. It seems that
608 the specificity of the root-associated microbial community of the central birch plants is
609 not related to the most abundant taxa. Interestingly, Durand *et al.* (2017) found that the
610 less abundant fungal OTUs present a higher co-occurrence network connectivity than the
611 most abundant OTUs. In the present study it was also observed that the most abundant
612 OTUs (both fungal and bacterial) showed a low number of bacterial and fungal
613 correlations. Fungi and bacteria share the same habitats and are therefore almost certain
614 to frequently interact in soil and plants (Boer *et al.*, 2005; Zhao *et al.*, 2014). Interactions
615 between co-occurring bacteria and fungi play a considerable part in determining their

616 respective roles, and investigating these interactions will lead to a more complete
617 understanding of microbial ecology (de Menezes *et al.*, 2017).

618 Surprisingly, the second indicator OTU belonged to the ericoid mycorrhizal group
619 *Oidiodendron maius* (Chambers *et al.*, 2008; Lukešová *et al.*, 2015); although no
620 *Ericaceae* plants are described at this landfill. Although less frequently, this mycorrhizal
621 type has indeed been found in association with other plant species (Chambers *et al.*, 2008)

622 Similarly to what was observed in the bacterial dataset (although not clearly
623 reflected by the ANOSIM analysis), *B. pendula* growing at the centre hosted a specific
624 fungal community while trees growing at the edges shared an important part of both root
625 community structure and composition. Bogar and Kennedy (2013) studied the relative
626 importance of host and neighbourhood effects on the ECM fungal communities
627 associated with *Alnus rhombifolia* (a host of specific ECM fungi) and *Betula occidentalis*
628 (a host of generalist ECM fungi). The authors found that while the host identity acts as a
629 primary filter on the composition and diversity of ECM fungal communities, proximity
630 to a closely related host can mediate significant changes in community structure. In the
631 same way, Hausmann and Hawkes (2009) found that the structure of arbuscular
632 mycorrhizal (AM) fungal communities on neighbouring grass plants depended upon
633 which pairs of host species were near each other. Neighbourhood effects can also be
634 driven by changes in abiotic conditions that favour the growth of certain ECM fungal
635 species, as demonstrated by Meinhardt and Gehring (2012); in that study, the authors
636 examined the influence of invasive *Tamarix* spp. on the mycorrhizal associations of
637 native *Populus fremontii* in an early-invasion field site and in the greenhouse and
638 laboratory. They found that the ECM fungal community structure on *P. fremontii* shifted
639 significantly in the presence of *Tamarix* spp., likely as a result of increases in soil nitrate
640 concentrations and electrical conductivity associated with soils affected by *Tamarix*.
641 Some ECM fungi, *Russula luteolus*, *Suillus bovinus*, and *Hebeloma crustuliniforme*, form
642 highly branched mycelial strands up to 40 cm (skinner and Bowen, 1974; Finlay and
643 Read, 1986a, b). In addition to the beneficial effects on the plant nutritive status, during
644 the scavenging process fungi will simultaneously colonise more than one plant found at
645 the site.

646 As previously described, abundances of both fungal and bacterial phyla were
647 highly variable. Ghosh and Das (2018) also found a high variability in the abundance of
648 different phyla and classes in the soil bacterial communities of a Mn-enriched tailing by
649 using a shotgun-based approach. These authors suggest that this could be the response of

650 heterogeneity in the environmental conditions and they highlight the importance of
651 studying and integrating results of bacterial diversity and biotic and abiotic related factors
652 for its correct interpretation. Although significant correlations were found between
653 bacterial and fungi OTUs and soil physicochemical properties or plant nutrients, these
654 were generally very weak (rho value usually below than 0.6). This could be due to the
655 lack of a strong gradient in soil properties and rather a random heterogeneity found in
656 these constructed waste landfills. Moreover, although this study involved the
657 characterisation of epispheic root microorganisms (which would be directly influenced
658 by soil properties) most of the analysed community belonged to the endophytic
659 community and therefore are not directly influenced by the soil physicochemical
660 properties. However, some interesting results were found, for example the correlation
661 between some bacterial and fungal OTUs and both soil CaCl₂-extractable Fe and Mn.
662 This could indicate the co-dissolution of Mn- and Fe-oxides from this soil leading to the
663 release of both elements. A deeper study involving the isolation of these bacterial strains
664 and characterisation of the biological pathways involved in this process would be
665 interesting for further exploitation of these bacterial strains in Mn-biomining. Das *et al.*
666 (2011) review the role of microorganisms in Mn-bioleaching operations for the recovery
667 of metals from low-grade ores that would be otherwise unfeasible using current
668 metallurgical technologies by their prohibitively high energy and capital costs. In general,
669 dissolution of Mn-rich minerals occurs through enzymatic reduction which is often
670 carried out by bacteria in the form of respiration where oxidised Mn acts as a terminal
671 electron acceptor or by the indirect release of reductive compounds resulting from
672 bacterial metabolism (Das *et al.*, 2011; Ghosh *et al.*, 2016). In both cases the biological
673 process occurs in the presence of organic carbon and energy sources (Das *et al.*, 2011;
674 Ghosh *et al.*, 2016). As it is well known, roots are a C-rich environment since up to 50 %
675 of the net photosynthetic carbon is released from plant roots into the rhizosphere as
676 rhizodeposits (Curl and Truelove, 1986). At the same time, it is known that the molecular
677 mechanisms behind Fe and Mn tolerance are frequently the same in bacteria
678 (Chandrangsu *et al.*, 2017).

679 Finally, several OTUs were correlated with either soil nutrient availability and/or
680 shoot nutrient concentrations. All trees growing at the area present shoot P and K
681 deficiency (according to the values reported by Marschner (1995)), and therefore plant-
682 associated microorganisms involved in the uptake and absorption of these elements can
683 play a key role in the growth of the vegetation at the site. At the same time, Zapata-

684 Carbonell *et al.* (2019) found a correlation between the distribution of *B. pendula* at the
685 site and CaCl₂-extractable P and organic matter, highlighting again the importance of
686 nutrients in the growth of the plants at this site.

687

688 **5. Conclusions**

689 To successfully implement a phytomanagement strategy it is vitally important to
690 characterise the plant-soil-microbial system. In this study we characterised the microbial
691 community associated with the roots of *B. pendula*, the most successful plant species
692 colonising a red gypsum landfill. The bacterial community associated with birch growing
693 in the centre of the landfill was very different from that of other tree species studied at
694 the mine but also that of *B. pendula* growing at the edges of the landfill. The most
695 abundant bacterial OTU (Otu 00001) belonged to the family *Streptomycetaceae* and was
696 widespread through all the studied roots. At the same time, according to the NMDS
697 analysis, this OTU seemed also to be related to the presence of birch at the centre of the
698 landfill. Other bacterial OTUs associated to birch roots (such as members of the order
699 *Rhizobiales*) are known to be involved in the N cycling, suggesting that this may be a
700 critical factor for effective growth of the plants at the site.

701 Data obtained from fungal sequencing showed a much higher replicate variability
702 than those obtained in bacterial sequencing. In the case of fungi, a less clear pattern was
703 observed although it seems also that birch selected some specific OTUs (e.g. OTUs
704 belonging to the genera *Oidiodendron* and *Russula*, and the order *Agaricales* and
705 *Hypocreales* were found to the indicators of the presence of *B. pendula*).

706 Several OTUs specifically associated to the birch growing at the centre of the area
707 are known to be involved in nutrient cycling (e.g. members of *Rhizobiales* or the fungal
708 species *Geopora*) and can therefore play a role in the high capacity of this plant to tolerate
709 the growth limiting conditions in the landfill.

710 Differences between microbial communities (bacterial and fungal) were based on
711 microbial structure but also on microbial composition and distribution: e.g. the Venn
712 diagram showed that the unique bacterial and fungal OTUs associated to each plant were
713 always present in a very low proportion in the total community (< 0.5% of abundance).
714 However, an analysis of the most abundant OTUs, generally shared by all plants (e.g.
715 heatmap or NMDS) also showed a specificity of the communities associated with *B.*
716 *pendula*. Finally, it is also important to remember that assemblages of host-associated
717 microbial communities are often function-based rather than structure-based (Burke *et al.*,

718 2011; Mendes *et al.*, 2014). Therefore, additional studies on the active part of microbial
719 communities (e.g. rDNA sequencing) or microbial ecosystem functionality (e.g., based
720 on commercial chips (e.g. GeoChip or Shotgun) or the *in vitro* characterisation of the
721 culturable community) can give a more complete view of these plant-soil-microorganism
722 systems.

723 **6. Funding**

724 This work was supported by the French National Research Agency
725 [PHYTOCHEM ANR-13-CDII-0005-01], the French Environment and Energy
726 Management Agency [PROLIPHYT ADEME-1172C0053], the Région Franche-Comté
727 [Environnement-Homme-Territoire 2014-069] and the Pays de Montbéliard
728 Agglomération [13/070-203-2015]. V.A.L. received a post-doc grant from the Région
729 Franche-Comté. C.Z. received a PhD grant from the French Ministry of Higher Education
730 and Research. We thank Dr. Petra KIDD for her thorough review of the English and her
731 useful comments on the manuscript.

732 **7. Conflict of interest**

733 Authors have no conflict of interest to declare

734

735 **8. Bibliography**

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1056 **Figure legends**

1057 **Figure 1** Map of sampling points for each plant species. *Betula pendula* sampled at the
 1058 center of the landfill and named as *B. pendula*. Plants of *P. tremula*, *P. nigra* and *S.*
 1059 *purpurea* were sampled at the edges. Plants of *B. pendula* growing in association with
 1060 these species at the edges were also sampled and named as *B. pendula*- (Pt), *B. pendula*-
 1061 (Pn) and *B. pendula*-(Sp). One soil sampling point was carried out for each plant pair.

1062 **Figure 2** Composition of the root-associated bacterial (a) and fungal (b) communities.
 1063 Only the 20 most abundant identified families are presented.

1064 **Figure 3** Heatmap and hierarchical cluster analysis of the most abundant bacterial and
 1065 fungal OTUs (>0.5%). The dendrogram represents linkage clustering using Euclidean
 1066 distance measures. OTU delineation was based on a threshold of <97% sequence
 1067 similarity. Assignments between brackets show the lowest taxonomic level associated
 1068 with the OTU: k kingdom, p phylum, o order, c class, f family, g genus, s genus_species

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1070 **Figure 4** Non-parametric multidimensional scaling (NMDS) plot of fungal and bacterial
1071 abundant OTUs (>0.5%) communities associated with the roots of *B. pendula*, *P. tremula*,
1072 *P. nigra* and *S. purpurea*. Each point represents the bacterial community of a given root.
1073 Each color represents one of the four trees.

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Table 1. Richness and diversity indices for the root-associated bacterial communities of the woody species sampled at the Thann site

	<i>B. pendula</i>	<i>P. tremula</i>	<i>B. pendula</i> (P.t)	<i>P. nigra</i>	<i>B. pendula</i> (P.n)	<i>S. purpurea</i>	<i>B. pendula</i> (S.p)
Mean number of sequences per sample	13,317	9,912	13,024	13,907	12,163	11,973	16,405
Subsample size	6,972	6,972	6,972	6,972	6,972	6,972	6,972
N° observed OTUs	257 ± 42 d	345 ± 31 a	310 ± 32 bc	329 ± 26 ab	292 ± 36 cd	316 ± 59 abc	287 ± 59 cd
Chao estimation	377 ± 70 d	505 ± 46 a	464 ± 62 abc	483 ± 42 ab	422 ± 48 cd	450 ± 94 bcd	443 ± 107 bcd
Shannon Index (H)	3.19 ± 0.31 c	3.73 ± 0.33 a	3.47 ± 0.38 abc	3.54 ± 0.24 ab	3.44 ± 0.31 abc	3.46 ± 0.49 abc	3.36 ± 0.42 bc
Inverse Simpson Index (1/D)	10.5 ± 2.8 b	16.2 ± 7.3 a	12.2 ± 5.0 ab	14.1 ± 3.6 ab	13.5 ± 5.3 ab	12.6 ± 5.4 ab	11.9 ± 4.5 ab
Shannon Index Evenness E	0.58 ± 0.04 b	0.64 ± 0.05 a	0.61 ± 0.06 ab	0.61 ± 0.04 ab	0.61 ± 0.04 ab	0.60 ± 0.07 ab	0.59 ± 0.06 ab

All diversity indices were calculated using an OTU threshold of $\geq 97\%$ sequence similarity on randomly sub-sampled data at the lower sample size (6,972 reads). Richness was calculated using the number of OTUs and Chao1 estimators. Diversity was estimated using the Shannon-Wiener (H), Inverse Simpson (1/D), and Shannon Index Evenness (E) indices. Mean values and standard deviations (mean \pm SD) are provided for the root samples. Values designated with the same letters were not significantly different (Kruskal Wallis test, $p < 0,05$).

Table 2. Richness and diversity indices for the root-associated fungal communities of the woody species sampled at the Thann site

	<i>B. pendula</i>	<i>P. tremula</i>	<i>B. pendula</i> (P.t)	<i>P. nigra</i>	<i>B. pendula</i> (P.n)	<i>S. purpurea</i>	<i>B. pendula</i> (S.p)
Mean number of sequences per sample	81,989	65,652	64,469	74,027	74,192	70,200	84,301
Subsample size	28,129	28,129	28,129	28,129	28,129	28,129	28,129
N° observed OTUs	52 ± 13 d	79 ± 16 ab	84 ± 14 a	58 ± 14 cd	70 ± 20 bc	70 ± 18 bc	79 ± 14 ab
Chao estimation	72 ± 24 b	103 ± 18 a	105 ± 23 a	81 ± 25 b	89 ± 25 ab	94 ± 30 ab	102 ± 18 a
Shannon Index (H)	1.33 ± 0.37 c	1.80 ± 0.30 ab	1.97 ± 0.36 a	1.56 ± 0.30 bc	1.56 ± 0.56 bc	1.52 ± 0.34 bc	1.66 ± 0.55 abc
Inverse Simpson Index (1/D)	2.96 ± 1.05 b	3.78 ± 1.15 ab	4.33 ± 1.93 a	3.31 ± 0.93 ab	3.74 ± 2.50 ab	3.18 ± 0.98 ab	3.88 ± 2.16 ab
Shannon Index Evenness E	0.34 ± 0.09 b	0.41 ± 0.06 ab	0.44 ± 0.07 a	0.39 ± 0.07 ab	0.37 ± 0.11 b	0.36 ± 0.07 b	0.38 ± 0.11 ab

All diversity indices were calculated using an OTU threshold of $\geq 97\%$ sequence similarity on randomly sub-sampled data at the lower sample size (28,129reads). Richness was calculated using the number of OTUs and Chao1 estimators. Diversity was estimated using the Shannon-Wiener (H), Inverse Simpson (1/D), and Shannon Index Evenness (E) indices. Mean values and standard deviations (mean \pm SD) are provided for the root samples. Values designated with the same letters were not significantly different (Kruskal Wallis test, $p < 0,05$).

Table 3. Analysis of similarities (ANOSIM) of the bacterial and fungal communities associated with the different trees and their interactions. An ANOSIM R value of 1 indicates complete dissimilarity between groups. Significant levels were estimated (p-value < 0.05)

Habitats	Bacteria		Fungi	
	R ²	p-value	R ²	p-value
Global	0.384	0.001**	0.311	0.001**
Intra-species				
<i>B. pendula</i> - <i>B. pendula</i> (Pt)- <i>B. pendula</i> (Pn)- <i>B. pendula</i> (Sp)	0.379	0.001**	0.327	0.001**
<i>B. pendula</i> (Pt)- <i>B. pendula</i> (Pn)- <i>B. pendula</i> (Sp)	0.405	0.001**	0.327	0.001**
<i>B. pendula</i> - <i>B. pendula</i> (Pt)	0.284	0.001**	0.621	0.001**
<i>B. pendula</i> - <i>B. pendula</i> (Pn)	0.617	0.001**	0.058	0.134
<i>B. pendula</i> - <i>B. pendula</i> (Sp)	0.174	0.028*	0.206	0.015*
Inter-species				
<i>B. pendula</i> - <i>P. tremula</i> - <i>P. nigra</i> - <i>S. purpurea</i>	0.48	0.001**	0.471	0.001**
<i>B. pendula</i> - <i>P. tremula</i>	0.539	0.001**	0.804	0.001**
<i>B. pendula</i> - <i>P. nigra</i>	0.607	0.001**	0.145	0.038*
<i>B. pendula</i> - <i>S. purpurea</i>	0.178	0.028*	0.12	0.041*
<i>B. pendula</i> (Pt)- <i>P. tremula</i>	-0.028	0.607	0.133	0.02*
<i>B. pendula</i> (Pn)- <i>P. nigra</i>	-0.031	0.613	-0.018	0.552
<i>B. pendula</i> (Sp)- <i>S. purpurea</i>	0.014	0.338	0.122	0.04*
<i>B. pendula</i> (Pt). <i>P. tremula</i> - <i>B. pendula</i> (Pn). <i>P.nigra</i> - <i>B. pendula</i> (Sp). <i>S. purpurea</i>	0.472	0.001**	0.472	0.001**
0 **** 0.001 *** 0.05 *				

Table 4. Indicator fungal and bacterial OTUs of *B. pendula* plants

	stat	p.value	Phylum	Class	Order	Family	Genus / Species	
BACTERIA	Otu01003	0.775	0.001 ***	Proteobacteria	Gammaproteobacteria	unclass	unclass	unclass
	Otu00390	0.721	0.004 **	Bacteroidetes	Cytophagia	Cytophagales	unclass	unclass
	Otu00260	0.655	0.001 ***	Proteobacteria	Alphaproteobacteria	Rhizobiales	unclass	unclass
	Otu01224	0.636	0.006 **	Proteobacteria	Alphaproteobacteria	Rhizobiales	unclass	unclass
	Otu00475	0.618	0.004 **	Proteobacteria	unclass	unclass	unclass	unclass
	Otu00331	0.612	0.005 **	Actinobacteria	Actinobacteria	Actinomycetales	Streptomycetaceae	unclass
	Otu00799	0.604	0.006 **	Proteobacteria	Alphaproteobacteria	Rhizobiales	Hyphomicrobiaceae	Rhodoplanes
	Otu00429	0.566	0.01 **	Proteobacteria	Alphaproteobacteria	Rhizobiales	Hyphomicrobiaceae	unclass
	Otu00480	0.566	0.012 *	unclass	unclass	unclass	unclass	unclass
	Otu00416	0.555	0.008 **	Proteobacteria	Alphaproteobacteria	Rhizobiales	unclass	unclass
	Otu00574	0.53	0.017 *	Proteobacteria	Alphaproteobacteria	unclass	unclass	unclass
	Otu01267	0.516	0.036 *	Proteobacteria	Alphaproteobacteria	unclass	unclass	unclass
	Otu00716	0.5	0.038 *	Actinobacteria	Actinobacteria	Actinomycetales	Micromonosporaceae	Catellatospora
Otu01261	0.474	0.041 *	unclass	unclass	unclass	unclass	unclass	
FUNGI	3_5515	0.993	0.001 ***	Ascomycota	Dothideomycetes	Incertae_sedis_8	Myxotrichaceae	Oidiodendron_maius SH217755.06FU
	41_79123	0.735	0.005 **	Basidiomycota	Agaricomycetes	Agaricales	Unclass	Unclass
	1_99723	0.577	0.002 **	Ascomycota	Sordariomycetes	Hypocreales	Unclass	Unclass
	4_35502	0.572	0.007 **	Basidiomycota	Agaricomycetes	Russulales	Russulaceae	Russula

Associations were calculated with the Dufrene–Legendre indicator species analysis routine in R. Significance levels: *P ≤ 0.05; **P ≤ 0.01.

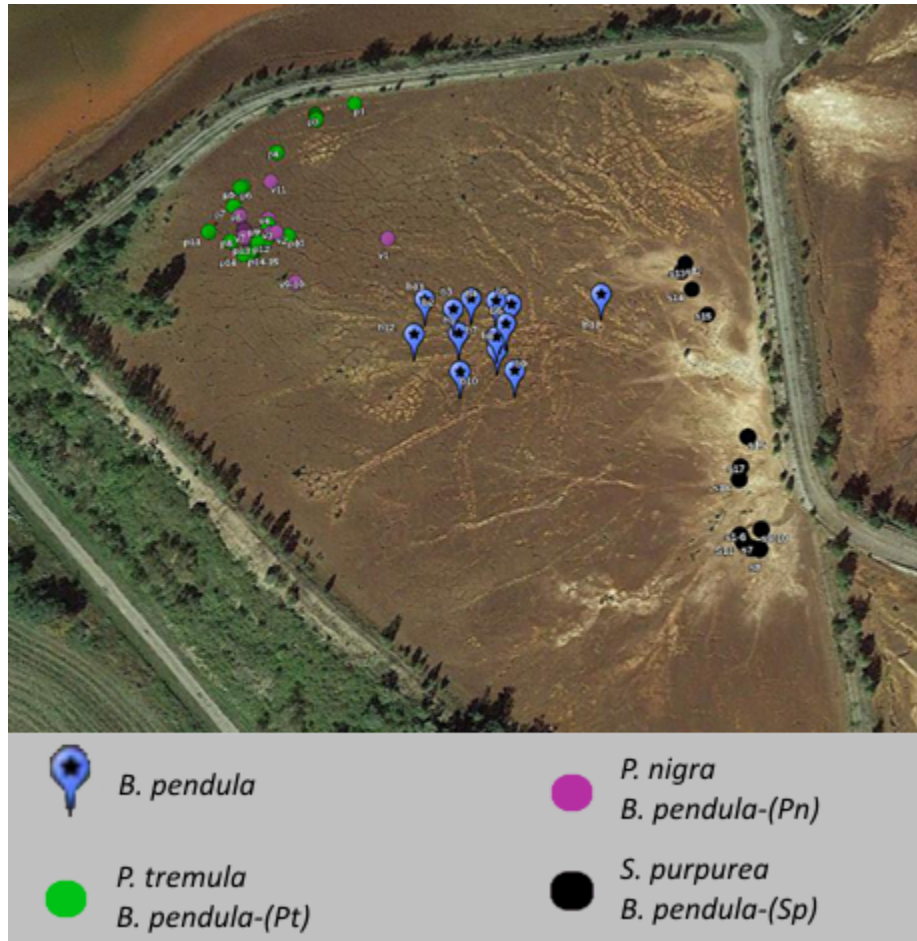


Figure 1 Map of sampling points for each plant species. *Betula pendula* sampled at the center of the dump and named as *B. pendula*. Plants of *P. tremula*, *P. nigra* and *S. purpurea* were sampled at the edges. Plants of *B. pendula* growing in association with these species at the edges were also sampled and named as *B. pendula-(Pt)*, *B. pendula-(Pn)* and *B. pendula-(Sp)*. Only one soil sampling point was carried out for each plant pair.

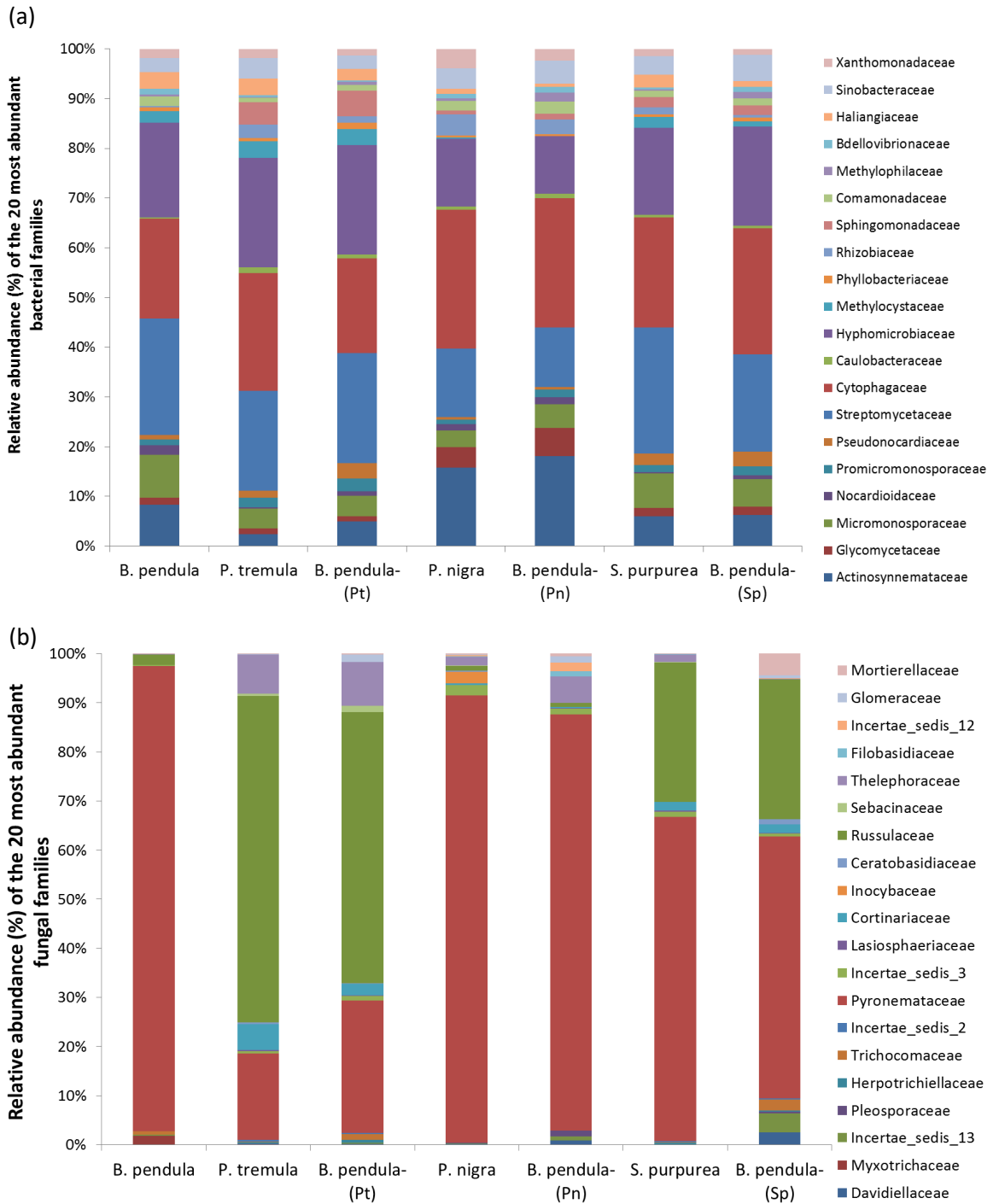


Figure 2 (a) Composition of the bacterial communities from the different roots at the family level and (b) composition of the fungal communities from the different roots at the family level. Only the 20 most abundant identified families are presented.

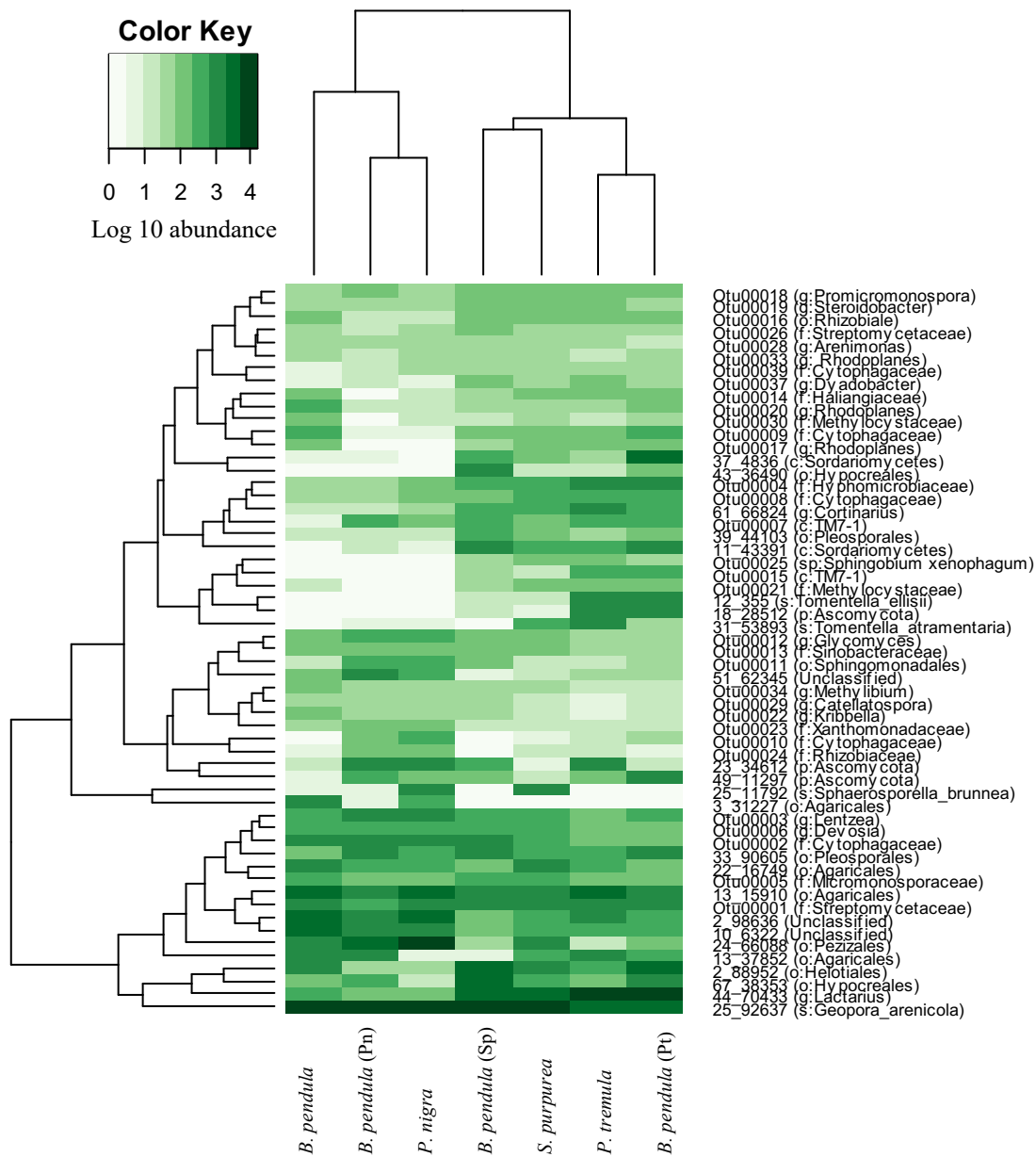


Figure 3 Heat map and hierarchical cluster analysis of the most abundant bacterial and fungal OTUs (>0.5%). The dendrogram represents linkage clustering using Euclidean distance measures. OTU delineation was based on a threshold of <97% sequence similarity. Assignments between brackets show the lowest taxonomic level associated with the OTU: k kingdom, p phylum, o order, c class, f family, g genus, s genus_species

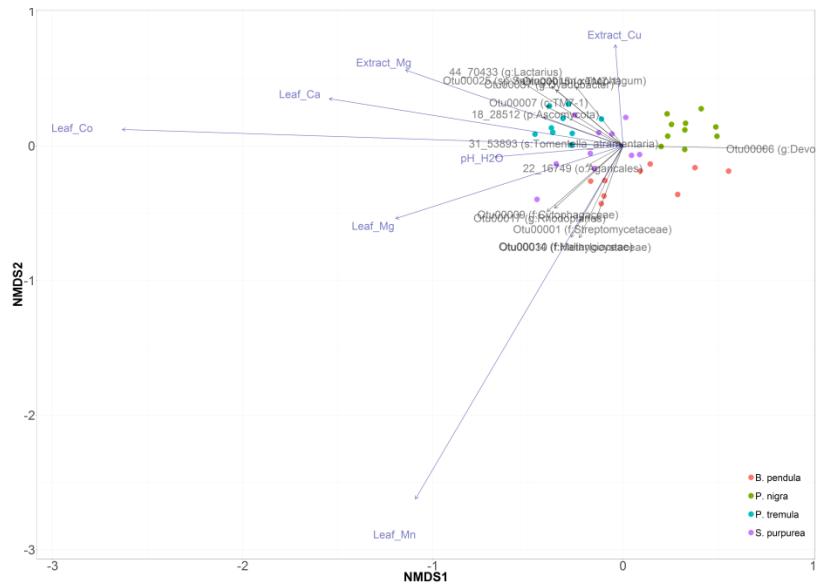


Figure 4 Non-parametric multidimensional scaling (NMDS) plot of fungal and bacterial abundant OTUs (>0.5%) communities associated with the roots of the *B. pendula*, *P. tremula*, *P. nigra* and *S. purpurea*. Each point represents the bacterial community of a given root. Each color represents one of the four trees.