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

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Keywords

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

Abstract

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

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

1. Introduction

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

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

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

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

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

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

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

2. Material and methods

2.1. Study site

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

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

2.2. Sampling procedure

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

2.3. Soil physicochemical properties and elemental composition of leaves

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

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

2.4. Molecular methods

The roots were carefully washed with tap water to remove visible soil and rinsed with sterile deionised water. The fine roots were then cut off and transferred to Eppendorf tubes and frozen at -20 °C. DNA was extracted from roots of 10 single individual trees from each plant species at each sampling point. To isolate DNA from the roots, plant cell lysis was first performed using 50 mg of frozen root samples that were pulverised with 3-mm tungsten carbide beads (Qiagen S.A.S., Courtaboeuf, France) in a Mixer Mill for 3 min at 30 Hz (model MM400; Retsch Inc., Newtown, PA). Root DNA was then extracted with a PowerSoil® DNA Isolation Kit (MO-BIO Laboratories, Inc., Carlsbad, CA USA). To improve DNA extraction, an additional step of 10 min at 60°C was realised after addition of C1 solution. The DNA quality and quantity were assessed by agarose gel electrophoresis and with the Quant-iT™ PicoGreen® dsDNA Assay Kit (Invitrogen, Carlsbad, CA, USA) using an FLX-Xenius spectrofluorometer (SAFAS, Monaco). Two-step PCR amplifications were performed with the bacterial primers 341F (5'- CCT ACG GGR SGC AGC AG -3') and 802R (5'- GAC TAC HVG GGT ATC TAA TCC -3') (Baker *et al.*, 2003; Beckers *et al.*, 2016) and the fungal primers ITS1F (5'- CTT GGT CAT TTA GAG GAA GTA A -3') and ITS2 (5'- GCT GCG TTC TTC ATC GAT GC -3') (White *et al.*, 1990; Gardes, 1993). The cycling conditions for 16S rRNA gene amplification involved an initial 3 min denaturing step at 94°C followed by 30 cycles of 25 s at 94°C, 25 s at 43.6°C, and 1:30 min s at 72°C. The cycling conditions for ITS genes included an initial 3 min denaturing step at 94°C followed by 30 cycles of 25 s at 94°C, 25 s at 45.4°C, and 1:30 min s at 72°C. The sequencing of the V3-V4 domains of 16S rRNA genes and the fungal ITS1 was performed with an Illumina MiSeq platform (Microsynth AG, Switzerland).

2.5. Data analysis

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

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

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

2.6. Statistical analysis

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

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

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

3. Results

3.1. Soil physicochemical properties and elemental composition of leaves

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

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

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

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

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

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

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

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

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

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

Figure 2 shows a clear pattern in the distribution of families between each pair of neighbouring trees (*P. tremula* - *B. pendula* (Pt), *P. nigra* - *B. pendula* (Pn) and *S. purpurea* - *B. pendula* (Sp)). However, due to the high data scattering, few of these results were significant. In the case of the bacterial dataset, the statistical differences were mainly associated to *P. nigra* and the neighbour birch (named as *B. pendula* (Pn)). These trees harboured a higher number of members of the families *Actinosynnemataceae* (Kruskal Wallis $\chi^2=37.9$, $p=1.2\times10^{-6}$), *Glycomycetaceae* (Kruskal Wallis $\chi^2=34.61$, $p=5.2\times10^{-6}$) and *Comamonadaceae* (Kruskal Wallis $\chi^2=20.6$, $p=2.1\times10^{-3}$) and lower members of the families *Pseudonocardiaceae* (Kruskal Wallis $\chi^2=32.4$, $p=1.4\times10^{-5}$) and *Methylocystaceae* (Kruskal Wallis $\chi^2=39.2$, $p=6.6\times10^{-7}$) than all the other trees. In the case of the fungal dataset the statistical differences were observed only for two families *Pyronemataceae* (Kruskal Wallis $\chi^2=24.4$, $p=4.4\times10^{-4}$) and *Sebacinaceae* (Kruskal Wallis $\chi^2=20.5$, $p=2.3\times10^{-3}$), which showed respectively a lower and higher number in roots associated to *P. tremula* and the nearby growing birch (*B. pendula* (Pt))

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

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

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

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

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

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

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

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

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

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

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

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

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

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

4. Discussion

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

5. Conclusions

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

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

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

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

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

6. Funding

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

7. Conflict of interest

Authors have no conflict of interest to declare

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Figure legends

Figure 1 Map of sampling points for each plant species. *Betula pendula* sampled at the center of the landfill 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). One soil sampling point was carried out for each plant pair.

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

Figure 3 Heatmap 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

1069

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.

1074

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)

| | Bacteria | | Fungi | |
|---|----------------------|----------------|----------------------|----------------|
| Habitats | R² | p-value | R² | 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 | Streptomyetaceae | 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.

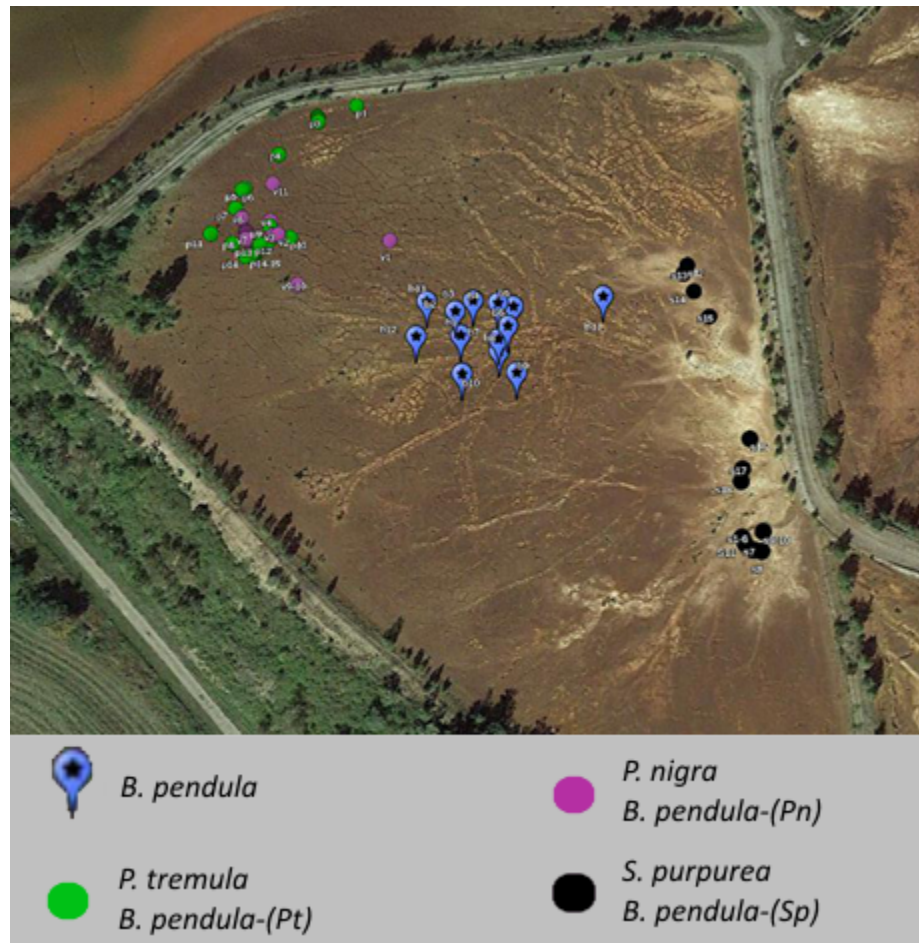


Figure 1 Map of sampling points for each plant species. *Betula pendula* sampled at the center of the dump and named as *B. pendula*. Plants of *P. tremula*, *P. nigra* and *S. purpurea* were sampled at the edges. Plants of *B. pendula* growing in association with these species at the edges were also 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.

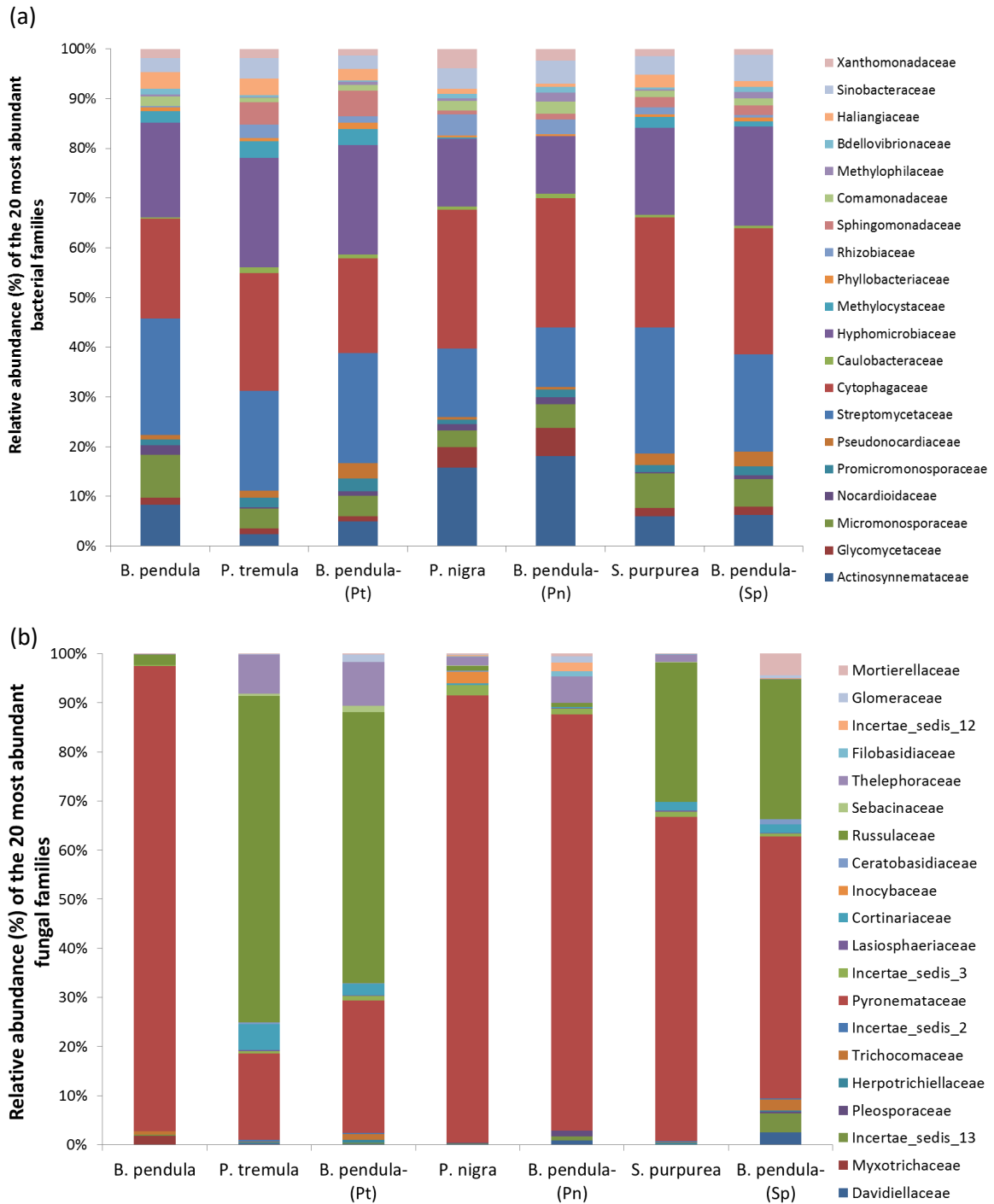


Figure 2 (a) Composition of the bacterial communities from the different roots at the family level and (b) composition of the fungal communities from the different roots at the family level. Only the 20 most abundant identified families are presented.

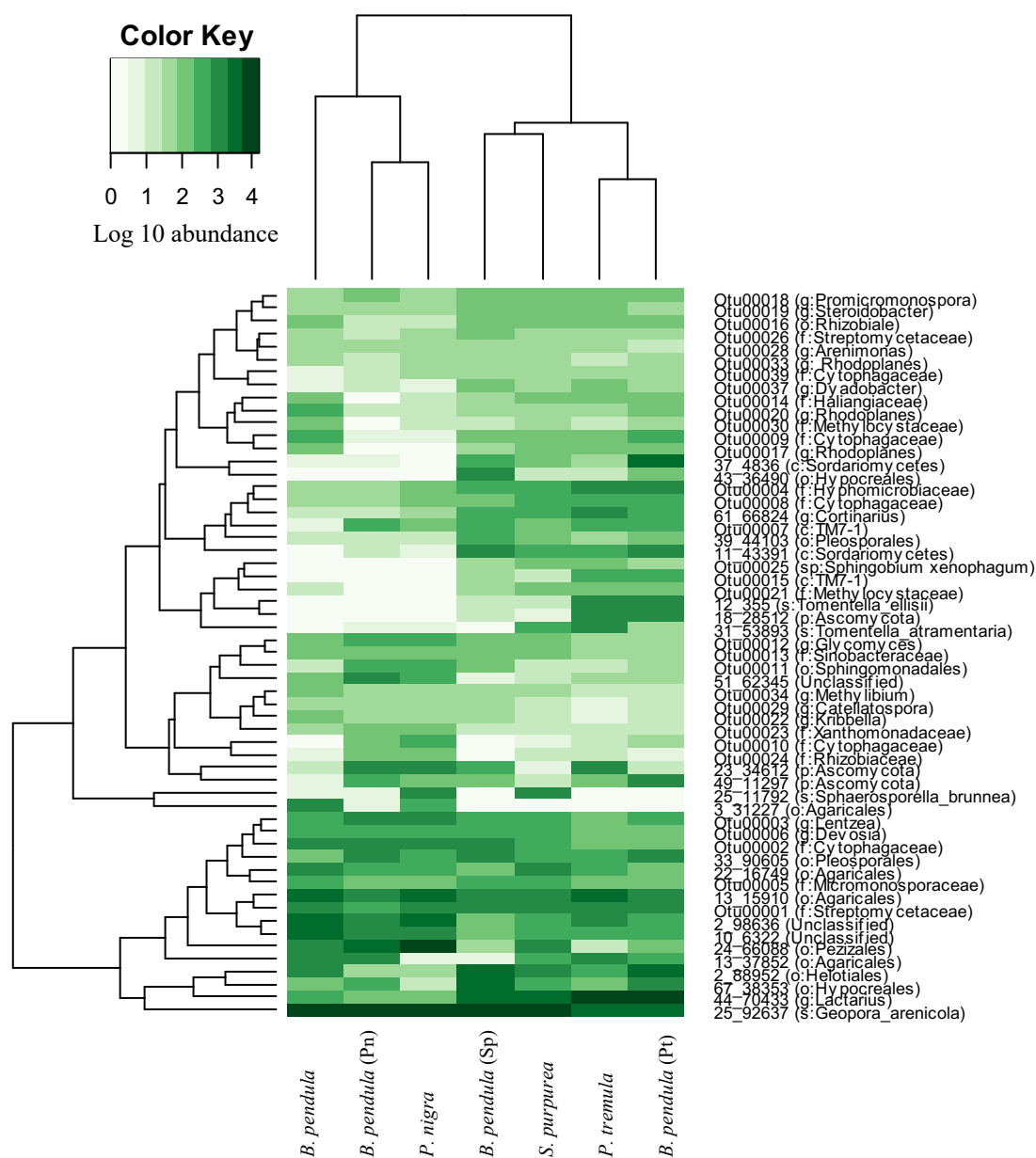


Figure 3 Heat map and hierarchical cluster analysis of the most abundant bacterial and fungal OTUs (>0.5%). The dendrogram represents linkage clustering using Euclidean distance measures. OTU delineation was based on a threshold of <97% sequence similarity. Assignments between brackets show the lowest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

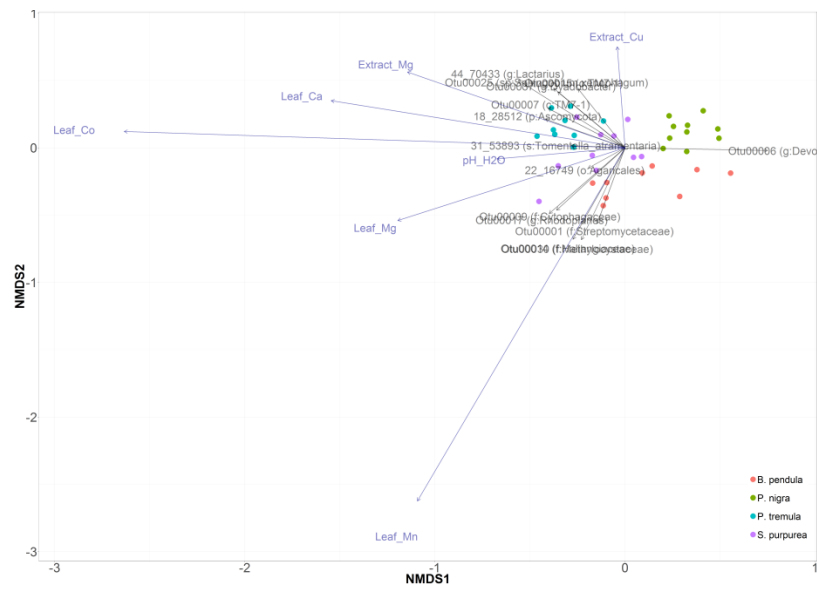


Figure 4 Non-parametric multidimensional scaling (NMDS) plot of fungal and bacterial abundant OTUs (>0.5%) communities associated with the roots of the *B. pendula*, *P. tremula*, *P. nigra* and *S. purpurea*. Each point represents the bacterial community of a given root. Each color represents one of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