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Bacterial succession in oil-contaminated soil under phytoremediation with poplars

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Abstract

Petroleum hydrocarbons (PHCs) continue to be among the most common pollutants in soil worldwide. Phytoremediation has become a sustainable way of dealing with PHC contamination. We conducted the off-site phytoremediation of PHC-polluted soil from an oil tanker truck accident, where poplars were used for the phytoremediation of the oil-polluted soil in a boreal climate during a seven-year treatment. The succession of bacterial communities over the entire phytoremediation process was monitored using microbial ecological tools relying on high-throughput 16S rRNA gene sequencing. Upon the successful depletion of PHCs from soils, endophytic communities were analyzed in order to assess the complete plant-associated microbiome after the ecological recovery. The rhizosphere-associated soil exhibited different bacterial dynamics than unplanted soil, but both soils had a bacterial community succession through the years, with diversity being negatively correlated with PHC concentration. In the relatively short growing season in North Europe, seasonal variations in environmental conditions were identified that contributed to the dynamics of bacterial communities. Overall, our study proved that phytoremediation using poplar trees can be used to assist in the removal of PHCs from soils in boreal climate conditions and provides new insight into the succession patterns of bacterial communities associated with these plants.

Introduction

Petroleum hydrocarbons (PHCs) are some of the most exploited and used chemicals worldwide, comprised of oil and various products refined from oil. Their common use inevitably results in them being widespread in the environment, including soil and ground water, and consequently in environmental problems causing severe economic losses (Wang et al., 2008). This distribution of oil not only strongly affects soil characteristics and overall soil health, vegetation and wildlife, but also microbial communities. PHC contamination is usually treated by physico-chemical methods which are not only expensive, energy-demanding, but have a negative impact on the soil structure and landscape. In contrast, bioremediation methods are cost-effective, environmentally friendly and do not cause damage to the soil structure and harm to microbiota living in soil (Salanitro et al., 1997; Romantschuk et al., 2000; Banks et al., 2003; Zhang et al., 2008; Tang et al., 2010; Lopez-Echartea et al., 2016).

PHCs are susceptible to microbial degradation, with the type of soil, nutrients, temperature, pH, and hydrocarbon fractions affecting their biodegradation (Whyte et al., 1998; Margesin and Schinner, 2001b; Chaîneau et al., 2003; Mukherjee et al., 2014). In a boreal climate, the biodegradation of hydrocarbons is diminished by low temperatures, making the whole process challenging (Atlas and Bartha, 1997; Whyte et al., 1998; Margesin and Schinner, 2001a), especially due to the increased oil viscosity (Atlas and Bartha, 1972; Whyte et al., 1998; Margesin and Schinner, 2001a) and retarded volatilization of short-chain alkanes (Atlas and Bartha, 1972; Margesin and Schinner, 2001a). Solidification at low temperatures is a challenge, since it hinders the bioavailability of PHCs (Whyte et al., 1998), reducing degradation rates (Leewis et al., 2013).

Vegetated soils host microbial communities that differ from those of unvegetated soils, often being more potent in terms of biodegradation (Anderson et al., 1993; Banks et al., 2003; Sipila et al., 2008; Musilová et al., 2016). The composition of soil microbial communities associated with plants depends on the plant species, plant nutrition, light supply and other factors which influence soil properties (Yang and Crowley, 2000; Berg and Smalla, 2009; Rídl et al., 2016). Rhizosphere microorganisms have been acknowledged for their important role in the degradation of organic pollutants for more than two decades (Donnelly et al., 1994; Siciliano et al., 2003; Slater et al., 2011; Toussaint et al., 2012; Sylvestre, 2013; Leewis et al., 2016). The rhizosphere acts as an inoculation and supplementation, providing nutrients for microbes and improving their proliferation (Yrjälä et al., 2017). Next-generation sequencing has enabled a better and more detailed understanding of the bacterial diversity in PHC-polluted soils and rhizospheres, allowing holistic environmental biotechnological studies of phytoremediation (Mukherjee et al., 2015).

More recently, endophytic microorganisms have been investigated in relation to phytoremediation (Germaine et al., 2009; Weyens et al., 2009a; Andreolli et al., 2013; Weyens et al., 2013). Plants growing in polluted environments are enriched in specific bacteria in the interior of their

roots in response to specific contaminants (Siciliano et al., 2001). Suitable endophytic bacteria with appropriate degradation pathways can then improve the degradation of pollutants with their host plant (Taghavi et al., 2005; Barac et al., 2009; Germaine et al., 2009; Weyens et al., 2009a). Endophytic bacteria are defined as those that inhabit the plant interior without causing disease and have plant-beneficial properties (Hallmann et al., 1997). An extended definition has been proposed that they are bacteria that spend at least part of their growth cycle within plants (Hardoim et al., 2015). Endophytes in plants are beneficial to the host through the mobilization of nutrients, production of phytohormones, and induction of plant defense mechanisms against phytopathogens. They enhance adaptation to harsh environmental conditions, thus improving plant growth (Schulz and Boyle, 2006; Puente et al., 2009; Hardoim et al., 2015; Truyens et al., 2015). The mechanisms by which plants select specific bacterial endophytes and vice versa is still not fully understood (Niu et al., 2017).

Poplar species have been used in a wide range of phytoremediation applications (Schnoor, 1997; Fillion et al., 2011; Isebrands et al., 2014; Šuman et al., 2018), and have many advantages: they are fast growing, geographically widespread, tolerant to contaminants and can decrease migration of contaminants (Schnoor et al., 1995). Poplars have been used in the remediation of sites contaminated with PHCs (Palmroth et al., 2002), trichloroethylene (Newman et al., 1997; Gordon et al., 1998; Weyens et al., 2009a), atrazine (Burken and Schnoor, 1997), 1,4-dioxane (Kelley et al., 2001), polyaromatic hydrocarbons (Andreolli et al., 2013), or combined pollution with PHCs, polychlorobiphenyls and heavy metals (Doni et al., 2012). Endophytic populations of poplar species have been studied for their suitability for phytoremediation purposes (Weyens et al., 2010; Yrjälä et al., 2010; Kang et al., 2012). In order to better understand phytoremediation processes, a succession of bacterial communities needs to be assessed over the entire period of site recovery. Such research has gained very little attention (Fierer et al., 2010) .

Remediation is especially challenging at high latitudes, due to the cold climate, lack of infrastructure, generally high expense of remediation and lower biodegradation rates (Leewis et al., 2013). Mean temperatures in Scandinavia, which belongs to the boreal climate region, range from +21 °C in July to −10 °C in January (https://en.climate-data.org/location/134283). For 5-6 months of the year, depending on the location, the medium temperature is below 0 °C (Yrjälä et al., 2017). With this in mind, we aimed to analyze the temporal progress of phytoremediation employing poplar trees for the decontamination of oil-polluted soil in outside field conditions, including the end of the process, where the contaminants have been degraded after seven years of phytoremediation. The process is here viewed as a secondary succession of bacterial communities in an ecologically-recovering environment without any nutrient supplements, which allowed us to evaluate the suitability of poplars (hybrid aspen) and observe the bacterial succession during the seven-year phytoremediation of PHCs. Upon the successful depletion of PHCs from soils, endophytic communities were analyzed in order to assess the complete plant-associated microbiome after the

ecological recovery. We hypothesized that the poplar trees were going to increase the efficiency of the remediation of oil-polluted soil and that the diversity of bacterial communities would be negatively correlated with PHC concentration. We also predicted that temperature and precipitation changes across the seasons would be significantly associated with bacterial community structure. We argued that the bacterial community at the end of phytoremediation would be very distinct from the soil community without plants. Finally, we expected that a portion of the soil bacterial populations during the remediation would stably colonize the plant and become endophytic.

Materials and Methods

Contaminated soil

The contaminated soil originated from an oil tanker truck accident in south of Finland in 2009. After the accident, the soil was excavated and transported to the facilities of the Finnish Forest Research Institute (METLA) in Haapastensyrja, Finland. The soil was classified as sandy soil and its pH remained neutral throughout the whole study. After homogenization, the soil was placed in a 3×12 m isolated plot with proper drainage and hybrid poplar clone seedlings (*Populus tremula* x *Populus tremuloides*) were planted in the soil in August 2009. The seedlings used in the study were obtained from propagation of woody cuttings performed at the former Finnish Forest Research Institute, METLA. A portion of the area of the plot was left unplanted and isolated from any vegetation, and was used as a control (3×0.8 m). The planted portion of the plot was arranged as follows: 5 replicates of one clone per width and 20 poplar seedlings per length, making a total of 100 poplar seedlings.

Soil sampling

Samples for chemical analyses were collected in the summer of 2010, 2013 and 2016 from the planted and unplanted plots. Samples from the planted plot consisted of 10 different subsamples from different parts of the plot and were performed in duplicates. The rhizosphere soil for microbial community analyses was sampled at the depth of 10 to 20 cm. Samples from the unplanted plot consisted of 4 different subsamples from different parts of this area. Approximately 600 grams of soil per replicate were sent for analyses. The rhizosphere soil samples were taken by shaking the roots of the poplar trees after removing bulk soil between roots. To study the response of the microbial community to seasonal changes, soil samples were taken in 2011 monthly from May until September.

PHC analyses and environmental data

The determination of PHC concentrations was commercially performed by MetropoliLab Oy (Finland), accredited by the Finish Accreditation Service T058 (EN ISO/IEC 17025). The analysis was performed by Gas Chromatography with a Mass Selective Detector.

Data on the precipitation and temperatures for the period of the experiment were obtained from the climate station Hyvinkää, Hyvinkäänkylä located 25 km from the Finnish Forest Research Institute in Haapastensyrja. The groundwater of the experimental plot was kept at +1 – +10 cm and watering was necessary only during hot weeks and was performed once or twice a week with a hosepipe. Biomass was not harvested until the termination of the study. No weeding or fertilization was carried out during the phytoremediation treatment. Nitrate and organic carbon concentrations were determined commercially by Eurofins (Finland).

Sampling of poplar clones

At the termination of the phytoremediation, samples from 12 poplar trees were collected. The stems were placed inside a flask with sterile water and agitated for several minutes to remove all dirt from the surface, flamed twice with ethanol and rolled over a PCA plate to verify the sterilization procedure. The PCA plate was checked for no growth up to three days. The next step consisted of homogenizing the stem material in a sterile mortar with liquid nitrogen. The homogenized plant material was used for DNA isolation, subsequent PCR amplification, and analysis of the endophytic populations.

DNA isolation, 16S rRNA gene sequencing and data processing

Genomic DNA from all soil samples was extracted with a FastDNA SPIN Kit for soil (MPBio, USA) following the standard protocol. Primers 515F 5′-GTGYCAGCMGCNGCGG-3′ and 926R 5′-CCGYCAATTYMTTTRAGTTT-3′ (Fraraccio et al., 2017) were used to target the V4–V5 region of the 16S rRNA gene. The PCR was performed in a final volume of 15 µL with: KAPA HiFi HotStart ReadyMix (Kapa Biosystems, USA) containing 0.02 U/µL of KAPA HiFi HotStart DNA Polymerase, 0.3 µM of each primer (Fisher Scientific Oy, Finland) and template DNA (~20 ng). The cycling program started with a 5-min denaturation of DNA at 95 °C, followed by 20 cycles of 20 s at 98 °C, 15 s at 56 °C, 15 s at 72 °C and a final extension for 5 min at 72 °C.

The same kit and primers were used for the genomic DNA amplification from the plant samples. The PCR was, however, performed with the addition of 0.3 µM of each anti-mitochondrial and anti-plastid peptide-nucleic acids (PNAs) (PNABio, USA) for the inhibition of mitochondrial and plastid 16S rRNA gene amplification. The cycling program started with a 5-min denaturation of DNA at 95 °C, followed by 20 cycles of 20 s at 98 °C, 15 s at 72 °C (annealing of the PNAs), 15 s at 56 °C, 15 s at 72 °C and a final extension for 5 min at 72 °C. The PCR products were analyzed by 1.5% agarose gel electrophoresis and excised from the gel using a Zymoclean Gel DNA Recovery Kit (ZYMORESEARCH, USA).

All PCR amplifications were performed in duplicates called later *technical replicas* and sent for library preparation and sequencing analysis on an Illumina MiSeq platform, which were performed in the DNA Core Lab of the University of Alaska Fairbanks, USA.

The processing of the sequence reads followed the same procedure described previously by (Lopez-Echartea et al., 2019) using DADA2 pipeline 1.8 (Callahan et al., 2016a) with some modifications. Briefly, sequence reads were subjected to a filtering step allowing 1 mismatch in the primer sequence, otherwise the whole read was discarded. The next step was trimming off the primer sequence from the sequence reads. To manage the diminishing quality of reads towards their ends, forward and reverse reads were shortened to a length of 257 and 146 nt, respectively. These values were calculated as the average positions where 75% of reads in samples had a quality score >= 25 while maintaining a hypothetical minimum of 25 bp overlap between the paired reads. In the filterAndTrim function, the argument matchIDs was set to true to remove the unpaired reads resulting from the primer filtering step and one mismatch was allowed when merging the forward and reverse sequence reads. Finally, the method used to detect and remove chimeric sequences was "pool" instead of the default. An additional refining step was made in which sequences differing by one base were clustered together, their counts were summed and the most abundant sequence was picked as the correct one. Technical replicas were merged, while keeping only those sequences that occurred in both of the technical replicas. Finally, a table of sequence variants was created with taxonomy based on the Silva reference database version 132. With the resultant data a phyloseq object (McMurdie and Holmes, 2013) was created, which was used for downstream statistical analyses. All sequencing reads were deposited in the NCBI Short Read Archive under SRA study number SUB5046937.

Multivariate Statistical Analyses

All statistical analyses and visualizations were performed in R project (R Development Core Team, 2009) using the packages phyloseq (McMurdie and Holmes, 2013), vegan (Oksanen et al., 2017), DESeq2 (Love et al., 2014), limma (Ritchie et al., 2015) and ggplots2 (Wickham, 2016). All samples were rarefied to an even depth of 18497 reads, except for the analyses performed with DESeq2. The maximum likelihood phylogenetic tree (GTR+G+I) was constructed with the help of the packages DECIPHER and phargnorn by following the steps described in Callahan et al. (2016b). A non-metric multidimensional scaling (NMDS) and distance-based redundancy analysis (dbRDA) were performed using weighted Unifrac distances. The observed number of sequence variants and Shannon diversity indexes were calculated for all samples in R project. The correlation between the diversity indexes and PHC concentrations were tested using the Pearson method at the confidence level of 0.95.

Venn diagrams were performed using the package limma (Ritchie et al., 2015) from R (Love et al., 2014). Finally, the unrarefied data of sequence variants were merged at the genus level (hereafter referred to as the genus-level phylotype) and tested for differential abundance of taxa using the DESeq2 package in R (Love et al., 2014). A false discovery rate cutoff of 0.01 and 1.2 fold change threshold were used for determination of statistical significance. For estimation of the size factor for the rhizosphere versus endospheric community, the *postcounts* method was used as some of the taxa were completely absent in the compared samples. The function lfcShrink was used to shrink the log2fold changes.

Results

Biodegradation of PHCs

The initial concentration of PHCs was 7300 mg/kg of contaminated soil. After 1 year, the concentration in the phytoremediation plot decreased to 3450 mg/kg, which accounts for a 53% removal, while the unplanted soil removal was 43%. By 2013, the removal in the planted and unplanted plots was 78% and 56%, respectively, compared with the initial concentration. By the end of the monitoring period in 2016, all PHCs were degraded in both the planted and the unplanted plots (Table 1).

Bacterial dynamics during bioremediation treatments

Non-metric multidimensional scaling (NMDS, stress = 0.06, Fig. 1) with weighted Unifrac distances showed that the bacterial community structure was significantly associated (P value < 0.001) with the concentration of PHCs and the remediation time (2010, 2013 and 2016). These 2 factors had an inverse correlation, as concentration of contaminants decreased with time. The diversity of the communities increased with time, while the concentration of contaminants decreased (Figure 2). A negative correlation was found between the diversity of the soil communities from planted and control plots and the concentration of PHCs, both in the observed number of sequence variants (Pearson correlation index -0.87 and -0.77, respectively) and the Shannon diversity index (Pearson correlation index -0.98 and -0.94, respectively).

Most abundant genera during the remediation treatments

The analysis of soil samples from the beginning, middle and end of phytoremediation enabled a study of the entire phytoremediation process and the dynamics of bacterial communities.

The most abundant genus-level phylotypes from the planted soil (Fig. 3) included: *Sphingomonas*, *Phenylobacterium*, *Burkholderia-Caballeronia-Paraburkholderia* and *Bradyrhizobium* detected throughout the entire phytoremediation period. In the unplanted control soil *Sphingomonas*, *Flavobacterium*, *Acidovorax* and *Bradyrhizobium* were among the most abundant genus-level

phylotypes. Interestingly, *Flavobacterium* and *Acidovorax* were found in 2013 but not anymore in 2016.

In 2010, at the early stage of phytoremediation, both the planted and unplanted soil shared *Sphingomonas* as the prevalent genus-level phylotype. Further abundant phylotypes in 2010 included *Thermomonas* and *Phenylobacterium*, found in both vegetated and unvegetated soil, but were relatively more abundant in the vegetated. *Sandaracinobacter* and *Massilia* were more abundant in the unplanted soil and scarce in the planted soil, and became even very rare over time in the planted soil. In the middle of the monitored phytoremediation, 2013, the most abundant phylotypes in both the planted and the unplanted soil were *Flavobacterium* and *Rhizobacter*. The phylotypes belonging to these genera increased between 2010 and 2013, but became almost nonexistent in 2016 in both soils, having their peak in the middle of phytoremediation. *Piscinibacter* became enriched specifically in the planted soil during the first phase of phytoremediation, but decreased by 2016. *Acidovorax* had a similar trend in the control soil in 2013 and was almost not detected in 2016. In 2016 *Bradyrhizobium* and *Bryobacter* became augmented in both the planted and the unplanted soil during the final phase of phytoremediation. Interestingly, *Burkholderia-Caballeronia-Paraburkholderia* became one of the most abundant phylotypes in the planted soil at the end of phytoremediation, but was not abundant in the unplanted soil. *Pyrinomonadaceae RB41* showed the same trend in the unplanted soil.

Response of microbial communities to seasonal changes

The seasonal variation in a boreal climate is large, with greatly varying temperature and light conditions. The response of the bacterial communities to seasonal changes was monitored in 2011, two years after the start of the experiment. NMDS (Fig. 4) using weighted Unifrac distances and subsequent fitting of environmental variables indicated that the composition of the bacterial communities was significantly associated with precipitation (P-value < 0.05), which was highest in the summer months (P value < 0.01), and season (P value < 0.01). A multiple-response permutation procedure analysis (MRPP) further confirmed that there were significant differences between the spring (May and June) and summer samples (July, August and September) with a $P < 0.05$ (observed delta 0.05871, expected delta 0.06496 and chance-corrected within-group agreement, A = 0.09614).

Summer samples had roughly twice as many bacterial sequence variants than spring samples (Fig. 5). The Kruskal-Wallis test did not find a significant difference in terms of diversity between the months.

The differential abundance analyses of bacteria identified the genera that significantly differed across seasons. Genera that were significantly enriched in the spring season were mainly from the phylum *Actinobacteria* and *Proteobacteria*. The most significantly enriched genus was *Dyadobacter* of *Bacteroidetes*. In the summer the communities were more diverse diverse, but the majority

belonged to the phylum *Proteobacteria*, with *Nitrosomonadaceae IS-44* and *Rhodoplanes* being extensively enriched in the summer. Other genera, such as *Shinella* or *Terracidiphilus*, were abundant in both seasons, but were more enriched during one season (Fig. 6).

Poplar endophytes and their relationship with the soil microbial community

The bacterial endophytic community in hybrid poplar stems was studied at the end of phytoremediation and compared to community in rhizosphere-associated soil. The Unifrac distance-based redundancy analysis showed that endophytic and soil communities are very different in terms of phylogenetic composition (Fig. S1), with the axis CAP1 explaining 47.7% of the variation in the community due to sample type (endospheric or rhizosphere). The axis MDS1 explained much lower variance (6.3%), which can mostly be ascribed to differences in the rhizosphere soil communities.

The most abundant taxa in the endophyte community belonged to unclassified genera across many classes, including *Alphaproteobacteria*, *Oxyphotobacteria*, *Bacteroidia* and *Phycisphaerae*. The other most abundant genera in the endophyte community were *Beijerinckiaceae*-1174-901-12, *Sphingomonas*, *Bryocella*, *Amnibacterium* and *Terriglobus.* The genus *Bryocella* (*Acidobacteria* subdivision 1) was exclusive to the endophytic community. The most abundant genera in the rhizosphere-associated soil were unclassified genera across several classes including: *Acidobacteriia, Blastocatellia (Subgroup 4), Gammaproteobacteria, Acidobacteria Subgroup 6* and *Alphaproteobacteria*. The other most abundant genera included *Bradyrhizobium, Burkholderia-Caballeronia-Paraburkholderia* and *Bryobacter*, with the latter being exclusive to rhizosphere soil and not found among endophytes.

Differential abundance analysis identified the genera that were significantly enriched/depleted in the endophytic versus rhizosphere community. In the endophytic communities, members of *Actinobacteria* and *Proteobacteria* with a few examples of *Bacteroidetes* and *Acidobacteria* and a single member of *Firmicutes* were significantly enriched (Fig. 8). *Bryocella* and *Frondihabitans* were only detected in the endophytic community. *Acidiphilium, Friedmanniella, Terriglobus* and *Amnibacterium* were enriched in the endophytic community and only sparse in the rhizosphere soil. As expected, the rhizosphere community had a high number of significantly more abundant taxa. The genera significantly enriched in the rhizosphere included members of multiple phyla, of which some members of *Verrucomicrobia, Plantinomicetes* and *Armatimonadetes* were solely present in rhizosphere soil, including *Candidatus Koribacter* and *Candidatus Soilbacter*. *Sphingomonas* and *Pseudomonas* were highly abundant in both environments, but their relative abundance was significantly higher in the endosphere. Genera such as *Burkholderia-Caballeronia-*

Paraburkholderia, *Acidithiobacillaceae-KCM-B-112* and *Aminobacter* were abundant in the rhizosphere soil, but also occurred in the endophytic community.

The abundance and community of retrieved sequences in the endosphere from the six studied poplars at the termination of the experiment was compared with those in the corresponding rhizosphere soil (Table 2) to enquire about the putative origin of endophytes in the stem. The endosphere had a comparably low number of observed sequence variants, on average 122, which accounted for 8 to 25% of the number of sequences in the rhizosphere soil. The average number of observed sequence variants in the rhizosphere was 812. Similarly, the Shannon diversity index was clearly lower as expected in the endosphere, 2.8 on average, while it was on average 5.7 for the rhizosphere.

Discussion

We investigated phytoremediation as a temporal microbial ecological process in Northern Europe by analyzing the dynamics of bacteria during different stages of remediation, spanning from the beginning, via the mid-phase up to the end of the phytoremediation, when contaminants had been removed. No nutrients were supplemented at the onset of phytoremediation so as to be able to detect the performance of plants in contaminated, nutrient poor sandy soil. The diversity of bacteria correlated negatively with the concentration of PHCs (Fig, 2), and a succession of bacterial communities could be detected with different taxa being more abundant at different stages of the 7-year phytoremediation (Fig. 3). A similar succession was detected in non-planted soil during bioremediation, but with different bacterial communities (Fig. 3). The boreal climate exhibited a seasonal variation of bacterial communities, so that a significant difference was observed between the spring and summer month communities. At the end of the phytoremediation, endophytic bacteria were analyzed. Surprisingly, *Acidobacteria* subdivision 1 genera were abundant endophytes together with typical endophytic genera of *Alphaproteobacteria*. Some bacterial endophytes were identified with no counterparts in soil, but many were typical rhizosphere bacteria that clustered especially with *Proteobacteria* (Fig. 7 and 8).

Phytoremediation has proven to be an effective, inexpensive and environmentally friendly method to treat PHC contamination. Poplar trees have been successful with a wide range of pollutants (Burken and Schnoor, 1997; Newman et al., 1997; Gordon et al., 1998; Kelley et al., 2001; Palmroth et al., 2002; Weyens et al., 2009c; Doni et al., 2012; Andreolli et al., 2013). The initial concentration of PHCs in the sandy soil in our study was 7300 mg/kg of soil, and after 3 years this concentration decreased by 78% in phytoremediation treatment. A similar poplar study, but at a higher annual temperature and using horse-manure-supplementation with initial concentrations of ~1150 mg/kg PHCs, (Doni et al., 2012) exhibited a decrease to ~200 mg/kg after one year of treatment. The increased organic C and N in the soil stimulated the indigenous microbial community and was reported

to increase the remediation rate. The removal of diesel fuel from boreal soil using poplar trees in an experiment with an initial concentration of ~5000 mg/kg soil (Palmroth et al., 2002) was effective, but with the use of several nutrient supplements. They concluded that plants accelerate the removal of hydrocarbons, but over time the removal in non-vegetated soil becomes similar to the vegetated. Our study is different from these studies in that we avoided the use of nutrients to better see the potential of the plant under stressful conditions. Data from 2010 and 2013 showed that the degradation proceeded faster in the vegetated soil despite the fact that the plants developed very slowly the first years of the study. This slow development can be ascribed to low nutrient levels in the soil and toxicity of oil contamination. Specifically, concentration of nitrates was below 10mg/l in both 2013 and 2016 and organic carbon decreased from 0.5% in 2013 to 0.1% in 2016. Mukaidani and Tamaki (2007) tested twelve different plant species; not including poplar trees, for their phytoremediation potential of PHCs, and observed degradation in the unvegetated control, but at slower rates than all the vegetated plots. Poplar trees and in general vegetated soils seem to increase the efficiency of the PHC removal from soils.

The concentration of PHCs was one of the key determinants of the bacterial community composition (Table 1) and correlated negatively with bacterial diversity according to our hypothesis (Fig. 2). A decrease in diversity after PHC-contamination has been found in several bioremediation studies (Röling et al., 2002; Hamamura et al., 2006) that did not include plants. We showed in a previous greenhouse study using poplars growing in boreal forest soil that the addition of oil caused an immediate and drastic drop in bacterial diversity (Mukherjee et al. 2013). The bacterial community recovered, however, at the end of the 2-month study done at room temperature and under controlled conditions. The predominant populations in our phytoremediation study included *Sphingomonas, Thermomonas, Phenylobacterium, Sandaracinobacter*, *Massilia, Rhizobacter*, *Flavobacterium, Bradyrhizobium, Bryobacter*, etc. (Fig. 3), some of which had been reported to be PHC degraders. Several other reported PHC-degrading bacteria, including *Pseudomonas*, *Rhodococcus*, *Gordonia, Bacillus, Burkholderia, Caulobacter, Flavobacterium, Nocardioides, Acidovorax, Massilia* and *Mycobacterium* (Prince et al., 2010; Chikere et al., 2011; McGenity et al., 2012; Yergeau et al., 2012; Omrani et al., 2018; Yang et al., 2019) were also detected in our study.

The temporal study of phytoremediation under field conditions gave us the opportunity to observe bacterial secondary succession (Mukherjee et al., 2013) in rhizosphere soil (phytoremediation) and nonplanted soil (bioremediation). The beginning, middle and end points of remediation had specific bacterial communities. Importantly, the rhizosphere soil had a very different succession pattern than that of the nonplanted soil. Given the fact that the majority of the PHCs were depleted within the first year, the most frequently detected taxa in 2010 are likely to have mostly been associated with PHC transformation and/or degradation. Phylotypes of the genus *Sphingomonas*, most abundantly detected at the beginning of the phytoremediation and in the nonplanted soil, have been

reported to be some of the most common PHC degraders (Gottel et al., 2011; Beckers et al., 2017). The other most frequently detected genera at the beginning of the treatment were *Thermomonas* and *Phenylobacterium* in the rhizosphere soil and *Sandaracinobacter* and *Massilia* in the unvegetated soil. Members of all of these genera were previously associated with the degradation of PHCs (Singleton et al., 2013; Wang et al., 2016; Li et al., 2017) or pesticides (Lingens et al., 1985) and linear alkylbenzene sulfonate (Ke et al., 2003). In 2016, at the end of our phytoremediation, where PHC concentrations were below the detection limits, both soils were dominated by *Bradyrhizobium, Bryobacter Burkholderia-Caballeronia-Paraburkholderia* (mainly in planted soil) and *Pyrinomonadaceae* RB41 (mainly in the unplanted soil), which are common soil heterotrophs involved in nitrogen fixation and/or the transformation of organic acids such as galacturonic and glucuronic arising from the decomposition of organic matter (Kulichevskaya et al., 2010). Thus, our results also indicated that ecological recovery of the site was achieved.

Distinct seasons are typical for the boreal climate, and during the winter season the soil is frozen for several months, which slows down the annual degradation (Leewis et al., 2013). Temperature plays a key role in the metabolic activity of bacteria. It was reported that the activity of an enzyme decreases by 50% with the decrease in temperature by 10 °C (Leahy and Colwell, 1990; Atlas and Bartha, 1997). For instance, a recent study found that individual alkanes and aromatics degrade approximately twice as fast at 13 °C than they do at 5 °C (Ribicic et al., 2018). Our results showed that there were significant differences in bacterial community composition between the months and the spring and summer seasons that typically exhibit fluctuating temperatures and precipitation patterns (Fig. 4). One of the drivers explaining the community structure was precipitation (Fig. 4), which reached its highest values during the summer season. Previous studies have also found that the microbial community structure changed significantly due to different watering patterns (Kaisermann et al., 2013; Chodak et al., 2015) and due to different water content (Uhlířová et al., 2005) in soil. Possible explanations for how precipitation might influence bacterial communities are either direct influence through osmotic pressure selecting more tolerable bacteria or through the regulation and availability of nutrients and pH (Chodak et al., 2015; Fierer, 2017; Bu et al., 2018). Despite a significant association being found between bacterial community structure and precipitation changes across seasons, our results did not show a significant relationship between bacterial community structure and temperature. Temperature influences certain taxa (Oliverio et al., 2017), but with current data it is difficult to establish its impact on the whole community. We also expected the community diversity to be higher during the summer months, but we did not find significant association between the monthly number of sequence variants and Shannon diversity indexes. Despite that, we still observed more than twice as many sequence variants that were unique to the summer months than to the spring months (Fig. 5). Similarly, Haas et al. (2018) found that the growing season (early June to October) of spruce trees had no effect on the alpha diversity of soil

and root bacteria. But unlike our study, they did not observe an effect on the community composition of soil and root bacteria related to the growing season, which we did.

Recent phytoremediation research has focused on broadening the knowledge and understanding of plant–microbe interactions in order to advance phytoremediation (Weyens et al., 2009b; Weyens et al., 2009c; Beckers et al., 2017). The study of endophytic bacteria and their interaction with their plant host has been of particular interest with respect to emerging technologies to remediate contaminated environments, including water and soil (Barac et al., 2009; Germaine et al., 2009). Several endophytic bacteria in poplars have been studied for their capacity to degrade organic compounds such as TCE and PAHs (Weyens et al., 2010; Kang et al., 2012; Andreolli et al., 2013), as well as for their plant-growth-promoting activities (Taghavi et al., 2009). We investigated the structure of endophytic communities at the termination of the phytoremediation in six poplar trees. We found that 8-25% of rhizosphere phylotypes (Table 2), were able to colonize the plant interior. The most commonly detected bacterial endophytic taxa have been reported (Hardoim et al., 2015) to belong to the following phyla: *Proteobacteria*, *Actinobacteria*, *Firmicutes*, *Bacteroidetes* and *Acidobacteria*. Endophytic bacterial communities of poplars heve been reported to be dominated by *Proteobacteria* and *Acidobacteria* (Gottel et al., 2011; Beckers et al., 2017). Our results agree with previous studies; observing those same phyla in the following proportions (on average): *Proteobacteria* 67%, *Actinobacteria* 10%, *Acidobacteria* 8%, *Bacteroidetes* 5% and *Firmicutes* 0.4%.

The most abundant bacterial genera found only in the endophytic community included *Bryocella, Frondihabitans, Kineococcus, Curtobacterium* and *Deinococcus*, also found in other studies as endophytes (Hardoim et al., 2015). The most abundant taxa in our endophyte community was interestingly *Bryocella*, which has only been found in one study as an endophyte (Trivedi et al., 2010). Their function as an endophyte is unknown, and the only described species is *Bryocella elongate* isolated from a methanotrophic enrichment culture. We also found a surprisingly high relative abundance of *Acidobacteria* in the poplar plants. Among the enriched endophytic *Acidobacteria*, the genus *Terriglobus* predominated. Populations of this genus are frequent inhabitants of tundra soil (Mannisto et al., 2011) and have previously been found as endophytes in arcto-alpine plants by (Nissinen et al., 2012). This brings up the fact that endophytic bacteria in our study partly represent taxa that are well acclimated to cold conditions. It would be of great interest to know if some psychrophiles also thrive in phytoremediation in a boreal climate. Some endophytic taxa in current study have previously been identified to be associated with poplar trees and phytoremediation, including *Clostridium, Enterobacter, Methylobacterium, Pseudomonas, Sphingomonas, Burkholderia* and *Arthrobacter* (Scott, 1984; Van Aken et al., 2004; Moore et al., 2006; Taghavi et al., 2009; van der Lelie et al., 2009). Some of these endophytes with a metabolic capacity to degrade organic pollutants have been inoculated into plants, which resulted in an increased phytoremediation efficiency (Barac et al., 2004; Taghavi et al., 2005).

The most abundant taxa in the endospheric and rhizosphere communities belonged to as-yet-unknown genera, which highlights the lack of knowledge surrounding these less studied environments. The most abundant taxa shared between these environments included (i) *Beijerinckiaceae*- 1174-901-12, some of which are characterized as obligate methanotrophs, chemoorganoheterotrophs, facultative methylotrophs and facultative methanotrophs, and importantly have capacity to fix nitrogen, which enables them to inhabit environmnets with low nitrogen levels (Marín and Arahal, 2014). (ii) *Sphingomonas and Burkholderia-Caballeronia-Paraburkholderia,* which are commonly isolated from soils, water, activated sludge, the plant phyllosphere, and rhizosphere (Glaeser and Kämpfer, 2014). Some of these taxa probably originated from rhizosphere soil and colonized the endosphere via the route soil-root-endosphere. Several studies support the theory that the rhizosphere is an important source of endophytes (Germaine et al., 2004; Compant et al., 2010; Hardoim et al., 2015), with root hairs playing an important role for inner colonization (Mercado-Blanco and Prieto, 2012). Taxa found exclusively in the endosphere of our poplars most likely originated from the original plant or from air and insects. This is the case for *Bryocella, Frondihabitans* and *Kineococcus.* In contaminated environments, in particular biodegradative populations are expected to colonize the endosphere (Compant et al., 2010; Hardoim et al., 2015). In conclusion our results showed the phylogenetic composition of endophytic communities established upon a successful ecological recovery of the contaminated soil. Further experiments are required to provide more insight into the succession of endophytic communities and their relationship over time with rhizosphere communities and pollutant removal. In addition, the resilience of the soil-originating biodegradative populations in the plant could be tested after the transfer of shoots to a new contaminated site to potentially improve phytoremediation.

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Figure 1. Non-metric multidimensional scaling (NMDS, stress = 0.06) of microbial community at genus level from planted and the unplanted plots in 2010, 2013 and 2016. The fitted vectors correspond to the direction and strength of the statistically significant (*P* value < 0.001) gradients of environmental variables.

Figure 2. Diversity indices of bacteria from soil samples taken in different years of phytoremediation based on 16S rRNA sequence data for all samples in the corresponding year.

Figure 3. Most abundant bacterial phylotypes at genus level in 2010, 2013 and 2016 in the phytoremediation of PHCs and control without plants.

Figure 4. Non-metric multidimensional scaling (NMDS, stress = 0.11) of bacterial communities from soil samples two years after planting using weighted Unifrac distances and subsequent fitting of environmental variables: precipitation (*P* value < 0.05), month (*P* value < 0.01) and season (*P* value < 0.01).

Figure 5. Two-way Venn diagram showing phylotypes from PHC-contaminated soil specific to spring and summer samples and those shared by spring and summer. The listed genera correspond to glomerated sequence variants in each season and the shared ones with those with more than 2% abundance.

Figure 6. Diagram of differential abundance analysis (DESeq) of soil bacteria in 2011, two years after the initiation of phytoremediation treatment. The diagram depicts genera enriched in soil sampled in the spring (lower part of diagram) versus summer (upper part of diagram).

Figure 7. Two-way Venn diagram showing phylotypes specific to endosphere and rhizosphere samples and those shared at the end of 7-year phytoremediation of PHCs. The listed genera correspond to glomerated sequence variants in each community. The shared genera represent only those that represented at least 1% of the total.

Figure 8. Differential abundance analysis of bacteria in rhizosphere soil and plant stems. The diagram depicts the genera enriched in the rhizosphere soil (left of diagram) and in the endosphere (right part of diagram).

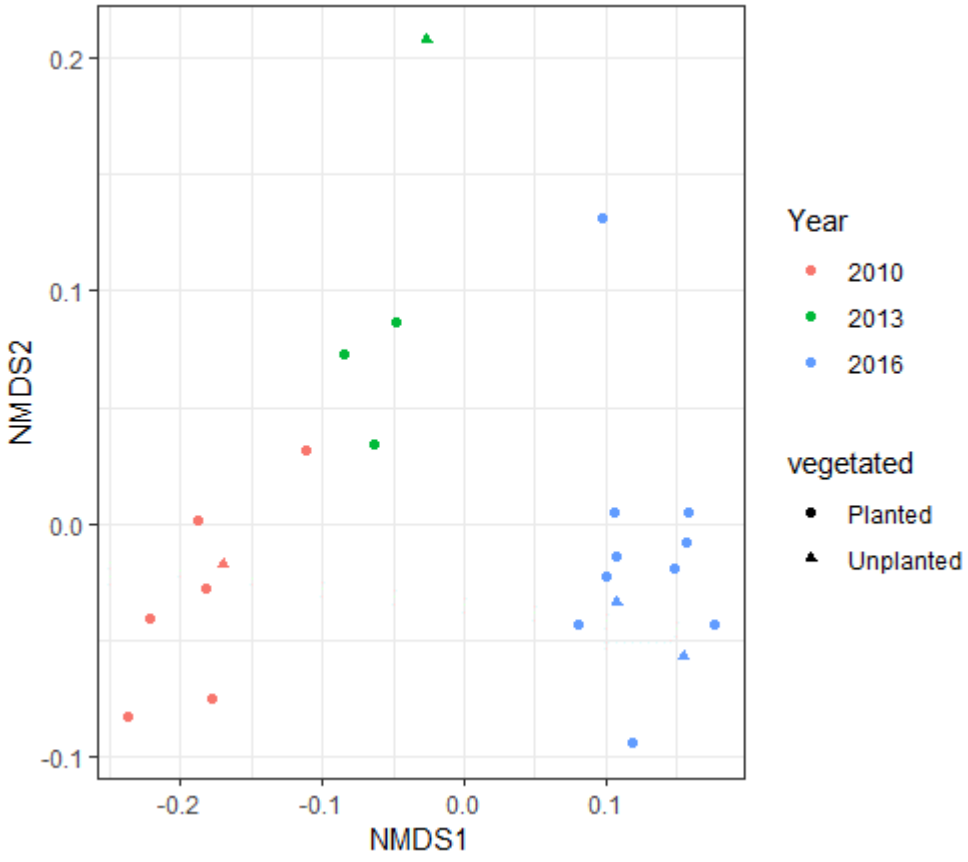
Figure S1. Unifrac distance-based redundancy analysis (dbRDA) of bacterial endophytes and rhizosphere soil at the genus level at the end of phytoremediation. The model used sample type (endospheric or rhizosphere soil) as the explaining factor, with *P* value < 0.001.

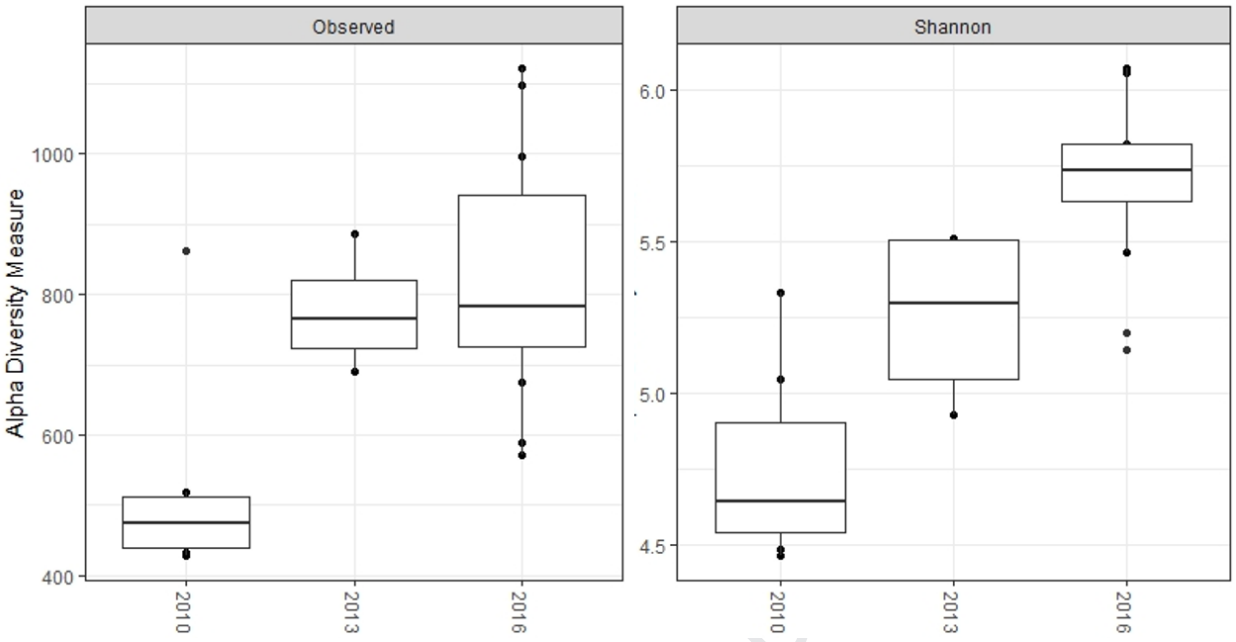
Table 1. Concentration of petroleum hydrocarbons (PHCs) in mg/kg in planted and unplanted soil from the beginning to the end of phytoremediation.

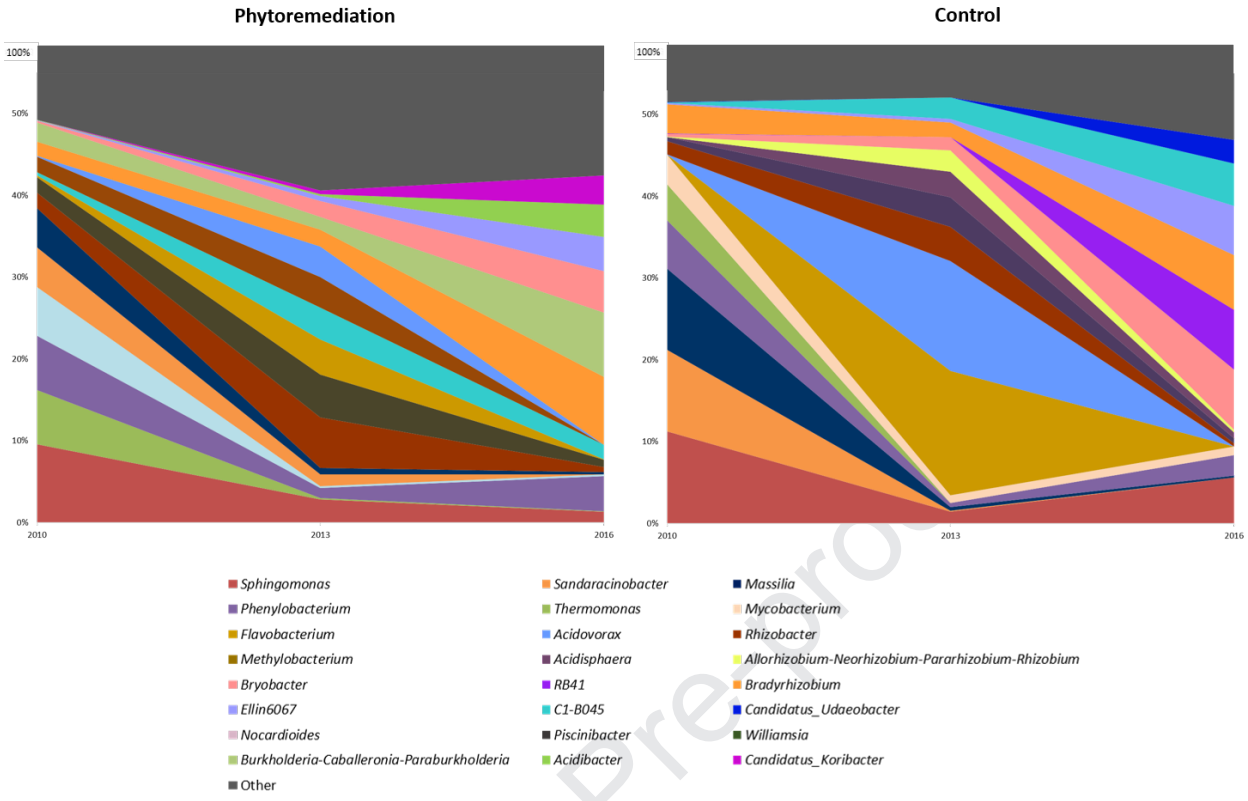
Treatment/Year	**mg/kg dry soil**			
	2009	**2010**	**2013**	**2016**
Planted	7300	3450	1600	<100
Unplanted	7300	4100	3200	<100

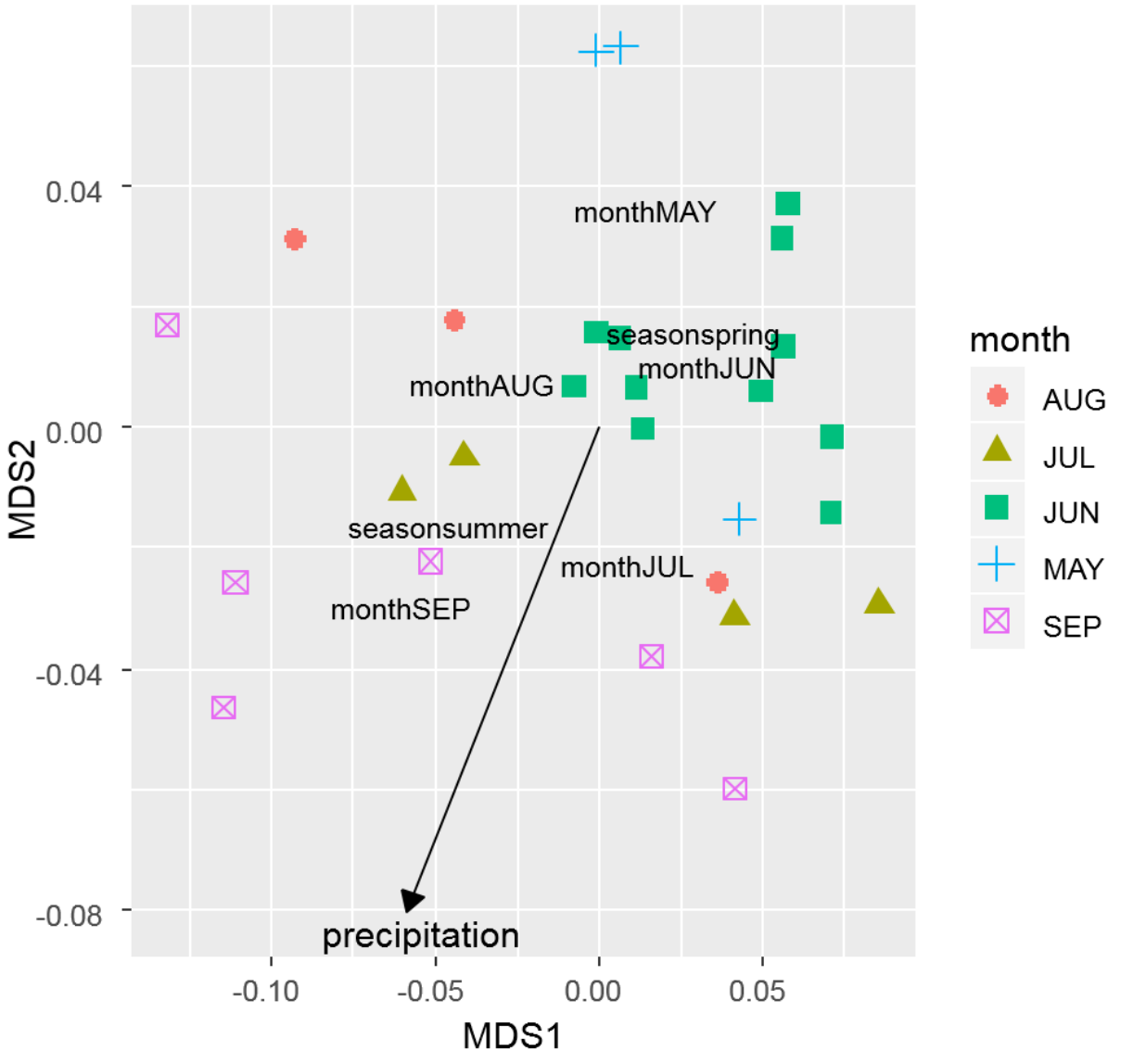
Table 2. Observed number of phylotypes and Shannon diversity indexes in six corresponding samples of the endosphere and rhizosphere of hybrid aspen.

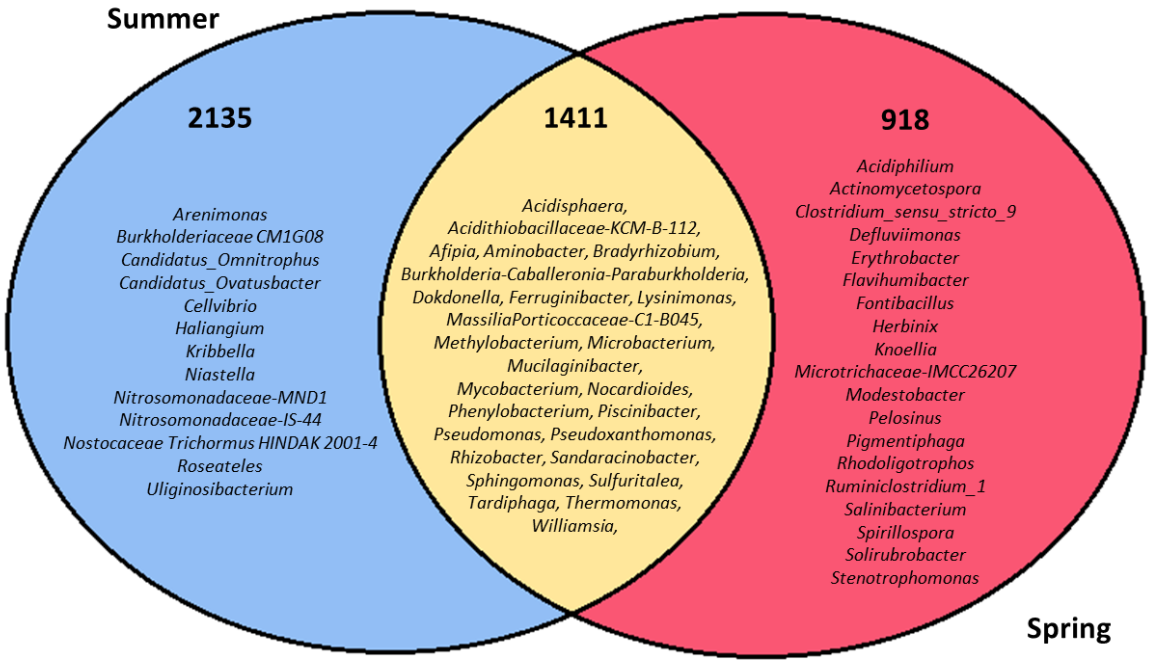
Sample type	Sample name	Observed	Shannon	Sample type	Sample name	Observed	Shannon	% of observed endophytes in rhizosphere
endosphere	endo1	186	3.6	rhizosphere	rhizo1	749	5.8	24.8
	endo2	125	3.4		rhizo2	845	5.8	14.8
	endo3	89	2.9		rhizo3	571	5.1	15.6
	endo4	116	1.8		rhizo4	1121	6.1	10.3
	endo5	83	2.1		rhizo5	996	5.8	8.3
	endo6	134	2.9		rhizo6	590	5.5	22.7
Averages		122	2.8	**Averages**		812	5.7	16.1

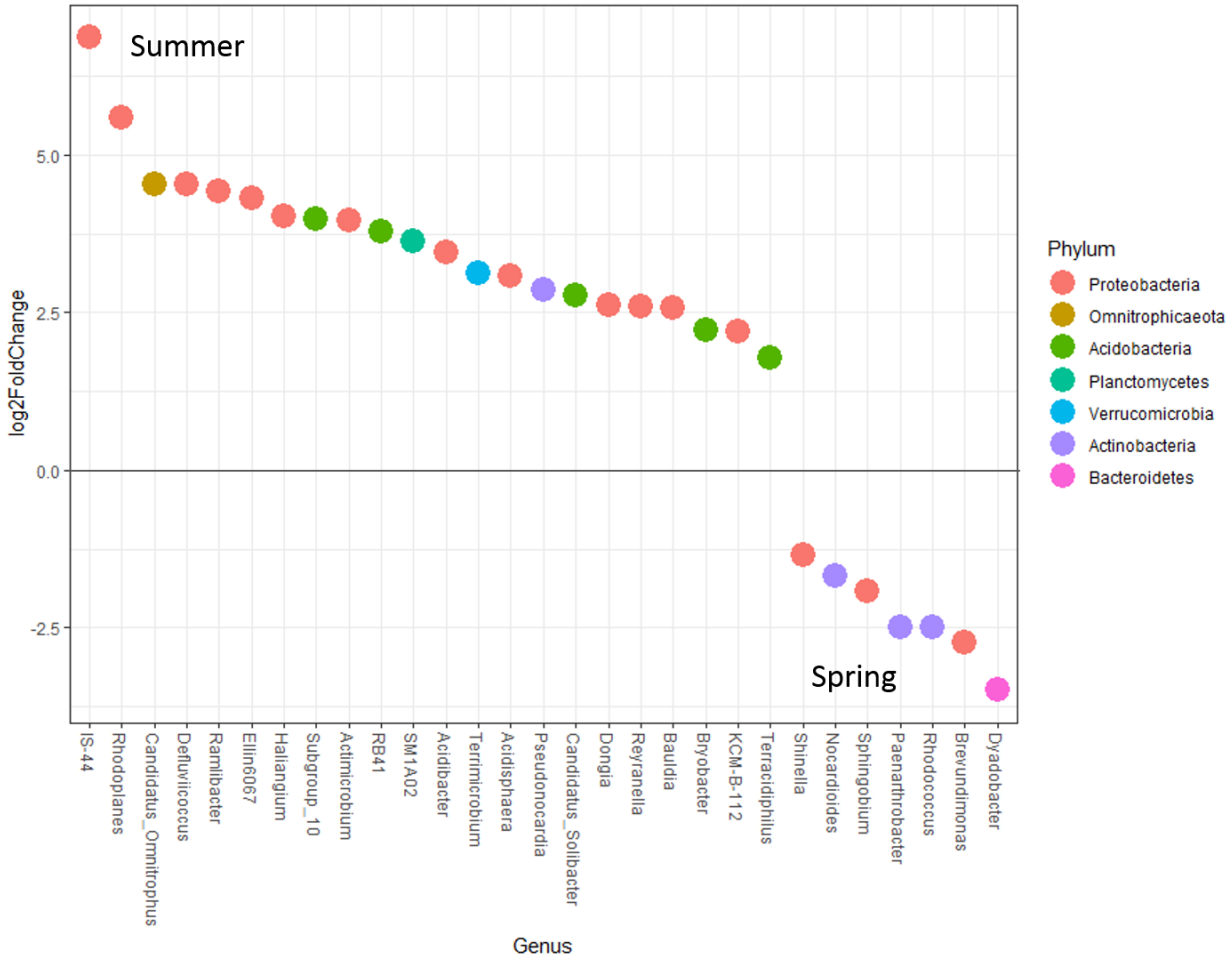

0.2
0.1
0.0
-0.1
NMDS2
-0.2
-0.1
0.0
0.1
NMDS1
Year
2010
2013
2016
vegetated
Planted
Unplanted

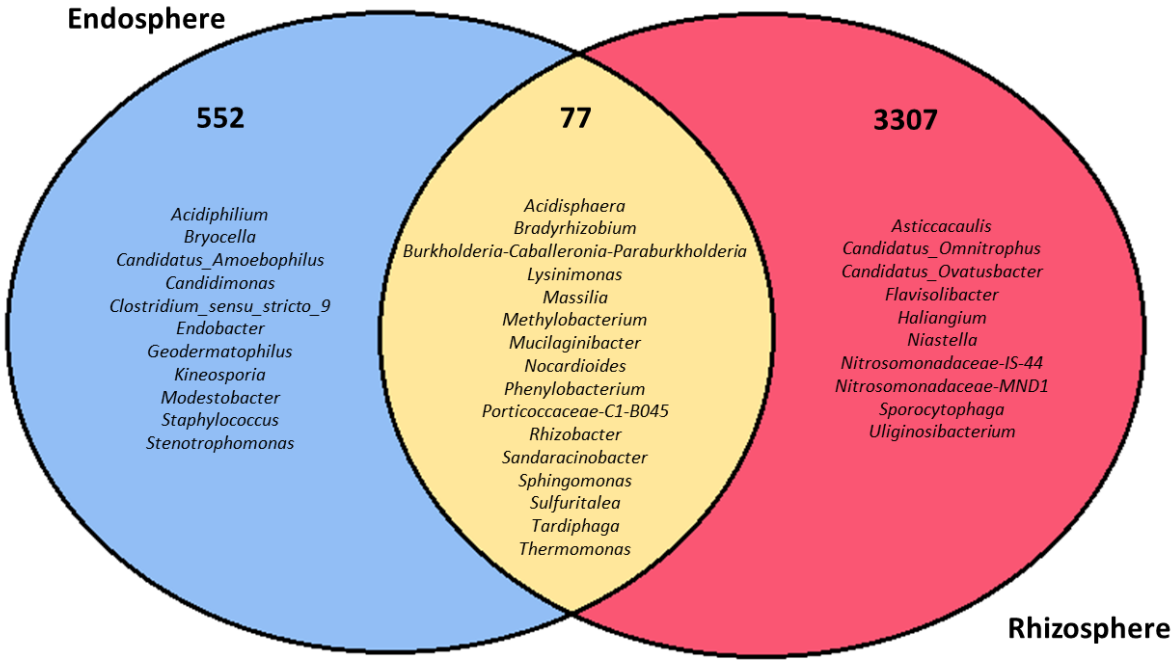


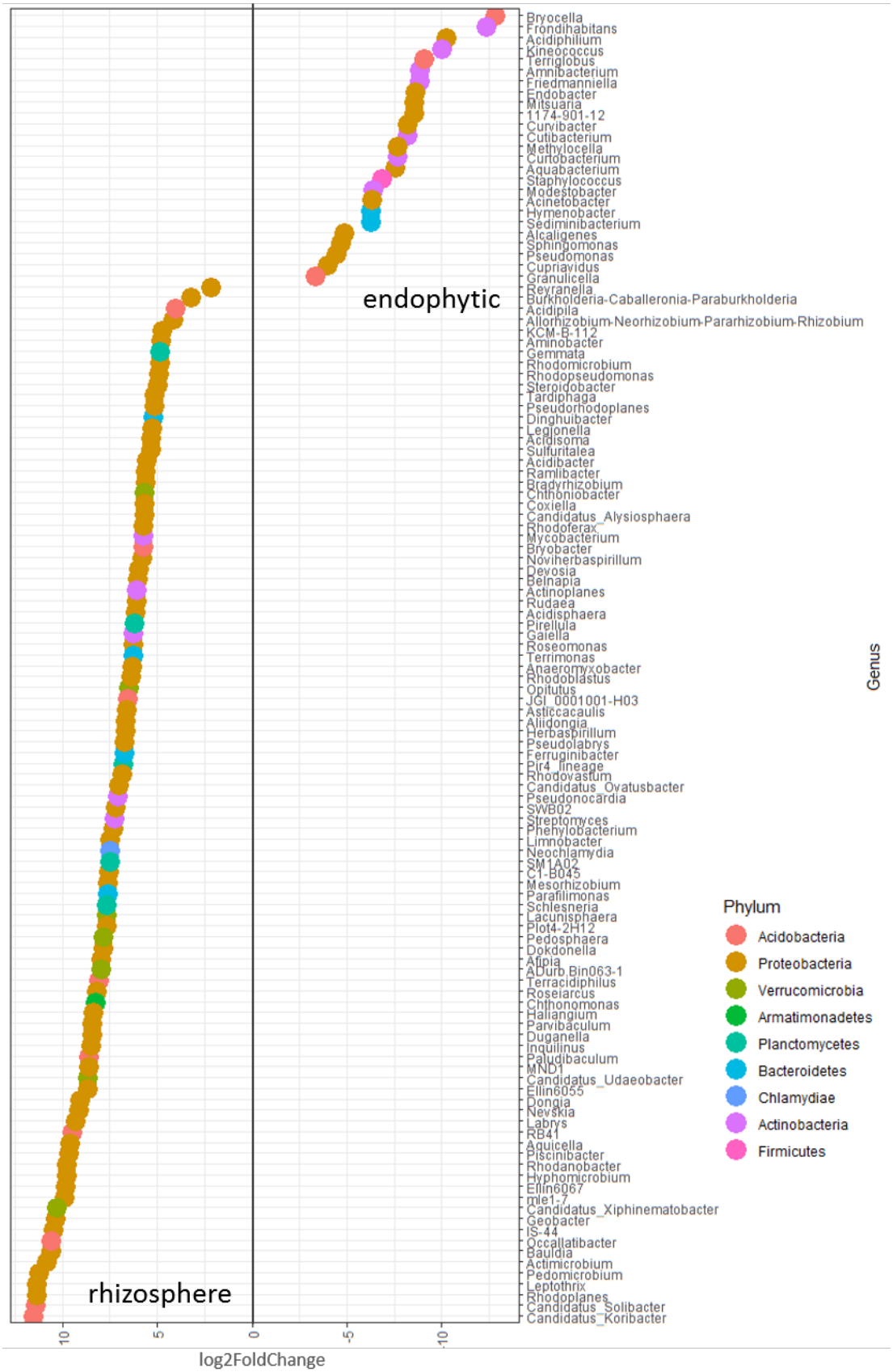



0.04
0.00
-0.04
-0.08
MDS2
-0.10
-0.05
0.00
0.05
MDS1
monthMAY
seasonspring
monthJUN
monthAUG
seasonsummer
monthJUL
monthSEP
precipitation
month
AUG
JUL
JUN
MAY
SEP


Summer
2135
Arenimonas
Burkholderiaceae CM1G08
Candidatus_Omnitrophus
Candidatus_Ovatusbacter
Cellvibrio
Haliangium
Kribbella
Niastella
Nitrosomonadaceae-MND1
Nitrosomonadaceae-IS-44
Nostocaceae Trichormus HINDAK 2001-4
Roseateles
Uliginosibacterium
1411
Acidisphaera,
Acidithiobacillaceae-KCM-B-112,
Afipia, Aminobacter, Bradyrhizobium,
Burkholderia-Caballeronia-Paraburkholderia,
Dokdonella, Ferruginibacter, Lysinimonas,
MassiliaPorticoccaceae-C1-B045,
Methylobacterium, Microbacterium,
Mucilaginibacter,
Mycobacterium, Nocardioides,
Phenylobacterium, Piscinibacter,
Pseudomonas, Pseudoxanthomonas,
Rhizobacter, Sandaracinobacter,
Sphingomonas, Sulfuritalea,
Tardiphaga, Thermomonas,
Williamsia,
918
Acidiphilium
Actinomycetospora
Clostridium_sensu_stricto_9
Defluviimonas
Erythrobacter
Flavihumibacter
Fontibacillus
Herbinix
Knoellia
Microtrichaceae-IMCC26207
Modestobacter
Pelosinus
Pigmentiphaga
Rhodoligotrophos
Ruminiclostridium_1
Salinibacterium
Spirillospora
Solirubrobacter
Stenotrophomonas
Spring


Summer
Spring
log2FoldChange
5.0
2.5
0.0
-2.5
IS-44
Rhodoplanes
Candidatus_Omnitrophus
Defluviicoccus
Ramlibacter
Ellin6067
Haliangium
Subgroup_10
Actimicrobium
RB41
SM1A02
Acidibacter
Terrimicrobium
Acidisphaera
Pseudonocardia
Candidatus_Solibacter
Dongia
Reyranella
Baudia
Bryobacter
KCM-B-112
Terracidiphilus
Shinella
Nocardioides
Sphingobium
Paenarthrobacter
Rhodococcus
Brevundimonas
Dyadobacter
Genus
Phylum
Proteobacteria
Omnitrophicaeota
Acidobacteria
Planctomycetes
Verrucomicrobia
Actinobacteria
Bacteroidetes


Endosphere
552
77
3307
Acidiphilium
Bryocella
Candidatus_Amoebophilus
Candidimonas
Clostridium_sensu_stricto_9
Endobacter
Geodermatophilus
Kineosporia
Modestobacter
Staphylococcus
Stenotrophomonas
Acidisphaera
Bradyrhizobium
Burkholderia-Caballeronia-Paraburkholderia
Lysinimonas
Massilia
Methylobacterium
Mucilaginibacter
Nocardioides
Phenylobacterium
Porticoccaceae-C1-B045
Rhizobacter
Sandaracinobacter
Sphingomonas
Sulfuritalea
Tardiphaga
Thermomonas
Asticcacaulis
Candidatus_Omnitrophus
Candidatus_Ovatusbacter
Flavisolibacter
Haliangium
Niastella
Nitrosomonadaceae-IS-44
Nitrosomonadaceae-MND1
Sporocytophaga
Uliginosibacterium
Rhizosphere


endophytic
rhizosphere
log2FoldChange
10
5
0
-5
-10
Genus
Bryocella
Frondihabitans
Acidiphilium
Kineococcus
Terriglobus
Amnibacterium
Friedmanniella
Endobacter
Mitsuaria
1174-901-12
Curvibacter
Cutibacterium
Methylocella
Curtobacterium
Aquabacterium
Staphylococcus
Modestobacter
Acinetobacter
Hymenobacter
Sediminibacterium
Alcaligenes
Sphingomonas
Pseudomonas
Cupriavidus
Granulicella
Reyranella
Burkholderia-Caballeronia-Paraburkholderia
Acidipila
Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium
KCM-B-112
Aminobacter
Gemmata
Rhodomicrobium
Rhodopseudomonas
Steroidobacter
Tardiphaga
Pseudorhodoplanes
Dinghuibacter
Legionella
Acidisoma
Sulfuritalea
Acidibacter
Ramlibacter
Bradyrhizobium
Chthoniobacter
Coxiella
Candidatus_Alysiosphaera
Rhodoferax
Mycobacterium
Bryobacter
Noviherbaspirillum
Devosia
Belnapia
Actinoplanes
Rudaea
Acidisphaera
Pirellula
Gaiella
Roseomonas
Terrimonas
Anaeromyxobacter
Rhodoblastus
Opitutus
JGI_0001001-H03
Asticcacaulis
Aliidongia
Herbaspirillum
Pseudolabrys
Ferruginibacter
Pir4_lineage
Rhodovastum
Candidatus_Ovatusbacter
Pseudonocardia
SWB02
Streptomyces
Phenylobacterium
Limnobacter
Neochlamydia
SM1A02
C1-B045
Mesorhizobium
Parafilimonas
Schlesneria
Lacunisphaera
Plot4-2H12
Pedosphaera
Dokdonella
Afipia
ADurb.Bin063-1
Terracidiphilus
Roseiarcus
Chthonomonas
Haliangium
Parvibaculum
Duganella
Inquilinus
Paludibaculum
MND1
Candidatus_Udaeobacter
Ellin6055
Dongia
Nevskia
Labrys
RB41
Aquicella
Piscinibacter
Rhodanobacter
Hyphomicrobium
Ellin6067
mle1-7
Candidatus_Xiphinematobacter
Geobacter
IS-44
Occallatibacter
Bauldia
Actimicrobium
Pedomicrobium
Leptothrix
Rhodoplanes
Candidatus_Solibacter
Candidatus_Koribacter
Phylum
Acidobacteria
Proteobacteria
Verrucomicrobia
Armatimonadetes
Planctomycetes
Bacteroidetes
Chlamydiae
Actinobacteria
Firmicutes

Bullet points:

- succession of soil bacterial communities during phytoremediation was monitored
- rhizosphere-associated soil exhibited different bacterial dynamics than bulk soil
- diversity was negatively correlated with petroleum hydrocarbon concentration
- endophytes were analyzed to assess the complete plant-associated microbiome
- poplars can be used to assist phytoremediation in boreal climate conditions

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: