Characterizing the root-associated microbial community structure after 5 years of phytoremediation on gold mine waste rock in Northern Quebec

Jinglin Chen
Department of Natural Resource Sciences
McGill University, Montreal

December 2018

A thesis submitted to McGill University in partial fulfillment of the requirements of the degree of Master of Science

ABSTRACT

Gold mining has historically been known to play a significant role in Quebec's economic development and also underpins the leading position of Canada in global metal production. As a result, the environmental impact on mined lands, such as ecosystem disturbance, metal contamination, and unappealing landscapes, have become a growing concern to the local community and regulatory authorities. Reclamation on the mined areas using plants and their associated beneficial microorganisms (i.e., phytoremediation) has been regarded as a cost-effective phytotechnology that holds promise in alleviating the impact of such metalliferous mining on the soil ecosystem, restoring soil sustainability and productivity, as well as improving the appearance of the landscape. As post-mining soil is nutrient-deficient and an inhospitable environment to establish plants, hardy native plants such as alders (*Alnus* spp.) and boreal conifers that naturally form symbioses with plant growth-promoting microbes are frequently chosen and applied with actinorhizal and mycorrhizal inoculations for phytoremediation effectiveness. In this project, we studied a phytoremediation field trial that was grown on a waste rock slope at the Sigma gold mine, Val-d'Or, QC, since 2012. The plantation consists of two alder species, green alder (*Alnus viridis* subsp. *crispa*) and speckled alder (*Alnus incana* subsp. *rugosa*), and two conifers, white spruce (*Picea glauca*) and jack pine (*Pinus banksiana*). Prior to transplantation in the mine site, seedlings of both alder species were greenhouse-inoculated with *Frankia* sp. strain AvcI1 alone and in combination with mycorrhizal fungal species, *Glomus irregulare* and *Alpova diplophloeus*; while white spruce was inoculated with *Hebeloma crustiliniforme* and *Paxillus involutus*, and jack pine was inoculated with *Suillus tomentosus* and *Laccaria bicolor*. To investigate the plant-microbe interactions during phytoremediation, the root-associated microbiome (rhizosphere and endophytic microbial communities) was characterized using amplicon sequencing, which targeted the 16S rRNA gene and the nuclear ribosomal internal transcribed spacer (ITS) region for exploring the bacterial/archaeal and fungal community structures (diversity and composition), respectively in environmental samples. The field responses (survival rates and seedling biomass production) of plants to the inoculation varied between types of plants (alders or conifers) and inoculation treatments. Our results indicated that neither inoculation of alders with *Frankia* nor the dual inoculation with *Frankia* and mycorrhizal fungi improved their performance in the mine. In the conifer trial, only the inoculated jack pine had higher survival rates (in 2017) and

significantly larger seedling biomass production compared with non-inoculated control plants. Amplicon sequencing results revealed that the plant rhizosphere and root endophytic microbial communities were primarily plant-driven rather than treatment-specific. Contrary to our expectation, not all inoculation treatments significantly increased microbial diversity in the plant rhizosphere and root compartments. Except for jack pine seedlings inoculated with the ectomycorrhizal (ECM) fungal species *Suillus* (*S. tomentosus*) and *Laccaria* (*L. bicolor*), the root-associated microbial composition was not significantly distinct from their uninoculated counterparts (control plants). The bacterial families Acetobacteraceae and Sphingomonadaceae displayed a higher relative abundance in both rhizosphere and bulk soils of ECM-inoculated jack pine than in the non-inoculated pine; in the root compartment, the Acidobacteriaceae (Subgroup 1) family was more abundant in the ECM-inoculated pine compared to the uninoculated pine. Moreover, a *Suillus* fungal genus was found dominating the rhizosphere, bulk soils and roots of ECM-inoculated pine seedlings whereas this genus was not present in the non-inoculated pine seedlings. Since one of the fungal inocula for jack pine seedlings also belonged to the genus *Suillus* and a positive response of jack pine to ectomycorrhizal fungal inoculation was also observed, we suspected that the identified *Suillus* could be the inoculum causing the positive impact. No inocula-like fungal genus other than *Suillus* was found in association with the other inoculated plants (white spruce and alders), which may explain the insignificant to negative effects of inoculation on plant seedlings growing in the gold mine waste rock. Regardless of inoculation effects, the planted soils in general improved soil characteristics of the mine leading to a neutral soil pH (7.0), higher moisture content, and a much higher microbial relative abundance.

RÉSUMÉ

L'exploitation de l'or a toujours été reconnue pour jouer un rôle important dans le développement économique du Québec et renforce également la position de leader du Canada dans la production mondiale de métaux. En conséquence, l'impact environnemental sur les terres minées, tels que la perturbation des écosystèmes, la contamination par les métaux et les paysages peu attrayants, est devenu une préoccupation croissante pour la communauté locale et les autorités responsables de la réglementation. La remise en état des zones minées à l'aide de plantes et de leurs micro-organismes bénéfiques associés (phytoremédiation) est considérée comme une phytotechnologie rentable susceptible d'atténuer l'impact de l'exploitation métallifère sur l'écosystème du sol, de restaurer la durabilité et la productivité du sol, ainsi que de contribuer à l'amélioration de l'apparence du paysage. Etant donné que le sol post-minier est pauvre en éléments nutritifs et qu'il est difficile d'y aménager des plantes, des plantes indigènes robustes telles que les aulnes (*Alnus* spp.) et les conifères boréaux qui forment naturellement des symbioses avec des microbes sont fréquemment choisies et inoculées à l'aide d'actinorhizes et de mycorhiziens pour augmenter l'efficacité de la phytoremédiation. Dans le cadre de ce projet, nous avons effectué un essai de terrain en phytoremédiation qui a été développé sur une pente de stériles de la mine d'or Sigma, à Val-d'Or, au Québec, depuis 2012. La plantation comprend deux espèces d'aulne, l'aulne vert (*Alnus* viridis subsp. *crispa*) et de l'aulne maculé (*Alnus incana* subsp. *rugosa*) et deux conifères, l'épinette blanche (*Picea glauca*) et le pin gris (*Pinus banksiana*). Avant la plantation sur le site de la mine, les deux espèces d'aulnes ont été inoculées dans des semis, avec la souche *Frankia* AvcI1 seulement et en combinaison avec les espèces fongiques mycorhiziennes, *Glomus irregulare* et *Alpova diplophloeus*; tandis que l'épinette blanche était inoculée avec *Hebeloma crustiliniforme* et *Paxillus involutus*, et le pin gris, avec *Suillus tomentosus* et *Laccaria bicolor*. Pour étudier les interactions plante-microbe au cours de la phytoremédiation, le microbiome associé aux racines (communautés microbiennes de la rhizosphère et endophytes) a été caractérisé à l'aide d'un séquençage d'amplicons ciblant le gène de l'ARNr 16S et la région d'espace transcrit interne (ITS) pour la caractérisation des communautés bactériennes/archaïennes et fongiques (diversité et composition), respectivement, dans des échantillons environnementaux. Les résultats ont démontré que le taux de survie et la biomasse de semis des plantes inoculées varient selon les types de plantes (aulnes ou conifères) et les traitements d'inoculation. Nos résultats ont indiqué que ni l'inoculation des aulnes avec *Frankia* ni la double inoculation avec *Frankia* et les champignons

mycorhiziens n'amélioraient la performance des plantes dans la mine. Dans l'essai sur les conifères, seul le pin gris inoculé avait des taux de survie significativement supérieurs et une production de biomasse de plantules supérieure à celle des plantes témoins non inoculées. Les résultats du séquençage des amplicons ont révélé que la rhizosphère des plantes et les communautés microbiennes des endophytes des racines étaient principalement dirigées par les plantes plutôt que par le traitement. Contrairement à nos attentes, tous les traitements d'inoculation n'augmentaient pas de manière significative la diversité microbienne dans la rhizosphère des plantes et les compartiments radiculaires. À l'exception des semis de pin gris inoculés avec les espèces fongiques ectomycorhiziennes (ECM) *Suillus* (*S. tomentosus*) et *Laccaria* (*L. bicolor*), la composition microbienne associée aux racines n'était pas significativement distincte de leurs équivalents non inoculés (plantes témoins). Les familles bactériennes Acetobacteraceae et Sphingomonadaceae ont montré une abondance relative plus élevée dans les sols à la rhizosphère (RZ) et en vrac (BK) de pins gris inoculés par la ECM par rapport aux pins non inoculés; dans le compartiment racinaire, la famille des Acidobacteriaceae (sous-groupe 1) était plus abondante dans le pin inoculé à la ECM que dans le pin non inoculé. De plus, un genre de champignon *Suillus* a été trouvé dominant dans la RZ, la BK et les racines de pins inoculés à la ECM, alors que ce genre n'était pas présent dans les plantules de pins non inoculées. Étant donné qu'un des inoculums fongiques pour les semis de pin gris appartenait également au genre *Suillus* et qu'une réponse positive du pin gris à l'inoculation de la ECM a également été observée, nous avons suspecté que le *Suillus* identifié pourrait être l'inoculum à l'origine de l'impact positif. Aucun autre type de champignon ressemblant à l'inocula de *Suillus* n'a été trouvé en association avec les autres plantes inoculées (épinette blanche et aulnes), ce qui peut expliquer les effets insignifiants ou négatifs de l'inoculation sur les semis de plantes poussant dans les stériles de la mine d'or. Indépendamment des effets d'inoculation, les sols plantés ont améliorés en général, les caractéristiques du sol de la mine, favorisant un pH du sol neutre (7.0), une teneur en eau plus élevée et une abondance microbienne relative beaucoup plus élevée.

ACKNOWLEDGMENTS

I would like to express my sincere thanks to my supervisor, Dr. Charles Greer, for giving me the opportunity to work on this project. His invaluable guidance, encouragements and support have helped me face and overcome many obstacles encountered during my masters. He was always generous with his profound knowledge and with his time to answer many of my questions, giving me the best advice and inspiring me to have a positive outlook on difficult situations. I do not think I could have had a better supervisor. I would also like to thank my co-supervisor, Dr. Lyle Whyte, for his help as an academic advisor at McGill. Thank you to Dr. Sébastien Roy and his lab group in the University of Sherbrooke, for their plantation experimental design, for the maintenance of the field site, and for their assistance during my field sampling campaigns at the Sigma gold mine in Val-d'Or, Quebec.

I want to also thank the Energy, Mining and Environment Research Centre (EME) of the National Research Council Canada (NRC) in Montreal for providing me with the research facilities to conduct my experiments. Thank you to all of the people in the EME group who provided me with technical assistance and made my masters a great pleasant learning experience: Christine Maynard, Claude Masson, Dr. David Juck, Danielle Beaumier, Danielle Ouellette, Jessica Wasserscheid, Julie Champagne, Julien Tremblay, Katrina Callender, Miria Elias, Nathalie Fortin, Sylvie Sanschagrin. In particular, I would like to extend a special thank you to Katrina Callender, for her extensive help in the lab, during sample collection, and for her useful suggestions for the preparation of my thesis. I can't thank her enough for that. Thank you to Nathalie Fortin for translating the thesis abstract into French. Special thanks to Julien Tremblay and Jessica Wasserscheid for their help with the bioinformatic analyses.

Thank you to all the friends I made during these years. I will always remember those unforgettable memories we shared together. I am thankful to Jennifer Sirosis and Sylvain Sirosis for being like a Canadian family to me. Thank you to my friends Jennifer and Heather for correcting my English grammar. Last but not least, to my beloved parents, thank you for always being there no matter what. This thesis could not have been accomplished without your love, encouragement, and endless support. Thank you.

CONTRIBUTIONS OF AUTHORS AND OTHER RESEARCHERS

The MSc. candidate Jinglin Chen, did the laboratory and field work, conducted all statistical analyses, and wrote all the content of this thesis, with invaluable suggestions, feedback and reviews provided by the thesis supervisor Dr. Charles Greer and the thesis co-supervisor Dr. Lyle Whyte.

Dr. Sébastien Roy and his laboratory team in the University of Sherbrooke designed this field experiment, conducted greenhouse inoculation of plant seedlings, initiated and maintained the plantation on the mine site.

Katrina Callender helped in sample collection and provided technical assistance in the lab. Christine Maynard, Julie Champagne, Miria Elias, Nathalie Fortin, Sylvie Sanschagrin also provided technical assistance and contributed greatly to my learning experience.

Julien Tremblay and Jessica Wasserscheid assisted with bioinformatics and statistical analysis.

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AC: green alder (*Alnus viridis* subsp. *crispa*)

ACI-FK: *Frankia*-inoculated green alder (*A. crispa*)

ACI-FK-ECM: *Frankia*-mycorrhizal fungi-inoculated green alder (*A. crispa*)

ACNI: uninoculated green alder (*A. crispa*)

Ad: alders' ectomycorrhizal fungal inoculum — *Alpova diplophloeus*

AM: arbuscular mycorrhizal fungi or arbuscular mycorrhizas

ANOVA: analysis of variance

AR: speckled alder (*Alnus incana* subsp. *rugosa*)

ARI-FK: *Frankia*-inoculated speckled alder (*A. rugosa*)

ARI-FK-ECM: *Frankia*-mycorrhizal fungi-inoculated speckled alder (*A. rugosa*)

ARNI: uninoculated speckled alder (*A. rugosa*)

BK: bulk soil

CTAB: hexadecyl trimethyl ammonium bromide

Ctrl: control

DNA: deoxyribonucleic acid

DSE: dark septate endophytes

E: white spruce (*Picea glauca*)

ECM: ectomycorrhizal fungi or ectomycorrhizas

E-ECM: ECM-inoculated white spruce (*Picea glauca*)

EMMs: estimated marginal means

E-NI: uninoculated white spruce (*Picea glauca*)

FK: alder bacterial inoculum — *Frankia* sp. strain AvcI1

Gi: alder arbuscular mycorrhizal fungal inoculum — *Glomus irregulare*

GLM: generalized linear model

Hc: white spruce ectomycorrhizal fungal inoculum — *Hebeloma crustiliniforme*

ITS: internal transcribed spacer

Lb: jack pine ectomycorrhizal fungal inoculum — *Laccaria bicolor*

NI: not inoculated

OTUs: operational taxonomic units

P: jack pine (*Pinus banksiana*)

PAHs: polycyclic aromatic hydrocarbons

PCBs: polychlorinated biphenyls

PCoA: principal coordinates analysis

PCR: polymerase chain reaction

PERMANOVA: permutational multivariate analysis of variance

PGPR: plant growth promoting rhizobacteria

PHC: petroleum hydrocarbon

Pi: white spruce's ectomycorrhizal fungal inoculum — *Paxillus involutus*

PNA: peptide nucleic acid; mPNA: mitochondrial PNA; pPNA: plastid PNA

POPs: persistent organic pollutants

PSMs: phosphate solubilizing microbes

PVI: plot volume index

RCD: root collar diameter

RDP: Ribosomal Database Project

SE: standard error

RNA: ribonucleic acid

rRNA: ribosomal ribonucleic acid

RZ: rhizosphere soil

St: jack pine's ectomycorrhizal fungal inoculum — *Suillus tomentosus*

SVI: seedling volume index

TCLP: toxicity characteristic leaching procedure

CHAPTER 1 : GENERAL INTRODUCTION

Mining activities have great economic, social and environmental impacts in Canada. Canada is one of the leading mining countries in the world, producing 13 major minerals and metals (Marshall 2017). The Canadian mining and mineral manufacturing sector, as reported by the Mining Association of Canada (MAC) (Marshall 2017), accounts for 3.5 % of the national GDP (approx. $57.6 billion) and 19 % of the country's total exports (approx. $92.8 billion) to international markets, indicating the significant contributions of mining industries to Canada's economy and global impact. In addition to its economic importance, contemporary mining also provides materials used in the construction of clean energy sources (e.g., nuclear, solar, wind) which leads Canada towards a low carbon future. Due to the growing demand for mine products (metals and minerals), mining operations are continuously being expanded throughout the country which also provides more employment opportunities. The MAC report also showed that mining industries currently employ more than 400,000 workers directly, with an additional roughly 200,000 employees hired indirectly by mining-associated companies. While the mining sector has created a profound economic and social impact on Canadian people's standard of living, mining extraction and processing activities have also resulted in great disturbance and contamination on the mined land, consequentially posing potential risks to human and environmental health (Rankin 2011). To comply with environmental standards and Canada's regulatory framework for mining operations, mining companies are now required to manage mine sites in an environmentally friendly manner, both during the mine life cycle and after mine closure.

Quebec is located on the Abitibi greenstone belt of the Superior Province, the Earth's largest Archean craton that is geographically specialized for mineral exploration and forms the core of the Canadian Shield (Percival et al. 2012). For this reason, metalliferous mining in Quebec has historically made substantial contributions to its provincial economic development, and this has also underpinned Canada's global leading position in metal and mineral production (Marquis 2004; Dupuis and Noreau 2017). Among the nearly 30 metallic minerals extracted throughout the province, the mining sector is still of particular interest in mining of precious metals such as gold (MERN 2017a). For more than 100 years, Quebec has undertaken large-scale gold mining activities, and currently, the province has 11 active gold-producing mines. In particular, gold

extraction is mainly done via open pit mining, a type of surface mining which can extract nearly 80% of ores, i.e., the gold bearing rocks from a mineral deposit (Rankin 2011). Open pit mining operations involve removing vegetation cover and upper soil layers (e.g., topsoil and overburden), and disposing of large amounts of mine spoils, often resulting in long-term soil perturbations on the mined sites (Jain et al. 2016; Kalucka and Jagodzinski 2016).

Additionally, the ratio of profitable component to waste rock in gold mining is significantly lower as compared to non-metallic mineral ores (Dudka and Adriano 1997). For this reason, the extraction and mineral processing of the ore generate vast amounts of metal-rich, sulfur- and cyanide-bearing solid wastes at or near the mining operation, which are directly disposed of and stockpiled without adequate waste management (Rankin 2011; Benarchid et al. 2018). These gold mine wastes have the potential to cause negative impacts on the ecosystem, such as acid generation, air pollution and water contamination (Lottermoser 2010; Lottermoser 2011). With the inherent metal and chemical pollutants, which are likely to migrate into surface- and underground water systems, or enter the food web, through soil erosion, wind weathering and water percolation, gold mine wastes are a great concern for the local community and authorities (MWC 2009; Hudson-Edwards et al. 2011; Rankin 2011). Therefore, multidisciplinary institutions, such as governments, mine companies and research institutions have a vested interest in post-mining reclamation on such waste repositories.

Currently, scientific studies regarding mine site rehabilitation have primarily focused on applying plant-based bioremediation, due to its solar-driven, cost-effective, aesthetically pleasing and environmentally-friendly advantages, outperforming conventional physical and chemical remediation technologies (Pierzynski et al. 1994). Unlike healthy soils, which maintain a well-balanced ecosystem with high biodiversity and productivity, in the mined soil, the physical (e.g., soil texture, moisture, porosity), chemical (e.g., pH, organic matter, mineral nutrients) and biological attributes are significantly altered (Cardoso et al. 2013). A lack of sufficient nutrients and essential biological processes is a major obstacle in the rehabilitation of mined land and regeneration of a self-sustaining vegetative cover. Soil microbial communities (diversity and relative abundance) are an integral element of successful reclamation because of their major roles in soil formation and stabilization, nutrient cycling (especially N and C), and plant growth

stimulation, which overall facilitates 90 % of soil ecosystem functions (Nielsen and Winding 2002; Macdonald et al. 2015; Adhikari and Hartemink 2016). Soil microbes are also more susceptible and rapid-response barometers to environmental changes than the aforementioned physical and chemical characteristics, and thus are supposed to forecast an early environmental disturbance (Cardoso et al. 2013; Muñoz-Rojas 2018). From this perspective, application of microbes for mine site rehabilitation should hold promise for improving soil conditions and the establishment and growth of vegetation in the mine sites.

Phytoremediation, a term coined around 20 years ago, is a widely adopted strategy in vegetative reclamation using vascular plants and their associated rhizosphere microorganisms to remove various organic (e.g., petroleum) or inorganic pollutants (e.g., metals) from, or to make them harmless in, different contaminated substrates including soils, sediments, groundwater and surface water (Singh et al. 2003; Vamerali et al. 2010; Nie et al. 2011; Wuana and Okieimen 2011). Recent research focusing on existing mine vegetation has found a diverse group of soil-derived microbes favoring phytoremediation processes from the metal tolerant plants' rhizosphere. The soil-root interface harbors the microbial community influenced by roots, suggesting that plant-microbe interactions are a crucial player in mine reclamation (Orłowska et al. 2010). The synergistic relationship of plants and microbes can be mutualistic to both partners: plants supply microbes with assimilated carbon (photosynthates), and in turn, plants obtain bioavailable nutrients (e.g., N, P) through the assistance of microbes (Berendsen et al. 2012). As rhizosphere microbes are host-specific, the composition structure and diversity of rhizosphere microbial communities, which are greatly influenced by the deposition of plant mucilage and root exudates, have been shown to be distinct from those of bulk soils, i.e., non-rhizosphere soils (Morgan et al. 2005; Bais et al. 2006). In addition to the profound impact of rhizosphere microbes on plant growth, the ability of plants to stabilize metals in the rhizosphere and the roots (i.e., phytostabilization), is also facilitated by specific fungal taxa, such as mycorrhizae (Wenzel 2009; Thavamani et al. 2017). Mycorrhizal fungi develop densely packed sheath and phenolic inter-hyphal material on mycorrhizal roots and are able to adsorb substantial amounts of metals, which protect roots from direct contact with the pollutant, greatly reducing the amount of bioavailable metals. The root-inhabiting microbes, i.e., root endophytes, were shown to interact with root exudates and regulate the fungal community in the rhizosphere for the benefit of the plants (Bais et al. 2006; Broeckling et al. 2008; Deng and

Cao 2017). In summary, microbial consortia in the rhizosphere and roots are both affected during phytoremediation, suggesting a need to characterize the root-associated bacterial and fungal communities that are selectively responding to, and responsible for, the reclamation process.

Plant species selected for phytoremediation of metalliferous wastes, which are termed metallophytes, should be resistant to a certain degree of metal concentrations. More importantly, they should not translocate metal pollutants to the above-ground biomass (i.e., hyperaccumulator plants) but stabilize them *in situ*, preferably in the root zone (i.e., non-hyperaccumulator plants) (Brooks et al. 1998; Lottermoser 2011). Alders (*Alnus* spp.), actinorhizal and mycorrhizal plant species that form symbioses with the nitrogen-fixing actinomycete, *Frankia*, and mycorrhizal fungi, respectively, have been widely used in land reclamation as pioneer species for amending soil nutrients, enhancing vegetation growth and initiating primary succession (Roy et al. 2007). The use of *Frankia* inocula with different alder species on oil-shale mine wastes demonstrated the capacity of alder-*Frankia* symbionts to degrade organic pollutants, facilitate plant growth, stimulate the indigenous microbial population, and accelerate phytoremediation efficiency (Greer et al. 2005; Lefrançois et al. 2010; Bissonnette et al. 2014). In a greenhouse study, *Frankia*-inoculated alders were also shown to thrive and promote seedling growth in gold mine wastes (Callender et al. 2016). In addition to *Alnus* spp., plants used in phytoremediation are usually native to the local environment. The boreal forest is one of the primary forest ecosystems in Quebec. Trees from the Canadian boreal forest generally include conifers such as white/black spruce, balsam fir, tamarack and jack pine, and deciduous plants such as white birch, trembling aspen, and willow (NRC 2017). Root-colonizing ectomycorrhizal (ECM) and arbuscular mycorrhizal (AM) fungi, which are naturally occurring plant microsymbionts for woody species, are believed to be involved in N and P cycling, they improve water and nutrient uptake by plants, and suppress the attack of plant pathogens (Gagné et al. 2006; Smith and Read 2008). Previous studies have demonstrated the efficacy of using ECM inoculants in enhancing the phytoremediation of mining disturbed soils (Bois et al. 2005; Huang et al. 2014; Zong et al. 2015).

Remediation strategies such as phytoremediation are considered a low-cost and efficient approach for mine reclamation and have gained popularity in sites with low to moderate metal contamination (Pilon-Smits 2005). The plant-microbe interaction is of pivotal importance for successfully

promoting plant survival and growth in nutrient-depleted environments. The introduction of robust plant species and the host-specific microbial consortium for plant enhancement are key in phytoremediation. However, very little information is available on this subject, and moreover, how a microbial community can be exploited to better plan soil rehabilitation remains largely unknown. The advent of high-throughput techniques such as 16S rRNA gene amplicon sequencing greatly simplifies microbial analysis in complex environments, which makes it possible to characterize plant-host microbial communities during mine reclamation. Therefore, the objective of our study was to contribute to the knowledge and understanding of the role of microbes in assisting revegetation of metal-contaminated soils, which will pave the way for future research to develop optimal and tailored phytoremediation strategies for mine site rehabilitation.

The study site for this project is a gold mine located adjacent to the town of Val-d'Or in Northern Quebec. The Sigma gold mine is stockpiled with large volumes of waste rock and tailings, which have moderate metal contamination (Co, Cr and Cu) and poor soil characteristics, including a slightly alkaline pH level (~ 8.6), low water retention and a low organic matter content (Beauregard et al. 2012; Callender et al. 2016). When a mine is located in densely populated areas such as the Sigma gold mine, rehabilitation of mine waste dumps usually becomes of great social interest. Therefore, in accordance with federal and provincial regulations for the development of sustainable mining and elimination of adverse environmental impacts (Laberge et al. 2016; MERN 2017b), phytoremediation trials have been initiated on site to restore the soil ecosystem function and stability through the reforestation of mine waste. This collaborative effort between three university teams involved the establishment of plantations on Sigma mine waste rock and overburden in 2012. The trials consisted of two alder species, green alder (*Alnus viridis* subsp. *crispa*) and speckled alder (*Alnus incana* subsp. *rugosa*), and two conifers, white spruce (*Picea glauca*) and jack pine (*Pinus banksiana*). Plant seedlings in the 2012 phytoremediation trial were inoculated with different combinations of *Frankia*, AM and ECM fungal inoculants.

The objectives of this study were to: 1.) evaluate differences in plant survival and growth between non- and inoculated plants. We hypothesized that plants that had been inoculated prior to outplanting will survive and grow better than non-inoculated plants; 2.) characterize the microbial communities (diversity and relative abundance) present in the plant rhizosphere and roots, corresponding to non- and inoculated plant seedlings. We hypothesized that the inoculated plants will have a more diverse microbial community over time than non-inoculated seedlings.

CHAPTER 2 : LITERATURE REVIEW

2.1 Gold mining in Quebec

Globally, Canada is known as a dominant mining nation. In all, there are 1,200 active mines, 65 of them are metal mines, 20 of which are in the province of Quebec, demonstrating the substantial contribution of Quebec to the national metal mining industry. In Quebec, the mineral sector is a driving force in its economy and also an important source of employment. An economic study funded by the Desjardins company indicated that Quebec's mining industry represented 1.4 % of the provincial GDP in 2015 and that the mining sector provided around 14,000 jobs throughout the year (Dupuis and Noreau 2017). The study also revealed that three regions dominate the province and have received the most regional mining investments: Nord-du-Quebec, Abitibi-Temiscamingue and Cote-Nord. Although nearly 30 metallic (e.g., iron, copper, zinc) and non-metallic minerals (e.g., peat, stone, silica, mica) are extracted in Quebec, the mining sector is still largely focused on mining of precious metals such as gold (Dupuis and Noreau 2017; MERN 2017a). Benefiting from a superior geological location within the largest gold deposit in the world, the Abitibi Greenstone Belt (AGB), gold mining activities are prevalent in the Abitibi-Temiscamingue region, which contains more than 70 % of Canada's mining sites (Benarchid et al. 2018) and has recognized gold potential within the Rouyn-Noranda – Val-d'Or axis (Figure 2.1). Moreover in 2017, this Abitibi-Temiscamingue region, where the amount of received mining investment ($1.2 billion) accounted for roughly 30 % of all exploration and deposit appraisal investment expenditures by Quebec mines, was considerably larger than that in the Cote-Nord and Nord-du-Quebec regions, at $657 million and $841 million, respectively (Madore and Caron 2018).

Due to the enriched gold reserves of Quebec, the development of gold mines has received considerable attention from both federal and provincial governments. Although gold mining activities (mineral extraction and processing) have contributed significant benefits in boosting the prosperity of the host province and local community, they can also cause serious environmental degradation (Lottermoser 2010; Benarchid et al. 2018). In recent years, with the global mining industry moving towards a more sustainable framework, the Canadian mining industry has also made substantial progress in its environmental management practices during the mine life cycle and after mine closure, by participating in a host of sustainability programs and initiatives

(Marshall 2017). In Quebec, the government and multidisciplinary research have made enormous collaborative efforts to foster the competitiveness of Quebec's mining sector and the mitigation of mining impacts on the environment in order to secure Quebec's leading position in modern and sustainable mining. Since 2013, the Ministere de l'Energie et des Ressources naturelles (MERN) invested $16.5 million over five years in a sustainable mining development research program entitled the Fonds de recherche du Quebec – Nature et Technologies (FRQNT) in order to help fund research projects which are carried out by universities and recognized research organizations, with the objective of optimizing Quebec's mineral potential (MRNF 2009; MERN 2017a).

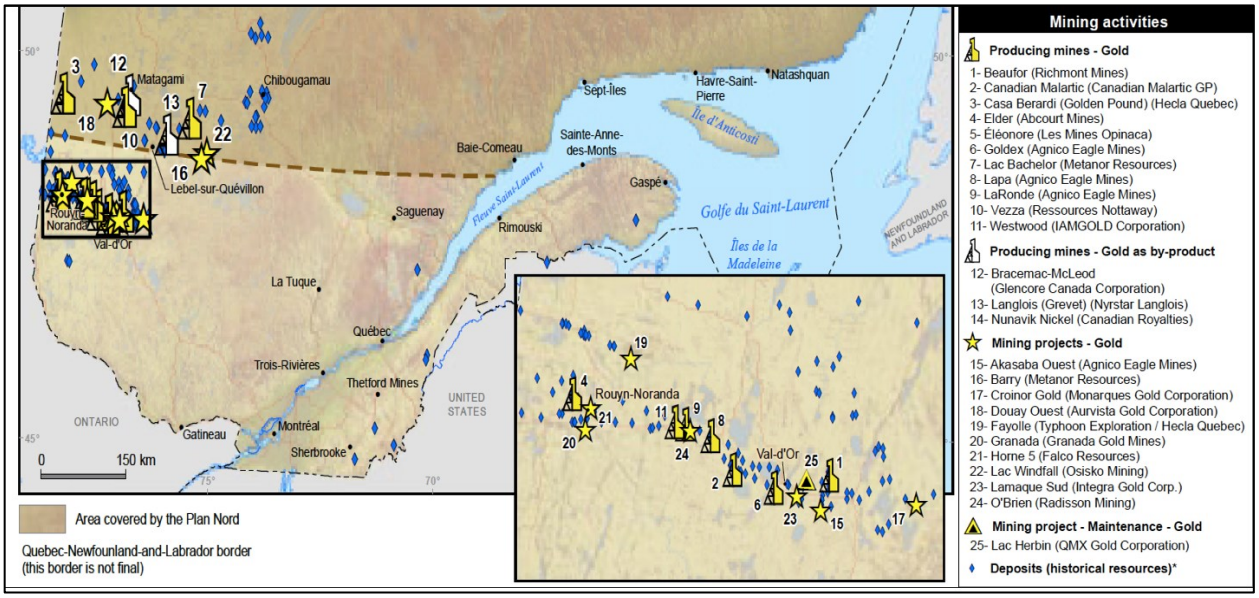

Figure 2.1. Map of the gold mining activity in Quebec. Source: Ministere de l'Energie et des Ressources naturelles (MERN), 2017

2.2 Environmental concerns arising from non-ferrous metal mining

The major environmental concerns of mining and mineral processing can be generally divided into six areas consisting of: air quality, water quality/quantity, acid mine drainage, land impacts, ecological impacts, and economic impacts (Jain et al. 2016). Although the impact of mining on each environmental attribute is variable depending on the mine site characteristics, and one attribute may be more severely affected than another at an individual mine, these six elements are frequently used in environmental assessment procedures for sustainably managing mining areas. In addition to major environmental concerns, the other related environmental impacts of mining

should also be indicated, including types of desired mineral or ore (metallic or non-metallic minerals), categories of target mineral deposit (placer or lode deposits), the extraction methods for mineral exploitation (e.g., surface mining, underground mining), and mineral processing (physical and chemical) (Rankin 2011; Jain et al. 2016).

The exploration of non-ferrous metallic ores (i.e., all metallic minerals other than iron), such as gold and copper, demonstrates the historical importance of metals in human society (Allan 1995; Armstrong et al. 2014; Sohn 2017). Non-ferrous metals occur in the earth's crust mainly as chemical compounds (minerals) such as sulfides, oxides, silicates, and carbonates (Sohn 2017). Since sulfides constitute a major proportion of rocks in non-ferrous metallic ore deposits, mining these metals can expose sulfides to air (oxygen) and water, which results in sulfuric oxidation and eventually generates the flow of acidic water comprising sulfates, heavy metals (Pb, Cr, As, Zn, Cd, Cu, Hg, Ni, etc.) and metalloids (Lottermoser 2010). This resulting outflow from mining operations creates acidic conditions at the mine site, and it is called acid mine drainage (AMD). AMD is regarded as a challenging environmental problem faced by many mining industries, because it poses a serious risk to the groundwater and aquatic ecosystem health (Lottermoser 2010). In addition to AMD, the release of acidic water laden with toxic heavy metals can also contaminate surface water and enter the food chain, exerting detrimental acute or chronic impacts on wildlife and humans (MWC 2009).

Mining extraction methods fall broadly into three categories: surface mining, underground mining and solution or *in situ* mining (Rankin 2011). The operation method of mining extraction usually depends on the depth of an ore body in the earth's crust. In general, underground mining is operated for deep and widely disseminated mineral deposits, whereas surface mining is applied when the ore body is located relatively close to the surface. In contrast to solution mining, which is not applicable for extracting metals on a commercial scale due to its requirement for the permeability of mineral deposits, surface and underground mining are the two mechanical mining operations that extract the metallic ore from mineral deposits. In a non-ferrous metallic mine, where the desirable ore only constitutes a small fraction of the total mined materials (Dudka and Adriano 1997), mining operations often involve the removal of vegetative cover, topsoil and the material lying over the mineral deposit (i.e., overburden) to access and extract the ore. This creates severe

perturbations on the mined land and generates a massive volume of solid wastes at or near the mine (Jain et al. 2016). In underground mines, passages are built to gain access to the ore, which reduces the amount of solid waste production. Particularly, in underground mines, the ratio of ores to waste rocks, i.e., the blasted rocks containing non-profitable mineral concentrations, is usually greater than 1 (Rankin 2011). By contrast, in surface mines, the mass of removed overburden and waste rocks from the sides of the open pit can be 1-4 times, or even up to 50-60 times larger than the amount of the extracted ore (Rankin 2011). As such, a large quantity of metal-containing waste rocks is brought to the surface after the ore extraction, which increases the contamination pathways of toxic metals to the ecosystem. The handling, storage and disposal of the solid wastes after surface mining is of primary concern for the mining sector, local community and regulators (Wuana and Okieimen 2011). Unlike organic chemical pollutants that can be degraded biologically (microbial action) or chemically into carbon dioxide and water, inorganic contaminants can persist in soils for a long time after their introduction (Barbour 1994; Wuana and Okieimen 2011). In addition to solid waste generation, in surface mines, air pollution, caused by the emission of dust and hazardous particulates from stripping the overburden (mineral drilling and blasting), has also become an ongoing environmental concern (Jain et al. 2016).

In terms of the mineral processing involved in a common metallic mine, the mined raw ore usually undergoes beneficiation processes at the mine in order to produce a concentrate of the valuable materials and save transportation costs. Beneficiation involves a series of physical and chemical separation steps, and because of the addition of water and chemicals during processing, the beneficiation process can impose significant negative impacts on water and the mining area (Rankin 2011; Jain et al. 2016). In the physical process, the mined material is crushed to particles less than 10-50 mm in diameter, and ground to reduce its size to 50-500 μm in mills. As more undersized materials of lower grade ore are left behind during the physical process, additional amounts of wastes are also produced around mining operations. In the chemical process, potentially toxic chemicals are often used to dissolve the valuable minerals from the raw ore (Rankin 2011). As a result, the leakage of tailings ponds and the erosion of waste piles can lead to large areas of land deterioration, and the contamination of surface and underground waters (Lottermoser 2010; Jain et al. 2016). In particular, the leachate residues from gold extraction and processing wastes often contain sodium cyanide (NaCN), which is a hazardous chemical substance

lethal to wildlife and humans if sufficient concentrations are taken up through inhalation, ingestion or skin absorption (Lottermoser 2010). Despite the poisonous character of cyanide-bearing wastes to humans and animals, cyanide has non-toxic effects on plants, especially cyanogenic plants, which have strong tolerance of elevated cyanide levels in the soil. Apart from cyanogenic plants, certain soil microorganisms, such as bacteria, fungi and certain arthropods, which are capable of using and/or decomposing cyanide, have been widely used in engineered biological oxidation of cyanide (Logsdon et al. 1999; Gessner et al. 2005). Regeneration of vegetation cover on the mined land, therefore, is supposed to immobilize cyanide in the waste repository thus avoiding it being transferred into plant tissues as well (Lottermoser 2010). In addition to cyanide pollution, the release of other toxic contaminants during gold mine operations, such as mercury (Hg), arsenic (As), and antimony (Sb), are also potential hazards to water and soil quality.

2.2.1 Land impacts of gold mines and waste management

Mining effects on land broadly include reduction of vegetation and biodiversity, soil contamination, land erosion, accumulation of solid waste, change of land use patterns (loss of forests), and disturbance to the natural ecosystem (Jain et al. 2016). Two primary solid wastes, waste rocks (coarse particles) and tailings (a slurry containing fine particles), form a total of 25 billion tonnes of solid wastes annually produced from mining and mineral processing activities throughout the world (Lottermoser 2010). In Canada, a rough estimate of the volume of solid wastes produced from mining is approximately 2 million tonnes per day (MWC 2009). As mentioned, gold mines, which are analogous to most metalliferous mines, produce very large quantities of solid waste during extraction and mineral processing of the ore (Dudka and Adriano 1997; Rankin 2011). As an example, 1 million tonnes of solid waste are generated during the production of 1 tonne of gold, consisting of 0.39 million tonnes of waste rock and unmined ores, and 0.59 million tonnes of dry mass from processing tailings and solution leachates (Rankin 2011).

In contemporary mining operations, the great majority of waste rock is either disposed of as waste rock dumps at the mine site or recycled at waste storage facilities as construction aggregates (Lottermoser 2011; Benarchid et al. 2018). The vast amount of waste rock that is not included in the waste management strategy can lead to potential environmental problems such as acid generation, the migration of contaminants (e.g., heavy metals, chemical residues) up the food chain

and their drainage into underground water systems. Therefore, careful handling of waste rock after mining and an adequate waste control process following mine closure have become crucial for environmental protection. As mentioned, part of the waste rock can be recycled for construction and reused as mine capping materials. However, due to the volumes produced and the hazardous substances inherent in the waste rocks, which in the long term can pose risks to the local community and the environment, the mining sector requires waste management alternatives other than reuse and recycle upon mine closure (Lottermoser 2011). In recent years, catering to a pressing need to foster the development of sustainable mining in Canada, the mining sector, government authorities and all stakeholders are collaborating to investigate efficient, economical and environment-friendly reclamation solutions for waste rock dumps when a mining operation ceases.

Complying with environmental requirements and regulations, all post-mining measures for mine wastes, whether through recycling, reuse or rehabilitation, are planned to return waste repositories and mined land to a standard allowing future land use. There are several similar definitions to describe the waste treatment and ecological management procedures following an anthropogenic disturbance on the soil environment: ‘remediation’, ‘reclamation’, ‘rehabilitation’ and ‘restoration’ (Finger et al. 2007). Remediation refers to the cleanup of a contaminated area, but remedial actions at mine sites can refer to the isolation or physical removal of contaminants from the terrestrial ecosystem (Finger et al. 2007). Reclamation, in a mining context, commonly defines the general process of returning the mined wasteland to a state with some beneficial use. Rehabilitation and restoration both indicate an ecological recovery of disturbed wasteland, but with different endpoints of ecological succession. The major difference is that, rehabilitation aims to recreate ecosystem processes, productivity, or services with regard to conditions existing prior to any anthropogenic disturbance, whether this progression is achieved or not; by contrast, restoration indicates a reinstatement of the original ecosystem (Li 2006). Recent scientific research reveals that restoration of mining-affected areas to the pre-mining ecosystem is difficult or impossible to achieve, so the terms rehabilitation and reclamation of mine wastes are used interchangeably in this context.

2.3. Phytoremediation on gold mine waste rock

The removal of vegetation cover and topsoil before gold mining operations severely affects the heterogeneity and sustainability of soils from the mined land. Since the functionality and sustainability of soils, to a large extent, are underpinned by the diversity and productivity of a well-developed plant community, revegetation on mined wastelands has proven to exert both direct and indirect influences on reclaimed mine soils (Yuan et al. 2018). The development of plant roots and the production of litter and root exudates directly affect the formation of soil aggregates, soil structure and other physicochemical properties of soils. The complex interactions of plants and soil biota (microflora and macrofauna) reveal the indirect effects of plants on soil fertility, as plants, assisted by soil biota, strongly accelerate the nutrient availability and the decomposition of organic matter. Metal contamination, soils characterized by high rock density, lack of nutrients and low infiltration, are typical challenges to revegetating gold mines (Yuan et al. 2018). In this case, metal-tolerant plant species (i.e., metallophytes) are generally selected to remove and stabilize contaminants in metalliferous mine spoils (Lottermoser 2011). Non-hyperaccumulator plants or metal-excluding plants, which do not concentrate metals in their above-ground biomass, are preferable in mite site rehabilitation, due to the decreased likelihood of transferring metals up the food chain. Additionally, the selected candidate plantation species should also be native to the local environment for better weed control. In terms of the nutrient deficiency in metalliferous mine wastes, one solution is to introduce N_2-fixing plant species to maintain the healthy growth and long-term persistence of vegetation (Li 2006). Overall, the use of vegetation as a basis for landscaping fulfills the objectives of visual improvement, long-term stabilization of metal pollutants, amelioration of mine substrates, minimization of soil erosion by water or wind, and restoration of ecosystem services, which cannot be realistically achieved by either chemical or physical remediation options (Johnson et al. 1994; Pierzynski et al. 1994; Li 2006).

Phytoremediation is a vegetative reclamation approach using plants and associated microbiota to remove, stabilize, or render organic or inorganic pollutants harmless (Schnoor 1997; Wuana and Okieimen 2011). Phytoremediation has several advantages over numerous physical-chemical remediation technologies (e.g., thermal desorption) and conventional bioremediation techniques (e.g., bioventing) (Khan et al. 2004). It is more cost effective, less environmentally disruptive, aesthetically pleasing, applicable over the long-term, and well-suited for large sites (Favas et al.

2014). Owing to distinct plant characteristics and the specific conditions at different treatment sites, phytoremediation can be generally divided into 5 different strategies, which are summarized in Table 2.1. Accordingly, plants used in the reclamation of metal-contaminated soils should demonstrate a capacity to withstand and thrive on the toxic metalliferous substrates, improve the quality of poor soils via restoring the ecosystem processes and, ultimately, aid in ecological succession.

Table 2.1. Five different types of phytoremediation.
Sources: Schwitzuébel 2004, Wuana and Okieimen 2011, Favas et al. 2014

Phytoremediation techniques	Mechanisms	Plant selection
Phytotransformation (Phytodegradation)	Uptake of organic contaminants from surface- or ground-water, and then degrade or transform them inside plant cells	Higher plant species possess complex enzyme activities to metabolize and detoxify xenobiotic compounds, e.g., *Populus* species
Phytovolatilization	Absorb and volatilize organic xenobiotics and certain metals/metalloids (e.g., Hg, Se and As) from soils, detoxify and release them into the atmosphere	
Phytoextraction (Phytoaccumulation)	Uptake of (organic or inorganic) contaminants from soils into plant tissues	Hyperaccumulator plants with rapid growth trait, high biomass yield and high bioaccumulation rate
Phytostabilization (Phytoimmobilization)	Absorb (organic or inorganic) contaminants into roots or precipitate them in the rhizosphere to reduce the bioavailability of metals	Non-hyperaccumulator plants with dense root systems
Phytofiltration (Rhizofiltration)	Plant roots (rhizofiltration) absorb or adsorb metal pollutants from groundwater and aqueous-waste streams	Plants are grown hydroponically for developing a large root system and acclimated to pollutants upon transplanting *in situ*

Phytostabilization and phytoextraction are two common types of phytoremediation in managing metal-contaminated soils (Schwitzguébel 2004). Unlike phytoextraction, which aims to remove metal pollutants from the contaminated soil, the reclamation purpose of phytostabilization is to stabilize metal pollutants and reduce their bioavailability in soils to prevent them from migrating

into water and the food chain via water percolation and soil/wind erosion (Schwitzguébel 2004; Favas et al. 2014). Metal pollutants can be stabilized in the contaminated soil for a long time during plant colonization and development at the mine. Plants are capable of sequestrating a great amount of metals in their roots thus reducing the mobility of metals in the mine substrate (Vamerali et al. 2009; Vamerali et al. 2010). Since vegetation is maintained at the contaminated site, phytostabilization also improves soil quality by increasing the amount of organic matter, nutrient levels, cation exchange capacity and biological activity (Arienzo et al. 2004).

2.4 Soil microbial community and its environment

Soil is a complex ecosystem composed of a large variety of microhabitats which result from the interaction of physicochemical gradients, fluctuating environmental conditions and the diversity of soil microbiota (Torsvik and Øvreås 2002). Soil-borne microbes are the unseen majority of microbial members that sustain a wide range of different life forms (Van Der Heijden et al. 2008). They are involved in key ecosystem services and serve as crucial drivers in maintaining soil productivity and sustainability. As such, soil microbes are frequently used as bioindicators to evaluate soil health, due to their high sensitivity and rapid response to any soil perturbation (Nielsen and Winding 2002; Muñoz-Rojas 2018). Investigations on the microbial component of the disturbed soil, as compared to soil physicochemical properties, are advantageous in providing in-depth and invaluable information for evaluating land reclamation efforts. While the soil microbial community is a driving force for sustaining key ecosystem services, its community structure (species diversity, richness and relative abundance) is also constrained by two categories of environmental factors: abiotic and biotic. Abiotic factors generally include soil characteristics, regional climate, salinity, water retention capacity, pH, and biotic factors mainly take the plant-microorganism interactions into account (Muñoz-Rojas 2018). There is no doubt that both factors, soil properties as well as plant-microbe interactions, strongly affect the structure and function of microbial communities. In this review section, we describe the contribution of soil microbiota to ecosystem stability, address the impact of soil physicochemical properties in shaping the community structure of soil microbiota, and reveal the intrinsic relationship between plants and microbes by focusing on the effect of plants on the community structure of rhizosphere soil microbiota.

2.4.1 Contribution of soil microflora to ecosystem stability

Microbial diversity and function, are major contributors to ecosystem processes, ensuring that organic compounds are recycled and retain ecosystem stability and resilience under fluctuating environmental conditions (Torsvik and Øvreås 2002; Adriano et al. 2004). Given that a declining pattern of microbial diversity is often associated with vulnerable lands destroyed through anthropogenic activities, the relationship between soil microbial diversity and terrestrial ecosystem functional stability is of particular interest in restoring soil sustainability. Whether ecosystem functions are carried out by specific microbial groups or by the interaction of diverse microbial species is still under debate, but soil habitats with a high diversity of bacterial and fungal species are likely to result in a more stable environment, and to have a higher resistance to anthropogenic disturbance. According to Wardle et al. (2004), food webs are also driven differently in soil habitats depending on whether the community is dominated by bacteria or fungi. Bacteria-dominated food webs stimulate the mineralization rate and bioavailability of nutrients to plants, whereas fungal-dominated food webs promote a slow and highly conservative cycling of nutrients.

Soil microorganisms also impact ecosystem stability through their dramatic influence on plant productivity and diversity, particularly when the affected plants are dominant or keystone species in a forest, such as *Quercus* (oak), *Acacia* and *Eucalyptus* (Burdon et al. 2006). Soil microorganisms are known to establish a mutualistic, neutral or pathogenic relationship with plants. The outbreak of certain plant pathogens can reduce the species richness of the plant community. For example, genera of *Phytophthora* sp., *Fusarium* sp. and *Pythium* sp. often target the keystone tree species in a natural forest ecosystem (Burdon et al. 2006). In contrast, soil microbes stimulate plant development by being involved in nutrient (e.g., N and P) cycling. Nitrogen (N) and phosphorus (P) are two important plant macronutrients. Although gaseous nitrogen (N_2) is unlimited and accounts for 78 % in the atmosphere (Elser 2011), this N is not directly accessible to plants. Similarly, P resources are sparingly available in soils and are often in insoluble phosphate forms, which are also not available for direct plant uptake. Microorganisms, however, can assist plants to acquire these two nutrients by increasing the bioavailability of N and P in soils. Soil bacteria are key drivers in N cycling which participate in several processes comprising (biological) N fixation, ammonification (i.e., N mineralization), nitrification, and denitrification (Nehl and

Knox 2006; Azcón-Aguilar and Barea 2015). During the N fixation, diazotrophs (i.e., some species in the families of bacteria and cyanobacteria), which possess the enzyme nitrogenase responsible for such activity, are able to transform ambient N_2 into plant assimilable N (ammonia) (Marschner 1995). According to the energy source and N fixation capability, the most studied N_2-fixing bacteria in terrestrial ecosystems can be categorized into three major types: symbiotic, associative, and free-living (Marschner 1995; Olivares et al. 2013). Of the three types, plant-symbiotic bacteria in general have the highest N_2-fixing efficiency because they are provided with energy from the host plants, both in the forms of carbohydrates and N metabolites (Marschner 1995). Free-living bacteria from diverse genera are also reported to contribute relatively low amounts of assimilable N to plants while a larger component of the N resource is retained for their own needs (Nehl and Knox 2006). Similar to N_2-fixing bacteria, soil microbes are also involved in P-cycling, which include P-solubilizers (inorganic phosphates) or P-mineralizers (organic phosphates) (Marschner 2008). Phosphate-mobilizing bacteria and fungi release plant-available P from inorganic or organic phosphates into soils through microbial solubilization or mineralization, respectively. As reviewed by Richardson et al. (2009), the fact that soil microbes are key players for the functioning of the P cycle has been shown by the increase of orthophosphate availability in the rhizosphere of plants with P mobilizing microbial inoculants, yet the effectiveness of P-mobilization by soil microbes in non-rhizosphere microhabitats is uncertain. Microbes that play a major role in mobilizing P resources include plant growth promoting rhizobacteria (PGPR), phosphate solubilizing microbes (PSMs), and symbiotic microbes such as arbuscular mycorrhizal (AM) and ectomycorrhizal (ECM) fungi (Antoun 2012).

2.4.2 Effects of soil property on microbial communities

The recent advent of various culture-independent technologies has enhanced our abilities to compare the dominance of bacteria and fungi present in the soil habitat at a taxonomic level, which provides new insights into how the microbial community structure is changing with soil properties. Soil properties related to bacterial community structure that are frequently analyzed include: pH, soil moisture content, cation exchange capacity, total carbon, total nitrogen, inorganic nitrogen (NH_4^+ and NO_3^-) and soil texture (Lauber et al. 2008; Kim et al. 2014). Among all measurable soil parameters, pH is considered the strongest factor influencing bacterial richness, diversity, and community composition. Supporting evidence regarding bacterial diversity is strongly correlated

with soil pH and has been confirmed in various microbial investigations conducted across different landscapes (Lauber et al. 2008), at both continental and local scales (Fierer and Jackson 2006), in both upper and lower soil layers (Kim et al. 2014). More specifically, bacterial diversity achieves the highest levels in neutral soils (pH ~7), decreases relatively in alkaline soils (pH > 7) and can drop to the lowest when the acidity in soil rises (pH < 7).

Another soil factor of interest impacting the bacterial diversity and community structure is soil texture, which is an overall indication of soil structure, spatial location, moisture content and nutrient status. While pH is commonly viewed as representing the cumulative effects of many chemicals, it is also necessary to be concerned with the role soil texture plays in influencing the bacterial community. Soil particle sizes ranging from 2 to 20 μm are assumed to be the most favourable growing condition for over 80 % of bacteria in terms of the great amount of available water, nutrients and high gas diffusion (Torsvik and Øvreås 2002). A similar observation was also made by Girvan et al. (2003); the authors found that shifts in the bacterial community were the consequences of changes in soil texture after obtaining nearly identical bacterial components among spatially diverse regions.

Anthropogenic changes in land-use often impose significant impacts on some soil properties such as soil texture and nutrient status, leading to a shift in the microbial community composition due to the altered edaphic characteristics. Since bacteria and fungi have their own distinct functions, the composition of bacterial and fungal communities also reflects the level of environmental disturbance. It was documented in Wardle et al. (2004) that the soil ecosystem with a populated bacterial community reflects high levels of disturbance, neutral to moderately acidic pH, an increase of nutrient availability and a reduction of organic matter content. The fungal-dominated soil ecosystem, however, demonstrates the site is less disturbed with acidic pH, slow successional progress, high organic matter content and low source quality. The change of soil nutrient status significantly affects the fungal community rather than the bacterial community. This can be explained by the fact that the population and diversity of saprotrophic fungi, which are the plant litter decomposers, are decreased when the soil organic matter content declines (Carlile et al. 2001).

2.4.3 Rhizosphere: a habitat for the plant-specific soil microbes

Soil microbial communities are the greatest reservoir of biological diversity in the world. A fraction of microbes that live in the vicinity of plant roots, termed 'rhizosphere', have developed synergistic relationships with plants. The German biologist Hiltner first defined the concept of the rhizosphere in 1904 to describe a narrow soil zone that is closely adhered to and immediately influenced by root secretions (Bais et al. 2006; Xu et al. 2012). Additionally, the surrounding soil beyond the rhizosphere soil is defined as bulk soil, literally meaning the massive amount of soil with no or minimal plant effects. As a consequence of the influence of root-derived compounds, the rhizosphere is a dynamic environment enriched with organic substrates to stimulate microbial growth (Egamberdieva et al. 2008).The microbial population in the rhizosphere is considerably more abundant compared with that in the bulk soil, a phenomenon known as the 'rhizosphere effect' (Davet 2004; Berg and Smalla 2009; Berendsen et al. 2012).The rhizosphere contains over 30,000 prokaryotic (bacterial and archaeal) species and up to 10^{11} bacteria per gram of root (Egamberdieva et al. 2008; Mendes et al. 2011). Since the number of microbial genomes in the rhizosphere is even larger than that of plants, the rhizosphere microflora is also called 'the second genome' of plants.

The rhizosphere, which serves as the root-soil interface, undergoes the most complex chemical, physical, and biological interactions occurring among plants, soil microbiota (flora and fauna) and the other abiotic factors. Plant roots excrete a variety of compounds into the rhizosphere, consisting of root exudates, mucilage and sloughed-off root cells, which is termed rhizodeposition (Davet 2004; Berendsen et al. 2012). The process of rhizodeposition represents a trophic supply from roots and a provision of ecological niches to sustain microbial growth (Buée et al. 2009a). Through the process, plants exert selective pressure to regulate the rhizosphere microbial composition, to ensure the supply of vital nutrients and to adapt to hostile soil environments (Davet 2004; Singh et al. 2004). For example, organic acids, low molecular weight compounds in root exudates, provide substrates for microbial metabolism and they are also the intermediates for soil biogeochemical reactions (Berg and Smalla 2009). In addition, organic acids can also assist in the solubilization of plant-unavailable Ca, Fe and Al phosphates in the soil, therefore enabling plants to absorb the inorganic phosphorus elements. The use of organic acids by plants is thought to be a response to nutrient deficiency (e.g., P-deficiency) (Bais et al. 2006).

The quality and quantity of rhizodeposition is related to the plant genome. Thus, there is good reason to believe that the genotype of plants has a more important effect on the rhizosphere microbial component compared to other soil compartments (Buée et al. 2009a). This host-specific characteristic is shown by less microbial diversity in the rhizosphere than in the bulk soil (Berg and Smalla 2009; Berendsen et al. 2012). Rhizosphere-dwelling microbes within the soil microbial community are extremely important in microbial research, as demonstrated by an increasing body of evidence showing that rhizosphere microbes are key determinants for plant fitness and nutrition (Haichar et al. 2008; Berendsen et al. 2012; Mendes et al. 2014). As previously described, rhizosphere (symbiotic) microbes assist plants in acquiring nutrients, typically nitrogen, which is not bioavailable for plants *per se* without N_2-fixing rhizobacteria and various actinomycetes (Bradshaw 1997). Šourková et al. (2005) found the alder-planted coal mine spoil heaps in Sokolov, Czech Republic, rapidly accumulated comparable amounts of organic carbon (C) and nitrogen (N) in the soil profile of reclaimed sites and had similar formation of the litter and fermentation layers as semi-natural alder forests. In particular, their study showed that the organic C and N content in a 40-year reclaimed mine site (the site had the least soil development) was only 26 % lower compared with the semi-natural sites; the authors attributed the fast accumulation of organic C and N to symbiotic N_2 fixation in alder roots. As for P-mobilization ability by rhizobacteria, although it has not been clearly elucidated to which extent they contribute to the amount of plant assimilable P, in general, scientists are in agreement that metabolization of organic acids by bacteria is 2-3 times faster than by plants *per se* and this results in an enhancement of P mineralization in the rhizosphere (Nehl and Knox 2006). In addition to assisting in the release of plant-required nutrients from inorganic forms, rhizosphere microbes also contribute to plant growth by immobilizing metal contaminants (Thavamani et al. 2017). Regarding physicochemical characteristics of the rhizosphere soil, which have been extensively studied in the past, many researchers have found that the rhizosphere differs significantly from the neighbouring soil with respect to a facilitated recycling of mineral elements, a lower redox potential, a reduction of plant allelopathy, an improvement of the soil compaction, the degree of moisture and the local pH level (Davet 2004; Singh et al. 2004; Berg and Smalla 2009; Buée et al. 2009a; Berendsen et al. 2012).

2.5. Root-associated microbial communities

Terrestrial plant roots define the interface between a plant and its soil habitat. They have garnered a great deal of attention because they support one of the richest microbial ecosystems on earth. In the development of such ecosystems, microfloral colonization includes the microbes living within root tissues and those in the rhizosphere compartment (i.e., the root surface). As previously described, rhizosphere microbes, typically rhizobacteria, play a critical role in reducing metal mobilization in soil and enabling plants to colonize hostile soil environments. One specific group of fungi, mycorrhizas, which proliferate in the interior and exterior of host plant roots, can also reduce metal mobility by adsorbing metals to their hyphae and thus preventing the pollutants from translocation to shoots (Pierzynski et al. 1994). While rhizosphere microorganisms substantially stimulate the development of a stabilized, decontaminated and nutrient enriched soil environment, root endophytes, which share an intimate relationship with host plants by residing inside of them, are also critical to plant survival and growth. Root endophytes are more plant-specific and the study of them certainly expands our understanding of plant performance throughout the reclamation stage. Overall, because the root-associated microflora affect the architecture and activity of roots dramatically, microbial investigations concentrated on the root aspect are of significant interest to developing biotechnologies that enhance plant growth (Xu et al. 2012). To understand the importance of the root microbiome in plant growth promotion, in this section, we introduce three types of root-associated microsymbionts: the root-nodulating actinomycete *Frankia* sp., mycorrhizal fungi, and root endophytes.

2.5.1 Root-nodulating bacteria: *Frankia* sp.

Actinomycetes, are gram-positive bacteria known for filamentous and branching characteristics, form complex bacterial morphological structures. The majority of Actinomycetes are free-living saprophytes that inhabit natural substrates and only a few of them are host-associated (Lechevalier and Lechevalier 1990). The bacterial genus *Frankia* is a well-known N_2-fixing and root nodulating actinomycete, belonging to the Frankiaceae family (Hahn et al. 1989). *Frankia* sp. are heterotrophic, aerobic or microaerophilic bacteria with filamentous growth. Initially, the genus *Frankia* was recognized as the heterogeneous population of “vesicle clusters” grown in plant root nodules and was simply defined as “root endophytes”. In the mid-1970s, with the advancements

in electron microscopy, Frankiae was confirmed with its actinomycetous morphology (Becking 1970; Becking 1974). In 1988 the symbiotic relationship to plants was also re-defined and changed from "plant endophyte" to "plant microsymbiont" at the 7th International Meeting on *Frankia* and Actinorhizal Plants in Storrs, Connecticut (Baker and Schwintzer 1990).

Frankia sp. are able to form the N_2-fixing symbioses with a taxonomically diverse group of perennial dicotyledonous plants from eight families (incl. 25 genera over 200 species), comprising Betulaceae, Casuarinaceae, Coriariaceae, Datiscaceae, Elaeagnaceae, Myricaceae, Rhamnaceae, Rosaceae (Baker and Schwintzer 1990; Lechevalier 1994). Due to their unique ability to harbor the actinomycete (e.g., Frankia*e*) in roots, the above indicated plant families are referred to as actinorhizal plants or actinorhiza (Baker and Schwintzer 1990). The role of Frankiae in plant roots was neither clearly described nor did it receive much attention. Not much progress had made before the first *Frankia* strain CpI1 was successfully cultivated *in vitro* at Harvard Forest (Callaham et al. 1978). Findings in Callaham et al. (1978), in which the sweet fern (*Comptonia peregrina*) was studied, not only significantly stimulated the subsequent research of *Frankiae* regarding their physiology and molecular genetics, but also provided strong evidence that *Frankiae* can be isolated, cultivated, and used to reinfect the original host plant.

With the improvement of culture isolation techniques, there has been a growing number of *Frankia* isolates obtained, using crushed nodule suspensions or pure-cultured cells (Torrey 1990). In most cases, *Frankia* strains are obtained from root nodules of their corresponding host plants. However, in some cases, *Frankia* strains have also been isolated from the rhizosphere of actinorhizal plants. Interestingly, it has been suggested that *Frankia* strains are not likely to be free-living in soils as indicated by unsuccessful strain isolations directly from the soil (Lechevalier and Lechevalier 1990), however, plants without greenhouse-inoculation of *Frankia* may also exhibit nodules on their roots in field trials. The same observations of *Frankia*-nodules grown on both inoculated and non-inoculated plants were confirmed in several phytoremediation studies (Greer et al. 2005; Lefrançois 2009; Callender 2015). Such indirect evidence suggests that some *Frankia* can grow in soils devoid of hosts, and this is also in line with the evolutionary assumptions made by a narrow range of actinorhizal plants (Baker and Schwintzer 1990; Mullin and An 1990).

2.5.2 Mycorrhizal fungi and the mycorrhizosphere

Mycorrhizal fungi belong to three divisions of true fungi: Zygomycota, Ascomycota and Basidiomycota (Smith and Read 2008). The group of mycorrhizae (i.e., mycorrhizal fungi) is ubiquitous and comprises around 50,000 species in forming mycorrhizal associations with approximately 250,000 terrestrial plants (Nehl and Knox 2006; Timonen and Marschner 2006). Mycorrhizal fungal hyphae are ten times thinner than plant roots and grow rapidly, and as a result, are able to access nutrients and water in deep soil horizons. In addition, mycorrhizae are known as vital contributors in N fixation and P mobilization, and that up to 80 % of N and P are made available to plants through mycorrhizal symbioses (Van Der Heijden et al. 2015). Specifically, the arbuscular mycorrhizal (AM) fungi and ectomycorrhizal (ECM) fungi that are present in vast numbers in the mycorrhizae, also exhibit an important ability to form mutualistic symbioses with a wide range of vascular plants (Kowalchuk et al. 1997). The arbuscular mycorrhizal fungi, comprising roughly 150 species of zygomycetes, form arbuscular mycorrhizal associations with most herbaceous plants and a few woody plants, whereas the ectomycorrhizal fungi, which consist of about 6,000 species of basidiomycetes along with a few ascomycetes and zygomycetes, primarily colonize woody plants (Mandyam and Jumpponen 2005).

The mycelia of root-colonized mycorrhizal fungi are able to readily penetrate soil, particularly arbuscular mycorrhizal fungi, which remarkably extend the root system with their mycelia, greatly expanding the root-colonizing space beyond the rhizosphere. The rhizosphere definition established in 1904 was restricted to describing the influence of plants upon the soil compartment, given that there was a limited understanding of the properties of plant-associated soil at the time (Timonen and Marschner 2006). Indeed, with more detailed information on the chemical and physical aspects of soil as well as the continuum of the root-mycelial system, the rhizosphere concept is no longer appropriate in fully revealing the function of mycorrhizal colonization in the soil ecosystem. Therefore, the concept of ‘mycorrhizosphere’ was proposed (Linderman 1992) to describe the unique ‘ecosystem’ formed by the plant-mycorrhizal associations.

It is evident that in the mycorrhizosphere, arbuscular mycorrhiza and ectomycorrhiza substantially alter the root exudation pattern via their hyphae and spores. In addition to the changes in root exudate composition, there are also fungal exudates which mycorrhizal fungi produce to regulate

the bacterial community, such as organic acids and antibiotics (Davet 2004; Deng and Cao 2017). Analogous to most fungi that exhibit filamentous growth, mycorrhizal fungi also show potential to change the physical environment of the rhizosphere. Their abundant mycelial filaments ensure soil particles assemble into stable and voluminous aggregates, which increases gas circulation and water retention (Azcón-Aguilar and Barea 2015). More recently, attention has concentrated on the selective pressure that mycorrhiza exert upon the competency of symbiotic and free-living rhizobacteria, which influence them to either promote or inhibit plant growth, although the extent of this influence is not clearly elucidated. The fact that mycorrhiza enhances the capacity of plant P uptake also mitigates the P deficiency in soils, which furthermore promotes root nodulation and invokes the synergistic interactions between root-nodulating bacteria (e.g., *Frankia*) and mycorrhizal fungi (Chatarpaul et al. 1989; Rojas et al. 2002; Tian et al. 2002).

2.5.3 Root endophytic microorganisms

Endophytic microorganisms are microbes (including bacteria and fungi) that reside inside the internal tissues of plants without causing visible disease symptoms on the host (Weyens et al. 2009). As emphasized by Smith et al. (2008), the vast majority of terrestrial plants possess microbial endophyte colonizations; an endophyte-free plant is an extremely rare exception in nature (Partida-Martinez and Heil 2011). Since plant roots are the main site of endophytic colonization, recent investigations have emphasized utilizing the capacity of root-inhabiting endophytes to enhance plant growth (Cao et al. 2002; Bulgarelli et al. 2012; Deng and Cao 2017). These studies have detailed many aspects of the plant root microbiome, including their role in plant growth and their interactive effects with rhizosphere microorganisms. Similar to rhizosphere microbes, root endophytic microbes also demonstrate their dependence on the plant host, but with less influence from environmental stress and nutrient competition. While it is well known that plants have a significant effect on selecting a variety of plant-beneficial microbial species to colonize the root-surface (i.e., rhizosphere microbes) and the root-interior (i.e., root endophtyes), the extent to which rhizosphere microbial communities differ from those in the root tissue is largely unknown. Some authors, as reviewed by Deng and Cao (2017), believe the community structure of root endophytes can be largely dissimilar to that of the rhizosphere microbiota, because the endophytic bacteria and fungi that proliferate within plant tissues are likely to establish a more intimate relationship with the host than their rhizosphere counterparts. Other authors, however,

hold the opinion that endophytic microbes are a subpopulation of the rhizosphere microorganisms, based on their research findings in which many root endophytic varieties are also common rhizosphere inhabitants (Palumbo and Kobayashi 2000; Marquez-Santacruz et al. 2010). Notably, the second argument has earned more advocates from the evolution and subspeciation points of view. Evidence was provided in the study of Bulgarelli et al. (2012), who showed that the benign bacterial endophytes from *Arabidopsis* plant roots were developed from soil bacterial species that had multiplied into roots to modulate plant growth. Other supporting evidence was reviewed by Santoyo et al. (2016), who demonstrated that the mechanisms whereby rhizosphere/soil bacteria faciliate and regulate plant development, such as nutrient acquisition and protection against pathogens, are also used by endophytic bacteria, in several microbial characterization studies (Marquez-Santacruz et al. 2010; Glick 2012; Lacava and Azevedo 2013).

Bacterial endophytes colonize both monocotyledonous and dicotyledonous plants, including a large number of bacterial species that also exert a positive effect on plant establishment and perform similar functions as plant growth promoting rhizobacteria (PGPR) (Palumbo and Kobayashi 2000). Nevertheless, unlike rhizosphere-dwelling microbes, these endophytes which have received less competition from soil environments and developed a specific adpation to inhabit plant tissues, also possess the potential to affect the synthesis of metabolites by plants (Brader et al. 2014). This metabolic interaction between endophytes and plants has expanded the practical use of endophytes in plant growth enhancement (de Oliveira et al. 2012). Moreover, the colonization of bacterial endophytes in plants also assists phytoremediation processes, and particularly are abundant on plant species that are highly tolerant of mine contamination and soil disturbance. Plants have been shown to recruit bacterial endophytes to survive in unfavorable environments (Santoyo et al. 2016). Specifically, Siciliano et al. (2001) showed that a substantial enrichment (several orders of magnitude higher than bulk soil concentrations) of hydrocarbon- and trinitrotoluene-degrading bacterial catabolic genotypes (*alkB* and *ndoB*, *ntdAa* and *ntnM*, respectively) were observed in the interior of plant roots in response to an increase of petroleum hydrocarbon (PHC) and trinitrotoluene contaminants, respectively. Similarly, endophytic fungi also show potential to improve phytoremediation efficiency (Deng and Cao 2017). Currrent scientific studies on fungal endophytes are mainly focused on their bioactive secondary metabolite

aspects and aimed at applying this knowledge for therapeutic use and biological control (Cao et al. 2002; Toju et al. 2013; Yamaji et al. 2016).

2.6 The plant-microbe symbiosis during phytoremediation

There is a growing interest in using microbial inoculation as part of phytoremediation approach on metal-contaminated sites, where the soils are nearly sterile materials deprived of suitable bacterial or fungal propagules. Siciliano et al. (2001) studied the plant selection pressure on bacterial endophytes in response to soil contamination and showed that plants inoculated with microbial inoculants in phytoremediation systems have a better growth effect than the uninoculated counterparts. One of the well-known plant-microbe symbioses that have been recognized and applied in plant growth enhancement is alder (*Alnus*)-*Frankia*. *Alnus* species can form symbiotic associations with *Frankia* which fix atmospheric nitrogen (N_2) in their root nodules (Baker and Schwintzer 1990; Sprent and Parsons 2000). Benefited by the nitrogen-fixing capacity of their actinorhizal microsymbionts, alders often serve as pioneer species for later introduction of plants and are great candidates for soil quality improvement and reforestation of degraded areas (Hibbs and Cromack 1990; Wheeler and Miller 1990; Schwencke and Carú 2001). Moreover, growing *Frankia*-inoculated alders for the phytoremediation of mine wastelands stimulated plant growth and soil fertility both in greenhouse trials (Greer et al. 2005; Quoreshi et al. 2007; Bissonnette et al. 2014; Callender et al. 2016) and in field trials (Moffat 2000; Lefrançois 2009). In the last 2-3 decades, the positive effects of dual inoculation with *Frankia* and mycorrhizal fungal species on alder field performance have been documented (Chatarpaul et al. 1989). Because the beneficial effects of such tripartite (plant-bacterial-fungal) interactions may vary with the combination and the selection of plant genotypes, *Frankia* and appropriate mycorrhizal strains (Isopi et al. 1994), future research is required to provide us with detailed insight into the multipartite symbioses among actinorhizal plants, *Frankia*, and mycorrhizal fungi.

Mycorrhizal plants are a group of plants in symbioses with mycorrhizal fungi, and they benefit from their fungal partners by having easier access to nutrients and water from deep soil layers. In addition to actinorhizal plants, mycorrhizal plants are frequently selected for phytoremediation. Boreal conifer trees such as jack pine (*Pinus banksiana* Lamb.) and white spruce (*Picea glauca*

(Moench) Voss) are two widespread species native to Canada. In northwestern Ontario especially, spruce and pine are of interest to forest managers due to their high commercial importance and their extensive use in forest regeneration programs (Parker et al. 1996). As natural hosts to many ectomycorrhizal fungi, members in the *Pinus* genus are often found to outcompete other conifers at outplanting if appropriate fungal inocula have been selected in a greenhouse setting or an operational nursery. As evidenced by the work of Richter and Bruhn (1989), jack pine seedlings inoculated with *Laccaria bicolor,* an ectomycorrhizal fungus that helps plants acquire N and P and protects them from plant pathogens, had on average 10% higher survival compared to (non-inoculated) control seedlings. Nadeau and Khasa (2016) described that *Amphinema byssoides* P. Karst (Atheliaceae), followed by *Tricholoma scalpturatum* (Fr.) Quél. (Tricholomataceae) and *Tomentella* sp.2, were the three most frequent ectomycorrhizal fungal species in the rhizosphere of white spruce growing on mining sites. Conifer seedlings were easily colonized by bacterial and fungal endosymbionts, or ectomycorrhizal fungal species in a greenhouse setting, and *A. byssoides* was reported as one of these nursery contaminants (Onwuchekwa et al. 2014). This finding meant that there were no such fungi-free nursery conifer seedlings, which often complicates the evaluation of inoculation treatments on the outplanted conifers.

2.6.1 Applications of alder-*Frankia* symbionts

Alders (*Alnus* spp.) are actinorhizal and mycorrhizal plants (trees or shrubs) belonging to the Betulaceae family; they form symbioses with the nitrogen-fixing actinomycete *Frankia* and mycorrhizas (Baker and Schwintzer 1990). Alders are robust perennial plants that thrive in Northern climates and early successional species that appear following natural or anthropogenic disturbances, which have been extensively studied as key organisms in disturbed ecosystems (Roy et al. 2007). Analogous to the interaction of rhizobia and leguminous plants, the colonization of alders by *Frankia* supplies their host plants with assimilable nitrogen sources by fixing gaseous nitrogen (N_2) into ammonia (NH_3) in their formed root nodules (Marschner 1995).This N_2-fixing capacity (nitrogenase activity) of *Frankia* shows great promise in assisting alders to establish on nutrient-deficient soils; moreover, the increased organic matter through alder litter decay can stimulate a diverse and abundant soil microbial community therefore reducing metal bioavailability in the soil (Roy et al. 2007). The application of alder-*Frankia* symbionts at contaminated mining sites has shown significant success in boosting the phytoremediation process,

especially in oil sands tailings with organic contamination (Greer et al. 2005; Lefrançois et al. 2010; Jaramillo 2012). In Greer et al. (2005), two robust native alder species, *Alnus rugosa* and *Alnus crispa*, were inoculated with *Frankia* sp. strain AvcI1 and grown in the greenhouse, using composite tailings (CT) sands obtained from an oil sands operation in northern Alberta. The researchers found that alders which received the *Frankia* inoculum had enhanced growth and also exerted a significant positive impact on the diversity, population and hydrocarbon degradation activity of the indigenous soil microbial community in the CT sands. In addition to greenhouse studies, *Frankia*-alder symbionts monitored in the field also demonstrated outstanding plant performance. A 5-year phytoremediation trial using *Frankia*-inoculated *A. crispa* seedlings in oil sands process-affected materials (OSPM), significantly facilitated plant biomass production and improved the soil quality at the reclamation site (Lefrançois et al. 2010). *Frankia*-inoculated alders were able to establish in the nutrient-deficient soil ecosystem without the addition of any fertilizer. The N_2-fixing ability of *Frankia* was also shown to be beneficial in the phytostabilization of metalliferous wastes (Callender et al. 2016). A 6-month greenhouse experiment of growing two *Frankia*-inoculated alder species (*A. crispa* and *A. glutinosa*) on a mixture of gold mine waste rock and fine tailings was conducted. Plant biomass, survival rates, nodulation, root length and weight showed no statistically significant differences between woodchip-amended mine residues (high N content) and the unamended controls, indicating that the plants were able to obtain their N requirements through the metabolic activity of their *Frankia* microsymbionts.

2.6.2 Ectomycorrhizae (ECM)-inoculated conifers

Ectomycorrhizal fungi and their symbiotic associations with mycorrhizal plants are perceived as main drivers of several key ecosystem functions. For instance, ectomycorrhizal fungi can help sustain the carbon and nitrogen cycling in terrestrial ecosystems and improve the tolerance of their plant partners in response to abiotic and biotic stresses to better colonize poor soil conditions (Kalucka and Jagodzinski 2016; Kumar and Atri 2018). From this perspective, the biodiversity and specificity of ectomycorrhizal fungal strains should be an integral consideration in rebuilding a self-sustaining and multifunctional local ecosystem. Since ectomycorrhizal fungi are naturally occurring microsymbionts of various woody plants including coniferous tree species, such as white spruce (*Picea glauca* [Moench] Voss), jack pine (*Pinus banksiana* Lamb.), larch (*Larix sibrica*), and various deciduous tree species, such as willow (*Salix* spp.), aspen (*Populus tremuloides* Michx.)

and balsam poplar (*Populus balsamifera* L.), they were frequently applied as microbial inoculants to improve the establishment and growth of plants in adverse soil environments by facilitating water and nutrient availability (Quoreshi and Khasa 2008; Quoreshi et al. 2008; Onwuchekwa et al. 2014; Nadeau and Khasa 2016). Several studies have demonstrated the benefits of nursery ectomycorrhizal inoculation in the rehabilitation of degraded soil environments such as oil sands tailings (Bois et al. 2005; Onwuchekwa et al. 2014), copper tailings (Zong et al. 2015), clear-cut sites (Gagné et al. 2006), reforested sites (Quoreshi et al. 2008), and landings, which are sites compacted as a consequence of the operation of large field equipment (Teste et al. 2004).

Ectomycorrhizal fungi are key components of healthy forest ecosystems. In mining-disturbed wastelands, however, the natural occurrence of ectomycorrhizae propagules is extremely low (Bois et al. 2005; Hawkins et al. 2015). Normally, the dispersal of ectomycorrhizal fungal propagules can be airborne or carried by animals, but to a large extent, it is via the vegetative spread of mycorrhizal networks on the basis of the vegetation present (Kalucka and Jagodzinski 2016). Hence, nursery inoculation with a single or pairs of appropriate ectomycorrhizal fungal strains can promote vegetation performance throughout the course of mine site rehabilitation. The efficacy of ectomycorrhizal fungal inoculum on plant growth enhancement is further confirmed in Bois et al. (2005), who reported that the increase of ectomycorrhizal fungal inoculum potential is in line with the age of reclaimed sites and the progress of succession stages.

The practical use of ectomycorrhizal fungi as a biotechnology strategy in bioremediation has been well-demonstrated, especially in the areas of facilitating the biodegradation or mineralization of several persistent organic pollutants (POPs), including polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs) and pesticides (Meharg and Cairney 2000). Mycorrhizal inoculation improves root architecture, particularly root length and root branching of seedlings, and is a technique often used in the phytoremediation of mine sites (Isopi et al. 1994). During the early plantation establishment period on reclamation sites, conifer seedlings rely largely on mycorrhizal symbionts to acquire nutrients, water, and resistance to some pathogens, all of which are considered essential to enhance outplanting performance (Quoreshi et al. 2008). In addition, the positive effects of ectomycorrhizal fungal inoculation on plant growth and survival were also examined on jack pine (*Picea glauca*) and white spruce (*Picea glauca*) seedlings planted in oil

sands reclamation sites of Alberta. ECM-inoculated conifers were found to be more tolerant of salinity and water stress (Onwuchekwa et al. 2014). A variety of studies have also shown that many introduced ectomycorrhizal fungal species can persist several years in the host and in the planting site, variably interacting with the indigenous soil microbial community (Quoreshi et al. 2008; Onwuchekwa et al. 2014). Ectomycorrhizal fungal inoculation is thought to increase the species richness of the ectomycorrhizal fungal propagule bank and subsequently facilitate the regeneration and plantation of seedlings in disturbed areas (Huang et al. 2018).

2.6.3 Tripartite relationships among plants, bacteria and fungi

Inoculation with either *Frankia* or ectomycorrhizal fungi for plant seedlings grown in the presence of mine substrates, as previously mentioned, is key to promoting plant performance. However, in harsh soil environments, the effect of co-inoculation with *Frankia* and mycorrhizae on plant colonization and biomass yield has not been adequately explored. Although some authors indicate that dual inoculation of mycorrhizas with *Frankia* on alder seedlings can cause a decrease of plant biomass during their early growth stages due to multiple micro-symbionts competing for the same resources such as photosynthates (Orfanoudakis et al. 2004), most scientists have agreed that symbiotic bacteria and mycorrhizal fungi can act synergistically to improve biomass production, mineral acquisition and the nitrogen-fixing ability of alder seedlings (Chatarpaul et al. 1989; Markham 2005; Oliveira et al. 2005; Yamanaka et al. 2005). Dual inoculation with *Frankia* and arbuscular mycorrhizal fungi have reportedly resulted in a better growth of actinorhizal plants as compared to the inoculation of *Frankia* alone (Tian et al. 2002). In Isopi et al. (1994), dual inoculation of *Alnus cordata* with the arbuscular mycorrhizal fungus, *Glomus* spp., and *Frankia* significantly increased alder growth after 5-months of growth in mine tailings by increasing the nodule's biomass and the mycorrhizal infection on the host plant. This suggests that the co-inoculation of *Frankia* and arbuscular mycorrhizas on *Alnus* species could be a good strategy to enhance revegetation of the reclamation sites. Yamanaka et al. (2003) inoculated *Alnus tenuifolia* with *Alpova diplophloeus* (an ectomycorrhizal fungus) and *Frankia* and found that the dual inoculation greatly facilitated the nitrogen-fixing ability of *Frankia* in alder roots after 5-months growth in a greenhouse. This result confirmed that the tripartite association among alders, *Frankia* and ectomycorrhizal mycorrhizas can also result in improved growth of plant seedlings. Nevertheless, not all of alder-*Frankia*-mycorrhizal fungal associations work in the same manner.

In Yamanaka et al. (2005), seedlings of *Alnus sieboldiana* grown in combination with *Frankia* and *Gigaspora margarita* (an arbuscular mycorrhizal fungus) showed no improvements in N-fixing activity or P nutrition, despite the fact that nodulated seedlings produced the highest biomass of shoots and root nodules compared to uninoculated controls and the single inoculation treatments. The result implies that the improved plant growth is not necessarily linked to the enhancement of plant nitrogen-fixation and mineral acquisition abilities but could be contributed by plant hormones produced or induced by arbuscular mycorrhizal fungi. The development of superior strains that are adapted to the host species and the planting site could be the key to optimize the effect of microbial symbiosis on plant seedling establishment and growth. There is therefore a need for more plant inoculation studies in order to fully understand and take full advantage of the plant-microbe symbiosis in the rehabilitation of disturbed terrestrial areas.

CHAPTER 3 : MATERIALS AND METHODS

3.1 Study site description

The study site is a plantation grown on a waste rock slope facing highway 117, located at the Sigma gold mine (48.107010°N, 77.765136°W) adjacent to Val-d'Or in Northern Quebec. The mine has been operated by the Century Mining Corporation since 1935, and is one of the richest gold-bearing mines in Quebec (Callender 2015). The mine has generated non-acidogenic sterile rock tailings (waste rock and fine tailings) during its operation. Despite the fraction of rock tailings being recycled in the gravel industry, massive volumes still remain at the mine site and are held in five retention basins (approx. 200 m × 1000 m). The mine was slightly alkaline with a pH of 8.3 for fine tailings and 8.6 for waste rock (Callender 2015). Moreover, as highlighted in a toxicity characteristic leaching procedure (TCLP) assay conducted in 2012, the Sigma mine was moderately contaminated by only a few trace metals such as Chrome (Cr) and Copper (Cu), for which, concentrations in the topsoil (soil horizon "A") were slightly higher than the background thresholds but were still below the maximum acceptable limits for domestic and industrial lands, as stated in the Quebec land protection guideline (Beauregard et al. 2012; Callender 2015; Laberge et al. 2016). The TCLP chemical report indicated a less hazardous and revegetation-applicable character of the studied gold mine wastes.

The studied phytoremediation field trial is one of the multiple large-scale revegetation projects funded by NSERC. The plantation was grown on a waste rock hill, which had previously been capped with fine tailings from an abandoned tailings pond southeast of the waste rock site. The plantation was spaced out over three random blocks, 120 m long by 8 m wide, and each block was subdivided into 9 plots, 12 m long by 8 m wide. Initially, 15 plants were transplanted into each plot. The phytoremediation field trial was interplanted with alder and conifer plants: green alder (*Alnus viridis* subsp. *crispa*, AC), speckled alder (*Alnus incana* subsp. *rugosa*, AR), white spruce (*Picea glauca*, E), and jack pine (*Pinus banksiana*, P). From May to August 2011, plant seedlings from this field trial were sown in the greenhouse nurseries of ArborInnov/University of Sherbrooke and University Laval, and were inoculated with different combinations of the bacterial strain *Frankia* sp. Avcl1 (FK) and mycorrhizal fungal species (names of inocula are described in Section 3.2). Subsequently in June 2012, plant seedlings including the uninoculated (control) and the

inoculated alders and conifers were planted on the mine waste rock hill. Based on the time that plant seedlings were transplanted on the mine, this phytoremediation field trial is referred to the 2012 plantation.

3.2 Inoculation treatments

The experimental design for the 2012 plantation was a randomized incomplete block design with unbalanced cell sizes (Dean et al. 2017), i.e., not every treatment is represented in each block nor are the number of replicate plots equal in every treatment (the conifer trial) (Fig. 3.1). In the six (3 × 2) treatments of the alder trial, non-inoculated green/speckled alder seedlings (ACNI and ARNI) were compared with alder seedlings inoculated with *Frankia* sp. strain AvcI1 alone (ACI-FK and ARI-FK), and in combination with two mycorrhizal fungal species, *Glomus irregulare* (Gi) and *Alpova diplophloeus* (Ad). Although *G. irregulare* is an arbuscular mycorrhizal (AM) fungus and *A. diplophloeus* is an ectomycorrhizal (ECM) fungus, the alder-bacteria-fungi treatment was termed the "FK-ECM" inoculation (ACI-FK-ECM and ARI-FK-ECM) (Fig. 3.1). In the four treatments of the conifer trial, white spruce seedlings were inoculated with two ectomycorrhizal fungal strains of *Hebeloma crustiliniforme* (Hc) and *Paxillus involutus* (Pi), whereas jack pine seedlings received a different pair of ectomycorrhizal fungal inocula, *Suillus tomentosus* (St) and *Laccaria bicolor* (Lb), which had been previously reported to increase plant survival and growth in oil sands reclamation sites in Alberta (Onwuchekwa et al. 2014). In the white spruce (E) trial, the uninoculated spruce treatment (E-NI) had two plot replicates, whereas there were three plot replicates of the ectomycorrhizal fungi-inoculated spruce (E-ECM) treatment (Fig. 3.1). Similarly, in the jack pine trial, the ectomycorrhizal fungi-inoculated pine treatment (P-ECM) was also triplicated while the uninoculated pine treatment (P-NI) did not have any replication (Fig. 3.1). While understanding the uninoculated jack pine plot was not replicated and the white spruce plot was only duplicated, which may reduce the reliability of statistical analysis in the conifer trial, we were not able to find another phytoremediation trial established as early as the 2012 plantation in the Sigma gold mine to evaluate the inoculation effect of microbial strains on plant field performance after several years of growth.

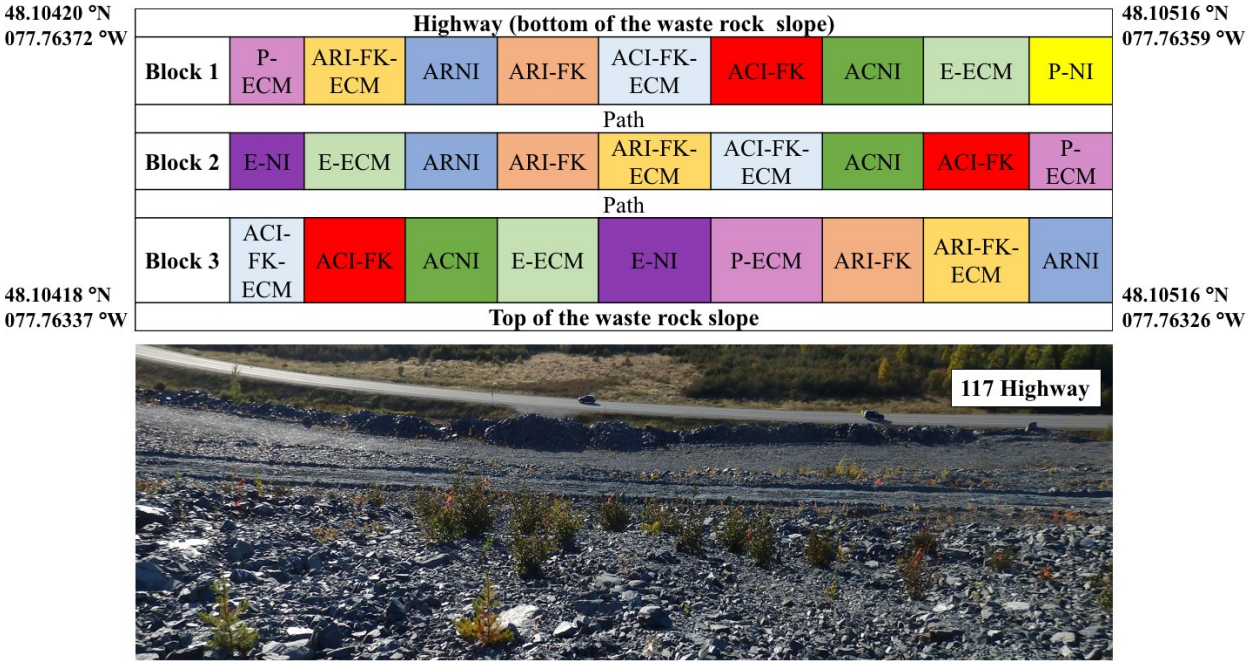

Figure 3.1. Field layout of the 2012 plantation. The plantation contains conifer and alder trials, comprising a total of 10 treatments, which are color-coded accordingly. Legend abbreviations: AC(R)NI=uninoculated green(speckled) alders, AC(R)I-FK=*Frankia*-inoculated green(speckled) alders, AC(R)I-FK-ECM=*Frankia*-mycorrhizal fungi (*G. irregulare* and *A. diplophloeus*)-inoculated green(speckled) alders, E-NI=uninoculated white spruce, E-ECM=white spruce seedlings inoculated with *H. crustiliniforme* and *P. involutus*, P-NI=uninoculated jack pine, P-ECM=jack pine seedlings inoculated with *S. tomentosus* and *L. bicolor*.

3.3 Field sampling

Two sampling events (September 2016 and 2017) took place during this project. Before the sampling time, plants from this phytoremediation trial had been grown on the mine waste rock substrates for four and five years. The first sampling event (September 2016) was a preliminary test to examine a real-time effect of plant establishment at the mine waste, in order to validate sampling and transport protocols. This was an important step as this work was being conducted on a remote site with limited/restricted accessibility and limited logistical support for storing and transporting samples (refrigeration/freezing, time for transport). In both sampling events, plant growth measurement and survival data were taken directly in the field, while samples destined for molecular/genomics analyses were transported back to the laboratory of the National Research Council Canada (NRC) in Montreal. In the first sampling event (September 2016), we took plant field measurements and carried out microbial analysis on the 36 (9 replicates × 4 treatments)

conifer rhizosphere soil samples from among the collected soils, including a preliminary study on processing time delay effects on the microbial community composition and diversity in the conifer rhizosphere. In the second sampling event (September 2017), in addition to plant field measurements, a complete microbial analysis was conducted on the soil (rhizosphere and bulk soils) and root samples harvested from both conifer and alder trials. In 2017, samples collected for the microbial analysis were immediately frozen (-20 °C/-6 °F) in a portable freezer on the site, remained frozen during transportation, and were stored at -80 °C upon arrival at NRC, Montreal.

3.3.1 Field measurements

In both sampling events (2016 and 2017), we counted the number of plants that survived from each treatment of each block and calculated the plant survival rates by treatment. For evaluating plant growth in the field, we measured plant height (cm) and root collar diameter (RCD, mm) at ground level by using a meter stick and a Vernier-Slide caliper, respectively. In the 2016 sampling, we measured the plant height and RCD data for the entire plantation, whereas in the 2017 sampling, measurements for five randomly-selected plants from each treatment were conducted, prior to harvesting the plant root and soil samples. The plant height and RCD parameters were used for calculation of seedling volume index (SVI) and plot volume index (PVI). SVI is an estimation of plant biomass calculated by combining the values of plant shoot height with the RCD, as the RCD is a more sensitive parameter to separate the treatment effects on growth response of conifer seedlings than plant height (Quoreshi et al. 2008). The SVI (cm^3) was calculated by multiplying the square of the RCD by the shoot height [$(RCD, cm)^2 \times$ plant shoot height (cm)] (Marx 1977). PVI (cm^3), which takes both the number of surviving plants per plot (treatment) and seedling volume index (SVI) into account to provide a general picture of plant growth, was calculated by multiplying the SVI (cm^3) and number of plants surviving per treatment [$(RCD, cm)^2 \times$ plant shoot height (cm) $\times$ number of surviving plants (Marx 1977; Quoreshi et al. 2008).

3.3.2 Sample collection

Plant root samples with associated soils were harvested for molecular analysis. In the 2016 sampling, we first identified nine average healthy-looking plants in each treatment based on survival counts, plant height and RCD values that had been taken from the entire plantation. Then,

for every selected plant, we excavated the rock surface around the plant by using small shovels and trowels, until it was deep enough to find roots growing outside the peat bag (15-45 cm depth). Once the fine roots of individual plants were well-exposed, we cut a segment of roots with closely-adhered soils, i.e., plant rhizosphere soils, by using sterile pruning secateurs, and placed the roots and rhizosphere samples in a sterile Whirl Pak bag (approx. 40-120 g/bag). To compare the effect of growing non- and inoculated plant seedlings on the soil properties of a planted site, we also randomly collected 12 Falcon tubes (50 mL/each) of bulk soil samples from unplanted sites at the mine. Soil pH and moisture content of the 90 (9 replicates × 10 treatments) plant rhizosphere samples and 12 unplanted bulk soil samples were immediately measured upon return to the lab. An aliquot for molecular analysis was stored at -20°C while the remaining soil was stored at 4 °C. For the processing time delay study, soil samples were stored at 4 °C for four (4), six (6) or 16 days before processing and molecular analysis in order to assess the sensitivity of microbes to post-sampling conditions, i.e., short and long-term storage at 4 °C. The 2016 data presented in Chapter 4 will hopefully enable us to better understand how soil microbial communities responded to processing time delays.

In the 2017 sampling, five replicates of rhizosphere soil per treatment were taken to provide an estimation of plant field performance, while plant bulk soils were also sampled to serve as a comparison (control) to plant rhizosphere soils. After the identification of five average-looking plants, we excavated the rock surface of individual plants and found actively living roots growing outside the peat bags. Roots were gently shaken to loosen the plant bulk soils into a sampling bag. Each bag was identified by treatment. Next, a segment of roots was cut (approx. 10 g or more) with rhizosphere soils using aseptic pruning secateurs, which had been sterilized with ethanol wipes each time prior to making the cut. Harvested root and rhizosphere samples were transferred into a sterile Whirl Pak bag and frozen immediately in the portable freezer (-20 °C) at the site. Bulk soil samples collected from replicated plots of the same treatment were mixed thoroughly in the same sampling bag. Thus, from ten bulk soil sampling bags (10 treatments), we collected replicates of the composite plant bulk soil samples in four Falcon tubes. Plant bulk soil replicate 1 was frozen immediately for molecular analysis while replicates 2-4 were stored at 4 °C for physicochemical analyses (pH and moisture content). We applied the same procedure to harvest plant rhizosphere and bulk soil samples for a total of 10 treatments. We also collected seven Falcon

tubes of composite bulk soil samples from five unplanted areas adjacent to the treatment blocks (from areas outside and between the treatment blocks). Two Falcon tubes were immediately frozen and stored at -20 °C for molecular analysis while the others destined for physicochemical analysis were stored at 4 °C.

3.4 Physicochemical analyses: soil pH and moisture content (%)

Soil pH was determined using 5 g of soil in a glass beaker, to which was added 25 mL of distilled and deionized water to make a 1:5 dilution. The obtained soil slurry was stirred for 2-3 mins and then left to stand for 30 mins. The slurry was swirled again while taking the pH measurement using an Accumet™ AB15 pH meter (Fisher Scientific). Values of soil pH were recorded when the readings became steady. Soil moisture content (%) was determined directly using an electronic moisture analyzer (OHAUS Corporation) or by measuring the difference between the wet (10 g) and dry weights of an aliquot of soil after completely drying overnight at 105 °C.

3.5 Preparation of the rhizosphere and root samples

Whirl Pak bags containing frozen rhizosphere and plant terminal roots were thawed on ice (4 °C) for about 10-20 mins before separating rhizosphere from roots. The rhizosphere and roots of the five replicates from uninoculated treatments (control) belonging to each plant species (AC, AR, E, P), were processed first to minimize cross-contamination. Plant treatments were processed in the following order: ACNI, ACI-FK, ACI-FK-ECM, ARNI, ARI-FK, ARI-FK-ECM, E-NI, E-ECM, P-NI and P-ECM. Under aseptic conditions, i.e., in a biological hood using sterilized spatulas and tweezers, one to two Falcon tubes of rhizosphere samples were collected from each Whirl Pak bag, removing any apparent roots. Rhizosphere soil aliquots were then frozen at -20 °C until nucleic acid extraction. A fraction of fine roots (approx. 5 g) were then transferred into Falcon tubes avoiding alder root nodules. The collected roots were subsequently subjected to a surface-sterilization protocol as described in Jaramillo (2012), with slight modification, as described below. Any portions of roots not immediately surface sterilized were refrozen at -20 °C.

3.5.1 Root surface sterilization

Roots from uninoculated treatments (controls) of the same type of plant treatments were surface sterilized first to minimize contamination. After roots were thawed, distilled and deionized water was added to the Falcon tube in which roots were completely immersed, and then, the tube was capped and gently swirled to wash off the remaining attached soils. This procedure was repeated 2-3 times until the rinsing water became clear. Rinsed roots were transferred into a new Falcon tube using ethanol-sterilized tweezers. Under the aseptic condition (in the hood), the rinsed root samples went through a sterilization workflow consisting of the following steps: 1 min of hand shaking in 100 % Ethanol, 1 min of hand shaking in 2.5 % NaClO, 10 mins in another addition of 2.5 % NaClO with a gentle shaking (at 115 rpm) on an orbital motion shaker, and less than 1 min (approx. 30 seconds) in 100 % ethanol by hand shaking. After that, autoclaved MilliQ water was added and the tube was shaken by hand for 1 min; this step was repeated 4 times. On the last rinse, 1 mL of the water from the Falcon tube was collected to confirm root sterility via 16S rRNA amplicon PCR on these 1 mL final washes.

3.5.2 Grinding of sterilized roots in liquid nitrogen prior to DNA extraction

Surface-sterilized root material (1-2 g) was ground manually to a fine powder using liquid nitrogen in a mortar and pestle that had been autoclaved (sterilized) and then chilled overnight (-80 °C). Root material was kept frozen while grinding in the liquid nitrogen and immediately processed for nucleic acid extraction. Ground samples not immediately processed were refrozen at -20 °C until used. The initial mechanical grinding of sterilized plant tissues with liquid nitrogen prior to DNA extraction, especially for fibrous tissues, proved to successfully access the nuclear material without causing degradation, and ultimately optimized the extraction method (e.g., CTAB) in obtaining high-quality genomic DNA (Murray and Thompson 1980; Doyle et al. 1990; Jaramillo 2012).

3.6 DNA isolation, quantification, and concentration/purification

3.6.1 Genomic DNA extraction using CTAB

Total genomic DNA was extracted from the collected bulk soils, rhizosphere and root samples, following a modified CTAB nucleic acid extraction (Ausubel 2002). The nucleic acid extraction method involved hexadecyl trimethyl ammonium bromide (CTAB), phenol, and chloroform steps. CTAB is a quaternary ammonium surfactant and a powerful cationic detergent used for isolating highly polymerized DNA from plant material (Surzycki 2000; Azmat et al. 2012). High concentrations of polysaccharides, phenolic compounds, and secondary metabolites present in plant material are common contaminants that impede the DNA isolation procedure (Murray et al. 1979; Murray and Thompson 1980; Fang et al. 1992; Azmat et al. 2012). This problem can be solved by using CTAB, which can form complexes with proteins and polysaccharides at high salt concentrations (at or above 0.5 M) thus removing them from DNA (Surzycki 2000; Sahu et al. 2012). The addition of chloroform and phenol during the extraction also simultaneously removes contaminants other than polysaccharides, such as beads, cell wall debris, and denatured proteins, by the end of DNA isolation procedure (Murray and Thompson 1980).

In our CTAB-based nucleic acid extraction protocol, the CTAB solution was adjusted to 10 % in 0.7 M NaCl and mixed with 240 mM phosphate buffer (1 M KH_2PO_4 and 1 M K_2HPO_4) in a 1:1 ratio, with the pH adjusted to 8.0 using NaOH. This CTAB-phosphate buffer was named ‘extraction buffer’ in the context. Five-hundred milligrams or 0.5 g of grinded root sample (BK, RZ, or root powder) were added to 15 mL polypropylene tubes (SARSTEDT, product number: 62.554.002) containing 250 mg (0.25 g) of bead mixture (a 1:1 mix of 0.1 mm and 0.5 mm zirconia-silica beads). In a chemical hood, 500 µL of extraction buffer and 500 µL of phenol: chloroform: isoamyl alcohol (25:24:1, Tris saturated, pH 8.0) were then added. The tubes were shaken with a Vortexer (Fisher Scientific) at maximum speed for 1 min and chilled on ice for 1 min. Tubes were then centrifuged at 10,000 g (or 4180 g/rcf) for 5 mins at 4 ºC and the supernatant containing nucleic acids was pipetted to a new conical base tube and then purified by adding 500 µL of chloroform: isoamyl alcohol (24:1) mixture. Tubes were centrifuged again at 10,000 g (or 4180 g/rcf) for 5 mins at 4 ºC and the supernatant was pipetted into a new 1.5 mL microcentrifuge tube. To precipitate nucleic acids 750 µL of cold isopropanol, 150 µL of 5 M NaCl, and 4.5 µL of glycogen (20 mg/mL) were added to samples and tubes were stored at -20 °C overnight. Nucleic

acid pellets were formed in tubes after 30 mins of centrifuging (12,000 x g, 4 ºC). The supernatant was cautiously discarded without destroying the DNA pellet, washed twice with 1 mL ice-cold 80% ethanol and air dried in the microcentrifuge tube for approximately 1 h. Pellets were then resuspended in 100 µL of nuclease-free water by gently flicking the tube or heating in a dry bath (55 °C for 10 mins). The nucleic acid (DNA and RNA) extracts were stored at -20 °C until PCR amplification. As for the unplanted BK soil samples, because the CTAB method generated low yield of genomic DNA, DNA isolation was done using the PowerMax® Soil DNA Isolation Kit (MoBio Laboratories, Carlsbad, California, United States), following the manufacturer's instruction with DNA eluted in 10 mM Tris-Cl. The DNA extracts were also stored at -20 °C before PCR amplification.

3.6.2 DNA quantification, concentration, and purification

DNA was quantified via the PicoGreen® dsDNA quantification assay (Invitrogen). For DNA extracts that had low concentrations, DNA concentration using the SpeedVac Concentrator (Thermo Fisher Scientific, CA) or DNA purification using the QIAEX II® Gel Extraction Kit (Qiagen, cat. nos. 20021 and 20051) was performed. The concentrated/purified DNA samples were re-quantified via the Qubit® dsDNA HS Assay Kits (Molecular probes, cat. nos. Q32851, Q 32854) with the Qubit® Fluorometer (2.0).

3.7 Library preparation and amplicon sequencing

3.7.1 A comparative PCR study

Since we extracted the genomic DNA directly from plant root tissue, there is a high possibility that host organellar sequences (mitochondrial and/or plastid), which share a common ancestor with bacterial lineages, could also be co-amplified when performing PCR (Fitzpatrick et al. 2018). The use of sequence-specific peptide nucleic acid (PNA) oligos in 16S rRNA gene amplicon PCR reactions has been proposed as an approach to block the amplification of host plant-derived mitochondrial (mPNA) and plastid (pPNA) sequences. Due to the rates of plastid contamination, as indicated by Fitzpatrick et al. (2018), which were variable across different host plant species,

we carried out a PCR study on three root samples that yielded the most DNA, to test the effect of host amplification.

We performed three different PCR reactions to test the co-amplification of the host sequences. We amplified the V4 and the V4-V5 hypervariable regions of the 16S ribosomal RNA (rRNA) gene by using the universal bacterial primer pairs: 515-F-Y(5'-GTGYCAGCMGCCGCGGTAA-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3') (Caporaso et al. 2012), 515-F-Y and 926R (5'-CCGYCAATTYMTTTRAGTTT-3') (Parada et al. 2015), respectively. All the template specific primers (515-F-Y, 806R, 926R) were attached to the overhang adapter sequence: CTGTCTCTTATACACATCT. PCR reagents in each 0.2 mL PCR tube included 8.5 μL of Nuclease-free H_2O, 12.5 μL of 2X Kappa HiFi HotStart ReadyMix (#KK2601), 0.625 μL of BSA (20 mg/mL, Roche Diagnostics #10711454001), 0.75 μL of each 20 μM forward (515F-Y) and reverse (806R) primer and 2-3 μL of 1:10 diluted root DNA template to a final volume of approximately 25 μL. We conducted a comparison between two different PCR reactions amplifying the V4-V5 region of the 16S rRNA gene; one PCR reaction included the plastid peptide nucleic acid (pPNA) oligos to block co-amplification of the chloroplast DNA sequences from the host plants, whereas another PCR reaction was carried out without adding pPNA. The PCR reaction without pPNA had the exact same reagents as described in the16S rRNA gene amplicon PCR targeting the V4 region, except the reverse primer was 926R. In our pPNA-added PCR reaction, we included the following reagents in a 25 μL PCR reaction: 9.19 μL of Nuclease-free H_2O, 12.5 μL of 2X Kappa HiFi HotStart ReadyMix, 0.5 μL BSA (20 mg/mL), 0.31 μL of 100 μM pPNA (PNABio), 0.25 μL of each 20 μM forward (515F-Y) and reverse primer (926R), and 2-3 μL of 1:10 diluted root DNA template (approx. 2 ng).

The PCR program for the reactions without pPNA involved an initial 3 mins denaturing step at 95 ºC, followed by 30 cycles of 30 s at 95 ºC, 30 s at 55 ºC, and 30 s at 72 ºC, a final elongation step of 7 mins at 72 ºC, with a hold at 4 ºC. As for the pPNA-added PCR reaction, we used the following PCR program at 30 cycles: 95 ºC pause, 5 mins 95 ºC - initial denaturation; cycle starts [45 s 95 ºC - denaturation; 10 s 78 ºC - PNA annealing; 1 min 55 ºC - primer annealing; 1 min 30 s 72 ºC -

extension] cycle ends; 10 mins 72 ºC - final extension, 4 ºC pause. Each batch of PCR reactions included a nuclease-free H_2O sample (negative control) and the commercially produced DNA of a known bacterium (positive control), *E. coli*. These two control samples were used to confirm the absence of contamination and check the efficacy of PCR reactions. After verifying the 16S rRNA gene amplicons via agarose gel electrophoresis, we purified the amplicon PCR product with 0.8 X AMPure XP beads (Beckman Coulter, A63880) to remove free primers and primer dimers. Following the manufacturer's instructions in Illumina's "16S Metagenomic Sequencing Library Preparation" guide (Part #15044223 Rev. B), we barcoded our purified amplicons with Nextera XT Index Primers (N7XX, S5XX) and carried out an 8 cycle PCR ("Index PCR") in an Eppendorf Mastercycler Pro (Eppendorf, 950030030). The indexed amplicon products were purified again, using 1.12 X AMPure XP beads. We quantified all the purified and indexed amplicons via the PicoGreen® dsDNA assay (Invitrogen, Burlington, ON). Based on the quantification result, we then added all the indexed amplicons from individual samples to a single microcentrifuge tube in an equimolar ratio (e.g. 4 nM). The concentration of the pooled library was quantified via the Qubit® dsDNA HS assay kit. The quality of the pooled library was assessed using the Agilent 4200 TapeStation System (G2991AA) with the High Sensitivity D5000 ScreenTape® TapeStation Analysis Software A.02.01 (Agilent Technologies, Inc. 2016). After verifying the quality, the pooled library was diluted to a concentration of 5 × 10 molecules/µL (approx. 1 ng/µL) for each sequencing run on the Illumina MiSeq platform.

3.7.2 Amplicon library preparation and sequencing (rhizosphere and root samples)

The characterization of rhizosphere and bulk soil microbial communities and root microbial endophytes was achieved by sequencing the 16S rRNA gene in bacteria/archaea, and the internal transcribed spacer (ITS) region of the ribosomal RNA gene (rRNA) in fungi. Specifically, the universal bacterial/archaeal primer pairs used to produce the 412 base pair 16S rRNA gene amplicons, were 515F-Y and 926R (described above), which amplify the V4-V5 region of the 16S rRNA gene. To characterize the fungal community in the soils and roots, the universal primers used to produce the 350-450 base pair ITS amplicons, were 1F (5'-CTTGGTCATTTAGAGGAAGTAA-3') and 58A2R (5'-CTGCGTTCTTCATCGAT-3') (Martin and Rygiewicz 2005). Reagents required for 16S/ITS PCR amplification included: 7.5 µL of

nuclease-free water, 10 µL of 2X Qiagen HotStartTaq *Plus* master mix (#203646), 0.5 µL of BSA (20 mg/mL), and 0.5 µL of each 20 µM 16S/ITS forward and reverse primers with the overhang adapter sequence (CTGTCTCTTATACACATCT). The above reagents (19 µL) were prepared in a biological hood and added with 1 µL of DNA template to make up a final volume of 20 µL in a 0.2 mL PCR tube. The 16S PCR program was: 5 mins DNA denaturation at 95 °C, 30 cycles of primer annealing, involving 30 s at 95 °C, 30 s at 55 °C and 45 s at 72 °C, and a final elongation step at 72 °C for 10 mins; the program was then held at 4 °C. Similarly, the amplification of the ITS fungal sequences followed a 30 cycle PCR program of: 5 mins denaturation at 95 °C, 30 cycles of annealing steps involving 30 s at 95 °C, 30 s at 45 °C and 30 s at 72 °C, and a final elongation step of 10 mins at 72 °C; and a hold at 4 °C. The 16S rRNA and ITS amplicon PCRs were carried out in at least triplicate of the root DNA samples.

The 16S rRNA gene amplicon PCR was conducted with pPNA, following the same PCR reaction and program as previously described. The reaction volume of the ITS amplicon PCR for roots was adjusted to 25 µL, including: 7.5 µL of nuclease-free H_2O, 12.5 µL of 2X 2G Robust HotStart Readymix (#KK5702) or 2X KAPA HiFi HotSart ReadyMix, 0.625 µL of BSA (20 mg/mL), 0.75 µL of each 20 µM ITS forward (1F) and reverse primer (58A2R) and 2-3 µL of 1:10 diluted DNA template. The 30-cycle program for the ITS PCR was as follows: 3 min denaturation at 95 °C, followed by 30 cycles of 30 s at 95 °C, 30 s at 45 °C and 30 s at 72 °C, and a final elongation step of 3 mins at 72 °C. For a few root DNA samples that could not be successfully amplified in the above-indicated ITS PCR reaction, a different ITS PCR reaction was applied: 18.25 µL of nuclease-free H_2O, 2.5 µL of 10X PCR buffer (illustra^{TM} r*Taq* DNA Polymerase, Lot. 4669591), 0.25 µL of Tag DNA Polymerase (5 units/µL), 1 µL of 25 mM $MgCl_2$, 1 µL of 5 mM mixed dNTPs (stock solutions with an equal volume of 100 mM A, G, C, T nucleotides), 0.5 µL of each 100 µM ITS forward (ITS-1F) and reverse (ITS-58A2R) primers, and 1-2 µL of 1:10 diluted DNA templates. The PCR program used was: 3 min of denaturation at 95°C, 30 cycles of: 95 °C/30 s, 55 °C/1 min, 72 °C/2 mins for final extension and the program was stopped at 4 °C. Libraries for sequencing were pooled by PCR type and transferred into 4 microcentrifuge tubes: 16S rRNA amplicon PCRs for soil and root samples, ITS amplicon PCRs for soil and root samples. The libraries were also prepared following Illumina's preparation instructions, as previously mentioned.

In some cases, after checking the pooled library quality on the TapeStation, some pooled libraries still contained primer dimers and overhang adapters. Therefore, an extra purification step with SPRISelect beads (40 µL per 50 µL sample) was performed to remove the primer and adapter dimers. The final concentration of the pooled libraries was quantified using the Qubit® dsDNA HS assay kits. The pooled libraries were sequenced on the Illumina MiSeq platform.

3.8 Downstream analysis

Sequences were analyzed through the National Research Council Canada's (NRC, Montreal, Canada) 16S rRNA and ITS amplicon bioinformatics pipeline as previously described (Tremblay et al. 2015). Sequencing reads were QCed, paired-end assembled and clustered at 97 % similarity. Taxonomic identities were assigned to operational taxonomic units (OTUs) using the Ribosomal Database Project (RDP) classifier (v 2.5) with the Greengenes (bacteria/archaea) and the UNITE (fungi) (https://unite.ut.ee) reference databases (DeSantis et al. 2006; Kõljalg et al. 2013). OTU tables, which were obtained by combining the abundance of each cluster to their resulting taxonomy, were analyzed in QIIME (v 1.9) and used to compute Shannon index and Bray-Curtis/Weighted UniFrac dissimilarity distance matrices, to determine the alpha- and beta-diversity of the microbial community, respectively (Caporaso et al. 2010). A tarball (.tar.gz) file containing alpha- and beta-diversity values, and taxonomic summaries was generated at the end of the pipeline run in QIIME, corresponding to each sequenced library. Downstream analyses were performed with in-house R scripts.

3.9 Statistical analysis

Plant survival and growth data from all trials (alder and conifer) was subjected to analysis of variance (ANOVA) using the R (R-3.5.1, https://www.r-project.org) and R-Studio software (v. 1.1.419 ©2009-2018 RStudio, Inc.). All plant data was checked for normal distribution and homoscedasticity in order to verify the assumptions for the ANOVA analysis. Normal distribution was confirmed in histogram plots of the residuals ('rcompanion' package in R) and the Shapiro-Wilk normality test as described in Mangiafico (2016). The homogeneity of variances was tested

using the residual plots in which no particular patterns were observed, suggesting that the assumption was confirmed. Plant survival count data was calculated by proportion and modelled as binomial in a generalized linear model (beta regression) using the '*betareg*' function in the 'betareg' package (Cribari-Neto and Zeileis 2010). Blocking variables (Block 1-3) on plant survival were treated as a fixed effect in the regression model. The difference among plant survival rates was analyzed using the '*Anova*' function of the 'car' package and selected the 'Type II' anova table (Zahn 2010). The SVI data and the PVI data for both alder and conifer trials were specified in a linear regression model using the R built-in '*lm( )*' function, and then analyzed using two-way ANOVA, in which plant types and inoculation were used as two independent factors to test their main effect on seedling biomass production, while the interaction between these two factors was tested as an interaction effect (Keppel and Wickens 2004). The effect of plant inoculation on soil pH and moisture content of the planted site was also specified in the linear regression model but tested via one-way ANOVA, in which the effects of inoculation and plant types were examined as an overall treatment effect on soil property. Tukey's HSD or Dunn-Sidak post-hoc analysis was applied to conduct multiple comparisons of estimated marginal means (EMMs) amongst treatments following the ANOVA analyses, using the 'emmeans' package in R, at a significant level of 0.05. The EMMs are the adjusted means from contrasting treatment groups. Instead of simply considering the average in the group, EMMs account for other factors in the model as well, assumed to provide a better estimation of differences in an unbalanced experimental design (Mangiafico 2016). The significant difference among the group means was shown by different letters using the 'multcompView' package, and the '*cldList*' function in the 'rcompanion' package in R (Piepho 2004). For determining the statistical difference of alpha-diversity (Shannon index) among the treatments, Kruskal-Wallis (nonparametric) test of one-way ANOVA followed by Dunn's multiple comparison test were conducted using the functions of '*kruskal.test ()*' and '*dunnTest ()*' in R, respectively (Dunn 1964; McDonald 2009; Hollander et al. 2013). The effect of inoculation treatments on the bacterial/archaeal and fungal community structure was assessed by permutational multivariate analysis of variance (PERMANOVA) using the '*adonis*' function of the 'vegan' package in R (Anderson and Walsh 2013). To assess the clustering of samples in each group of comparisons, principal coordinates analysis (PCoA) plots were drawn using the 'vegan' package in R based on the OTU matrices generated by the QIIME scripts.

CHAPTER 4 : RESULTS AND DISCUSSION

4.1 Plant survival and biomass production in the field

The field performance of plants in the 2012 phytoremediation trial following 4 years (2016) and 5 years (2017) of growth on the mine site was estimated by their survival rates and seedling growth measurements collected from the two sampling events (September 2016 and 2017). Numbers of surviving plants in each replicate plot were calculated in proportion (%) to the initial number of plant seedlings (15 plants/plot) transplanted on the mine. Field responses of plants (survival and seedling biomass production) to the effects of the selected phytoremediating plants, inoculation treatments, and the interactions between host plants and inoculation treatments, were predicted by a beta regression model that is similar to a binomial generalized linear model (GLM). Then, the model was used to perform two-way ANOVA analysis. The effect of the blocks was treated as a fixed factor in the beta regression model. Plant biomass production was evaluated by seedling volume index (SVI) and plot volume index (PVI). Following the two-way ANOVA, multiple comparison via Tukey HSD or Dunn-Sidak post-hoc analysis was conducted on the estimated marginal means (EMMs) of plant survival by averaging blocks, and the EMMs of plant growth variables by averaging the treatment replicates.

4.1.1 Alder survival and growth in 2016 and in 2017

In both years, the inoculation was found to have a significant effect on the survival rates of green alder and speckled alder (2016: $p=0.007991$, 2017: $p=0.01345$). In contrast, in both years, no significant ($p>0.05$) survival difference was detected corresponding to the species of alder used, nor the interactive effect between the alder species and the inoculation. By testing the inoculation effect within the same alder species via the Tukey post-hoc analysis, we found that the survival rates of the inoculated green alder were not significantly ($p>0.05$) different from their uninoculated counterparts in both years (Fig. 4.1). In the speckled alder plots, the uninoculated speckled alder had relatively higher survival rates than the *Frankia*(FK)-inoculated seedlings, and significantly higher survival rates than the *Frankia*-fungi(FK-ECM) dual inoculated seedlings (2016: $p=0.0495$, 2017: $p=0.0042$), indicating that the inocula used did not improve the survival of speckled alder in the field (Fig. 4.1). As for alder seedling growth, based on the seedling volume index (SVI), no significant seedling growth difference was found in 2016, whereas a significant seedling growth

difference ($p<0.001$) was detected in 2017, corresponding to the alder species used. Moreover, a slight increase of alder seedling growth over time was observed (Fig. 4.2). In Figure 4.2 (B), Tukey HSD post-hoc analysis conducted on the entire alder field trial in 2017 revealed that the *Frankia*-inoculated green alder produced significantly more above-ground seedling biomass than the *Frankia*-inoculated speckled alder ($p=0.0033$) and the dual inoculated speckled alder ($p=0.0291$). Seedling growth of the uninoculated green alder was also found to be significantly larger ($p=0.0069$) than the growth of *Frankia*-inoculated speckled alder seedlings. No significant seedling growth difference was detected within the same alder species (green alder or speckled alder) in both years. When combining the number of surviving plants and seedling volume index, which results in the plot volume index, we found that there was no significant difference of plot volumes observed among inoculation treatments, nor between the two alder species. Although no significant inoculation effect was found comparing the PVI data within the same alder species, based on the rapid seedling growth of green alder and the negative effect of inoculation on the survival of speckled alder, we conclude that green alders have outcompeted speckled alders on the mine site (Fig. 4.3).

In the present study, the inoculation treatments neither improved survival nor seedling growth of the alder species selected (*A. crispa* and *A. rugosa*). This result was in accordance with the findings in a previous greenhouse study from our lab, in which similar survival rates and seedling biomass production of the *Frankia*-inoculated *A. crispa* and *A. glutinosa* in the Sigma gold mine waste rock amended with woodchips relative to unamended treatments were observed (Callender et al. 2016). Interestingly, we noticed alders in tripartite symbiosis with *Frankia*, arbuscular mycorrhizal fungus (*G. irregulare*) and ectomycorrhizal fungus (*A. diplophloeus*) showed the lowest survival rates and plot seedling biomass among treatments in both years, which can be explained by a dramatic energy (photosynthates) consumption of active microsymbionts on their plant hosts leading to a negative impact on plant survival and growth (Roy et al. 2007). In addition, Roy et al. (2007) reviewed that the genus *Glomus* is able to increase the growth and nutrient acquisition of some alder species during their early establishment in nature; however, as alders continue to grow, this arbuscular mycorrhizal symbiosis is outcompeted by ectomycorrhizal fungi, which in general have high host specificity. In this sense, our results can be interpreted as seedling inoculation with

A. diplophloeus was not capable of enhancing either the survival or the biomass growth of *A. crispa* and *A. rugosa*. Possible explanations for this result are either the loss of infectivity of *A. diplophloeus* strains over several years of in vitro subculturing, or the failure of this ectomycorrhizal fungal species to form ectomycorrhizae on roots of green or speckled alders in the present study. Similar observations were also made by Beaudoin-Nadeau et al. (2016), who found that none of their four different *A. diplophloeus* strains improved the seedling fitness of green and speckled alders on Canadian oil sands tailings. In contrast to the findings in Yamanaka et al. (2003), in which dual inoculation of thin leaf alder (*Alnus incana* (L.) Moench ssp. *tenuifolia* (Nutt.) Breitung) with both *Frankia* sp. and *A. diplophloeus* resulted in an increase of alder seedling biomass growth and rock solubilization, our results suggest that alders in tripartite associations with *Frankia*, *A. diplophloeus* and *G. irregulare* did not boost alder survival and growth in the metal-contaminated mine substrate.

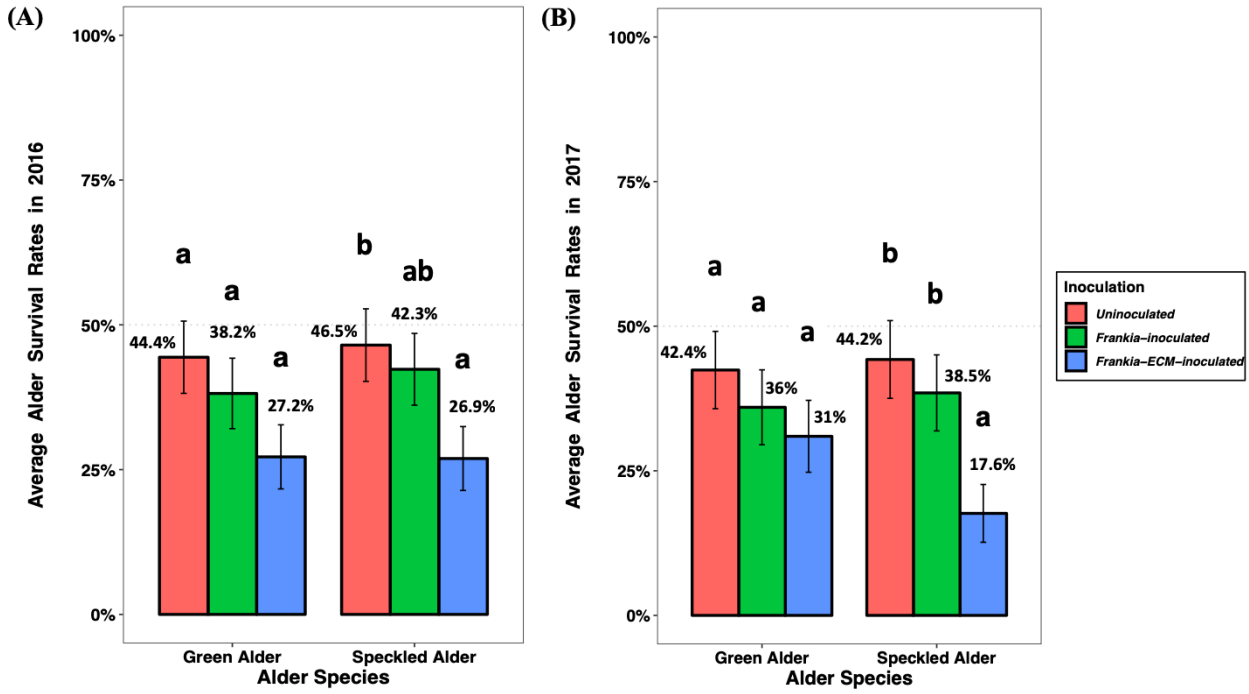

Figure 4.1. Average plant survival rates of non-, *Frankia*-, and *Frankia*-mycorrhizal fungi-inoculated alder seedlings comparing (A) 2016 and (B) 2017 via two-way ANOVA. Bars (mean ± SE) labelled with different letters indicate significant ($p<0.05$) differences among alder survival via Tukey's HSD post-hoc analysis.

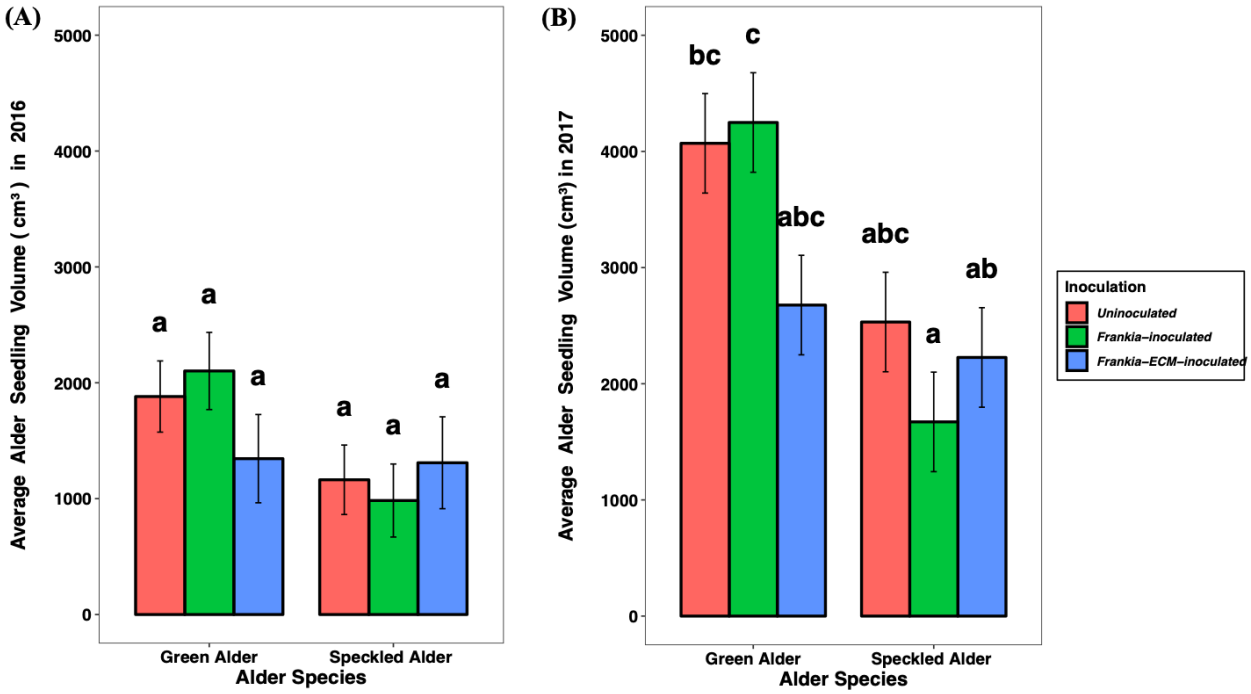

Figure 4.2. Average plant seedling growth of non-, *Frankia*- and *Frankia*-mycorrhizal fungi-inoculated alders comparing (A) 2016 and (B) 2017 via two-way ANOVA. A) SVI mean ± SE (n = 102) in 2016; (B) SVI mean ± SE (n = 30) in 2017. Bars labelled with different letters indicate significant ($p<0.05$) differences among alder seedling growth, as determined by Tukey's HSD post-hoc analysis.

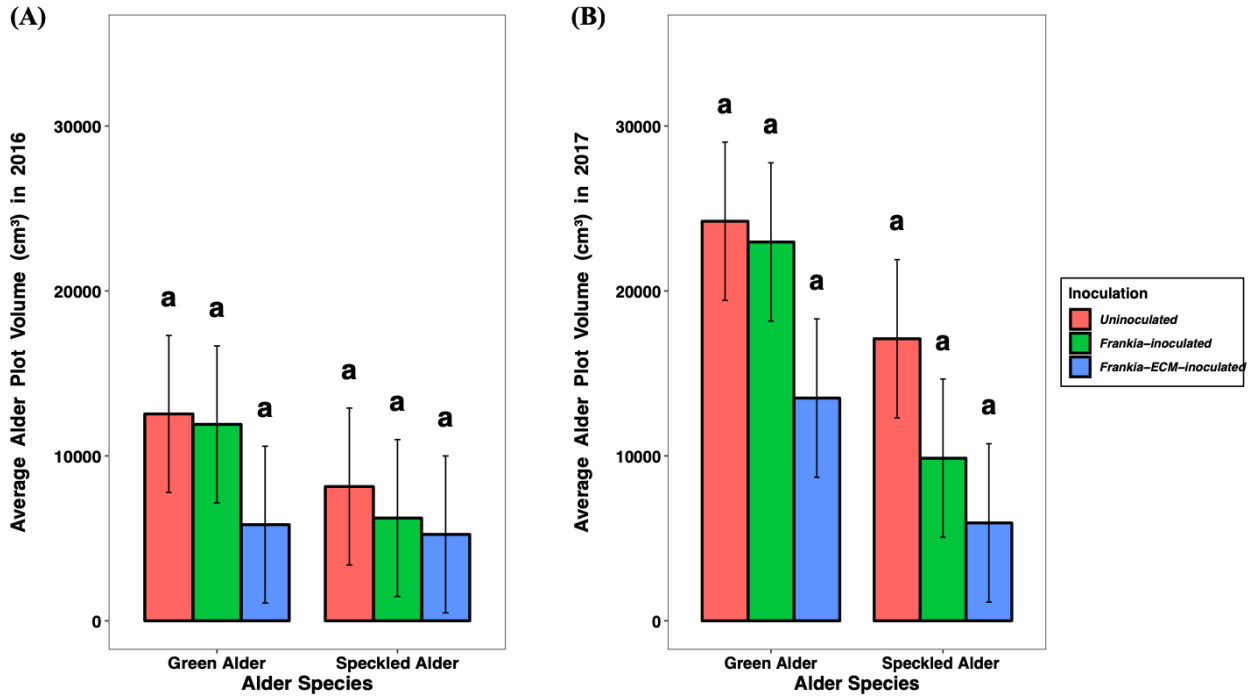

Figure 4.3. Average plot volume index (PVI) of non-, *Frankia*- and *Frankia*-mycorrhizal fungi-inoculated alders comparing (A) 2016 and (B) 2017 via two-way ANOVA. Bars (mean ± SE) labelled with the same letters indicate that no significant ($p>0.05$) difference was observed among the plant seedling biomass production in plots, as determined by Tukey's HSD post-hoc analysis.

4.1.2 Conifer survival and growth in 2016 and in 2017

ANOVA analysis conducted on the beta-regression model of conifer survival rates indicated a significant ($p<0.001$) survival difference between white spruce and jack pine. The analysis also showed that neither the inoculation nor the interaction between conifers and inoculation had a significant effect on conifer survival. Dunn-Sidak post-hoc analysis (p values were adjusted by "sidak") carried out following the two-way ANOVA revealed that jack pine, in general, had higher survival rates than white spruce in both years (Fig. 4.4). As specifically demonstrated in Figure 4.4 (A), in 2016, the survival rates of the ectomycorrhizal (ECM) fungi-inoculated white spruce, which had 51.1 % survival in the field, were significantly lower than the uninoculated jack pine ($p=0.0326$) and the ECM-inoculated jack pine ($p=0.0018$), at 73.5 % and 71.2 % survival, respectively. In addition, a significant ($p=0.03$) seedling survival difference was also observed between the non-inoculated white spruce, at 52.8 %, and the ECM-inoculated jack pine. In 2017, a slight decline of plant survival from 2016 was observed in the conifer trial. In 2017, the survival rates (66.9 %) of the ECM-inoculated jack pine were higher than that of uninoculated jack pine (58.2 %), and were significantly higher than the seedling survival of the uninoculated white spruce ($p=0.0048$), at 41 %, and the seedling survival of the ECM-inoculated white spruce ($p=0.0035$), at 44.3 % (Fig. 4.4 B). In terms of the effect of ECM inoculation on conifer survival, our results showed that, in both years, there was not a significant seedling survival difference observed within the same type of conifer plants. For the conifer seedling growth, the SVI showed that the type of conifer, the ECM inoculation, and the interaction between the conifer species and the inoculation all had significant effects on conifer above-ground seedling growth in both 2016 ($p<0.001$) and 2017 ($p<0.01$). As shown in Figure 4.5, the ECM-inoculated jack pine had the largest seedling biomass among all the treatments in both years, at 249.2 cm^3 in 2016 and 338.1 cm^3 in 2017. In addition, we found that conifers with ectomycorrhizal fungal inoculation generally produced larger seedling biomass than uninoculated controls, except for the ECM-inoculated white spruce in 2016, which had lower seedling biomass than the uninoculated counterparts, although this difference was insignificant (Fig. 4.5 A). As mentioned, the ECM-inoculated jack pine had significant larger seedling growth than the uninoculated jack pine, both in 2016 ($p<0.0001$) and in 2017 ($p=0.0001$), which is so far the only significantly positive effect of inoculation on plant seedlings. In Figure 4.6, although no significant difference of the conifer plot volumes was found in both years, the

inoculation of ectomycorrhizal fungi to jack pine plants still displayed an outstanding host growth enhancement as compared to non-inoculated jack pine.

Our findings revealed that the ectomycorrhizal fungal species that were used to inoculate jack pine seedlings, *S. tomentosus* (St) and *L. bicolor* (Lb), seemed to work well and stimulate jack pine performance on the metal-contaminated mine residues. It is known that *Laccaria* sp. and *Suillus* sp. are the most compatible ectomycorrhizal fungal symbionts for inoculation of pine (*Pinus*) seedlings (Quoreshi and Khasa 2008). In the present study, the ectomycorrhizal fungal colonization by *H. crustiliniforme* (Hc) and *P. involutus* (Pi) did not substantially affect white spruce survival and seedling growth, while jack pine with ectomycorrhizal fungal inoculation by St and Lb resulted in higher survival (in 2017) and significantly greater seedling growth (both 2016 and 2017) among all the treatments of conifers, including the uninoculated control. This result was partially in agreement with the conclusions reached by Onwuchekwa et al. (2014), who studied the same conifer species with ectomycorrhizal fungal inoculations of Hc, St, and Lb in oil sands reclamation sites of Alberta and found that the inoculation combinations of St and Lb with jack pine seedlings also significantly increased its SVI, but neither improved the conifer seedling survival nor the PVI. Moreover, Onwuchekwa et al. (2014) also found the seedling biomass (SVI and PVI) of white spruce, to a large extent, was not significantly enhanced by inoculating with an individual ectomycorrhizal fungal strain or the combinations of three ectomycorrhizal fungal species. Another phytoremediation study examining outplanting performance of inoculated conifer and hardwood plants found that the survival rates of the majority of conifers did not significantly differ from their non-inoculated controls (Quoreshi et al. 2008). In addition, Quoreshi et al. (2008) found that except for *L. bicolor*, most of the introduced ectomycorrhizal fungal strains, which were supposed to colonize plant roots, were replaced by indigenous ectomycorrhizal fungal species after 5 years of growth in the field. *L. bicolor*, as noted by Parladé and Alvarez (1993), is known to be an aggressive root colonizer capable of increasing total plant biomass. In the previous studies, the survival and growth of conifer seedlings observed on different field sites often varied greatly with different inoculation treatments depending on the plant and fungal species involved. Many researchers have referred the ineffectiveness of fungal inocula in forming mycorrhiza in tested plant roots to the plant-fungus incompatibility and the particular soil characteristics in the field (Quoreshi et al. 2008; Smith and Read 2008; Onwuchekwa et al. 2014).

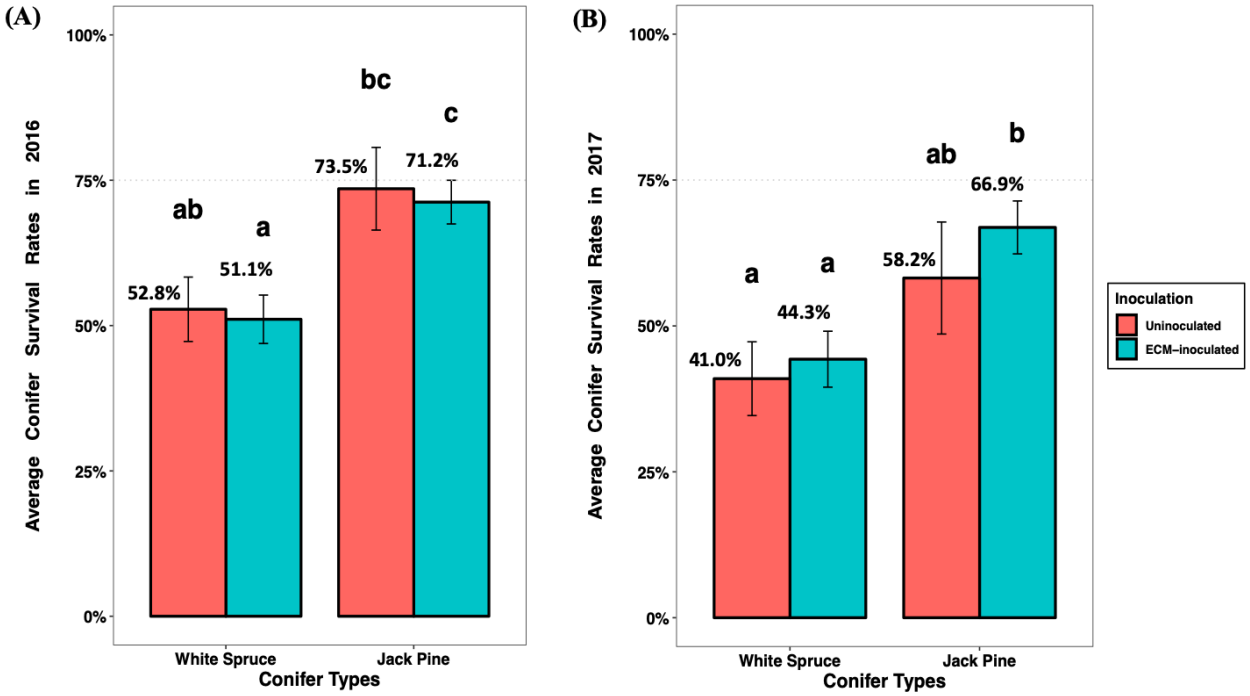

Figure 4.4. Average survival rates of non- and ECM-inoculated conifers comparing (A) 2016 and (B) 2017 via two-way ANOVA. Bars (mean ± SE) labelled with different letters indicate significant ($p<0.05$) differences among plant survival rates, as determined by Dunn-Sidak post-hoc analysis.

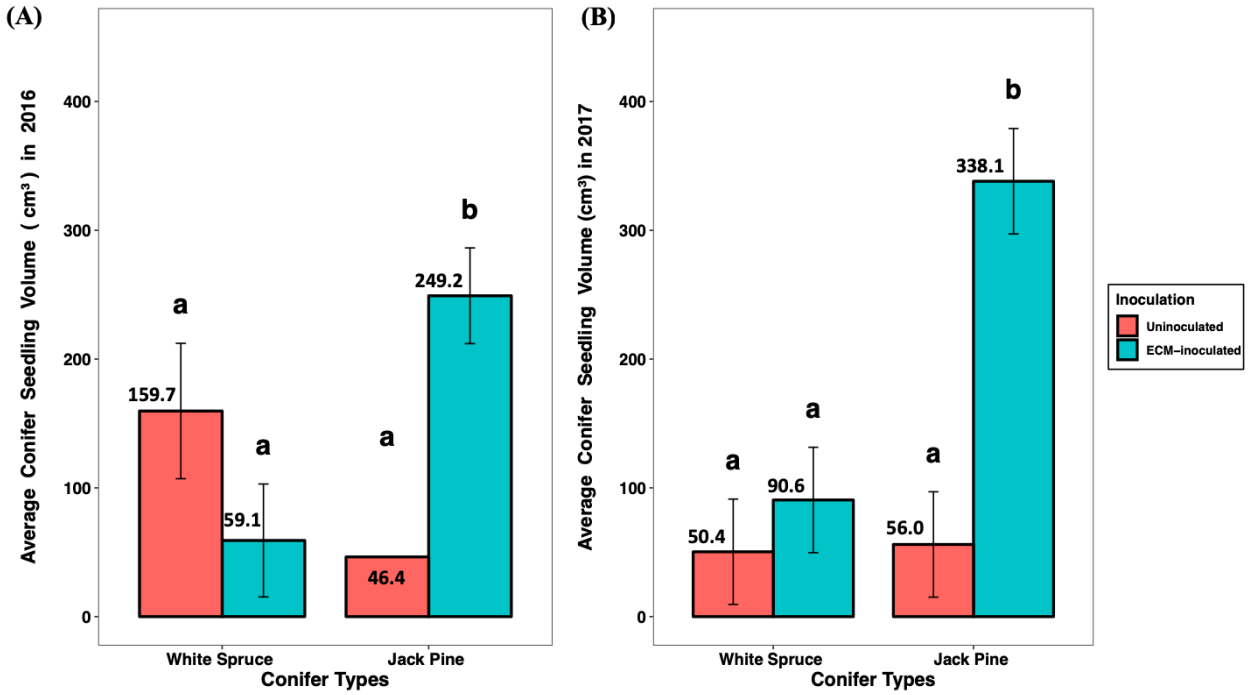

Figure 4.5. Average plant seedling biomass production of non- and ECM-inoculated conifers comparing (A) 2016 and (B) 2017 via two-way ANOVA analysis. A) SVI mean ± SE (n=82) in 2016; (B) SVI mean ± SE (n=20) in 2017. Bars labelled with different letters indicate significant ($p<0.05$) differences among plant seedling growth, as determined by Dunn-Sidak post-hoc analysis.

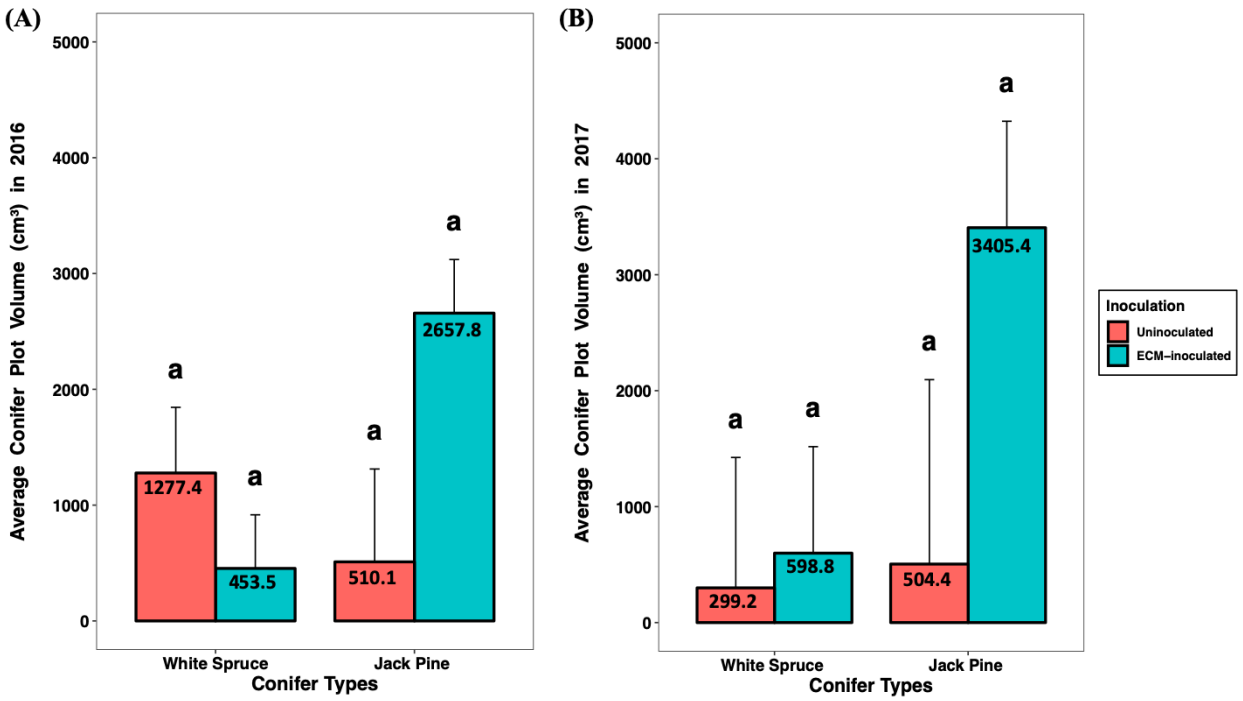

Figure 4.6. Average plot volume index (PVI) of non- and ECM-inoculated conifers comparing (A) 2016 and (B) 2017 via two-way ANOVA. Bars (mean + SE) labelled with same letters indicate non-significant differences ($p>0.05$) among the average conifer seedling biomass production in plots, as determined by Dunn-Sidak post-hoc analysis.

4.2 Effect of phytoremediation on mine site soil properties

After growing on the mine site for 4-5 years, most surviving plants that we found were able to extend their roots outside of the original peat bags, thus having a substantial impact on the soil characteristics at the mine. We conducted a physicochemical test on two common soil parameters, pH and moisture content (%), and the results are presented in this section. In general, measurements of soil pH and moisture content (%) were compared between unplanted sites and planted sites with uninoculated and inoculated seedlings, in order to evaluate the effect of phytoremediation on the soil. Statistical analysis was conducted using one-way ANOVA, in which the plant-inoculum interaction was considered as an overall treatment effect on the planted site. For a clear demonstration, the results of soil pH analysis (Fig. 4.7) were separated from the soil moisture analysis (Fig. 4.8), in order to compare their changes between two growth seasons (2016 and 2017). For clarification, the tested soil samples harvested from the planted sites in 2016 were the plant rhizosphere while in 2017 were the plant bulk soils.

4.2.1 Effect of phytoremediation on soil pH over time

As illustrated in Figure 4.7, the pH of the unplanted bulk soil, which represents the initial soil condition after mining, was significantly ($p<0.0001$) higher (alkaline) than the planted sites, which in contrast, had near-neutral pHs in both years. Specifically, the fact that the pH in the unplanted bulk soil remained steady between 2016 and 2017, at 8.4 and 8.5 respectively, suggests that the Sigma gold mine substrates without human-assisted revegetation are likely to remain unchanged (alkaline) for quite some time. Interestingly, although the pH values in the planted soil did not dramatically differ across treatments, we observed a slight increase in the pH over the two years, from an average of 6.5 in 2016 (Fig. 4.7 A) to a pH ranging from 7.0 to 7.8 in 2017 (Fig. 4.7 B). The inoculation effect on plant seedlings was not obvious, as inoculated seedlings did not exert a significant impact on the soil pH of their planted sites compared to the uninoculated control plants on their planted sites (Fig. 4.7). This suggests that soil pH was largely affected by revegetation and it was neither a sensitive indicator specific to the type of plant, nor to the introduced microbial inocula.

4.2.2 Effect of phytoremediation on soil moisture content (%) over time

The Tukey's HSD test indicated the moisture content (%) in the unplanted bulk soil, which represents an initial post-mining condition of the site, was significantly ($p<0.0001$) lower, at 4.0 % and 4.2 %, in 2016 and 2017 respectively, compared to that in the planted sites (Fig. 4.8). The significant difference of soil moisture content observed between unplanted and the planted sites can be explained by the contribution of plants that stabilize soils and decrease water erosion. As for the impact of inoculation on soil moisture content, in 2016, we observed a non-significant ($p>0.05$) difference of soil moisture content across inoculation treatments within the same type of plants (Fig. 4.8 A). In 2017, however, we found the moisture content in the bulk soils of the inoculated plants was significantly lower than that in the uninoculated plant bulk soils, except for the green alder treatment, in which the uninoculated green alders were associated with a significantly ($p=0.0408$) higher soil moisture content than their FK-inoculated seedlings, but this difference was not significant ($p>0.05$) with their FK-ECM-inoculated seedlings (Fig. 4.8 B). As a decreasing trend of soil moisture content was observed in the planted sites between 2016 and 2017 (Fig. 4.8), and the difference of soil moisture content between uninoculated and the

inoculated seedlings was not significant in 2016 but became significant in 2017, these results were viewed as a positive sign that both plants and microbes are thriving at the studied mine site, since the water availability in soil is necessary for both plants and microbes.

The physicochemical results showed that the revegetated sites had a significantly less alkaline pH in comparison to the unvegetated sites. This result indicated a positive impact of phytoremediation in changing the pH and nutrient availability in the soil, because alkaline mine substrates are frequently characterized by deficiencies in trace elements, such as copper, manganese, iron and zinc (Lottermoser 2010). Arienzo et al. (2004) also emphasized that a high soil pH could be one of the most important parameters that limits the availability of trace metals to plants. The lower soil pH in the reclaimed soils are also beneficial for the introduction of late-successional plant species, since many plants are not able to tolerate an alkaline pH greater than 8.5 (Lottermoser 2010). In addition, a near-neutral pH (7.0) level in the soil also promotes the colonization of soil microbes (Davet 2004). From the results, pH values in the plant rhizosphere soils were closer to the neutral pH and also had less variation in the different inoculation treatments compared to that in the plant bulk soils. Plants, especially those with N_2-fixing symbionts, can neutralize soils with organic acids and also lower (acidify) soil pH by amending the soil with organic matter (Hibbs and Cromack 1990; Fisher and Binkley 2012). Alders as N_2-fixing symbiotic plants are found to acidify soils. In contrast to what was reviewed in Roy et al. (2007) and observations made by Callender et al. (2016), the alder species we used for this phytoremediation study did not demonstrate a significant acidification effect on the soil compared to the conifers. We did find a significantly higher moisture content in the reclaimed (revegetated) soils, which can be viewed as an added benefit of plants in altering soil compaction and reducing soil erosion (Davet 2004). Comparing the moisture content between the plant rhizosphere and bulk soils, it is apparent that plant rhizosphere soils had higher water availability than the bulk soils, which can be explained by microbes (e.g., mycorrhizal fungi) colonizing the vicinity of roots enhance the water uptake ability of plants (Smith and Read 2008). As there were also significant moisture differences in plant bulk soils observed in different inoculation treatments, in which soils grown with the inoculated seedlings generally had lower moisture content, a possible explanation is that microbial activity, in general, was higher in soils with inoculated seedlings, leading to higher water consumption (Davet 2004).

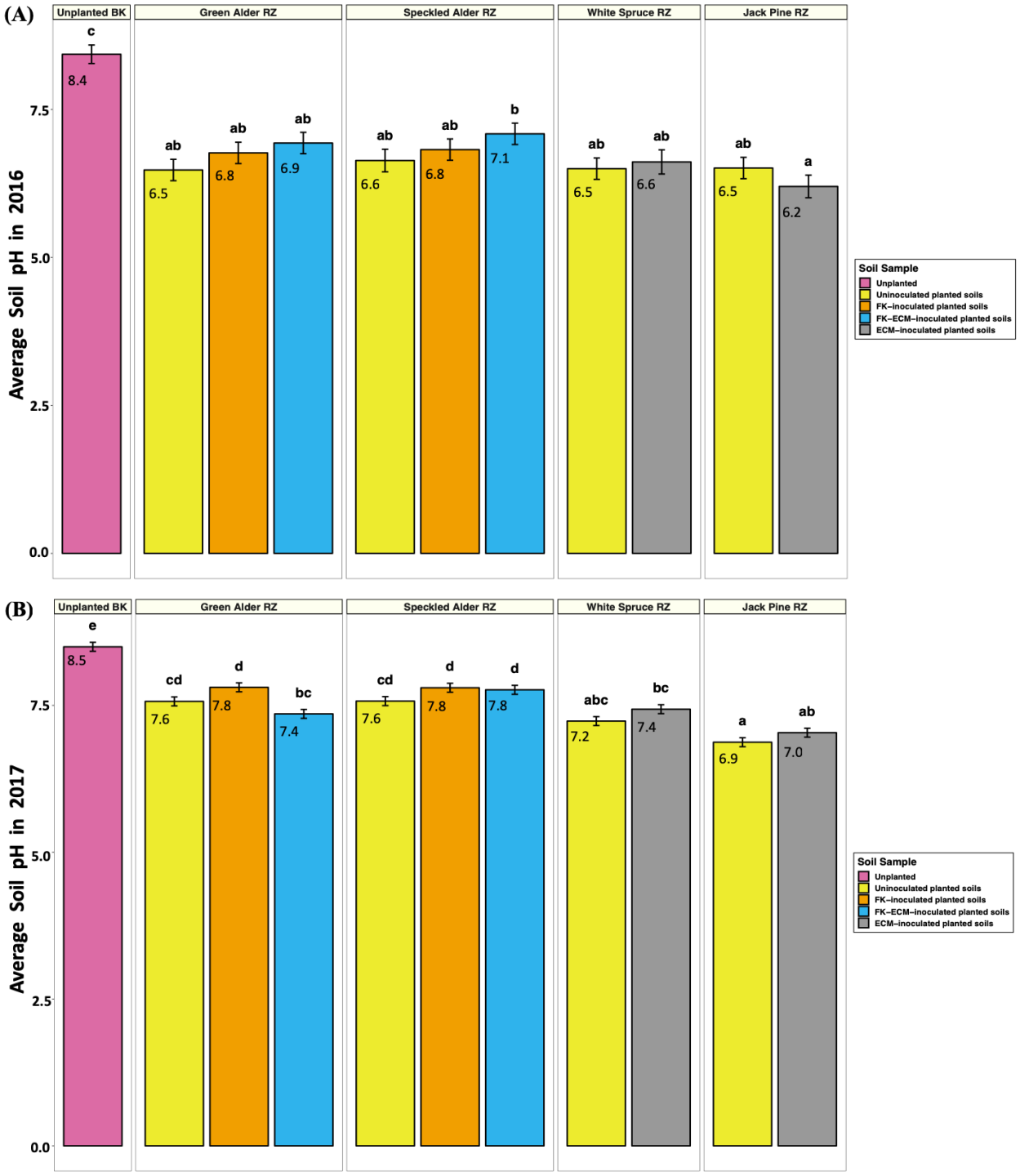

Figure 4.7. Soil pH comparing the unplanted bulk soil, the non- and the inoculated seedlings planted rhizosphere and bulk soils over two growth seasons (2016 and 2017). (A) pH mean ± SE (n=98) in 2016; the planted soil tested was plant rhizosphere soil. (B) pH mean ± SE (n=44) in 2017; the planted soil tested was plant bulk soil. Bars labelled with different letters indicate significant differences at $p<0.05$ by Tukey's HSD. Abbreviations: BK=bulk soils, RZ=rhizosphere soils, FK= *Frankia* sp. strain AvcI1, FK-ECM= plants inoculated with *Frankia* and mycorrhizal fungi (*G. irregulare* and *A. diplophloeus*), ECM=ectomycorrhizal fungi (*H. crustiliniforme*, *P. involutus*, *S. tomentosus*, *L. bicolor*).

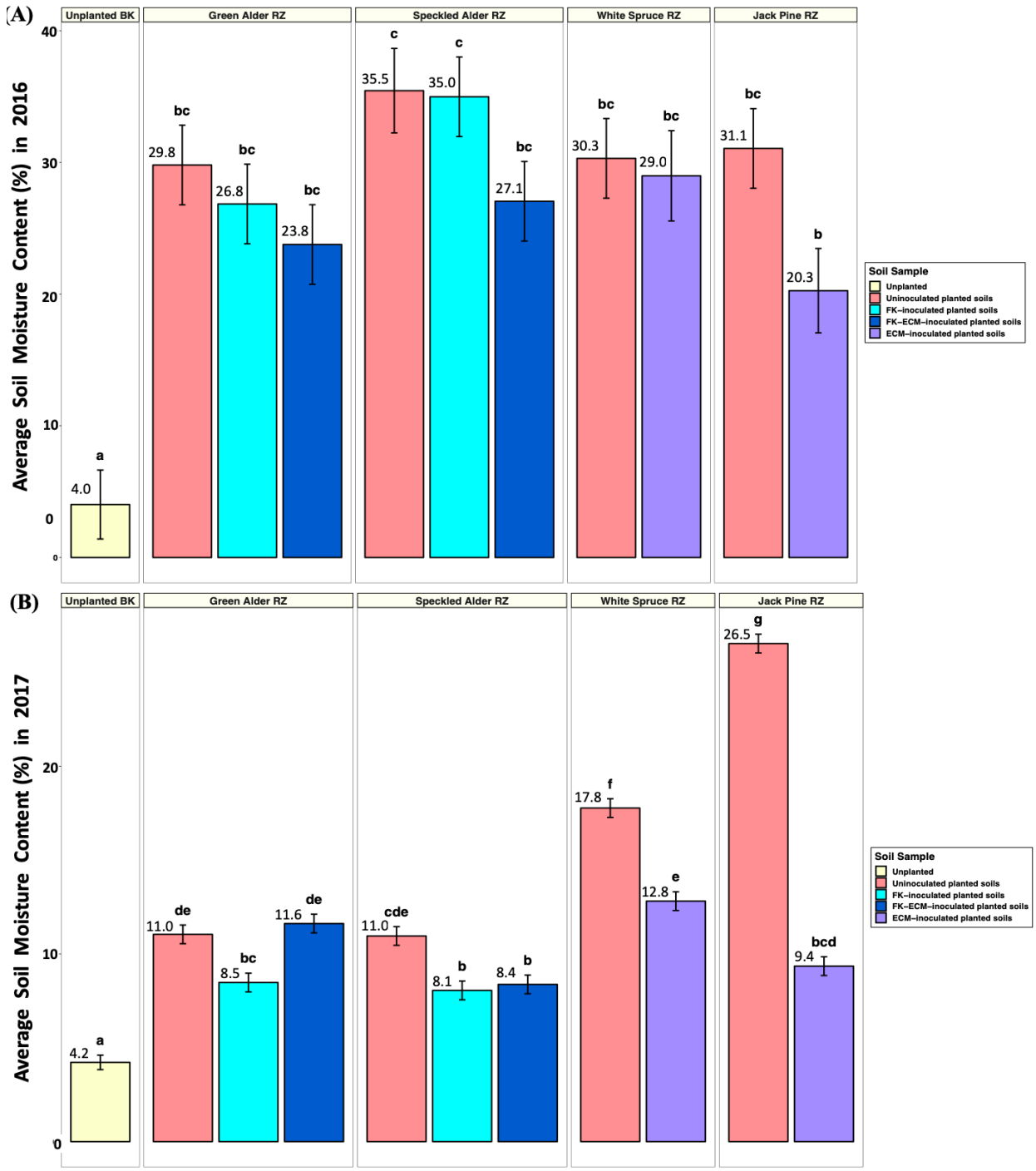

Figure 4.8. Soil moisture content (%) comparing the unplanted bulk soil, the non- and the inoculated seedlings planted rhizosphere and bulk soils over two growth seasons (2016 and 2017). (A) moisture mean ± SE (n=98) in 2016; the planted soil tested was plant rhizosphere soil. (B) moisture mean ± SE (n=35) in 2017; the planted soil tested was plant bulk soil. Bars labelled with different letters indicate significant differences at $p<0.05$ as determined by Tukey's HSD. Abbreviations: BK=bulk soils, RZ=rhizosphere soils, FK= *Frankia* sp. strain AvcI1, FK-ECM= plants inoculated with *Frankia* and mycorrhizal fungi (*G. irregulare* and *A. diplophloeus*), ECM=ectomycorrhizal fungi (*H. crustiliniforme*, *P. involutus*, *S. tomentosus*, *L. bicolor*).

4.3 Effect of the processing time delay on the microbial community structure (2016 data)

We retrieved a total of 462,231 and 1,881,067 high quality sequences of the V4-V5 hypervariable region of the bacterial and archaeal 16S rRNA gene (approx. 412 bp) and the ITS region of the fungal rRNA gene (approx. 420 bp), respectively, on the Illumina MiSeq platform. After quality filtering and rarefication, the obtained 16S rRNA and ITS gene sequences were converted into operational taxonomic units (OTUs; sequence identity cutoff of <97 %) and then classified into different bacterial/archaeal and fungal taxa. The alpha-diversity (species richness and evenness) within the microbial community was estimated by Shannon index. According to He et al. (2013), the Shannon index is considered a reliable and stable indicator for microbial diversity, less susceptible to sequencing errors. Significant differences among treatments, which are displayed as different letters in the graphs, were analyzed using the global Kruskal–Wallis test followed by Dunnett's multiple mean comparisons with P-values adjusted by the Bonferroni–Holm method. In general, bacteria prevailing in the conifer rhizosphere samples were more diverse than fungi (Fig. 4.9). Surprisingly, the time delay in sample processing had little effect on the bacterial diversity in the conifer rhizosphere samples as compared to the fungal diversity (Fig. 4.9). No significant differences of bacterial diversity were observed among the majority of conifer rhizosphere samples, except for the ectomycorrhizal (ECM) fungi-inoculated spruce treatments (E_ECM_4d/16d), which had significantly lower ($p<0.001$) Shannon diversity compared with the other treatments (Fig. 4.9 A). In contrast, the fungal diversity in the conifer rhizosphere samples was strongly affected by the processing time delay, as a substantial decline was observed in the Shannon diversity across the majority of samples as processing time delays increased (Fig. 4.9 B). Specifically, the fungal diversity in the jack pine treatments (P_NI_6d/16d and P_ECM_4d,6d,16d) had significant ($p<0.001$) time delay responses among the rhizosphere samples processed on different days (4d/6d, 16d). The decreased fungal diversity of conifer rhizosphere with the delay in sample processing indicated that the species richness of the fungal community was more sensitive to storage effects. Conifer rhizosphere samples stored for up to 16 days at 4 °C (i.e., treatments of E_ECM_16d, E_NI_16d, P_NI_16d, P_ECM_16d) displayed a sharp drop in fungal diversity as compared to those that had been stored under the same conditions for a shorter time (4 or 6 days). We assumed that a similar decline with storage time would occur in the bacterial diversity of the conifer rhizosphere samples, but this trend was not observed. In fact, the significant

distinction in the bacterial diversity of two ectomycorrhizal (ECM) fungi-inoculated white spruce treatments (i.e., treatments of E_ECM_4d/16d), demonstrated that the bacterial diversity in the rhizosphere samples of our conifer plants was not impacted to the same degree as the fungal diversity.

To investigate the beta (β)-diversity among microbial communities, we performed principal coordinates analysis (PCoA) using the weighted UniFrac distance matrices computed between each community composition in the conifer rhizosphere samples sequenced (n=36), on the basis of OTU counts. The weighted UniFrac distance matrix provides a phylogenetic measure of between-communities diversity (β-diversity) based on the abundance on each microbial taxa (Lozupone et al. 2007). PCoA is a scatterplot-like ordination plot in which samples (treatments) with high similarity cluster closely, whereas dissimilar samples are distributed further apart. Visualization of the weighted Unifrac dissimilarity distances on the PCoA plots (Fig. 4.10) indicated that more sample variation resulted from the effect of inoculation treatments on conifer rhizosphere samples than those resulting from the effect of time delays. In Figure 4.10, specifically, we observed a clear separate clustering of E_ECM treatments on both PCoA graphs, which were depicted by blue circles (E_ECM_16d) and triangles (E_ECM_4d). PERMANOVA highlighted that, compared to the other experimental groups, ectomycorrhizal (ECM) fungi-inoculated white spruce (E_ECM_4d/16d) possessed dissimilar compositions of both bacterial ($F=21.09$, $p<0.001$) and fungal ($F=5.7$, $p<0.001$) communities, accounting for 39 % ($R^2=0.39$) and 14 % ($R^2=0.14$) of the statistical variance, respectively. The difference in microbial community compositions in the conifer rhizosphere soils processed between different days, however, was not evident on the PCoA plots. Although PERMANOVA indicated a significant processing delay effect on both communities (bacterial composition: $F=4.7$, $R^2=0.23$, $p<0.001$; fungal composition: $F=2.55$, $R^2=0.07$, $p=0.0065$), the significant result is likely due to the highly variable data dispersion.

Taxonomic graphs of relative microbial abundance, which were generated by assigning our classified 16S rRNA and ITS gene OTUs to the international nucleotide sequence reference databases, Greengenes and UNITE, respectively, provide detailed information of microbial dominance within the community in each sequenced sample. Figure 4.11 reveals the bacterial

community composition whereas Figure 4.12 demonstrates the fungal community composition. In both taxonomic graphs, relative abundance of microbial taxa presented in a given rhizosphere sample was depicted by an average stacked bar (n=9) for that sample. When looking at the dominant bacterial and fungal taxa within each community and comparing the difference across all the conifer rhizosphere samples, we found that the E-ECM treatments (E_ECM_4d and E_ECM_16d) possessed a very different bacterial and fungal community from other conifer rhizosphere samples sequenced (Fig. 4.11 and Fig. 4.12). In Figure 4.11, the top 20 bacterial taxa (families) that were dominant, represented approximately 50 % of the bacterial community composition in the conifer rhizosphere samples sequenced. The conifer rhizosphere samples were dominated by the phyla Acidobacteria, Bacteroidetes, Planctomycetes, and Proteobacteria. Our preliminary results also showed that the composition of bacterial families was highly consistent across treatments, except for the E-ECM treatments, which comprised much higher relative abundances of the Acidobacteriaceae, Sphingobacteriaceae, and Acetobacteraceae families relative to their abundances in the other treatments (Fig. 4.11). The Acetobacteraceae, as reviewed in Komagata et al. (2014), comprises two groups, including the acetic acid bacteria such as *Acetobacter* and *Gluconacetobacter*, and the acidophilic bacteria such as *Acidiphilium* and *Roseomonas*. The acetic acid bacteria belonging to the genera of *Gluconacetobacter*, *Swaminathania*, and *Acetobacter* were reported to have nitrogen-fixation capability for promoting plant growth. The family Acetobacteraceae is often found inhabiting the plant rhizosphere and internal plant tissues (Fuentes-Ramírez et al. 2001; Dutta and Gachhui 2006). Acidobacteriaceae, possesses some well-known abilities such as the ability to quickly respond to changes in macro-, micro-nutrients and acidity in the soil (Kielak et al. 2016). The last abundant bacterial group dominating the E-ECM treatment was the family Sphingobacteriaceae, which also comprises diazotrophic bacterial members, which were reported to fix nitrogen in rice plants grown in Brazil (Videira et al. 2009). Based on the literature, these three bacterial families appear to help plants uptake nitrogen.

Unlike the bacterial community, the fungal community structure (genus level) in each given sample had more variability, which made it difficult to explore the main variables (inoculation or processing time delays) causing the compositional dissimilarities (Fig. 4.12). Amongst the 20 most abundant fungal OTUs, 18 of these belonged to the Dikarya subkingdom (Ascomycota and

Basidiomycota). In particular, Sordariomycetes and Agaricomycetes were the most predominant classes corresponding to the Ascomycota and Basidiomycota, respectively. Fungal members in the Agaricomycetes class were found to be predominant in every treatment, which was in agreement with the taxonomic analyses shown by Buée et al. (2009b), that the ectomycorrhizal fungal species from the Boletales, Agaricales and Thelephorales were abundant in their forest soil samples. Noticeably, the genus *Tylospora*, which is classified in the family Atheliaceae, was present in the groups of E_NI_16d and P_NI_16d, as opposed to the absence of this genus in the contrasting groups (E_NI_6d and P_NI_6d). In addition, the ectomycorrhizal (ECM) fungi-inoculated jack pine treatments (P_ECM_4d, 6d, 16d) were also greatly affected by the processing delays (Fig. 4.12). The dominant fungal phyla in the P_ECM treatment encountered a sharp shift as the processing time delay increased, from the Ascomycota in P_ECM_4d, to Zygomycota (genus *Mortierella*) in P_ECM_6d, and the Basidiomycota (genus *Amphinema*) in P_ECM_16d treatment. Moreover, the relative abundance of the genus *Suillus* (not the spruce's ectomycorrhizal fungal inoculum) was substantially higher in the ectomycorrhizal (ECM) fungi-inoculated white spruce treatments as opposed to the other treatments.

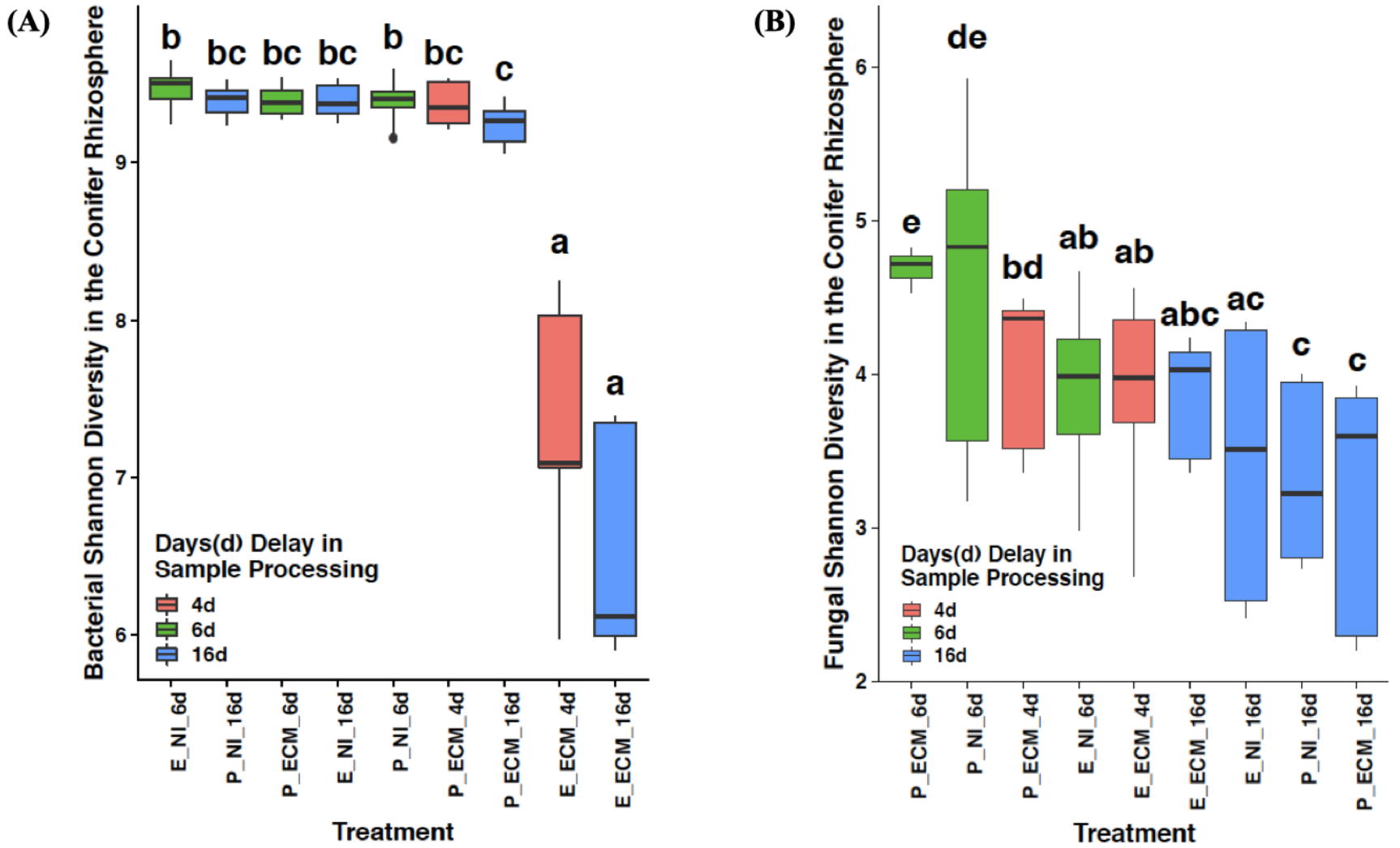

Figure 4.9. Within-sample diversity (α-diversity) shown by the Shannon index in the conifer rhizosphere samples collected in 2016. Shannon indices described the change of species richness and evenness within the (A) bacterial and (B) fungal community in the conifer rhizosphere corresponding to the impact of processing delays in days (4d, 6d, 16d). The diversity indices were shown in boxplots and arranged in a descending order of the mean (the horizontal line within each box). Mean diversity values with different letters on the top of bars were statistically different at the significant threshold of 0.05. E = white spruce, P = jack pine, NI = not inoculated, ECM = plants inoculated with ectomycorrhizal fungi.

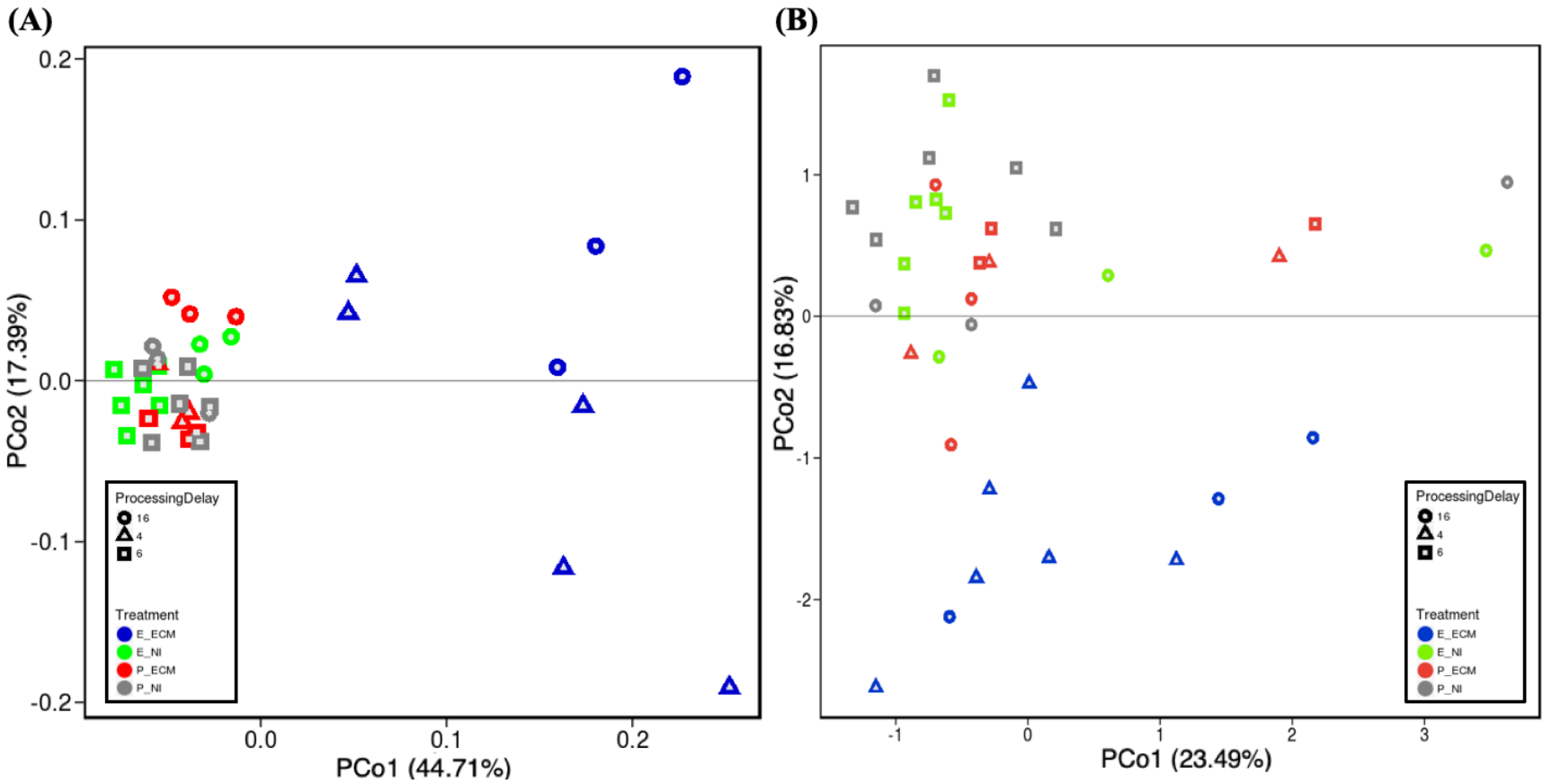

Figure 4.10. Between-sample diversity (**β** diversity) comparing the (A) bacterial and (B) fungal community composition in the conifer rhizosphere samples collected in 2016. Each point in the principal coordinates analysis (PCoA) plot represents a sample (n=36), with distances between samples calculated using weighted Unifrac as a measure of community composition dissimilarity. Plant-inoculum treatments are shown in different symbol colors and the processing time delay in days is shown by different symbol shapes. The variation of each microbial community is explained by the % variances of the principle coordinates shown on the X and Y axes. E= white spruce, P= jack pine, NI= not inoculated, ECM= plants inoculated with ectomycorrhizal fungi.

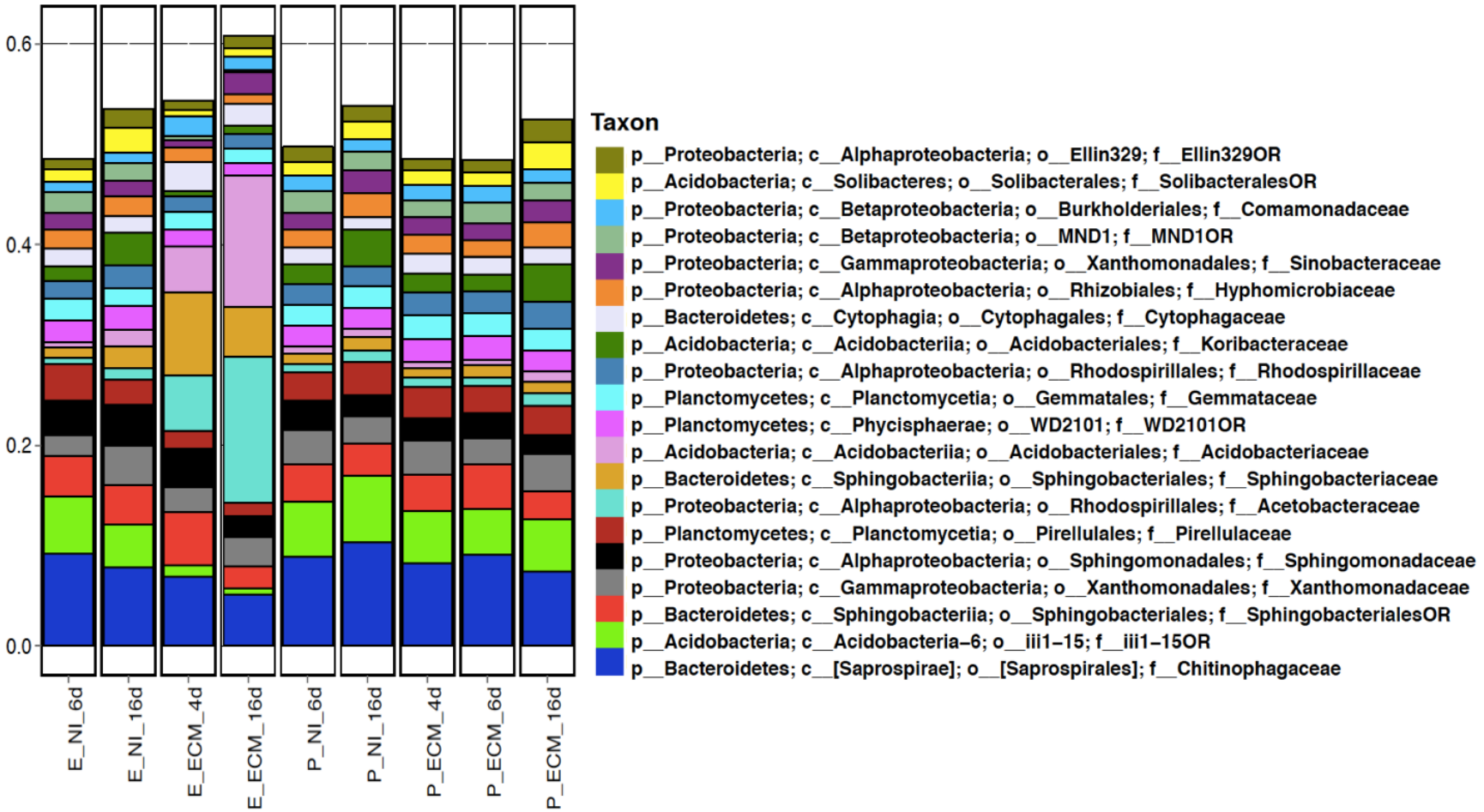

Figure 4.11. The family-level bacterial community composition of conifer rhizosphere soils collected from the year of 2016. The stacked bars represent the 20 most abundant bacterial taxa in a given sample from the 16S rRNA gene amplicon sequencing. X-axis indicates treatments while Y-axis indicates relative abundance of the bacterial taxa. Treatments on the X-axis are shown as a combination of plant-inoculum and the processing time delay in 4 days (4d), 6 days (6d), and 16 days (16d). E= white spruce, P= jack pine, NI= not inoculated, ECM= plants inoculated with ectomycorrhizal fungi. The letter before the name of each taxon represents the identification level of that particular organism (p-phylum, c-class, o-order, f-family, g-genus).

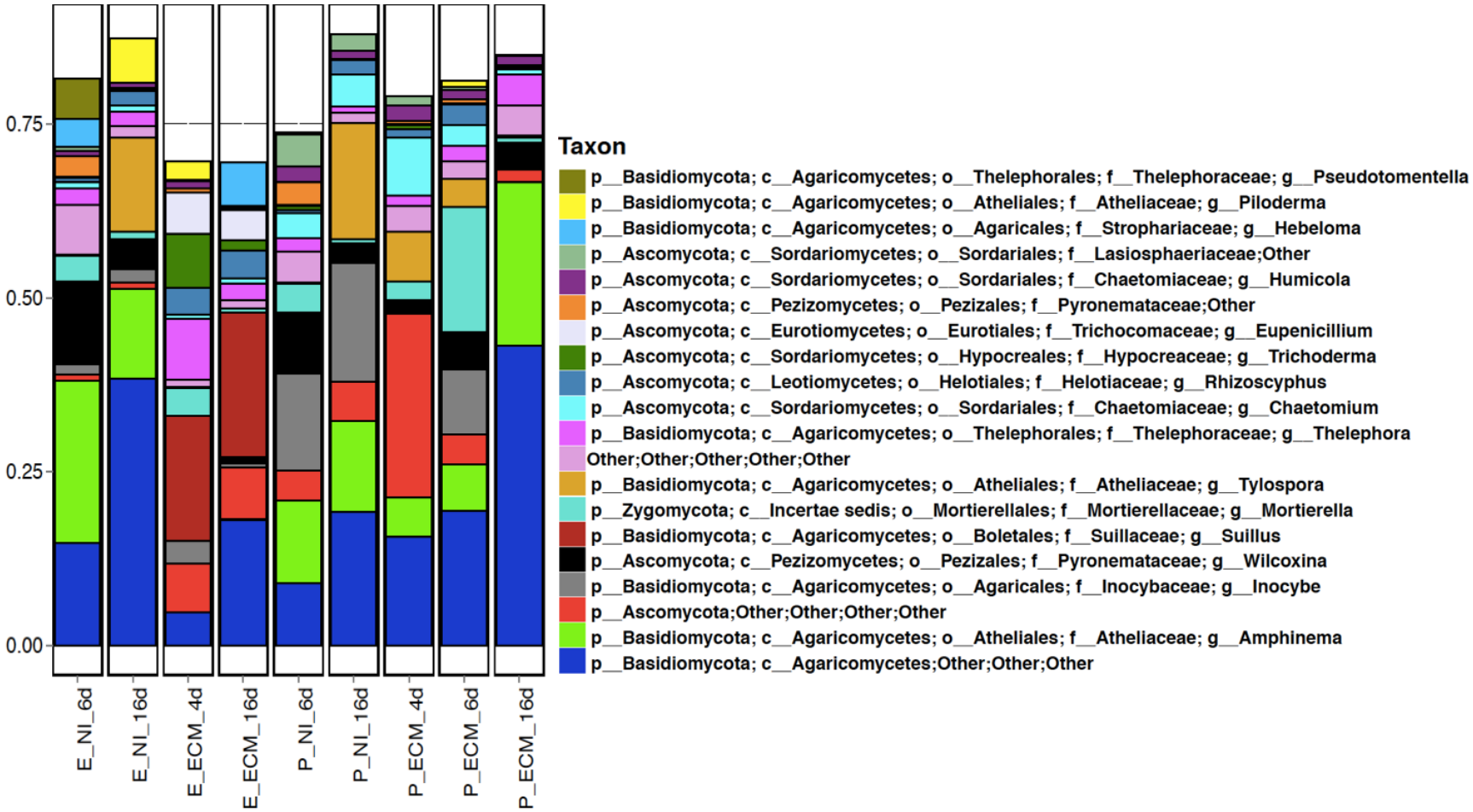

Figure 4.12. The genus-level fungal community composition of conifer rhizosphere soils collected from the year of 2016. The stacked bars represent the 20 most abundant fungal taxa in a given sample from the ITS amplicon sequencing. X-axis indicates treatments while Y-axis indicates relative abundance of the fungal taxa. Treatments on the X-axis are shown as a combination of plant-inoculum and the processing time delay in 4 days (4d), 6 days (6d), and 16 days (16d). E= white spruce, P= jack pine, NI= not inoculated, ECM= plants inoculated with ectomycorrhizal fungi. The letter before the name of each taxon represents the identification level of that particular organism (p-phylum, c-class, o-order, f-family, g-genus).

4.4 Bacterial rhizosphere and endophytic communities (2017 data)

The root-associated bacterial microbiomes were investigated in the rhizosphere and root compartments of the plants. We performed 16S rRNA gene amplicon (the V4-V5 region) sequencing on the Illumina MiSeq platform to characterize bacterial communities. From the plant rhizosphere samples that were sequenced, we obtained a total of 1,835,558 high-quality sequences from the platform and following quality assessment those sequences were reduced to 17,634 clusters (OTUs) based on a 97 % similarity threshold. From the plant root samples sequenced, we obtained a total of 655,886 high quality sequences that were clustered into 5,990 bacterial OTUs, on the basis of the same sequence similarity threshold (>97 %). In each (rhizosphere/root) compartment, we analyzed the within-community diversity (α-diversity) using the Shannon index, the between-community diversity (β-diversity) using the weighted Unifrac distance matrix, and characterized the bacterial community structure across different plant-inoculum treatments. Prior to the characterization of bacterial endophytes in the plant roots, we conducted a PCR study on three root samples using three different 16S rRNA gene amplicon PCR reactions: one used primer pairs 515-F-Y and 806 R to amplify the V4 region of 16S rRNA gene; one used primer pairs 515-F-Y and 926 R to amplify the V4-V5 region; one also amplified the V4-V5 region using the 515-F-Y and 926 R, but included plastid peptide nucleic acid (pPNA) oligos in the PCR reaction. The PCR study was conducted to evaluate the possible co-amplification of organellar sequences (e.g. chloroplast sequences) from the host plants that can impede the efficiency of 16S rRNA gene amplicon sequence analysis. In this comparative PCR study, a total of 222,535 high-quality sequences (245,223 QCed reads) were retrieved from the sequencing that gave 2,717 clusters (OTUs). The sequencing results and analyses of the PCR study are also presented in this section.

4.4.1 PCR study using plastid peptide nucleic acid (pPNA) to block host amplification

The alpha-diversity (Shannon index) of the three selected root samples indicated a greater variation among the individual samples than the PCR treatments. As shown in Figure 4.13, we found that the root sample ACI_FK_1 had a substantially higher bacterial diversity value, which ranged from 7.8 to 8.3 on the Shannon index, across all PCR reactions. The root sample ACI_FK_ECM_1 had

a diversity value that ranged from 7.2 to 7.4. Finally, the root sample P_ECM_3 had a low bacterial diversity, which ranged from 6.4 to 7.0. The difference in α-diversity among the three tested root samples was shown to be significant after performing the Kruskal-Wallis (nonparametric) test ($p<0.001$). Although it appears that the PCR amplification of the V4 region of the 16S rRNA gene (16S_V4) yielded relatively higher bacterial diversity values compared to the PCR amplifying the V4-V5 region (16S_V4-V5) (Fig. 4.13), the difference was only shown to be marginally significant ($p=0.04307$) by the Kruskal-Wallis rank sum test but was not detected by the multiple mean comparisons using the Dunnett's method. This suggests that the difference of bacterial diversity observed among different PCR experiments was, overall, not significant. For comparing the bacterial endophytic communities corresponding to different PCR experiments, we graphed an ordination plot (PCoA) using bacterial OTUs and also performed PERMANOVA analysis, using the weighted UniFrac dissimilarity matrix, which measures the phylogenetic distance among different root endophytic bacterial communities. As shown in Figure 4.14, we found that root samples amplified in the V4 region clustered separately (blue symbols) from the samples amplified in the V4-V5 region (green and red symbols), regardless of whether the pPNA was included in the PCR reaction or not. However, results obtained from the PERMANOVA analysis demonstrated that the differences observed among PCR experiments were not statistically significant ($R^2=0.37$, $p>0.05$). We concluded that including the pPNA in the PCR reactions of our tested plant samples did not have an observable impact on the community structure of bacterial endophytes.

From the taxonomic graph (Fig. 4.15), which provided detailed information of the dominant top 20 bacterial genera in the root endophytic community of each sample, we observed a different bacterial composition in the root samples amplified with the V4 region compared to those amplified with the V4-V5 region. When comparing specific taxa across the three PCR reactions, we found the genera *Bryobacter*, *Ohtaekwangia*, *Niastella*, *Terrimonas*, *Mucilaginibacter*, *Pir4 lineage*, and *Granulicella* and the family *Chitinophagaceae* were much less abundant or virtually absent in the samples amplified with the V4 region (16S:V4), whereas the genera *Actinospica*, *BD1-7 clade* and *Actinoplanes* were much more abundant in that (16S:V4) sample (Fig. 4.15). When comparing the dominant bacterial taxa in the root samples with pPNA (16S: V4-V5+pPNA) or without pPNA (16S: V4-V5) (Fig. 4.15), we found that the pPNA oligos had minimal effects

on the bacterial community composition in plant roots. Apparently, the V4-V5 amplified 16S rRNA gene sequences with or without pPNA detected a very similar community of root endophytic bacteria across the individual samples (Fig. 4.15). This result shows a strong agreement with the α-diversity (Fig. 4.13) and β-diversity (Fig. 4.14) analyses.

The present PCR comparative study was to determine whether non-target 16S rRNA (chloroplast) sequences would be co-amplified with our plant root samples. Approaches to eliminate the influence of such plant-derived (chloroplast and mitochondrial) sequences include the optimization of PCR amplification by using the sequence-specific peptide nucleic acids (PNAs) as blockers to those co-amplified sequences (Fitzpatrick et al. 2018), and the selection of optimal 16S rRNA primer pairs to minimize non-target DNA contamination (Beckers et al. 2016), followed by the removal of recognized "Chloroplast" and "Mitochondria" sequences from sequencing datasets (Beckers et al. 2017). Fitzpatrick et al. (2018) concluded that, while the mismatch of universal PNA to target plastid DNA sequences happens occasionally, the PNA clamps are still considered valuable tools in preserving the amplicon sequencing efficacy for future microbial community studies. Other researchers who used the primer pair 799F-1391R (amplifying the V5-V7 region) found that only small numbers of chloroplast/plastidal 16S rRNA sequences co-amplified during PCR (Beckers et al. 2017). Comparing their results to ours, the use of PNA had little effect, possibly due to the primers we used for amplification. However, the importance of verifying non-target DNA sequences in the design of amplicon sequencing libraries remains a major concern for downstream data analysis.

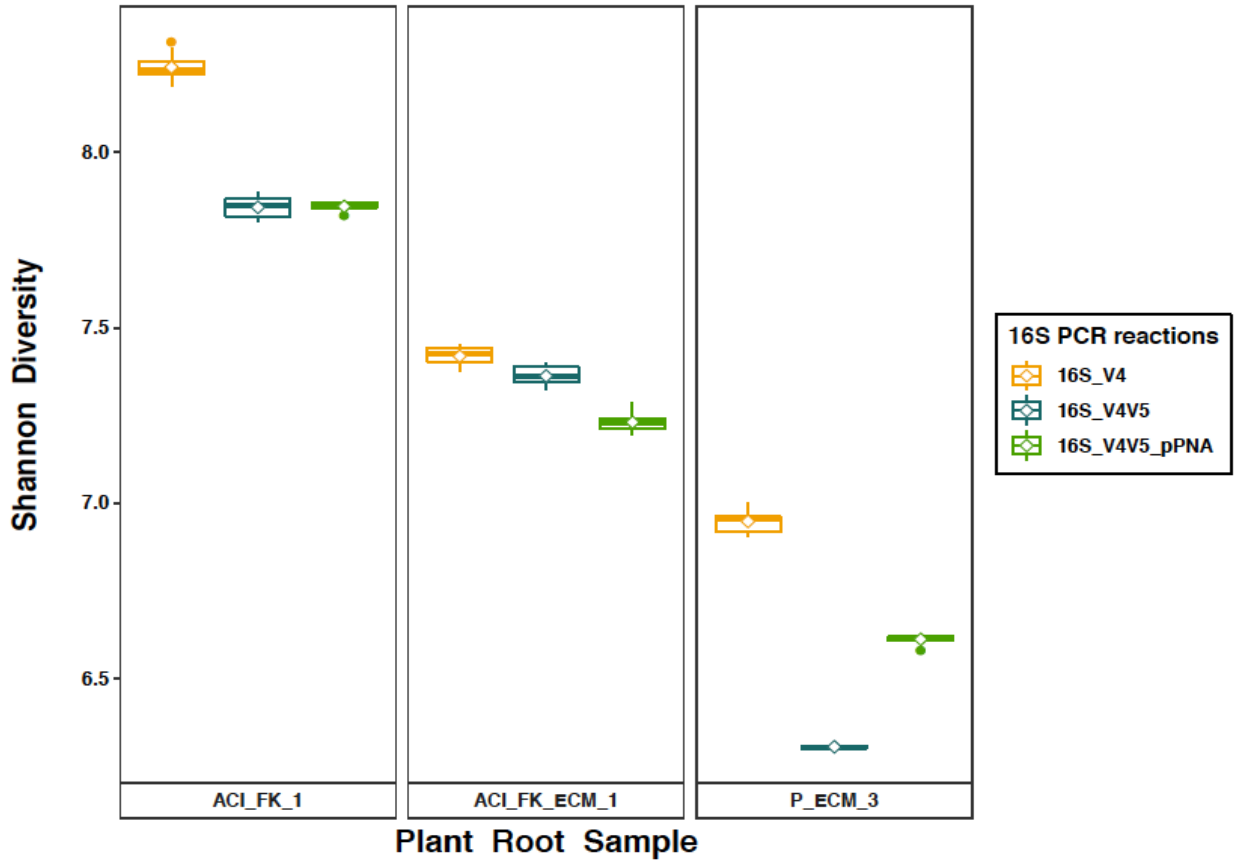

Figure 4.13. Comparison of alpha (**α**)-diversity among three 16S rRNA gene amplicon PCR reactions on the tested root samples (ACI_FK 1, ACI_ECM 1, P_ECM 3). Three PCR reactions were employed: 16S_V4, 16S_V4V5, and 16S_V4V5_pPNA. The three experiments compared the sequences from two amplification primer sets (V4 region and V4-V5 region), with and without plastid peptide nucleic acid (pPNA) oligos. A Kruskal–Wallis (nonparametric) test was performed on the Shannon indices to evaluate the statistical difference among treatments.

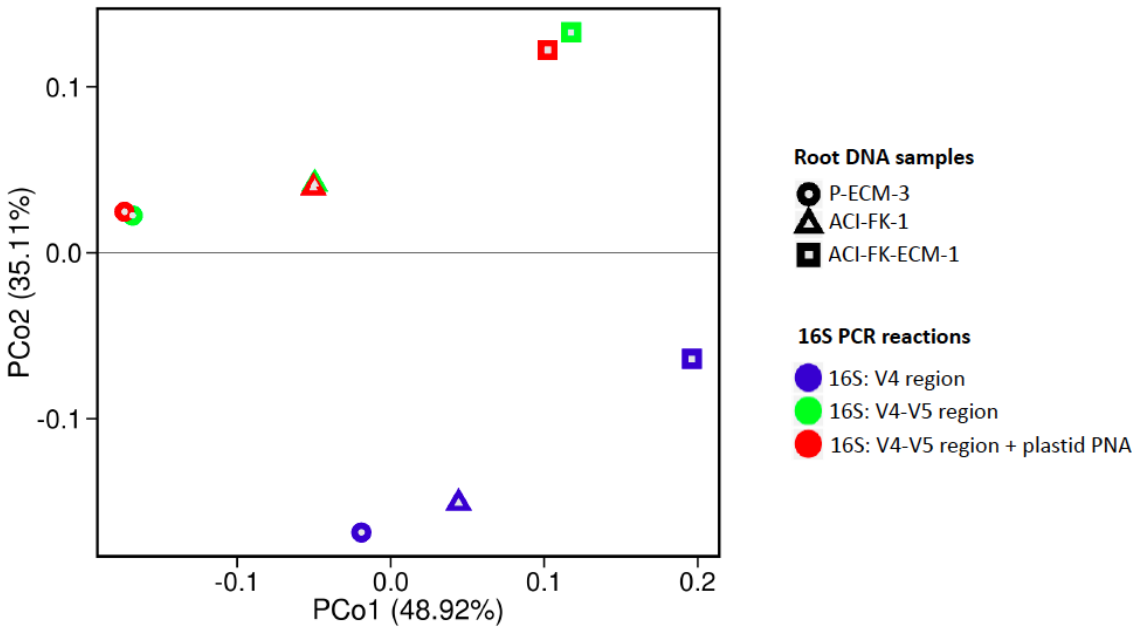

Figure 4.14. Principal Coordinates Analysis (PCoA) plot using weighted UniFrac dissimilarity distances, with 48.92 % and 35.11 % variance explained by the principal coordinates. The plot illustrates the community composition differences of endophytic bacteria in root samples using three different 16S rRNA gene PCR reactions. The three selected root samples are indicated by different symbols, while the three PCR reactions are shown in different colors.

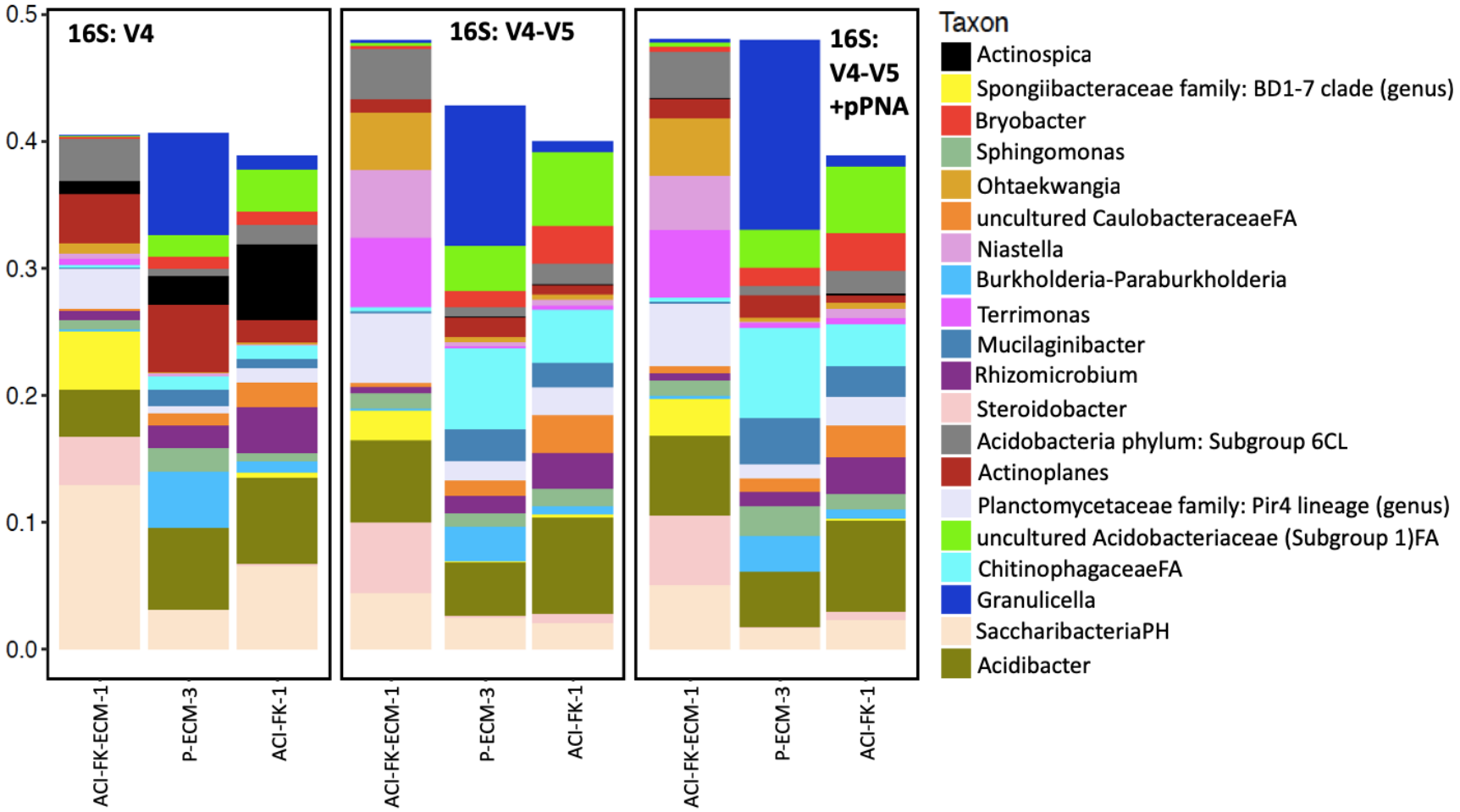

Figure 4.15. Relative abundance of bacterial root endophytic genera in the tested root samples compared among the three 16S rRNA gene amplicon PCR experiments. We amplified the V4 region (16S:V4), V4-V5 (16S: V4-V5) region of the 16S rRNA gene from the root samples, using the 515F-Y and 806R primers, the 515F-Y and 926R primers, respectively. Plastid peptide nucleic acid (pPNA) oligos were added in the PCR reactions amplified for the V4-V5 region (16S: V4-V5+pPNA).

4.4.2 Alpha (α)-diversity of bacterial communities in the plant rhizosphere versus roots

We investigated bacterial diversity in the plant rhizosphere, bulk soils and roots using Shannon index. When comparing the diversity values between the plant rhizosphere and bulk soils, we found that bacterial diversity in the majority of plant bulk soil samples was insignificantly ($p>0.05$; Dunnett's test) different from the plant rhizosphere soils, except for the ACNI and ACI-FK treatments (See Appendix 1: Fig. A.1). In addition, we observed significantly higher ($p<0.001$) bacterial diversities in the majority of plant bulk soils, compared to the unplanted soil (control), except for the four treatments: ARI-FK, ACI-FK-ECM, ARI-FK-ECM and P-ECM (Fig. A.1). From these results, we conclude that, after a 5-year growth following the transplantation in the mine waste rocks, our plants are able to enrich the soil with a great diversity of bacterial species. Among the plant rhizosphere (Fig. 4.16 A), compared to the unplanted control, four plant-inoculum treatments possessed a statistically indifferent ($p>0.05$; Dunnett's test) bacterial diversity: uninoculated and *Frankia*-inoculated speckled alders, uninoculated green alders, and ectomycorrhizal (ECM) fungi-inoculated jack pine. Uninoculated, ECM fungi-inoculated white spruce and uninoculated jack pine had the highest ($p<0.001$) bacterial diversities among the plant-inoculum treatments and the unplanted control. When comparing the diversity values across inoculation treatments within the same type of plants, we found that *Frankia*-inoculated and *Frankia*-mycorrhizal fungi-inoculated green alders had relatively higher species richness than their uninoculated counterparts (Fig. 4.16 A), and the difference between the uninoculated and *Frankia*-mycorrhizal fungi-inoculated green alders was significant ($p<0.001$). No significant differences of bacterial diversities were observed between uninoculated and the inoculated speckled alders, nor between the uninoculated and the inoculated white spruce. In our treatments of jack pine, non-inoculated pine had a significantly ($p<0.001$) higher bacterial diversity than the ECM fungi-inoculated pine (Fig. 4.16 A). In the root compartment, we observed that Shannon diversity values were significantly higher ($p<0.001$) in alder roots, at an average of 7.5, than in conifer roots, which had a diversity value ranging from 5 to 7 (Fig. 4.16 B). When exploring the inoculation effect on the diversity of the root endophytic bacteria, we found that the mean bacterial diversity in the inoculated green alders and the inoculated jack pine were slightly higher than their uninoculated counterparts (Fig. 4.16 B). However, the difference of bacterial diversity found among inoculation treatments on the same type of plants, was not statistically significant ($p>0.05$).

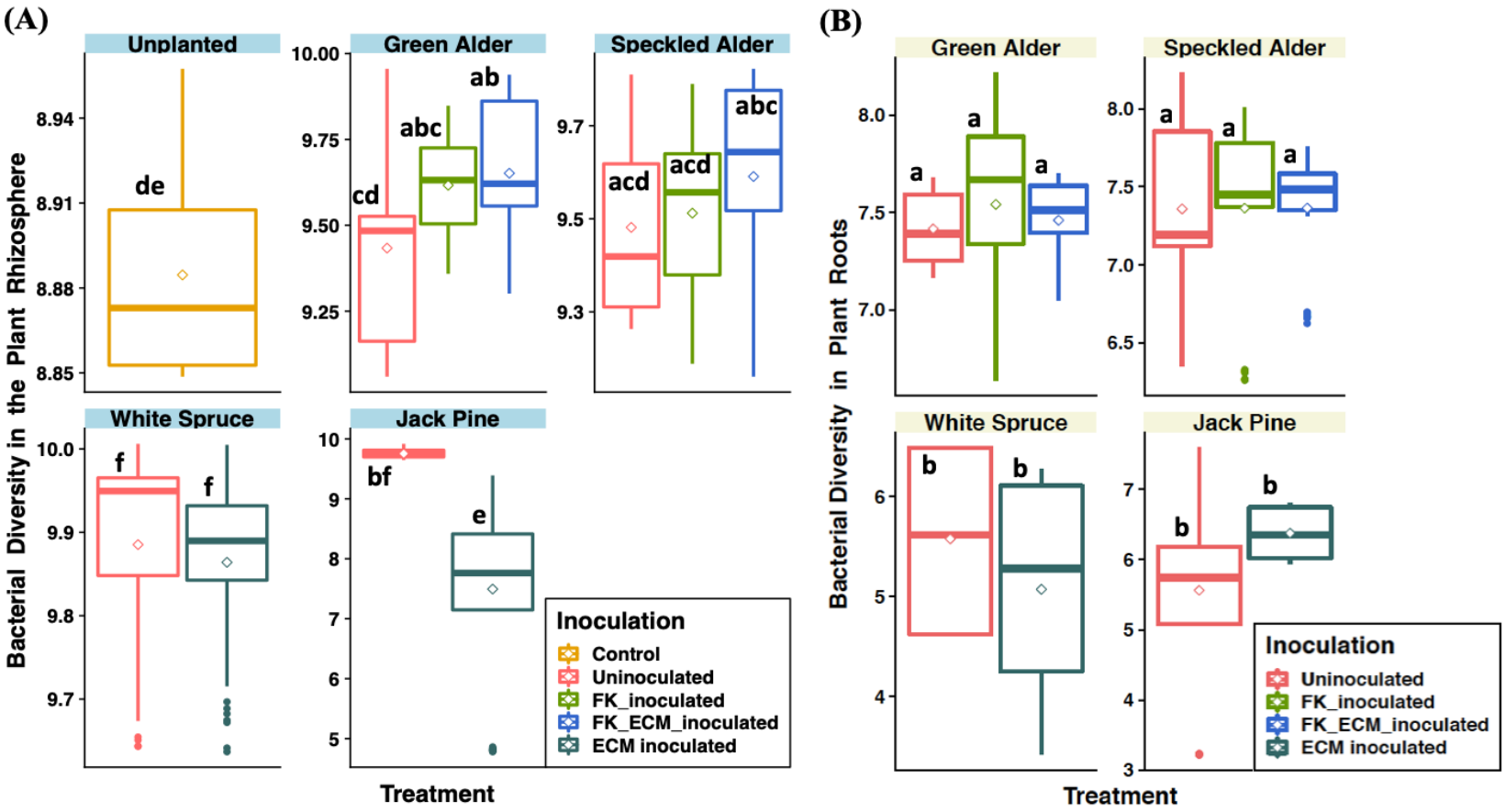

Figure 4.16. Alpha (α)-diversity (Shannon index) of bacterial communities comparing (A) the plant rhizosphere and (B) roots. In the root compartment, boxplots show the Shannon diversity values of different investigated plants (green alder and speckled alder); in the rhizosphere compartment, bacterial diversities of the plant rhizosphere are also compared to the unplanted bulk soil (control). The horizontal line within each box displays the median and the diamond indicates the mean. We performed statistical analyses using Kruskal-Wallis tests followed by Dunnett's multiple mean comparison with P-values adjusted by the 'Bonferroni–Holm' method. Bars bearing different letters are significantly ($p<0.05$) different from each other. FK=*Frankia* sp. strain AvcI1, ECM=ectomycorrhizal fungi.

4.4.3 Beta (β)-diversity of bacterial communities in the plant rhizosphere versus roots

We performed PCoA using the weighted UniFrac distance, a measure widely used to compare the similarity/dissimilarity of microbial communities, to analyze the β-diversity (between-communities diversity) of bacterial communities in the plant rhizosphere (Fig. 4.17 A) and in the roots (Fig. 4.17 B). In Figure 4.17 (A), we observed a clear separation of the 'Control' data point (the blue diamond), which represents our unplanted soil sample, and a well-defined cluster of the ectomycorrhizal (ECM) fungi-inoculated jack pine (P-ECM: green squares) from the rest of the dataset. PERMANOVA tests based on the weighted UniFrac distance matrix revealed that no significant ($p>0.05$) difference of bacterial community compositions was discerned among different types of plants (green/speckled alders, white spruce, jack pine and the unplanted bulk soil), nor between the bulk soils (the planted and the unplanted bulk soils) and the plant rhizosphere soils ($p>1$), nor the control and the other samples ($p>0.05$). A significant difference ($p<0.001$) was found comparing the rhizosphere and the bulk soils collected from the ectomycorrhizal (ECM) fungi-inoculated jack pine to the others (the control, the rhizosphere and bulk soils from other plants). In Fig. 4.17 (B), PCoA ordination plotted on the weighted Unifrac distance matrix revealed that the compositional difference of root endophytic bacterial communities, to a large extent, was dependent on the type of host plant, except for an outlier (E-ECM-1). Although the pattern was not evident in the graph (Fig. 4.17 B), we do observe a separation of the alder treatments from the conifer treatments by drawing a dash line in between. In general, the remediation plants used in our project can be categorized into two plant families: Betulaceae (green and speckled alders) and Pinaceae (white spruce and jack pine). Alders used in our field belong to the same plant genus—*Alnus,* which are likely to establish symbiotic relationships with similar endophytic bacteria. Our observation was confirmed by conducting PERAMONVA analysis on the weighted Unifrac distance matrix. The analysis indicated that the bacterial composition difference observed between the alders and the conifers was statistically significant ($p<0.001$). Moreover, for comparing the root endophytic bacterial composition to the plant rhizosphere, we also performed the analysis on the group of ectomycorrhizal (ECM) fungi-inoculated jack pine (P-ECM) and found that the bacterial compositional difference between the uninoculated and ectomycorrhizal (ECM) fungi-inoculated pine was also marginally significant ($p=0.0027$).

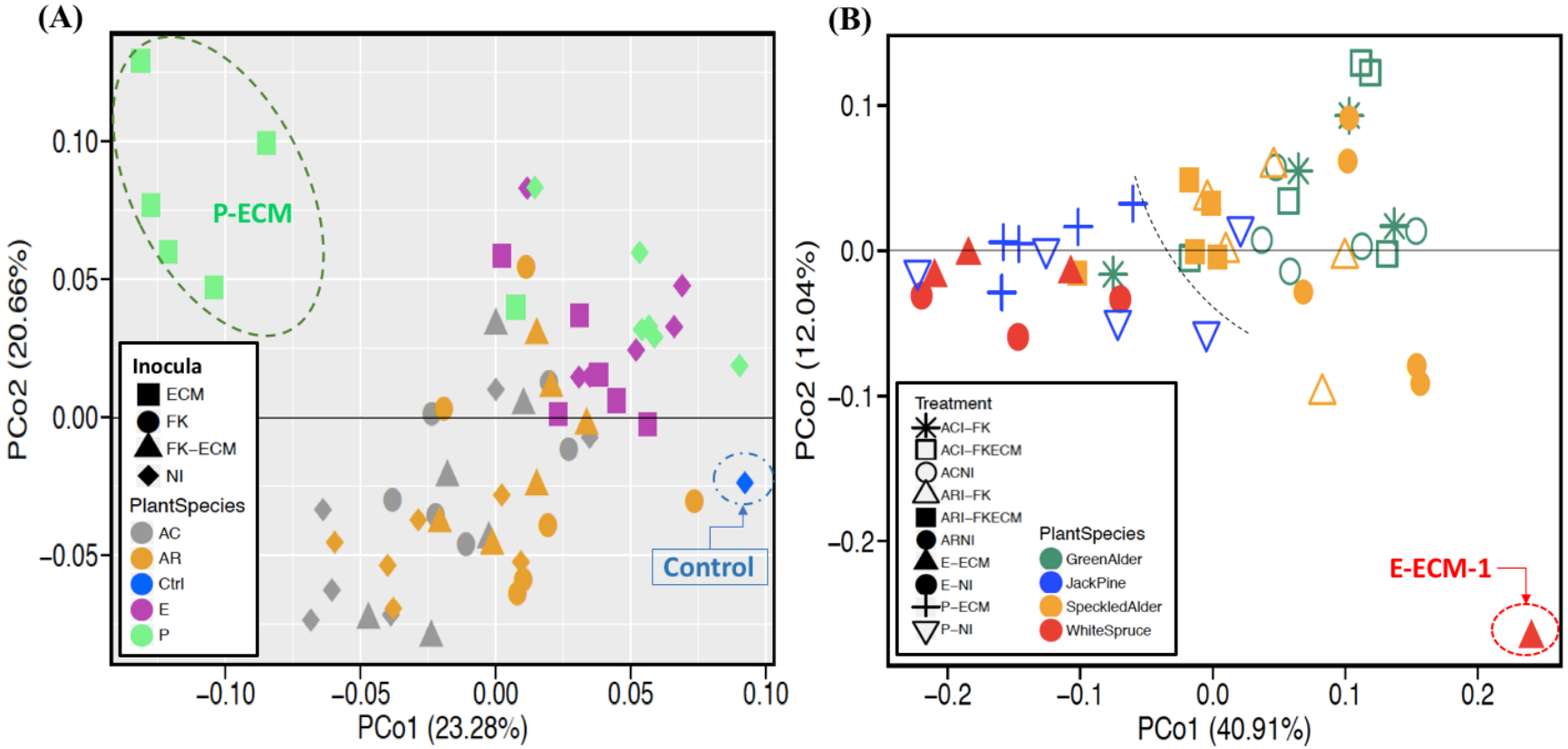

Figure 4.17. PCoA plots of weighted UniFrac computed distances between bacterial community compositions in (A) the plant rhizosphere and in (B) roots. (A) Different colored points represent different types of soils that are associated with plants or without plants (control), while different shapes identify the different inocula used. (B) Different colored symbols represent different types of plants, while different symbol shapes describe different combinations of plant-inoculum treatments. Abbreviations: AC=green alder, AR=speckled alder, Ctrl=control, E=white spruce, P=jack pine, ECM=ectomycorrhizal fungi, FK=*Frankia* sp. strain AvcI1, FK-ECM or FKECM= co-inoculation of *Frankia* sp. strain AvcI1 and mycorrhizal fungi, NI=not inoculated.

4.4.4 Bacterial taxonomic composition in the plant rhizosphere versus roots

The 16S rRNA amplicon sequencing results revealed approximately 50 % of bacterial families in the soil sample sequenced, the 20 most abundant of which were Coxiellaceae, Planctomycetaceae, Comamonadaceae, Acetobacteraceae, Cytophagaceae, Sinobacteraceae, Rhodospirillaceae, Koribacteraceae, Xanthomonadaceae, Sphingomonadaceae, Hyphomicrobiaceae, Pirellulaceae, Gemmataceae, Chitinophagaceae and unclassified families belonging to the bacterial orders of Ellin651, Sphingobacteriales, Myxococcales, WD2101, Ellin329, and iii1−15 (Fig. 4.18 A and Fig. 4.19 A). Compared with the bacterial community composition in the plant (alder and conifer) rhizosphere and bulk soils, we found that the unplanted bulk soil had lower relative abundance of the bacterial families Koribacteraceae, Rhodospirillaceae, Acetobacteracea*e* and Sinobacteraceae, and the bacterial orders Ellin6513 and Ellin329 (Fig. 4.18 A and Fig. 4.19 A). In addition, a consistent prevalence of the 20 dominant bacterial families was observed in rhizosphere and bulk soils across the six alder treatments (Fig. 4.18 A); similarly, the conifer-associated rhizosphere and bulk soils also resembled each other, except for the ectomycorrhizal (ECM) fungi-inoculated jack pine, which had a bacterial composition greatly differing from the other conifer soils (Fig. 4.19 A). Specifically, the Acetobacteraceae and Sphingomonadaceae families were very abundant in the rhizosphere of the ectomycorrhizal (ECM) fungi-inoculated jack pine compared to their uninoculated counterparts (Fig. 4.19 A). Members in the Acidobacteriaceae (Subgroup 1) family have been reported as aerobic chemoheterotrophs to solubilize inorganic phosphates (Srivastava et al. 1996; Campbell 2014; Kielak et al. 2016), which increase the bioavailability of P to plants. Sphingomonadaceae, in general, grow over a wide pH range, from pH 4.5 to 10, with optimum growth between pH 6.0–7.0 (Glaeser and Kämpfer 2014). Members of the family Sphingomonadaceae were found to grow in a variety of habitats, including aquatic systems, soils associated with plants, and habitats contaminated with recalcitrant (poly)aromatic contaminants of natural or anthropogenic origin (Stolz 2009; Glaeser and Kämpfer 2014). The genus *Sphingomonas,* which currently includes 61 species and represents the largest genus of *Sphingomonadaceae*, were isolated from the rhizosphere of several crops such as millet, sorghum and rice (Hebbar et al. 1992; Engelhard et al. 2000), seeds of rice plants (Mano et al. 2006), and surface-sterilized rice plant roots and above-ground tissues (Videira et al. 2009). In Videira et al. (2009), the isolated strains of *Sphingomonas* spp. showed *nif*H genes that resulted in acetylene

reduction, i.e., a way of measuring nitrogen fixation; while in Engelhard et al. (2000), the nitrogen fixation ability of the isolated sphingomonads was not verified.

According to the 16S rRNA amplicon sequencing, approximately 60 % of plant (alder and conifer) root endophytic members, at the family level, were examined. The 20 most abundant bacterial families were Sphingobacteriaceae, Rhizobiales Incertae Sedis, Solibacteraceae (Subgroup 3), Hyphomicrobiaceae, Rhizobiaceae, Micromonosporaceae, Bacillaceae, Haliangiaceae, Acidobacteriaceae (Subgroup 1), Brucellaceae, Comamonadaceae, Xanthomonadaceae, Caulobacteraceae, Sphingomonadaceae, Cytophagaceae, Xanthomonadales Incertae Sedis, Planctomycetaceae, Chitinophagaceae, the unidentified bacterial family in the Subgroup 6 class of Acidobacteria, and the others (Fig. 4.18 B and Fig. 4.19 B). In terms of the inoculation effect on the alder seedlings, we could not find any specific bacterial taxa corresponding to our inoculation treatments, as the dominant bacterial families were similar across all the alder roots (Fig. 4.18 B). In Nickel et al. (2001), who inoculated *Frankia* strains into soils, the introduced *Frankia* strains remained infective and competitive for nodulation in the absence of plants. Although no *Frankia* (Actinobacteria, Frankiales, Frankiaceae) was detected in the alder rhizosphere or in roots (excluding nodules), it is unclear whether the introduced *Frankia* strains affected the nitrogen level in the alder-planted soil. In fact, as our plots of non-inoculated alders were planted adjacent to the alders with *Frankia* inoculum treatments (single- and dual-inoculation), the soil property of three consecutive alder-planted plots are assumed to be alike during the course of phytoremediation, presumably by the amendment of leaf litter from the three alder treatment plots (NI, FK, FK-ECM). Bacterial endophytes in the conifer roots, however, had a different community structure in the ectomycorrhizal (ECM) fungi-inoculated jack pine, which had a higher relative abundance of Acidobacteriaceae (Subgroup 1) in their roots, compared to the uninoculated jack pine and white spruce, and the inoculated white spruce (Fig. 4.19 B). Moreover, when comparing the bacterial taxonomic profile in the plant (alder and conifer) roots with that in the plant rhizosphere (Fig. 4.18 and Fig. 4.19), we found that they had seven bacterial families in common: Chitinophagaceae, Comamonadaceae, Cytophagaceae, Hyphomicrobiaceae, Planctomycetaceae, Sphingomonadaceae, and Xanthomonadaceae.

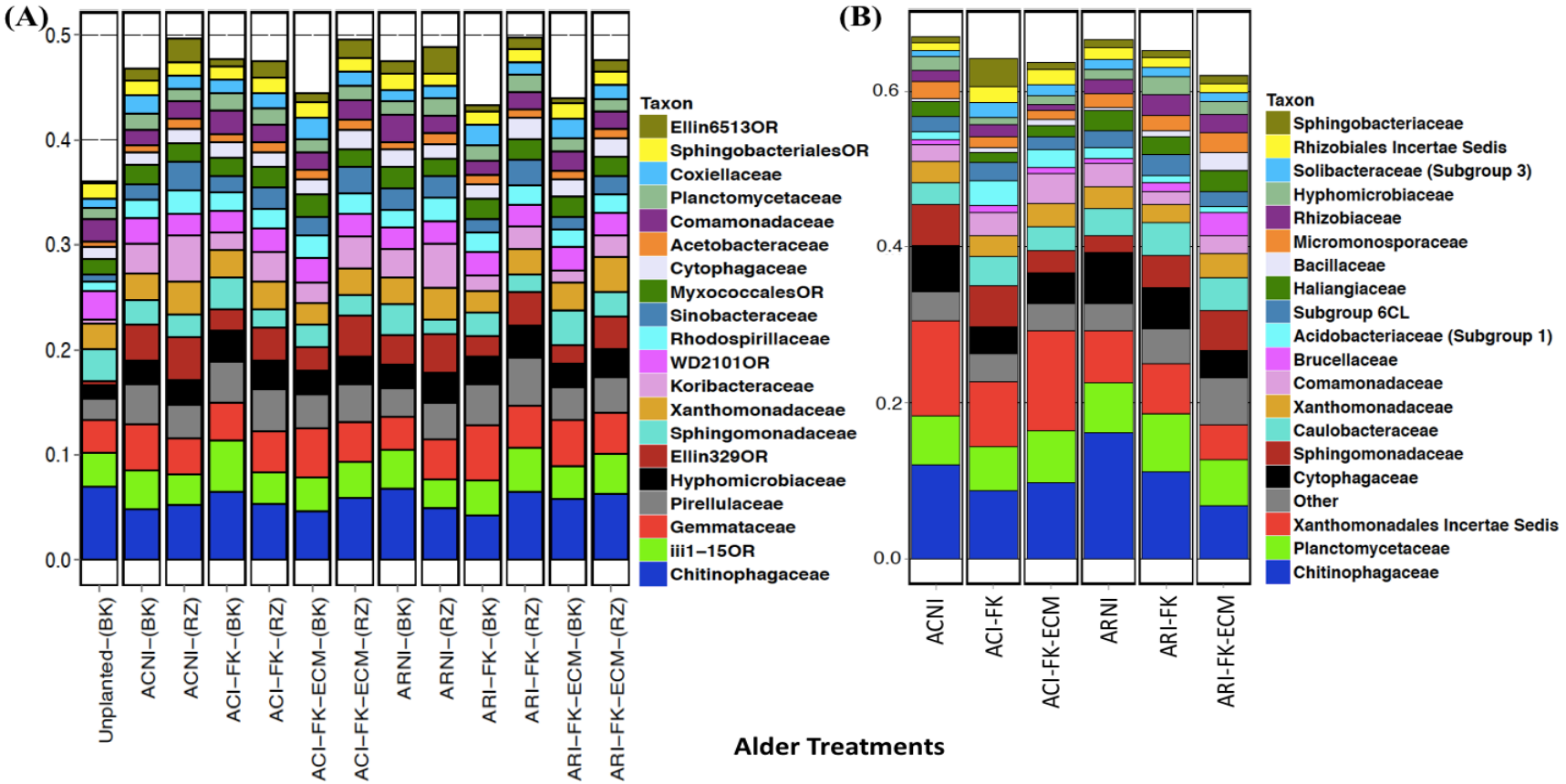

Figure 4.18. The family-level bacterial community compositions comparing the (A) rhizosphere and the (B) roots of alders. The stacked bar depicts the 20 most abundant bacterial families in the averaged sample replicates from the 16S rRNA gene amplicon sequencing. In both graphs, Y-axes indicate the relative abundance of bacterial families in a given sample. X-axis in (A) displays the unplanted bulk soil, six each of the alder-associated rhizosphere and bulk soils, while the X-axis in (B) indicates the six treatments of alders. Abbreviations: AC(R)NI=uninoculated green(speckled) alders, AC(R)I-FK=*Frankia*-inoculated green(speckled) alders, AC(R)I-FK-ECM= the inoculated green(speckled) alders with *Frankia* sp. strain AvcI1 and mycorrhizal fungi (*Glomus irregulare* and *Alpova diplophloeus*), BK=bulk soils, RZ=rhizosphere soils.

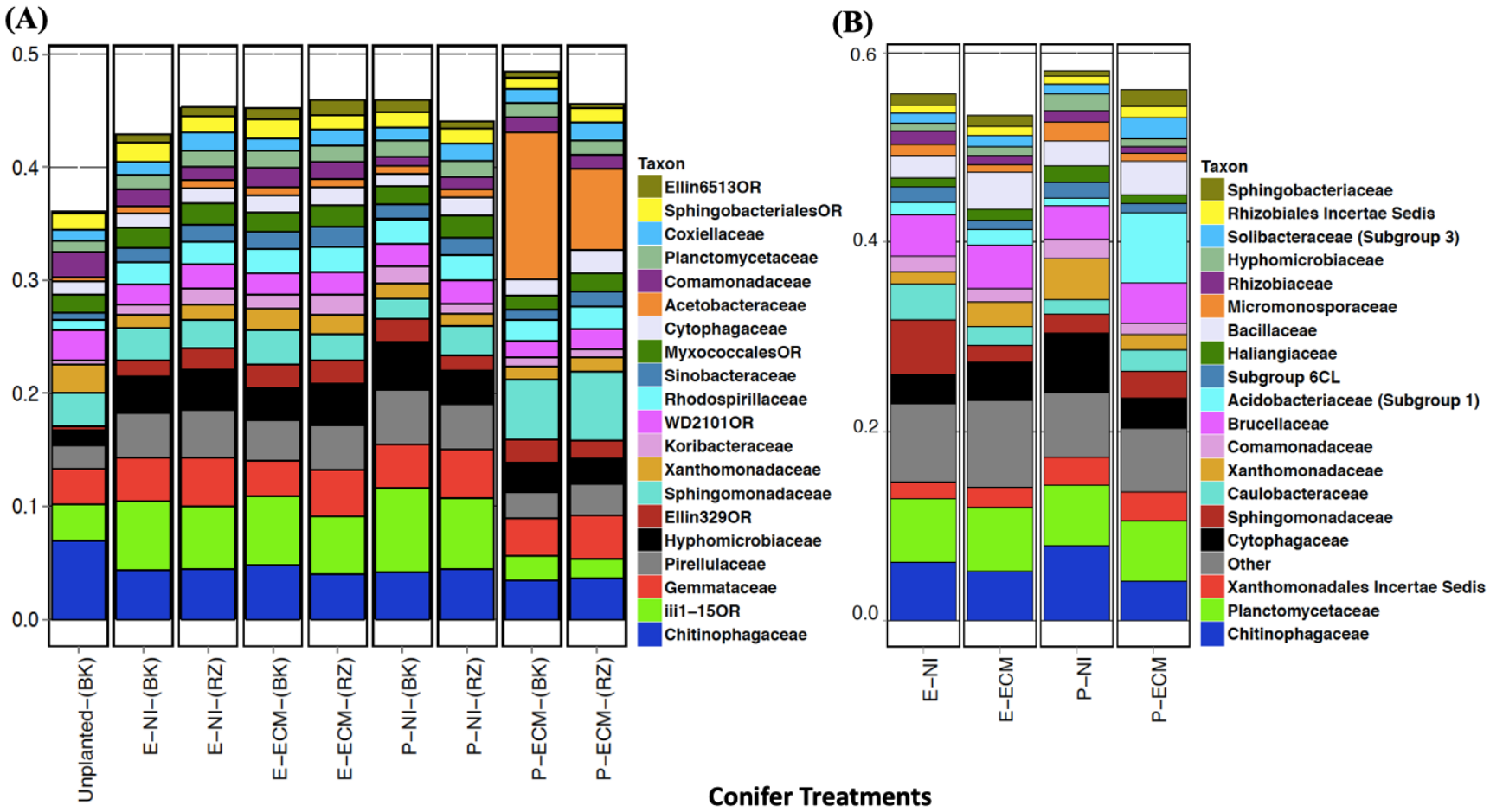

Figure 4.19. The family-level bacterial community compositions comparing the (A) rhizosphere and the (B) roots of conifers. The stacked bar depicts the 20 most abundant bacterial families in the averaged sample replications from the 16S rRNA gene amplicon sequencing. In both graphs, Y-axes indicate the relative abundance of bacterial families in a given sample. The X-axis in (A) displays the unplanted bulk soil, four each of the conifer-associated rhizosphere and bulk soils, while the X-axis in (B) indicates the four treatments of conifers. Abbreviations: BK=bulk soils, RZ=rhizosphere soils, E-NI= uninoculated white spruce, E-ECM= white spruce inoculated with *Hebeloma crustiliniforme* and *Paxillus involutus,* P-NI= uninoculated jack pine, P-ECM= jack pine inoculated with *Suillus tomentosus* and *Laccaria bicolor*.

4.5 Fungal rhizosphere and endophytic communities (2017 data)

We studied the root-associated fungal microbiome by investigating the fungal communities in the rhizosphere and the root compartments of plants. We sequenced the internal transcribed spacer (ITS) region of the rRNA gene (ITS amplicon sequences) on the Illumina MiSeq platform to characterize the fungal communities. From the plant rhizosphere and bulk soil samples sequenced, we obtained a total of 4,849,480 high quality sequences from the platform and later these fungal sequences which clustered into 4,772 OTUs based on a 97 % similarity threshold. From the plant root samples sequenced, we obtained a total of 2,001,533 high quality sequences which clustered into 997 fungal OTUs, on the basis of the same sequence similarity threshold (>97 %).

4.5.1 Alpha (α)-diversity of fungal communities in the plant rhizosphere versus roots

Shannon diversity values comparing the plant-associated bulk soils and the rhizosphere soils indicated a significant difference ($p<0.05$; Dunnett's test) of fungal diversity found in 5 out of the 10 treatments, including uninoculated green alders (ACNI), treatments of speckled alders (ARNI, ARI-FK, and ARI-FK-ECM), and uninoculated white spruce (E-NI) (See Appendix 2: Fig. A.2). By contrast, an insignificant difference ($p>0.05$) in fungal diversity was found between the rhizosphere and the bulk soils of the inoculated green alder treatments (ACI-FK and ACI-FK-ECM), ectomycorrhizal (ECM) fungi-inoculated white spruce (E-ECM), and treatments of jack pine (P-NI and P-ECM). In addition, we observed an insignificant ($p>0.05$) difference between all the plant bulk soils and the unplanted soil (control). As indicated in Figure A.2, we found relatively lower fungal diversities in the majority of plant rhizosphere soils compared to the unplanted soil (control), except for the treatments of jack pine (P-NI and P-ECM). These results suggest that, after 5 years of growth following planting in the Sigma gold mine waste rock, our plants were incapable of stimulating an increase of fungal diversity in the mine soil residues. When focusing on the plant rhizosphere soils (Fig. 4.20 A) and comparing their fungal diversities against each other and with the unplanted control (Ctrl), we found that fungal diversities in the treatments of jack pine (P-NI and P-ECM) were significantly low ($p<0.001$). In terms of the inoculation effect on plant seedlings, as shown in Fig. 4.20 (A), the same type of plant was clearly differentiated by color, in most cases, we neither observed significant differences between the uninoculated and the inoculated plants, nor among different inoculations, except for the *Frankia*-inoculated green alders

(ACI-FK), which had a significantly ($p<0.001$) higher fungal diversity than the *Frankia*-mycorrhizal fungi-inoculated green alders (ACI-FK-ECM). In the fungal root endophytic communities, diversities were strongly related to the type of inocula applied to the plant (Fig. 4.20 B). As boxes in Figure 4.20 (B) were colored by the type of inoculation treatment, we observed an evident trend that the green and speckled alders applied with tripartite (plant-bacteria-fungi) inoculations had a higher fungal diversity in their roots than the alders inoculated with the *Frankia* sp. strain AvcI1 alone. Likewise, we also found a similar pattern in the conifer group, that the ectomycorrhizal (ECM) fungi-inoculated conifers (white spruce and jack pine) had significantly higher ($p<0.001$) fungal species richness than their uninoculated counterparts (Fig. 4.20 B). Although the difference of fungal diversity between the tripartite inoculation and *Frankia* inoculation was only statistically significant ($p<0.001$) in the speckled alders, and not in the green alders ($p>0.05$), and the tripartite inoculated alders also did not significantly differ from the uninoculated alders (Fig. 4.20 B), these results still provide us some perspectives that the fungal inocula we used were able to colonize plant roots while leading to a greater diversity of endophytic fungi inside the roots.

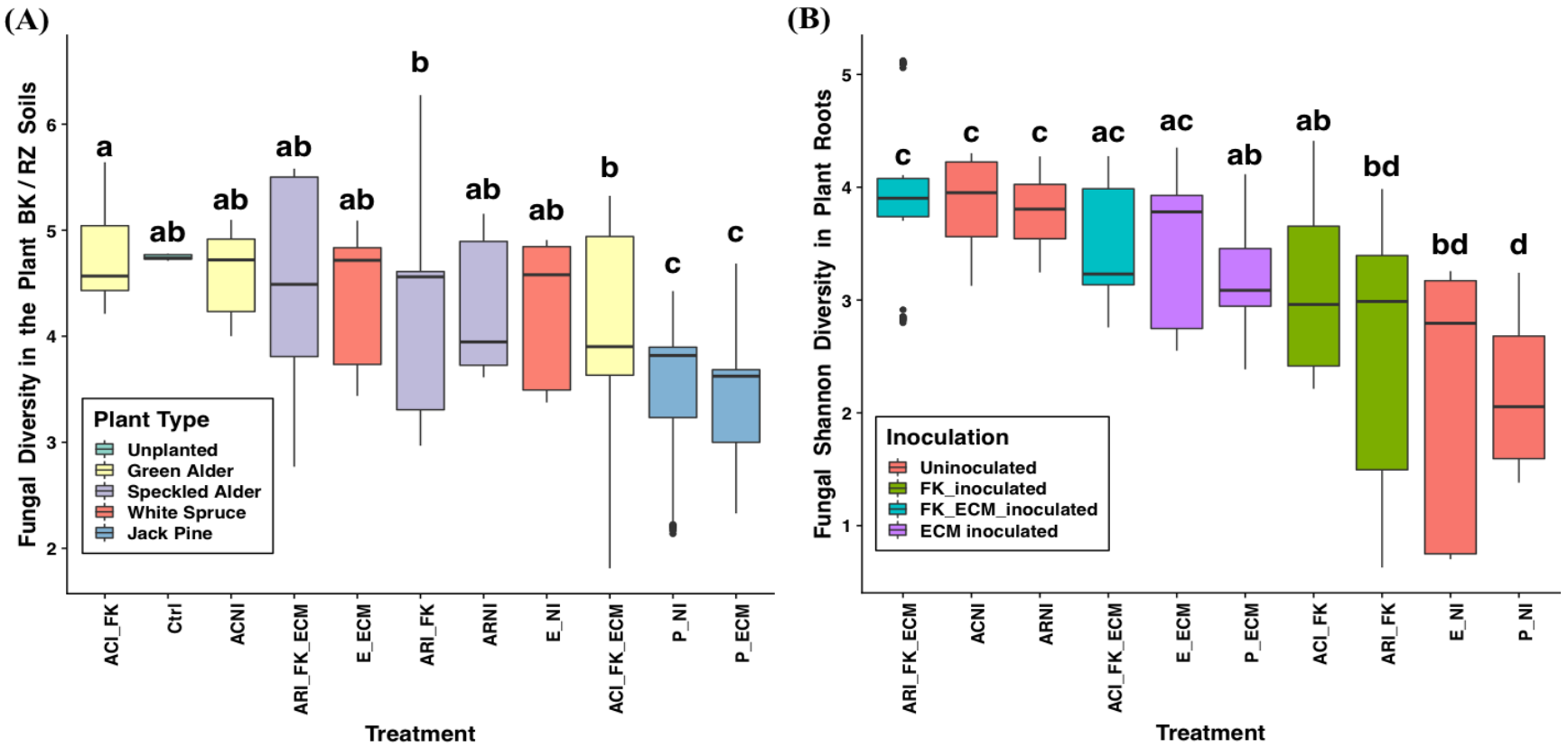

Figure 4.20. Alpha (α)-diversity (Shannon index) of fungal communities comparing (A) the plant rhizosphere and (B) roots. The diversity indices were shown by boxplots in a descending order of the mean (the horizontal line within each box). (A) Boxes are colored by different plant types, including the unplanted site. (B) Boxes are colored by different inoculation treatments, including uninoculated. We performed statistical analyses using Kruskal-Wallis tests followed by Dunnett's multiple mean comparison with P-values adjusted by the 'Bonferroni–Holm' method. Bars bearing different letters are significantly different from each other ($p<0.05$). Abbreviations: Ctrl=control, AC=green alder, AR=speckled alder, E=white spruce, P=jack pine, NI=not inoculated, FK=*Frankia* sp. strain AvcI1, FK_ECM= co-inoculation of the *Frankia* sp. strain AvcI1 and mycorrhizal fungi, ECM=ectomycorrhizal fungi.

4.5.2 Beta (β)-diversity of fungal communities in the plant rhizosphere versus roots

We performed β-diversity (between-communities diversity) analysis, based on the weighted UniFrac distance, to compare the difference of fungal community compositions across the soil and root samples. In Figure 4.21 (A), we observed two well-defined clusters (denoted by two dashed ellipses) in the PCoA analysis, and a clear separation of 'Control' (unplanted soil) from all the planted soil samples. These separations, however, were considered weak groupings in the PCoA plot, because the groupings only explained approximately 29 % of the total variability between the plant rhizospheric fungal communities along the two principal coordinates (PCo1: 17.22 %; PCo2: 11.42 %), and the remaining 71 % of fungal variations were unmeasured. By carrying out the PERMANOVA test on our dataset, we found that the separation of 'Control' data (unplanted soil) was not statistically significant ($p>0.05$), while the separation of conifers from alders was significant ($p<0.001$). In addition, the analysis also revealed that the fungal community structure compared among the unplanted soils (control), the plant rhizosphere and bulk soils was not significant ($p>0.05$). In the root compartment (Fig. 4.21 B), the PCoA ordination did not reveal any strong groupings of the plant-inoculum treatments. As illustrated in the graph (Fig. 4.21 B), the combined effect of plant and inoculum variables, together, explained roughly 42 % of the fungal community variation in the roots on the two primary axes (PCo1: 28.61 %; PCo2: 12.8 %), suggesting that unmeasured biotic or abiotic factors are needed to explain the remaining 58 % of the variation. Although a less evident separation of the ectomycorrhizal (ECM) fungi-inoculated jack pine (blue crosses) from the uninoculated jack pine (blue inverted triangles) was seen in the weighted UniFrac-based PCoA plots by drawing a dashed line in between, analysis of the distance matrix suggested no significant (PERMANOVA; $p>0.05$) differences between these two inoculation treatments of jack pine. Additionally, after removing the outlier (ARI-FK-2), the analysis also showed a weak difference between conifers and alders ($p=0.0268$), among four different types of plants ($p=0.0119$) and different combinations of plant-inoculum treatments ($p=0.0154$).

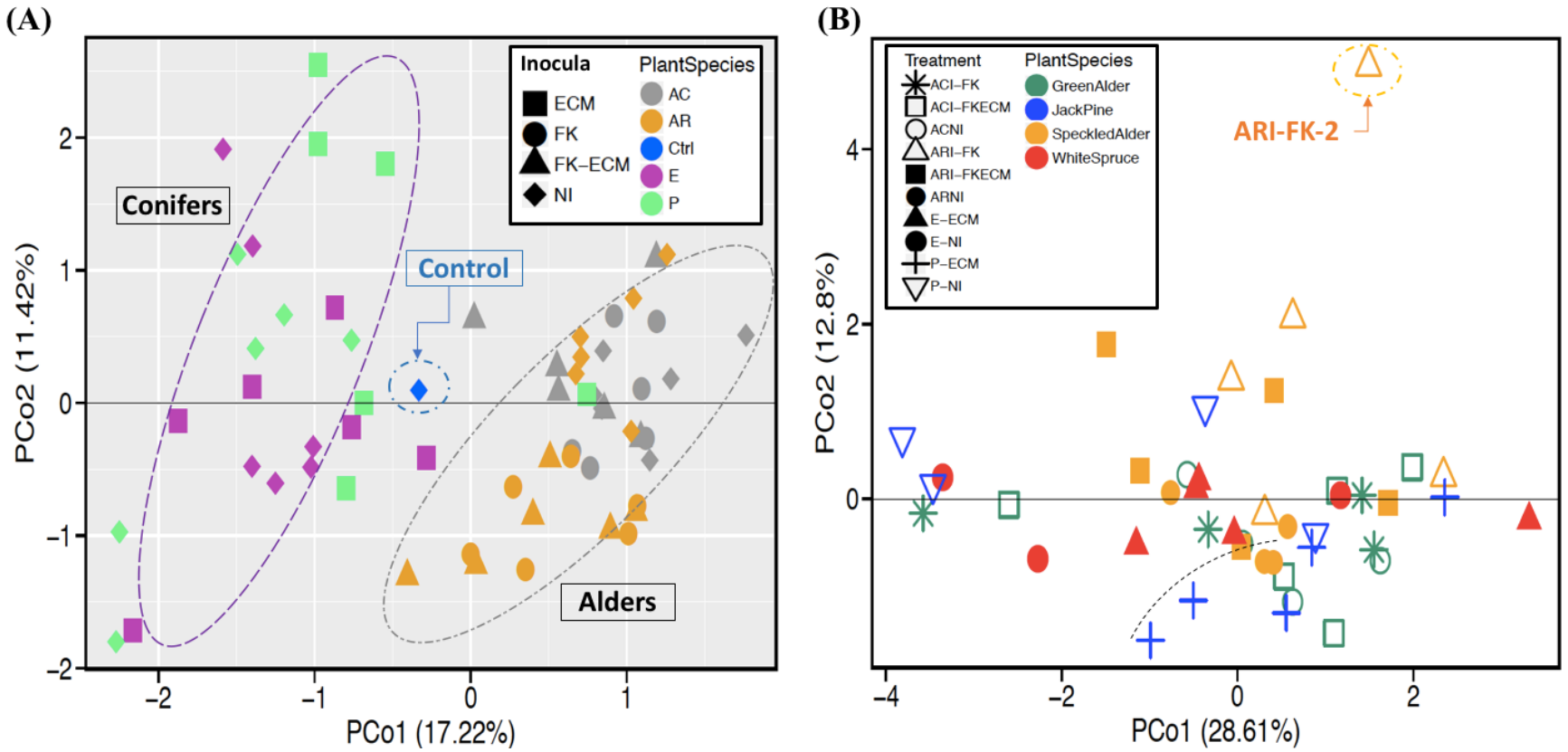

Figure 4.21. PCoA analysis plots of weighted UniFrac computed distances between fungal community compositions in (A) the plant rhizosphere and in (B) roots. (A) Different colored symbols represent different types of soils that are associated with plants or without plants (control), while different symbol shapes describe different inocula involved or the lack of inoculation. (B) Different colored symbols represent different types of plants, while different symbol shapes describe different combinations of plant-inoculum treatments. Abbreviations: AC=green alder, AR=speckled alder, Ctrl=control, E=white spruce, P=jack pine, ECM=ectomycorrhizal fungal strains, FK=*Frankia* sp. strain AvcI1, FK-ECM or FKECM= co-inoculation of the *Frankia* sp. strain AvcI1 and ectomycorrhizal fungi, NI=not inoculated.

4.5.3 Fungal taxonomic compositions in the plant rhizosphere versus roots

Based on the ITS amplicon sequencing, we were able to analyze around 60 %-80 % of fungal taxa, at the genus level, in the rhizosphere and bulk soil samples sequenced (Fig. 4.22 A and Fig. 4.23 A), and moreover, 75 % of endophytic fungal communities on average in plant roots were explained (Fig. 4.22 B and Fig. 4.23 B). In general, the 20 most abundant fungal genera in the characterized soil samples (the unplanted bulk soil, the alder and conifer's rhizosphere and bulk soils) were *Tylospora*, *Phialocephala*, *Trichocladium*, *Inocybe*, *Tomentella*, *Mortierella*, *Suillus*, *Trichosporon*, *Wilcoxina*, *Chaetomium*, *Rhizoscyphus*, *Amphinema* and *Thelephora*, and six unidentified fungal members in the families of Sporormiaceae and Pyronemataceae, the Agaricales order, the classes of Sordariomycetes and Agaricomycetes, the Ascomycota phylum, and another unidentified fungal phyla. When comparing the composition of fungal communities in both rhizosphere and bulk soils of plants (both alders and conifers) to that in the unplanted bulk soil (Fig. 4.22 A and Fig. 4.23 A), putative fungal taxa dominating roughly 25 % of the fungal community in the unplanted soil belonged to the Ascomycota phylum (4-5 %), the *Mortierella* genus (2-3 %), the Agaricomycetes class (<1 %), and other phyla (17-18 %). In Figure 4.22 (A) we found that, within the same plant-inoculum treatment, the fungal community compositions in the alder-associated bulk soils were not different from their rhizosphere soils. Likewise, a similar prevalence of the dominant fungal taxa was also observed when comparing the conifer-associated bulk soils with their rhizosphere soils (Fig. 4.23 A). Notably, in Figure 4.22 (A), although the green alder and the speckled alder associated rhizosphere and bulk soil samples both had many of the same fungal taxa, the genus *Trichosporon* was only present in the green alder planted soil samples, and the genus *Phialocephala* was also much more abundant in the green alder planted soils than in the soils planted with speckled alders. The genus *Phialocephala*, which is among the best-characterized dark septate endophytes (DSE), preferentially colonizes roots of many woody plant species, especially conifers, and is also widespread over the Northern Hemisphere (Sieber and Grunig 2013). In addition, the two genera were found to more associate with green alders and more variations of fungal communities were found in the treatments of speckled alders than in green alders. When looking at the *Frankia*-inoculated and the *Frankia*-mycorrhizal fungi-inoculated speckled alders (ARI-FK and ARI-FK-ECM), we found that the Sporormiaceae family was more abundant while the genus *Tomentella* was less abundant in their bulk soils than their rhizosphere soils (Fig. 4.23 A). In terms of the inoculation effect on the conifers (Fig. 4.23 A), we

found that ectomycorrhizal (ECM) fungi-inoculated jack pine (P-ECM) harbored a different fungal community in their associated rhizosphere and bulk soils. In particular, the Sordariomycetes class, and the genera *Inocybe* (<10 %) and *Suillus* (<40 %), had a higher relative abundance in the ectomycorrhizal (ECM) fungi-inoculated jack pine than their uninoculated counterparts (P-NI). In contrast, the relative abundance of the genera *Tylospora, Amphinema*, *Wilcoxina*, *Tylospora* and the family Pyronemataceae were higher in the P-NI treatment than the in the P-ECM treatment (Fig. 4.23 A). The genus *Suillus* found in the inoculated jack pine's rhizosphere is likely to be our ectomycorrhizal fungal inoculum for jack pine — *Suillus tomentosus*. Except for the difference detected between the inoculated and uninoculated jack pine, we also found a relatively higher abundance of the genus *Inocybe* and the class Sordariomycetes, lower abundance of the genus *Tylospora* and the order Agaricales in the uninoculated white spruce (E-NI), than in their inoculated counterparts (E-ECM).

As for the root endophytic communities, after removing the outlier (ARI-FK-2) in the six treatments of alders (Fig. 4.22 B), we found that green alder roots harbored a similar fungal endophytic community across the different inoculation treatments, whereas the fungal community structure in speckled alder roots varied largely by inoculation treatment. The genus *Leptosphaeria* was more abundant, while the genera *Neonectria* and *Phialocephala* were less abundant in the speckled alder roots than in the green alder roots. Interestingly, we observed in each inoculation treatment of speckled alders, that there was a noticeably dominant fungal genus closely associated with it; they were the genera *Rhizoscyphus*, *Adisciso*, and *Neonectria*, in the uninoculated, *Frankia*-inoculated, *Frankia*-mycorrhizal fungi-inoculated speckled alders, respectively. In conifer roots (Fig. 4.23 B), we found the composition of fungal endophytes was similar among the uninoculated conifers (white spruce and jack pine) and ectomycorrhizal fungi-inoculated white spruce. In particular, the genera *Cosmospora*, *Neobulgaria* and *Leptosphaeria* were more abundant, while *Amphinema* was less abundant, in the ectomycorrhizal fungi-inoculated white spruce than in the uninoculated white spruce. Compared with the uninoculated jack pine, the ectomycorrhizal fungi-inoculated jack pine had a much higher relative abundance of the genera *Suillus* and *Rhizoscyphus*, and a substantially lower relative abundance of *Parastagonospora*, *Amphinema*, *Neonectria*, and *Wilcoxina*.

We found that both root and rhizospheric fungal communities had six dominant genera in common: *Amphinema*, *Phialocephala*, *Rhizoscyphus*, *Suillus*, *Thelephora*, and *Wilcoxina*. The fact that a much more relative abundance of *Phialocephala* was present in the roots of green alder (25 %) than in speckled alder (10 %), and in the roots of ectomycorrhizal fungi-inoculated jack pine (10 %) than in non-inoculated jack pine (5 %), suggests the colonization of the root endophyte *Phialocephala* spp. is able to comparatively stimulate plant performance in the post-mining waste. Moreover, as the members of *Phialocephala fortinii* s.l. species, together with *Acephala applanate* (i.e., PAC species complex), were previously reported to be confined to forest ecosystems (Brenn et al. 2008; Grünig et al. 2008), the presence of *Phialocephala* in the plant roots could be viewed as a positive sign of phytoremediation on the mine waste towards reforestation.

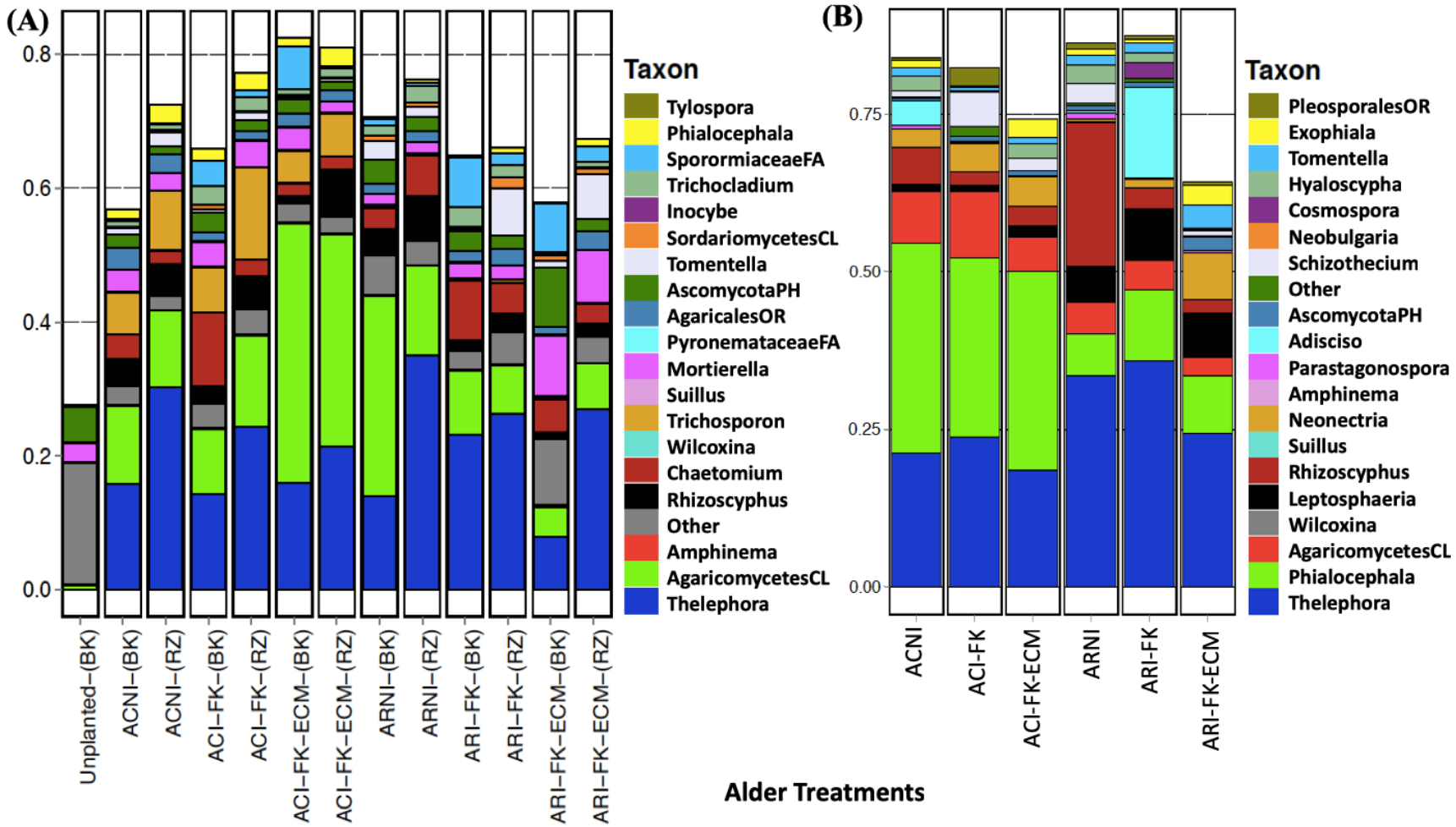

Figure 4.22. The genus-level fungal community compositions comparing the (A) rhizosphere and the (B) roots of alders. A stacked bar depicts the 20 most abundant fungal genera in the averaged sample replications from the ITS amplicon sequencing. In both graphs, Y-axes indicate the relative abundance of fungal genus in a given sample. X-axis in (A) displays the unplanted bulk soil, six each of the alder-associated rhizosphere and bulk soils, while X-axis in (B) indicates the six treatments of alders. Abbreviations: AC(R)NI=uninoculated green(speckled) alders, AC(R)I-FK=*Frankia*-inoculated green(speckled) alders, AC(R)I-FK-ECM= the inoculated green(speckled) alders with *Frankia* sp. strain AvcI1 and mycorrhizal fungi (*Glomus irregulare* and *Alpova diplophloeus*), BK=bulk soils, RZ=rhizosphere soils.

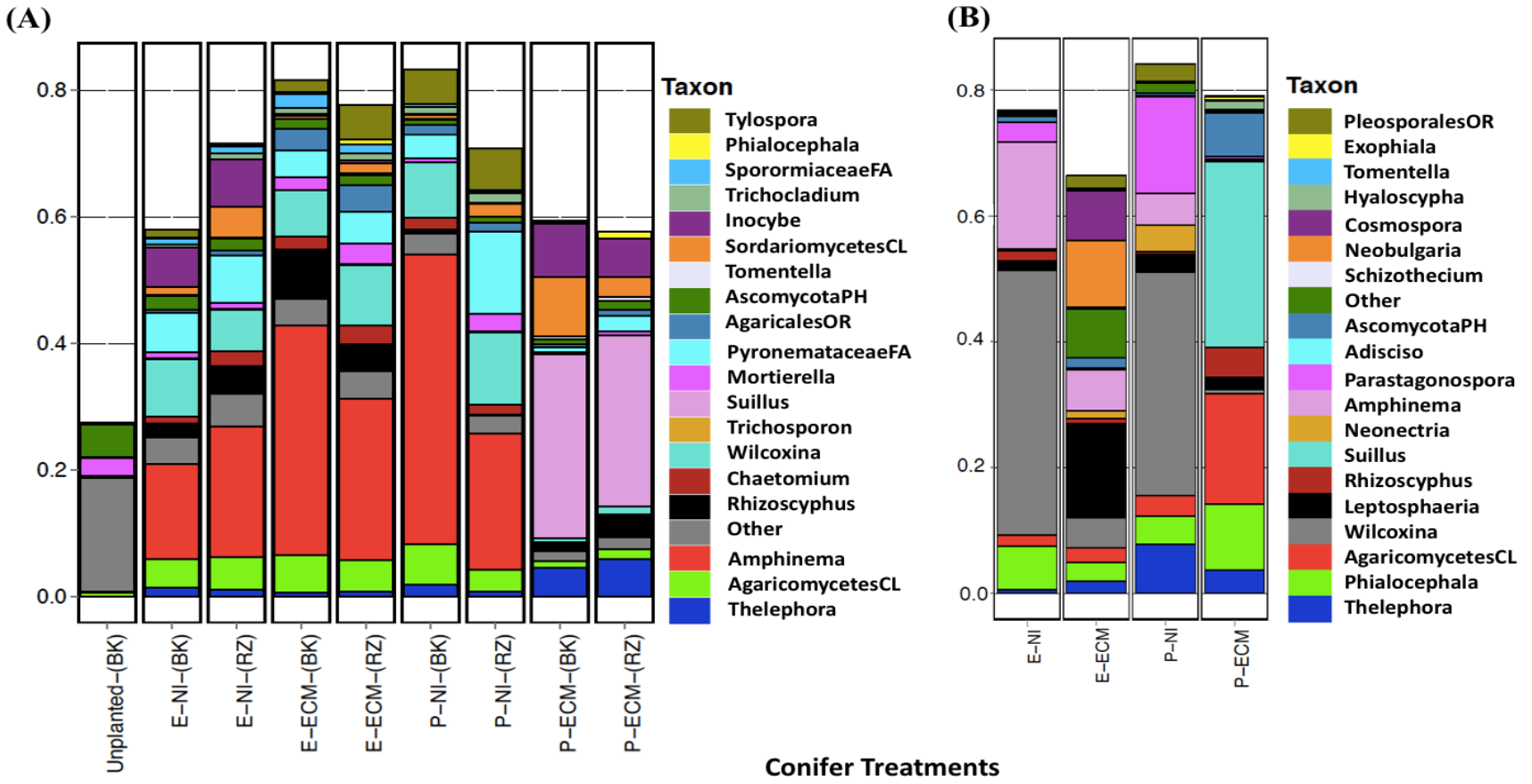

Figure 4.23. The genus-level fungal community composition comparing the (A) rhizosphere and the (B) roots of conifers. The stacked bar depicts the 20 most abundant fungal genera in the averaged sample replications from the ITS amplicon sequencing. In both graphs, the Y-axes indicate the relative abundance of fungal genera in a given sample. The X-axis in (A) displays the unplanted bulk soil, four each of the conifer-associated rhizosphere and bulk soils, while the X-axis in (B) indicates the four treatments of conifers. Abbreviations: BK=bulk soils, RZ=rhizosphere soils, E-NI= uninoculated white spruce, E-ECM= white spruce inoculated with *Hebeloma crustiliniforme* and *Paxillus involutus,* P-NI= uninoculated jack pine, P-ECM= jack pine inoculated with *Suillus tomentosus* and *Laccaria bicolor*.

CHAPTER 5 : CONCLUSIONS AND FUTURE PERSPECTIVES

5.1 Conclusions

Alder-*Frankia* symbionts, which have been extensively studied in the past and reported to positively affect seedling performance in different mine residues, and significantly change the indigenous soil microbial community (Greer et al. 2005; Lefrançois et al. 2010; Callender et al. 2016), did not have a significant treatment-specific effect on the alder performance and microbial community structure in the present research. In the current phytoremediation field trial, inoculation with *Frankia* alone, or the tripartite inoculation with *Frankia*, *A. diplophloeus* (an ectomycorrhizal fungus) and *G. irregulare* (an arbuscular mycorrhizal fungus) of the two alder species studied, *A. crispa* and *A. rugosa*, did not exert a positive effect on seedling survival and growth compared to non-inoculated control seedlings, after 5 years of field growth. In contrast to the findings in previous studies (Chatarpaul et al. 1989; Yamanaka et al. 2003; Greer et al. 2005), and also our hypotheses, alder seedlings had a negative growth response to co-inoculation of *Frankia* and the two selected mycorrhizal fungi, compared to the controls and the seedlings with the single inoculation of *Frankia*. These results indicated that the ectomycorrhizal (ECM) fungus *A. diplophloeus* likely failed to form ectomycorrhizae on roots of either alder species growing in the gold mine, as the arbuscular (AM) fungus *G. irregulare*, which is known to only support early stage seedling establishment, was likely replaced by other ectomycorrhizal fungi in later stages of plant development (Roy et al. 2007). In general, green alders (shrubs) outcompeted speckled alder (trees) in the studied site as shown by plant survival rates and the growth parameters (seedling volume and plot volume indices).

Slightly increased bacterial diversity was observed in the rhizosphere soil of inoculated alders relative to the unplanted the rhizosphere soils of non-inoculated alders. Similarly, the diversity of bacterial root endophytes was also slightly higher, although not significant, in inoculated versus non-inoculated alders. Regardless of inoculation treatments or alder species, similar compositions of dominant bacterial families were observed in soils (rhizosphere and bulk soils) as well as root compartments of alders. While no marked fungal diversity differences were found between the rhizosphere soil of inoculated alders and uninoculated alders, alder roots without inoculation and the tripartite inoculation of *Frankia* and mycorrhizal fungi possessed relatively higher diversity of

endophytic fungi than alders with *Frankia* alone. Similar fungal communities were observed in green alder rhizosphere soil, bulk soil, and roots, while fungal community composition was more variable in both soil and root compartments of speckled alders. In particular, *Phialocephala* spp., a common plant growth promoting dark septate endophyte (DSE) (Sieber and Grunig 2013), were found to colonize green alder roots, and were also slightly more abundant in green alder associated soils, compared to speckled alder treatment groups. *Trichosporon* spp., which have been previously reported to prime plants to resist viruses (Chiu et al. 2018), were also found to be dominant in green alder associated soils. As green alders had a better growth performance than speckled alders in the mine site, it could be suggested that the high biomass production of green alders was stimulated by the colonization of *Phialocephala* and *Trichosporon* fungal genera.

Ectomycorrhizal (ECM) fungal inoculation of coniferous plants (white spruce and jack pine) revealed that the effectiveness of inoculating ectomycorrhizal fungal strains on plant seedlings for growth enhancement largely depends on the compatibility and host-specificity between the tested fungal strains and plants. In the present project, a negative growth response of white spruce seedlings inoculated with *H. crustiliniforme* and *P. involutus* was observed, in comparison to non-inoculated spruce seedlings. Evidence from the literature also showed that the effect of nursery inoculation with different ectomycorrhizal fungi on the formation of ectomycorrhizae and/or the postplanting performance of different spruce species varies from positive to insignificant, and may even reduce seedling growth (Hodson and Wilkins 1991; Quoreshi et al. 2008; Onwuchekwa et al. 2014; Repáč et al. 2015; Repáč and Sendecký 2018). In contrast to white spruce, jack pine seedlings inoculated with *S. tomentosus* and *L. bicolor* had higher survival among all the introduced plants (both conifers and alders), and also produced significantly greater seedling biomass compared to the non-inoculated pine seedlings.

Molecular analyses of the collected conifer-associated bulk soil, rhizosphere soil and root samples demonstrated that bacterial diversity in the rhizosphere soils of non- and inoculated white spruce, and uninoculated jack pine was significantly higher than the ectomycorrhizal fungi-inoculated jack pine. Although the species richness of bacterial endophytes in conifer roots was significantly lower relative to alders, there was not a remarkable bacterial diversity difference among conifer roots. With regards to the bacterial community composition in the conifer plantation, the main difference

was that the Acetobacteraceae and Sphingomonadaceae families were much more abundant in the rhizosphere and bulk soils of the ectomycorrhizal fungi-inoculated jack pine, and the relative abundance of Acidobacteriaceae (Subgroup 1) was much higher in the roots of the ectomycorrhizal fungi-inoculated jack pine. The presence of these bacterial families may explain the enhanced growth of the ectomycorrhizal fungi-inoculated jack pine. For instance, many genera in the Acidobacteriaceae (Subgroup 1) family are aerobic chemoheterotrophs (*Acidicapsa*, *Bryocella*, *Granulicella*, *Edaphobacter*), which are responsible for phosphate solubilization (Srivastava et al. 1996; Campbell 2014; Kielak et al. 2016). In the Acetobacteraceae family, three acetic acid bacterial genera, *Gluconacetobacter*, *Swaminathania*, and *Acetobacter*, are capable of fixing nitrogen (Pedraza 2008). The other family Sphingomonadaceae also contains genera (e.g., *Sphingomonas*) capable of nitrogen fixation (Glaeser and Kämpfer 2014).

Although no significant difference of fungal diversity was observed in the rhizosphere soils between non- and the ectomycorrhizal fungi-inoculated conifers, root endophytic fungi were significantly more diverse in the ectomycorrhizal fungi-inoculated conifers versus uninoculated control seedlings. With regards to fungal species composition, a *Suillus* spp. was predominant in both soils and roots of the ectomycorrhizal fungi-inoculated jack pine, while no such genus was found to be associated with the non-inoculated control seedlings. Since *Suillus tomentosus* was one of the fungal inocula for jack pine plants, we suspected that detection of this genus was highly suggestive of its colonization in pine roots. The genus *Suillus* has been frequently reported to exhibit high host-specificity and preference for the conifer family Pinaceae*,* typically *Pinus* (Cairney and Chambers 1999). Although *Suillu*s species had intraspecific variations in forming mycorrhizal symbioses with different pine species (Liao et al. 2016), our results suggested that the jack pine seedlings inoculated with *S. tomentosus* were able to perform well on gold mine waste rock, leading to increased rates of revegetation and phytostabilization. We thus conclude that selection of a *Suillus*-*Pinus* combination could be of practical use for the phytoremediation of gold mine waste rock or other alkaline mine residues with minor metal contamination.

In summary, our results showed that the ectomycorrhizal (ECM) fungi-inoculated jack pine had better performance than non-inoculated jack pine on gold mine waste rock, partially in agreement with our previous hypotheses indicating inoculated plants had better survival and growth than non-

inoculated control plants. Partially in line with our second hypothesis, bacterial diversity in the rhizosphere soil and roots of inoculated alders was slightly higher, although not significant, than the uninoculated alders. Moreover, fungal root endophytes were found to be significantly more diverse in the ectomycorrhizal fungi-inoculated conifers compared to the uninoculated conifer group. The relative abundance of several microbial taxa, including the bacterial families Acidobacteriaceae (Subgroup 1), Acetobacteraceae, and Sphingomonadaceae, and the fungal genus *Suillus*, were higher in ectomycorrhizal fungi-inoculated jack pine relative to non-inoculated jack pine, suggesting that those microbes play a vital role in seedling growth enhancement of jack pine. As predicted, the two soil parameters measured, pH and moisture content, were substantially improved during phytoremediation, which aid the establishment of late-successional plant species that are less tolerant of the adverse soil conditions of mine substrates.

5.2 Future perspectives

The present study evaluated the field performance of several plant species following different inoculation treatments. Overall, since the performance of the ectomycorrhizal fungi-inoculated jack pine seedlings in this phytoremediation trial was outstanding, this suggests that the use of *Pinus*-ECM fungus symbionts could improve the long-term reclamation of gold mine waste rock. However, plant seedling response to fungal inoculation is complex and variable, particularly in adverse soil conditions and disturbed sites, in which the plant-microbe interactions may change over time. Currently, the lack of understanding of inoculation success for planted seedlings under different soil conditions, and fewer long-term monitoring field studies compared to greenhouse experiments, will necessitate more research in order to clearly investigate plant-microbe interactions in mine waste and other challenging environments. In the present project, amplicon sequencing of the 16S rRNA gene (bacteria/archaea) and the nuclear ribosomal ITS gene (fungi) allowed us to explore microbial taxonomic composition to the genus level, although there are still some unidentified taxa in our samples. Other advanced next-generation sequencing techniques such as metagenomics and metatranscriptomics, which reveal detailed insights into gene identification and expression, can further describe the functional potential of using ectomycorrhizal fungi and identify more effective ectomycorrhizal fungal strains that hold promise for phytoremediation practices. Although in the current research, neither the *Frankia*-alder

symbionts nor the tripartite symbioses of *Frankia*, mycorrhizal fungi and alders exhibited a pronounced impact on alder performance in the mine site, contrary to previous findings, co-inoculating green alders with the *Frankia* sp. strain AvcI1 as well as the identified fungal pathogen *Trichosporon* and the DSE fungal genus *Phialocephala* could be used to improve the success of future phytoremediation strategies.

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APPENDIX 1

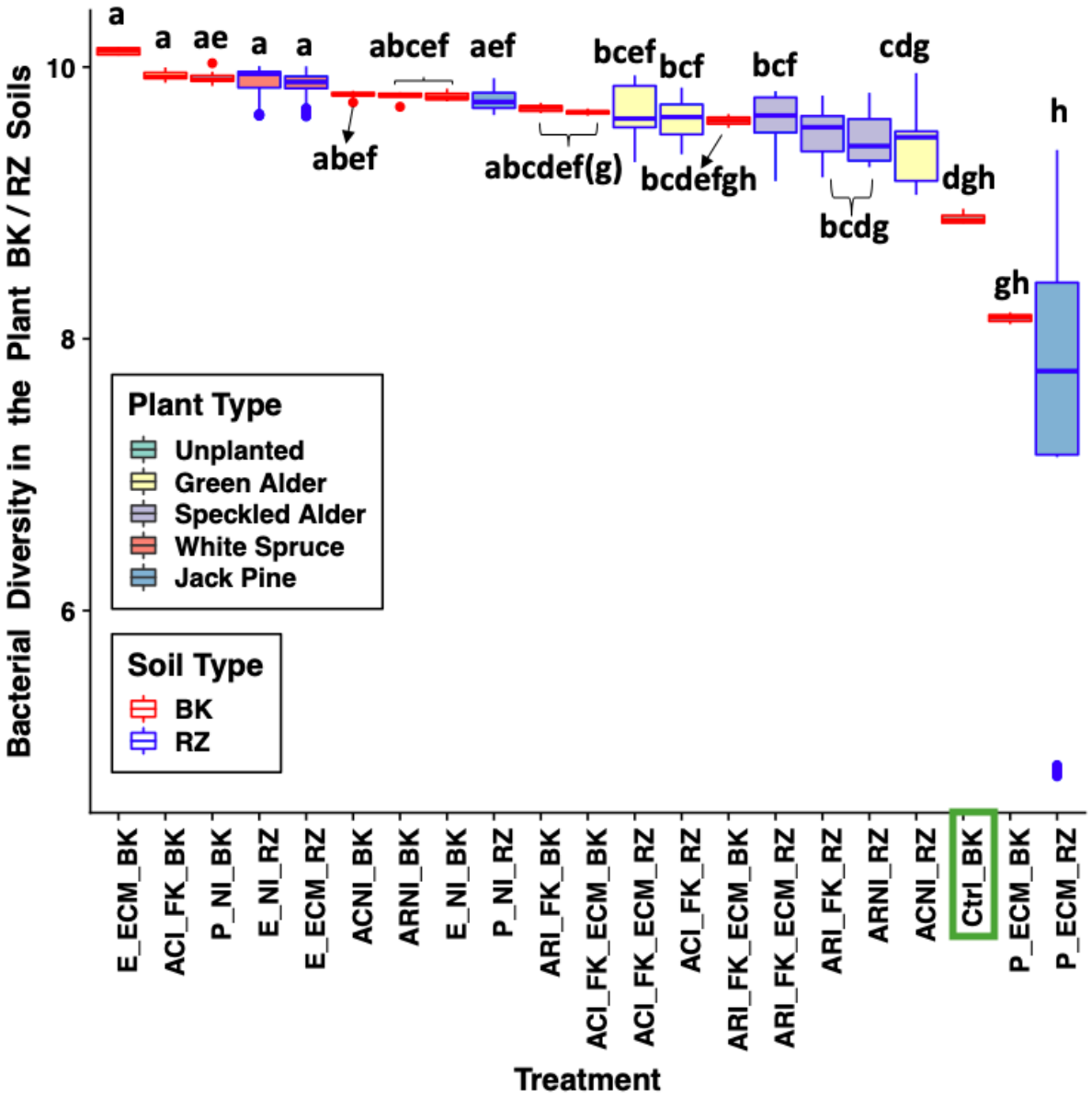

Figure A. 1 Boxplots of Shannon indices indicating bacterial diversities in the bulk soils and the rhizosphere soils sampled from different plant-inoculum treatments. Soil sampled from the unplanted site serves as the control to the plant bulk and rhizosphere soils. The lower and upper hinges of a box correspond to the 25th and 75th percentiles, while the median is displayed as the horizontal line within the box. Outliers are shown by dots beyond the end of the whiskers. Statistical analyses were performed using Kruskal-Wallis test followed by Dunnett's multiple mean comparison with P-values adjusted by the 'Bonferroni–Holm' method. Bars bearing the same letters are not significantly ($p>0.05$) different from each other. BK=bulk soils, RZ=rhizosphere soils, AC=green alder, AR=speckled alder, E=white spruce, P=jack pine, NI=not inoculated, FK=*Frankia* sp. strain AvcI1, AC(R)I_FK_ECM= alders received co-inoculation of *Frankia* sp. strain AvcI1 and mycorrhizal fungi (*Glomus irregulare* and *Alpova diplophloeus*), ECM=ectomycorrhizal fungi, Ctrl=control.

APPENDIX 2

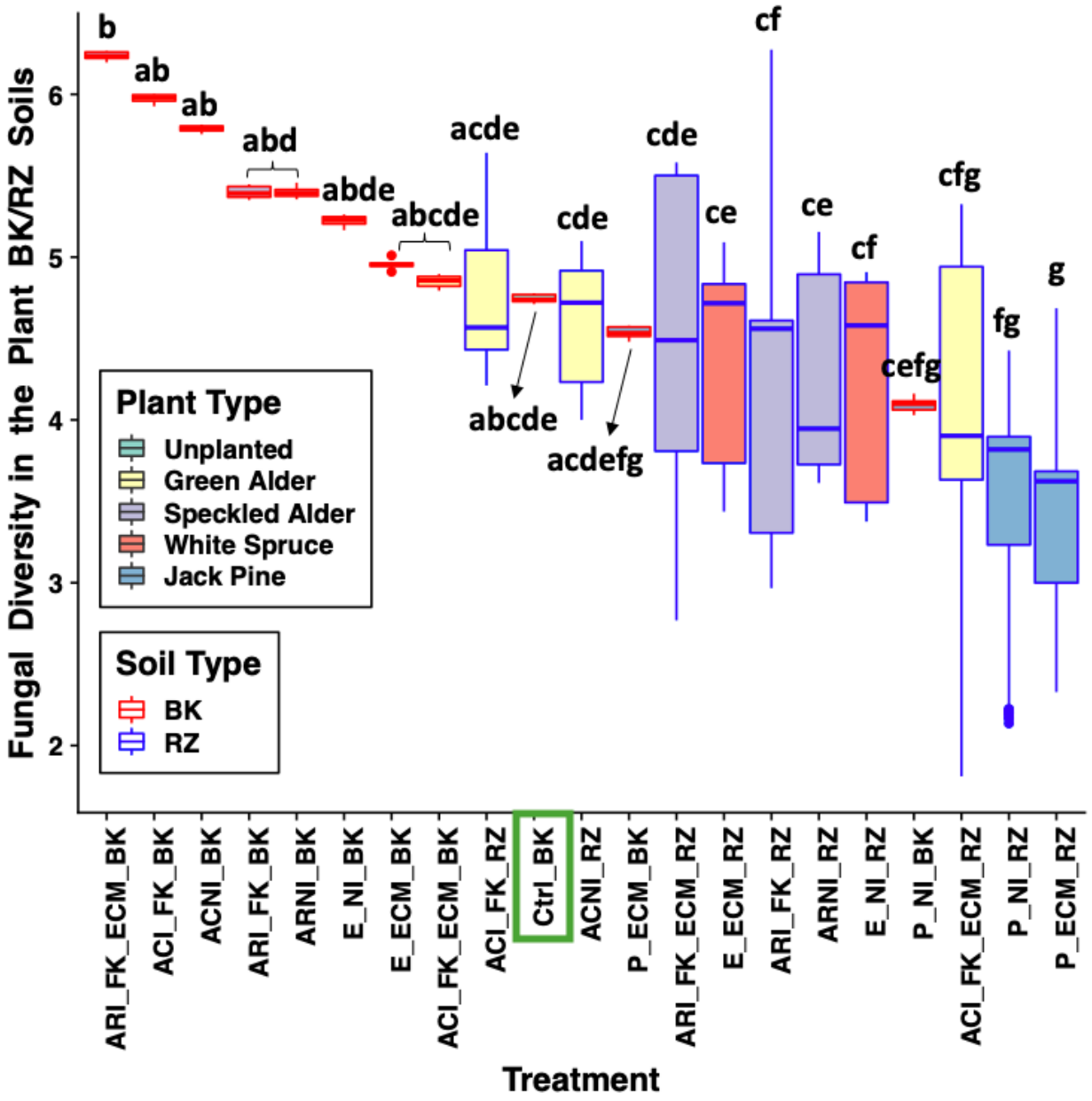

Figure A. 2 Boxplots of Shannon indices indicating fungal diversities in the bulk soils and the rhizosphere soils sampled from different plant-inoculum treatments. Soil sampled from the unplanted site serves as the control to the plant bulk and rhizosphere soils. The lower and upper hinges of a box correspond to the 25th and 75th percentiles, while the median is displayed as the horizontal line within the box. Outliers are shown by dots beyond the end of the whiskers. Statistical analyses were performed using Kruskal-Wallis test followed by Dunnett's multiple mean comparison with P-values adjusted by the 'Bonferroni–Holm' method. Bars bearing the same letters are not significantly different from each other ($p>0.05$). BK=bulk soils, RZ=rhizosphere soils, AC=green alder, AR=speckled alder, E=white spruce, P=jack pine, NI=not inoculated, FK=*Frankia* sp. strain AvcI1, AC(R)I_FK_ECM= alders received co-inoculation of *Frankia* sp. strain AvcI1 and mycorrhizal fungi (*Glomus irregulare* and *Alpova diplophloeus*), ECM=ectomycorrhizal fungi, Ctrl=control.