Development, production and application of alder-*Frankia* symbionts for the remediation and revegetation of oil sands process-affected materials (OSPM) in Athabasca

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ABSTRACT

Symbiotic alders are potential candidates for use in the remediation and revegetation of oil sands reclamation sites, and greenhouse inoculation has been shown to help ensure successful out-planting in the field. For successful nodule formation and growth, the time of inoculation (plant age) and N input are factors to be considered. In the present study, symbiosis was induced between *Alnus crispa* and *Frankia* strain AvcI1. Seedlings were grown, inoculated and exposed to different growing conditions that consisted of combining the presence or absence of *Frankia*, three different plant ages and five N treatments, to determine the best method for enhancing plant nodulation and growth. Results indicated that inoculation of 9-week-old seedlings with *Frankia* improved seedling growth, promoted nodule formation and ensured efficient N_2 fixation. Fertilization with 100ppm of N was counterproductive for plant health, while the lower concentration of N, 10ppm, did not fulfill the N requirements of seedlings, suggesting the need to apply higher concentrations of N that do not surpass 100ppm.

Greenhouse inoculated alders were subsequently used in a large-scale field trial to evaluate their ability to improve soil quality and metabolic activity of the indigenous microbial community in an oil sands reclamation site. In addition, the inoculated *Frankia* was monitored to determine if it remained present as part of the endophytic community in alder nodules. Results showed that inoculated alders were capable of very active growth, out performing non-inoculated plants, producing up to five-fold

more biomass within the 3 growing season monitoring period. Alders also promoted the proliferation of heterotrophic and hydrocarbon degrading bacteria in the rhizosphere. Indigenous *Frankia* strains, that shared molecular similarities with the symbiont used in this study, were found in the nodules of non-inoculated alders, as well as in some of the soil samples indicating that a molecular detection approach requires further validation.

RÉSUMÉ

Les aulnes symbiotiques ont un excellent potentiel pour la restauration et la re-végétalisation des anciens sites d'exploitation des sables bitumineux, et la pré-inoculation en serre a été démontrée comme aidant à assurer le succès de la plantation sur le terrain. Pour une croissance et une formation optimale des nodules, plusieurs facteurs sont à considérer : le moment de l'inoculation (âge de la plante) et les sourcesd'N. Dans la présente étude, *Alnus crispa* a été inoculé avec la souche *Frankia* AvcI1. Les semis ont été exposés à différentes conditions incluant la présence ou l'absence de *Frankia*, trois moment d'inoculation et cinq traitements d'N, afin de déterminer la meilleure méthode pour induire la nodulation des plantes et optimiser la croissance. Les résultats ont indiqué que l'inoculation avec Frankia à 9 semaines d'âge a amélioréla croissance des semis, la formation de nodules et la fixation de N_2. La fertilisation avec 100 ppm d'N a été nocive pour la santé des plantes, tandis que la fertilisation avec 10 ppm d'N n'a pas remplis les besoins en azote des semis, ce qui suggère la nécessité d'appliquer des concentrations plus élevées d'N ne dépassant toutefois pas 100 ppm.

Les semis d'aulnes inoculés en serre ont ensuite été utilisés dans un essai à grande échelle sur le terrain afin d'évaluer leur capacité à améliorer la qualité des sols et l'activité métabolique de la communauté microbienne indigène sur un ancien site d'exploitation des sables bitumineux. En outre, la souche de *Frankia* inoculée a été surveillée pour déterminer si elle est restée présente parmis la communauté

endophyte des nodules d'aulne. Les résultats ont démontrés que les aulnes inoculés étaient capable d'une croissance beaucoup plus active que lesplantes non inoculées, produisant jusqu'à cinq fois plus de biomasse au cours des trois saisons de croissance de l'essai sur le terrain. Les aulnes ont aussi favorisé la prolifération des bactéries hétérotrophes et des bactéries dégradant les hydrocarbures leur rhizosphère. Dessouches de *Frankia* indigènes qui partageaient des similitudes moléculaires avec le symbiote utilisée dans cette étude, ont été retrouvées dans les nodules des aulnes non inoculés, ainsi que dans certains des échantillons de sol indiquant qu'une approche de détection moléculaire nécessite une validation plus poussée.

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ABBREVIATIONS

BK	Bulk soil
C	Non-inoculated *Alnus crispa*
CEC	Cation exchange capacity
CF	F9-inoculated *Alnus crispa*
F9	*Frankia* strain AvcI1
F9-In	Alders inoculated with F9; soil planted with F9-In alders.
HS-1, HS-2, and HS-3	Health status of alder seedlings
HTB	Heterotrophic bacteria
J09	June 2009
L	Leaves
MD5	Millenium Dump site 5
MN	Macronutrients
MPN	Most probable number technique
Ni	Non-inoculated alders (controls)
NOD	Nodules
Nod-0	Alder seedlings with nodules
Nod-1	Alder seedlings without nodules
OM	Organic matter
OSPM	Oil sands processes affected materials
P1, P2 and P3	Plant age treatments (planting groups)
PAH	Polycyclic aromatic hydrocarbons
PAHB	Polycyclic aromatic hydrocarbon degrading bacteria
R	Non-inoculated *Alnus rugosa*
RF	F9-inoculated *Alnus rugosa*
RHZ	Rhizosphere
RTC	Root trainer cells
S	Stem
SVI	Seedling volume index
S10	September 2010
S11	September 2011
TE	Trace elements
UF9	Unplanted area adjacent to the F9-In plot
UNi	Unplanted area adjacent to the Ni plot

INTRODUCTION

The current work is part of a line of investigations on the use of alders for the revegetation and remediation of oil sands process-affected materials (OSPM). The work comprises two parts: a greenhouse experiment, in which the development and production of alder-*Frankia* symbionts was studied, and a field experiment in which alder-*Frankia* symbionts were employed to revegetate and remediate an overburden reclamation site located in the Athabasca oil sands region.

The symbiotic relationship of alders with *Actinobacteria* of the genus *Frankia* permits the plants to be pioneer organisms on disturbed soils that are characterized as being hostile environments for the establishment of most plants. Conditions of disturbed soils include lack in essential elements like N, little or no organic matter, and the presence of toxic compounds (Huss-Danell, 1986; Paschke, 1997; Roy et al., 2007). The employment of alder trees and shrubs include the large-scale production of alder-*Frankia* symbionts, which involves the growth of alder seedlings that are inoculated with a suitable *Frankia* strain under controlled growing conditions (Berry and Torrey, 1985; Périnet et al., 1985; Quoreshi et al., 2007; Stowers and Smith, 1985). The early stages of growth are crucial since alder seedlings, that have already formed symbiotic associations with an infective and effective *Frankia* strain, are more likely to survive and be successful colonizers (Berry and Torrey, 1985). In addition to inoculation, two other factors are to be considered in the production of symbionts: the uptake of N by the plants and the age of the plant. The *Frankia* infection and nodulation processes require a certain amount of N before nodules mature and are capable of fixing N_2 on their own (Valverde and Huss-Danell, 2008). On the other hand, early inoculation was found to be the most appropriate method to ensure a successful infection process (Stowers and Smith, 1985). But how early should seedlings be inoculated? The formation of nodules may be affected by the pre-inoculation conditions to which plants are exposed. Exposure to an artificial source of N may aid the plants during the pre-inoculation stage, where seedlings would use N for their immediate growth needs. This aids the photosynthetic activity, which in turn stimulates N_2 fixation, thus leading to nodulation (Bethlenfalvay et al., 1978; Stewart and Bond, 1961). In addition, continuously providing the plants with the right amount of N after inoculation can maintain this dynamic, resulting in a well-nodulated stock of healthy alder-*Frankia* symbionts that can be successfully out-planted in disturbed soils. As symbiotic alders are capable of N_2-fixation, their employment represents a cost-effective strategy for N accumulation, as they supply from 60 – 320 kg N ha^{-1} a^{-1} to soil (Newton et al., 1968) through leaf litter, root decay and root exudates, all rich in N and other essential plant nutrients (Huss-Danell, 1986). Whenever symbiotic alders are used, other plant species benefit from the accumulated N in the soil (Bradshaw, 1997). Microbial activity is also essential for the rehabilitation of disturbed lands as it promotes soil formation. Microbes decompose the organic matter and make essential nutrients in the soil matrix available to plants, enabling their growth (Cundell, 1977; Insam and Domch, 1988; Mott and Zuberer, 1997). It is known that the microbial community in soil is highly affected by the presence of plants and they gather in great numbers in the root zone, referred to as the rhizosphere, where plants provide them with carbon-rich exudates. The chemical structure of these exudates may be similar to certain contaminants and microorganisms can take them up and degrade them, reducing their toxicity and concentration in the soil matrix (Leahy and Colwell, 1990). This type of interaction between plants and microorganisms at the rhizosphere level is known as the rhizosphere effect, and it is considered one of the primary mechanisms responsible for the degradation of petroleum compounds in contaminated soils (Frick et al., 1999). Opting for the establishment of robust pioneer plants like alders may boost the indigenous microbial community found in the soil cover as they deposit organic matter in the form of leaf litter and root decay. At the same time, root exudates can increase the metabolic activity of bacteria capable of degrading hydrocarbon contaminants. As a result, the decomposition of organic matter is accelerated helping to speed up forest floor formation, and the concentration of residual hydrocarbons is simultaneously reduced, making the soil less hostile (Roy et al., 2007). The present work was based on the following hypothesis: alders that have been pre-inoculated with a selected *Frankia* strain will have a higher survival rate and grow more effectively than non-inoculated alders when out-planted on oil sands sites. Soil quality will improve due to the presence of alder-*Frankia* symbionts, and the indigenous microbial population in their rhizosphere and its metabolic activity will be positively affected, potentially increasing the number of beneficial hydrocarbon-degrading bacteria.

OBJECTIVES

1) Alder-*Frankia* symbionts have already been used for small-scale planting of disturbed lands, however, it was of interest to develop an efficient protocol for the production of well-nodulated alder seedlings prior to large-scale out-planting in the field for the remediation and revegetation of disturbed lands. The hypothesis to be tested was that alders, inoculated under controlled conditions (greenhouse) would be more efficiently nodulated than non-inoculated alders, and therefore survive and grow better when out-planted on harsh sites.

2) Alder-*Frankia* symbionts have previously been tested for their performance on hydrocarbon-contaminated soils (Greer et al., 2005; Lefrançois et al., 2010). In this study, I wanted to evaluate the efficiency of symbionts in a large-scale field trial for the remediation and revegetation of an overburden reclamation site in Athabasca. The hypothesis was that greenhouse inoculated alders would perform more effectively than non-inoculated alders when out-planted on oil sand sites. I evaluated the performance of both inoculated and non-inoculated plants and determined their impact on soil quality and on the soil microbial community, specifically with regards to indigenous hydrocarbon-degrading bacteria.

CHAPTER ONE: Literature review

1.1.) Oil sands in Alberta

1.1.1.) Description

Although the estimation of global oil reserves is challenging, when one includes oil sands, Canada has the second largest stated reserve after Venezuela. Most of this reserve is located in the province of Alberta, and it extends eastward into Saskatchewan. The oil sands are a non-conventional source of petroleum, consisting of water-wetted sand and fine soil particles (mostly clay) surrounded by a layer of bitumen. The oil sands from Alberta are 70% sand and fine soil particles, 10% water, and from 0% to 18% petroleum (Kleindienst, 2006). The total reserve is divided into four major deposits that together comprise an estimated 1.7 to 2.5 trillion barrels of bitumen. These deposits are located in the following regions: Athabasca, Wabasca, Cold Lake, and Peace River (Government of Alberta, 2009). Distribution of the deposits is shown in Figure 1.

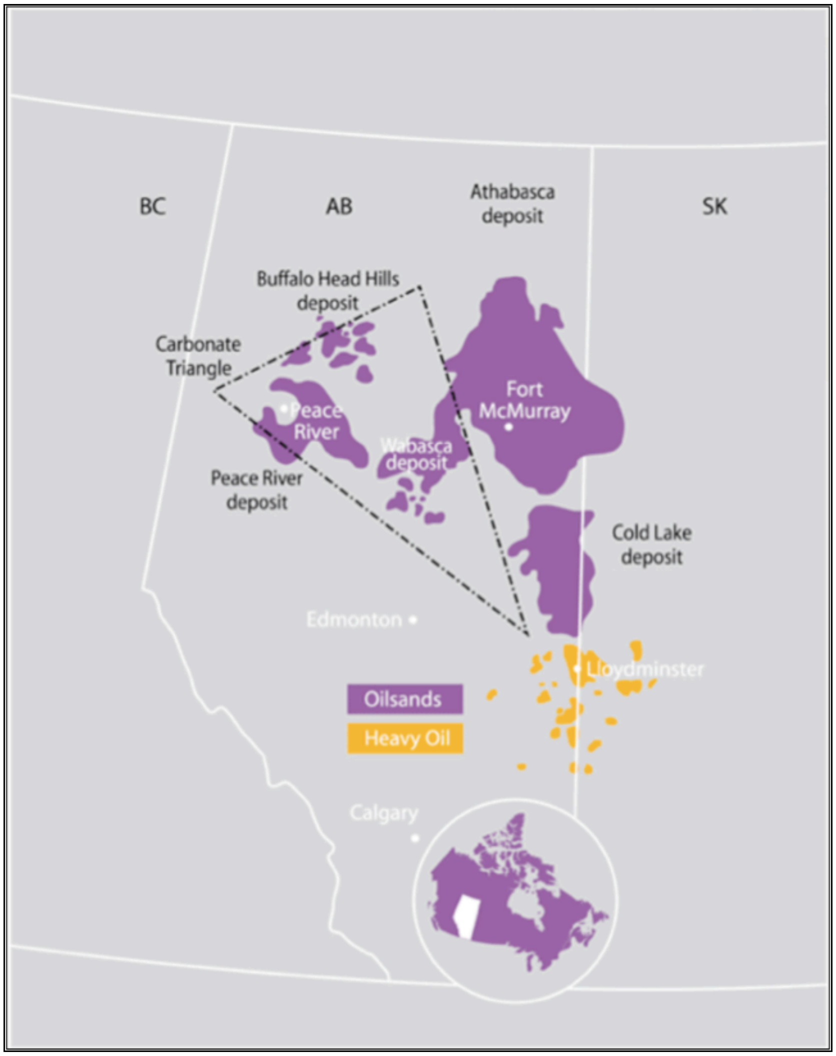

Figure 1. Oil sands deposits in western Canada. Source: Canadian Centre for Energy Information (modified picture).

1.1.2) The Canadian boreal forest

The oil sands deposits in Alberta are found within the territories of the boreal forest, a natural zone comprised of vegetated areas and wetlands. The forest covers 30% of the Canadian landmass; here, 77% corresponds to forestland and over 60% borders on inland water areas (Brandt, 2009). The green lands of the boreal forest support a high biodiversity of plants and wildlife; in addition, these lands are the ancestral homelands of First Nation communities. The forest stores a large amount of carbon and acts as a climate regulator, or shield, against global warming phenomena (IBCC, 2011; Woynillowicz et al., 2005). The boreal forest is characterized by having short cool summers and long severely cold winters, with annual precipitation ranging from 200 to 600 mm (Molles, 2002). The plant community is comprised of coniferous and deciduous cold-tolerant trees, peat, muskeg and lichens. Notable tree species include members of the groups: fir, larch, spruce, pine, poplar and birch (Bonan and Shugart, 1989; Brandt, 2009). The wildlife includes 327 animal species comprising 40 fish, 5 amphibian, 1 reptile, 236 bird and 45 mammal species (Grant et al., 2008). Included amongst the native mammals are found emblematic species that are representative of the Canadian territory, such as the moose, wolf, caribou, bear, rabbit, lynx, cougar, mink, wolverine, and river otter. First Nation communities have made use of the resources found within the Boreal forest including the oil sands, which were used by aboriginal peoples primarily to repair their canoes. Some of the communities found in the area are the Athabasca Chipewyan First Nation, the Chipewyan Prairie First Nation, the Fort McKay First Nation, the Fort McMurray First Nation, and the Mikisew Cree First Nation (Gosselin et al., 2010). All of these communities are settled in the areas surrounding the Athabasca oil sands region, where the main recovery of oil sands takes place.

1.1.3) Geology of the oil sands deposits in Alberta

According to geological studies, the materials extracted from Alberta's oil sands region are naturally oil-enriched and have a saline-sodic nature. The oil sands originated from the Cretaceous period and are a delta deposit of an ancient tropical sea in which sediment deposition occurred over thousands of years. The formed layer was then compressed by extensive glacier formations and was followed by heavy oil material migrating from elsewhere (Mossop, 1980). The upward migration of oil through the soil layers filled the current "ore zone" which contains the highest percentage of quartz sands. In the past, this characteristic made the migration of oil through the sand particles an easy task. However, the overlying soil consisting of finer particles and clay-rich materials, acted as a barrier to the upward migration of the oil to the surface. These particles still ended up absorbing some of the oil, giving rise to the formation of what are known as lean oil sands, an unrecoverable type of oil (Berkowitz and Speight, 1975; Kleindienst, 2006).

The oil sands deposits in Athabasca can be differentiated from each other mainly in how extensive they are and in terms of the depths at which the ore is localized, which in turn plays an important role in how the oil resource can be exploited and the techniques that are necessary for its recovery (see section 1.2). The Athabasca deposit is located in northeast Alberta and lies over the McMurray geological formation. It is the largest of the four deposits, encompassing more than 42,340 km^2, and is the only one in which the oil sands are localized at shallow depths of less than 75 m. This unique characteristic permits the exploitation of the resource through conventional surface mining techniques. The Wabasca deposit is a westward extension of the Athabasca deposit covering ~6,500 km^2 of territory. Here, the ore is localized at depths that range from 100-700 m. The Cold Lake (Clearwater geological formation) and Peace River (Bluesky geological formation) deposits cover territories of 9000 and 5200 km^2, respectively. These two deposits have the most deeply buried ore: 400-600 m depths at Cold Lake, and 500-700 m depth at Peace River (Berkowitz and Speight, 1975; Dusseault, 2001).

1.2) Oil sands exploitation

1.2.1) Mining operations in Alberta

There are two processes by which oil sands are extracted: through surface mining operations and *in situ* operations. Surface mining started in the late 1960's, and has been the most commonly used, as well as the most damaging way to recover oil sands. I*n situ* operations have only been implemented in recent years. Only 20% of the total reserve is close enough to the surface to allow its extraction through surface mining; the rest of the reserve requires the extraction of the oil sands via *in situ* methods since the ore is too deep to be reached by conventional techniques (Thompson, 2009). Currently, surface mining and the *in situ* technique known as Steam Assisted Gravity Drainage (SAGD) are performed at the Athabasca and Wabasca deposits, while the SAGD and Cyclic Steam Stimulation (CSS) techniques are exclusively used at the Cold Lake and Peace River deposits (Government of Alberta, 2009). The present study focuses on surface mining operations, however *in situ* techniques will be broadly explained in this section.

The surface mineable zone is limited to the north part of the Athabasca deposit, in the region of Fort McMurray (Mossop, 1980) where the ore is localized at depths of less than 75 m, outcropping extensively along the banks of the Athabasca River. Here, surface mining consists of removing soil layers that lie above the oil sands and the subsequent stripping of the ore once it has been reached. Trees and other cover vegetation are harvested, while the forest floor, comprised of the organic and mineral horizons, is stripped away and stored for later use in reclamation practices. Oil sands are then taken to facilities for separation and upgrading of the bitumen. Each of the processes carried out during mining operations, extraction, treatment of the sands and upgrading of the bitumen, accumulate materials and by-products, some of which are used in reclamation practices (Government of Alberta, 2009; Johnson and Miyanishi, 2008). These materials are known as oil sands process-affected materials (OSPM), and have characteristics that make them a challenge for the restoration of the Athabasca natural landscapes once mining operations are completed (see section 1.1.3).

In situ operations are based on the injection of hot steam directly into the deep buried deposit to facilitate its recovery. The SAGD technique employs two parallel wells that are drilled through the deposit. One well injects hot steam (injection well) while the other is used to recover the oil (production well), the latter being larger than the former. When the substrate is heated, the oil becomes more malleable so it can be pumped up through the production well. To maintain the stability of the landscape, water is injected to fill up the space previously occupied by the oil. The CSS technique is based on a continuous cycle that applies high-pressure in addition to hot steam to fracture and melt down the bitumen. As a result, the bitumen flows easily through a production well and up to the surface for recovery (Kleindienst, 2006). *In situ* techniques are expected to be used for the recovery of a large part of the oil sands reserves in Alberta. As the use of these techniques avoids the disturbance of above ground ecosystems, it attempts to provide of a better way of exploiting the resources without leaving extensive damaged areas that represent a challenge for landscape restoration. It should be stressed that these projections are in question, as greenhouse gas emissions and unseen soil disturbances may lead to subsequent negative ecological effects (Greenpeace, 2011).

1.2.2) Oil sands process-affected materials (OSPM) obtained from surface mining operations.

The composition of the oil sands makes it necessary to treat them so that their oil fraction can be separated from the soil and water fractions. Once separated, the bitumen is upgraded to produce light, sweet synthetic oil (Government of Alberta, 2009). OSPM are the materials that accumulate during the mining and extraction processes that are further used in reclamation practices or recycled for continuing refinery operations. These comprise the overburden material, tailings sands, and mature fine tailings sands, which are further treated to produce composite tailings, and water (Gosselin et al., 2010). The overburden is stripped and stored during oil sands mining. It is found below the forest floor and directly above the ore. The stripped overburden is used to fill pits left by completed operations, or piled up in extensive areas known as overburden dump sites, which are reclaimed at a later date. The mined overburden of the Athabasca oil sands area is highly alkaline with a saline-sodic nature due to its geological origin (see section 1.1.3). It is comprised of recent fluvial deposits, a variety of glacial deposits, bedrock formations and hydrocarbon-enriched materials (OSVRC, 1998). Following overburden removal, the oil sands are extracted and tailings sands are produced during the extraction of the oil fraction, which is separated from the soil and water fractions. Tailings sands are the OSPM that are produced during surface mining operations. The process consists of treating the sands with hot water and caustic soda, which separates the soil fraction, eliminating it afterwards as a slurry-like material that contains water (from the process), coarse and finer soil particles, unrecovered hydrocarbons and other potentially toxic by-products. The slurry is pumped into settling basins that cover large areas of land, known as tailings ponds. The coarse solids (<75%), comprised mostly of sand particles, are of little economic value and do not represent an environmental risk as they settle quickly to the bottom of the ponds, creating a solid surface that further slurry waste is poured onto. The finer particles, clay and silt, which represent ~30% of the waste do represent a major risk because of their extremely slow settling, their hydrocarbon content and accumulation of acute toxic compounds like naphthenic acids. The slurry is left in the basins for about two years while the coarse fraction settles and the suspended finer particles are treated with gypsum to create a consolidated dense material known as composite tailings. Eventually, part of the released water is recycled and re-used in the bitumen extraction process (Grant et al., 2008).

1.3) Land reclamation practices in Athabasca

1.3.1) Lands disturbance in Athabasca

The prolonged surface mining practices in Athabasca have generated extensive areas of disturbed lands and have markedly changed the landscape of the region, generating controversy over whether these lands will ever be fertile and self-sustaining again (Grant et al., 2008; Greenpeace, 2011). Three oil companies work within the minable zone in Athabasca: Suncor Energy Inc., Syncrude Canada Ltd., and Albian Sands Energy Inc. It was calculated that by March of 2009, a total of 602 km^2 of land had been disturbed as a result of the active mining operations of the three companies (Government of Alberta, 2009). It is known that surface mining drastically alters the physical and biological nature of forestlands by destroying vegetation, polluting air, and water, and damaging the soil (Johnson and Miyanishi, 2008). In Athabasca, the extraction and upgrading of bitumen are energy demanding processes that produce greenhouse gas emissions ranging from 62 to 164 Kg CO_2 eq/bbl (carbon dioxide equivalent/barrel) compared to 27-58 Kg CO_2 eq/bbl of crude from conventional oil production (Greenpeace, 2011). Leaching of toxic components contained in the temporary constructed structures, overburden dumps and tailing ponds, are a major risk for the surrounding rivers streams and groundwater (Gosselin et al., 2010). The soil ecosystem suffers the most, as the extensive areas that have been damaged may take hundreds of years to recover if this process is left to occur under natural conditions.

1.3.2) Obligations of oil companies in Athabasca

As established by the Land Surface Conservation and Reclamation Act of 1973 and the Environmental Protection and Enhancement Act of 1992 (Government of Alberta, 1999), oil companies working in Athabasca are forced to reclaim lands that have been mined and return them to a state that resembles the undisturbed landscapes that existed before. This may include the restoration to a state similar to the undisturbed lands that surround the mining zone or the creation of productive green areas of commercial forest within the natural range of ecotypes found in the Central Mixed Wood Sub-Region of the Boreal forest (CEMA, 2006; Johnson and Miyanishi, 2008; OSVRC, 1998; Purdy et al., 2005). Reclamation is chosen when the extent of damage is high and landscapes are re-shaped and soils replaced in a way that can ensure the initiation of natural succession or that shortens the time needed for their rehabilitation to begin. These processes take place once the disturbed land is no longer part of active operations.

The current study was conducted at Suncor Energy Inc., at an area where they carry out oil sands extraction through surface mining operations. Suncor is a pioneer oil company that started its reclamation practices in 1971 by the revegetation of dry lands that were created using stripped overburden material. From its opening in 1967 to the end of 2010, Suncor has disturbed approximately 19,737 km^2 of land, having reclaimed 7% of the total (1,294 km^2) with erosion control as its primary goal (Anderson et al., 1998; Suncor, 2011). The reclamation practice of the company involves the seeding of grasses and legumes that can grow quickly to change the landscape, providing vegetative cover for bare soils (Johnson, 2008). Currently, Suncor and the other oil companies operating in Athabasca are working on developing self-sustained ecosystems, in addition to controlling erosion, by following the steps found in the guidelines for the reclamation of disturbed lands in Athabasca created by the OSVRC (1998) that involves the use of native tree species of the boreal forest to accelerate the formation of new ecosystems.

1.3.3) Re-shaping landscapes

Although the potential for reclaiming disturbed lands is theoretically possible, changes in soil texture and structure, the mixing of geological layers, presence of contaminants and reduction of water infiltration are significant obstacles to this goal (Singh et al., 2002). Reclamation prescriptions are based on the creation of a new forest floor that will act as a structure-holding cover for the OSPM destined for reclamation purposes. The OSPM (see section 1.2.2) will act as building materials or parent materials, in the construction of new natural-like structures, while a peat-mineral mix, originally obtained from the removed forest floor, acts as the new soil cover. The peat-mineral mix becomes the main source of organic matter and nutrients for the revegetation process (Danielson et al., 1983); seeds, grasses, woody and root debris, and an indigenous microbial community are found in this new surface, meaning there is a great potential for a new ecosystem to develop (OSVRC, 1998). Revegetation practices take place once the new natural-like structures have been constructed and seeded with grasses and legumes, followed by the planting of seedlings of selected tree species.

1.3.4) Revegetation in Athabasca

The revegetation of reclamation sites in Athabasca involves two major steps: 1) erosion control with grasses and legumes, and 2) the planting of selected tree species. Tree planting in particular is thought to have major effects on the soil as it may shorten the recovery time due to how trees affect the physicochemical characteristics, nutrient cycles and microflora in the soil. Only Canadian boreal tree species native to the undisturbed lands of Alberta can be used for revegetation, as established by the OSVRC (1998). Trees commonly found growing in the areas surrounding the mining sites include white spruce (*Picea glauca*), trembling aspen (*Populus tremuloides)*, jack pine (*Pinus banksiana*), poplar (*Populus balsamifera*), black spruce (*Populus mariana*), balsam fir (*Abies balsamea*) and tamarack (*Larix laricina*) (Gosselin et al., 2010; Lazorko, 2008; Rowe, 1972). Some of these species have been extensively used in reclamation practices. One good example is the coniferous tree jack pine (*Pinus banksiana*), which was first found to be a good candidate for the remediation of tailing sands by successfully growing in shallow overburden and peat-overburden (over tailings). Nitrogen was found to be a limiting factor for its growth, but P was not (Danielson et al., 1983), thanks to a symbiotic association with mycorrhizal fungi that were present in the substrates. A more recent study (Bois et al., 2005) corroborates these findings by demonstrating how jack pine survival could be ensured if inoculated with the appropriate mycorrhizal symbiont prior to planting. Other species that have been evaluated for reclamation and have been identified as potentially good candidates include the actinorhizal trees silverberry (*Elaeagnus commutata*) the buffalo berry (*Shepherdia canadensis*)

(Visser et al., 1991), and more recently alders (*Alnus* spp.) (Lefrançois et al., 2010). Visser et al. (1991) tested the dual inoculation of silverberry and buffalo berry with mycorrhizal fungi and *Frankia* for use in the revegetation of tailing sands. The experiment showed that after two years of growth in the field, overall growth performance (shoot height and weight, root weight, nodule weight and root length) of the inoculated plants was up to six times greater than from non-inoculated plants and these results were highly correlated with the presence of nodules. Lefrançois et al. (2010) obtained similar results, finding that the presence of *Frankia*-inoculated alders boosted the metabolic activity of the indigenous soil microflora and increased the number of PAH degraders.

1.4) Alders

1.4.1) Description and characteristics

Alder is the common name given to a group of plants that belong to the genus *Alnus*, which is part of the *Betulaceae* family. The group is comprised of trees and shrubs that are found in nature in symbiotic relationships with the nitrogen-fixing actinobacterium *Frankia* (Akkermans and Van Dijk, 1976). Alders are found within the phylogenetically diverse group of "actinorhizal plants", in which members are characterized by their engagement in symbiotic relationships with *Frankia* strains. The actinorhizal group is comprised of plants belonging to eight families, 25 genera, and over 200 species (Lechevalier, 1994).

Certain species of alders are natives of the Canadian Boreal forest, including two that were used in this study (*Alnus viridis ssp crispa*-green alder and *Alnus incana spp.*

rugosa-grey alder), and grow naturally in the areas surrounding the oil sands. These native plants are included among the species that can be used for the revegetation of reclamation sites in Athabasca (OSVRC, 1998).

1.4.2) Importance of alders

When it comes to the revegetation of reclamation sites in the Athabasca oil sands region, selected plant species should be capable of surviving the harsh conditions found in these new environments: high salt content, alkaline pH, low levels of available nutrients and residual hydrocarbons. Alders have proven to be a good choice for the task. Studies have demonstrated that in their presence, the rehabilitation of highly altered lands is possible since they can accelerate the recovery of soil and reduce the concentration of hydrocarbon contaminants. From a selected group of boreal species, alders performed the best when growing in controlled saline-sodic conditions (Khasa et al., 2002). They have been used to aid hardwood species in the revegetation of coal mine spoils in Europe; here, alders decreased the pH of the soil while positively affecting the nutrient content of the soil matrix by accelerating the accumulation of essential nutrients: carbon, nitrogen and phosphorus (C, N and P). Interplanted species benefited from the N input that alders provided through the decay of leaf litter (Plass, 1977; Šourková et al., 2005). More importantly, their potential to remediate hydrocarbon-contaminated sites was first reported by Godwin and Thorpe (2000), and more recently by Greer et al. (2005) and Lefrançois et. al. (2010), who demonstrated that rhizoremediation with alders is a good strategy for the decontamination of hydrocarbon-enriched materials.

1.5) *Frankia*

1.5.1) Description and characteristics

Frankia is the only genus within the *Frankiaceae* family. Its members are characterized as sporulating filamentous *Actinobacteria* that are capable of fixing atmospheric nitrogen (N_2), either as free-living microorganisms (saprophytic) (Chaia et al., 2010) or in symbiosis with actinorhizal plants (Wall, 2000). Current classification of the genus *Frankia* was developed based on comparison of the complete 16S rRNA gene sequence of representative strains (Normand et al., 1996). The classification is comprised of three major groups or clusters, which are: group 1 (strains infective for the *Betulaceae*, *Myrica* and *Casuarina* families), group 2 (non-isolated strains infective for *Rosaceae*, *Datiscaceae*, *Coriariaceae* and *Rhamnaceae* families) and group 3 (strains infective for the *Elaeagnaceae* family) (Benzon et al., 2004). Strains that naturally infect alders fall into group 1 and are known as *Frankia alni* infective strains (Normand et al., 2007).

1.6) Alder-*Frankia* symbionts

1.6.1) Colonization of *Frankia* in alder roots

Frankia strains belonging to the *Frankia*-alni infective group colonize alders by root hair infection (Berry and Torrey, 1983; Berry et al., 1986; Valverde and Huss-Danell, 2008). The process consists of the interaction of hyphae cells of saprophytic *Frankia* with root hairs of the plant. The interaction promotes the deformation of the root hairs, their branching and curling, followed by the hyphae entering through the hairs and becoming entrapped within the root's structure. During the infection process, plant cells grow and surround the hyphae in a process known as encapsulation. *Frankia* remains encapsulated from this point on leading to the formation of prenodules. From here, and under N limited conditions, *Frankia* hyphae start to differentiate into specialized cells called vesicles that contain the nitrogen fixing enzyme dinitrogenase (Meesters et al., 1987; Tjepkema and Winship, 1980), which is extremely sensitive to oxygen (O_2). Inactivated vesicle cells keep the enzyme isolated from exposure to O_2 via a thick layer of lipids. The differentiation into vesicles leads to the complete formation of mature nodules: plant organs that resemble lateral roots where N fixation takes place and that, in the case of alders, are perennial (Lechevalier, 1994). Only infective and effective *Frankia* strains can promote the formation of N_2-fixing active nodules (Baker and Torrey, 1980).

1.6.2) N_2-fixation in root nodules

As a free-living microorganism, *Frankia* takes up nutrients from the soil and fixes N_2 for its own growth, but in symbiosis, the metabolism of both *Frankia* and its host are modified (Valverde and Huss-Danell, 2008). The development of nodules and the activation and maintenance of the dinitrogenase enzyme are dependent on the supply of organic C compounds obtained during photosynthesis by the plant (Gordon and Wheeler, 1978). This C supply travels through the phloem and reaches the roots and nodules. At the same time, easily assimilated N, coming from the nodules, travels through the xylem up to the rest of the plant. This N supply is then transformed into ammonium (NH_4^+), which is taken up by the plant and enhances photosynthetic activity. Afterwards, part of this N is sent to the nodules where *Frankia* make use of it. This continuous cycle ensures the exchange of nutrients between host and symbiont (Bethlenfalvay et al., 1978).

CHAPTER TWO: Materials and Methods

2.1) Plant age, nitrogen input and inoculation effects on the production and development of alder-*Frankia* symbionts.

2.1.1) Experimental set-up

The experiment lasted 19 weeks and was divided into four stages: pre-inoculation, inoculation, post-inoculation and harvesting. The seeds of *Alnus viridis ssp. crispa* (subsequently referred to as *A. crispa*) from Obed Summit, Alberta, were obtained from the National Tree Seed Centre, Fredericton, New Brunswick. (#8360546.3), and had a germination rate of 57.5%. Root trainers (8 x 4 model) from Beaver-plastics (Spencer-Lemaire Industries Ltd; Edmonton, Alberta) were used to contain the medium where seedlings of *Alnus crispa* grew throughout the experiment. The root trainers were placed in plastic trays to provide support and to avoid excessive water loss. The soil-medium was a 3:1 peat moss-vermiculite mixture, previously found to be suitable for the germination of alder seeds (Berry and Torrey, 1985; Stoeckeler, 1949). Tap water was employed to water the seeds and seedlings. Plant age treatments, also referred to here as planting groups, were obtained by sowing three batches of seeds at different times. There were five N treatments (A-E), which consisted of Hoaglands solution (Hoagland and Arnon, 1950) containing three different N concentrations (100ppm N, 10ppm N and 0ppm N) with sodium nitrate ($NaNO_3$) as the N source. Each N treatment had a specific application pattern, further detailed in Table 1. The N treatments are referred to as fertilizers. Alder seedlings were inoculated (at the inoculation stage) with *Frankia* strain AvcI1, designated here as F9 (Greer et al., 2005).

Fertilizers			
N treatments	N concentration	Application pattern	
A	100ppm	Continuing	Pre-inoculation/Post-inoculation
B	100ppm	Pre-inoculation	Pre-inoculation only
C	10ppm	Continuing	Pre-inoculation/Post-inoculation
D	10ppm	Pre-inoculation	Pre-inoculation only
E	0ppm	Control	Pre-inoculation only

Table 1. Fertilizer treatments. Description of the N treatments applied in the experiment and their application patterns.

2.1.2) Pre-inoculation stage

The pre-inoculation stage lasted 11 weeks, covering the sowing of the first batch of seeds until the day before inoculation with F9. A starting group was set, which consisted of the assembly of the root trainers in a way to obtain the three planting groups: P1, P2 and P3 (rows), each exposed to the five N treatments A-E (columns) (Figure 2.) The soil-medium was moistened with tap water a few days before planting of the seeds. For planting P1, 5-6 seeds per root trainer cell were put directly onto the soil-medium and monitored to record the time that it took for most of the seeds to germinate (one week). At this point the process was repeated two more times for the second and third batch of seeds, obtaining the planting groups P2 and P3. Application of the fertilizers and watering of the plants followed a weekly-interspersed pattern. Fifteen mL of N solution was applied to the seedlings using pre-sterilized 30 mL syringes, a different one for each of the solutions (a new syringe was used on each fertilization-day). Tap water was initially applied with a plastic atomizer for a gentle watering of the seeds and young seedlings. Once seedlings were well rooted, a plastic can was used for the watering. On weekdays seeds and young seedlings were watered once or twice a day. On weekends, water was poured into the trays for slow absorption. This watering regime was necessary to avoid desiccation problems.

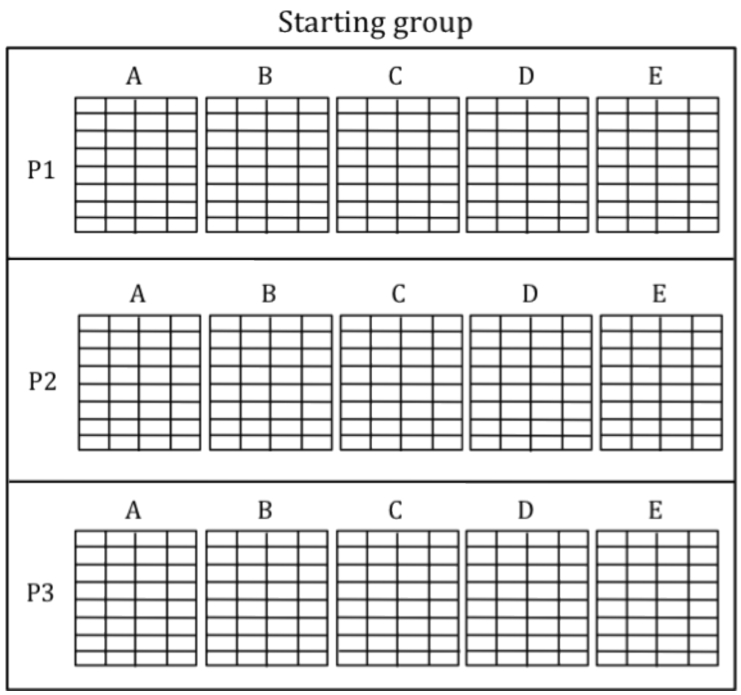

Figure 2. Setting up of the starting group. Eight rows of root trainers of four cells each were used to assemble the starting group. The plantings: P1, P2 and P3, were exposed to five different N treatments (A-E, see Table 1).

2.1.3) Inoculation

The day before inoculation the groups: F9 inoculated seedlings (F9-In) and control seedlings (Ni) were created, by splitting the starting group in two (Figure 3). Seedlings were thinned, leaving one per root trainer cell to ensure effective infection and plant development. The new groups were kept well separated from one another to avoid cross-contamination, and water was poured into the trays to avoid desiccation. Inoculation was performed on week 11 as follows: *Frankia* F9 was grown in liquid Qmod B medium (Schwencke, 1998) for ca. 6 weeks at 27°C. The culture was concentrated by centrifugation, transferred into a sterile Falcon tube, sonicated at 1 sec intervals for 12 secs, and the tube was then centrifuged. The supernatant was removed and the pellet transferred to a graduated sterile tube containing a small portion of the supernatant. The tube was centrifuged and the volume (μl) of packed cells (pcv) was determined. Inoculum was diluted with sodium pyrophosphate (0.1%) to a target of 0.4 μl pcv/ml and 5 ml was dispensed per plant. Plants were lightly watered after inoculation to insure inoculum penetration into the root zone. Non-inoculated plants were kept separated from inoculated plants at all times. Alder seedlings were 11 (P1), 9 (P2) and 7 (P3) weeks old when inoculation was performed.

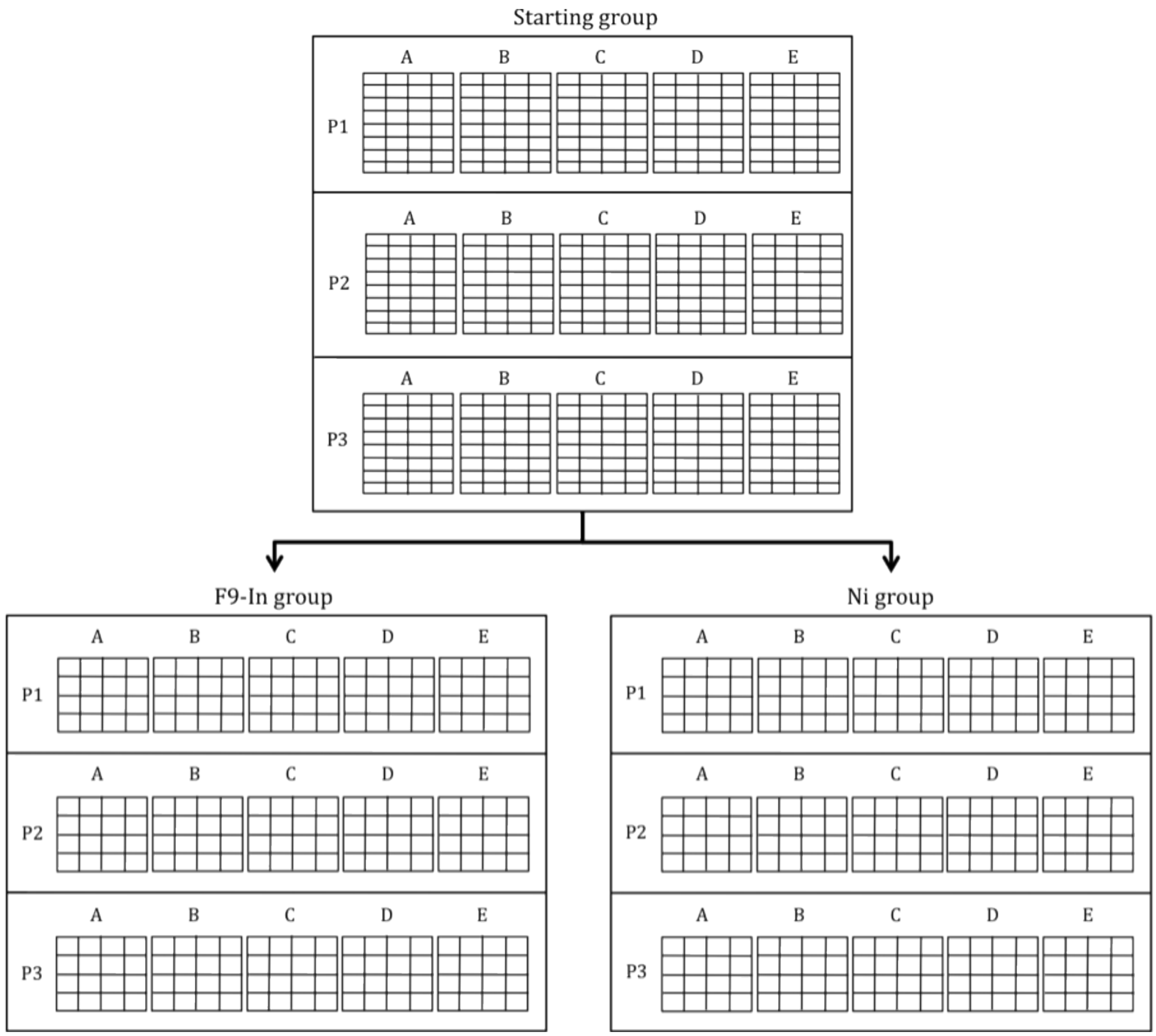

Figure 3. Experimental design. The starting group was split in two: the F9-In group and the Ni group, each of which received a different plant age (plantings P1, P2 and P3) and N treatments (A-E, see Table 1).

2.1.4) Post-inoculation stage

The post-inoculation stage lasted 8 weeks. From this point, watering and fertilization were restarted on weeks 12 and 13, respectively. To reduce the risk of contamination, watering and fertilization were performed starting with the Ni group each time.

2.1.5) Harvesting

Harvesting was carried out during week 19, 8 weeks after inoculation. Seedlings were sacrificed by taking them out of the root trainers and gently removing the soil from their thin roots, followed by washing them with running tap water. By the end of the experiment seedlings were 19 (P1), 17 (P2) and 15 (P3) weeks old.

2.1.6) Data collected

Two groups of data were collected: the first at the end of the pre-inoculation stage, and the second at harvesting. Fifteen different treatments were obtained at the end of the pre-inoculation stage, consisting of the pairing of the three planting groups with the five N treatments (Table 1). To obtain the first group of data, the number of root trainer cells containing plants at the end of the pre-inoculation stage was recorded. At harvesting, a total of 30 different plant age-N input-inoculation treatments were obtained (Table 2). From the harvested seedlings, the following parameters were measured: nodulation, shoot height and health status. Nodulation was determined by examining the presence/absence of nodules on each seedling and classifying them into one of two categories: nodulated (Nod-1), and not nodulated (Nod-0); shoot height was obtained by measuring each seedling in cm from the root collar to the tip bud, without counting the leaves; for the parameter health status, seedlings were identified as belonging to one of three health status categories: green and healthy seedlings (HS-1), green-yellowish seedlings (HS-2), dried or dead seedlings (HS-3). Green foliage indicated either good N_2 fixation capacity, or that non-nodulated plants were using the N provided during treatment.

Green-yellowish seedlings indicated poor N_2 fixation capacity or exhaustion of the N source provided during fertilizer treatment. Dried or dead seedlings indicated that the conditions were unfavorable and/or that the concentration of N provided had a toxic effect on the seedlings (Mackay et al. 1987).

First group of treatments		
Plant age	N input	Treatments
P1	A: 100ppm N/100ppm N	P1A
	B: 100ppm N/ X	P1B
	C: 10ppm N/10ppm N	P1C
	D: 10ppm N/X	P1D
	E: 0ppm N/X	P1E
P2	A: 100ppm N/100ppm N	P2A
	B: 100ppm N/ X	P2B
	C: 10ppm N/10ppm N	P2C
	D: 10ppm N/X	P2D
	E: 0ppm N/X	P2E
P3	A: 100ppm N/100ppm N	P3A
	B: 100ppm N/X	P3B
	C: 10ppm N/10ppm N	P3C
	D: 10ppm N/X	P3D
	E: 0ppm N/X	P3E

Table 2. The different treatments obtained from the combination of the factors plant age and N input at the pre-inoculation stage.

Second group of treatments					
F9-In (F)			Ni (N)		
Plant age	N input	Treatments	Plant age	N input	Treatments
P1	A: 100ppm N/100ppm N	FP1A	P1	A: 100ppm N/100ppm N	NP1A
	B: 100ppm N/ X	FP1B		B: 100ppm N/ X	NP1B
	C: 10ppm N/10ppm N	FP1C		C: 10ppm N/10ppm N	NP1C
	D: 10ppm N/X	FP1D		D: 10ppm N/ X	NP1D
	E: 0ppm N/X	FP1E		E: 0ppm N/X	NP1E
P2	A: 100ppm N/100ppm N	FP2A	P2	A: 100ppm N/100ppm N	NP2A
	B: 100ppm N/ X	FP2B		B: 100ppm N/ X	NP2B
	C: 10ppm N/10ppm N	FP2C		C: 10ppm N/ 10ppm N	NP2C
	D: 10ppm N/X	FP2D		D: 10ppm N/ X	NP2D
	E: 0ppm N/X	FP2E		E: 0ppm N/X	NP2E
P3	A: 100ppm N/100ppm N	FP3A	P3	A: 100ppm N/ 100ppm N	NP3A
	B: 100ppm N/X	FP3B		B: 100ppm N/ X	NP3B
	C: 10ppm N/10ppm N	FP3C		C: 10ppm N/ 10ppm N	NP3C
	D: 10ppm N/X	FP3D		D: 10ppm N/ X	NP3D
	E: 0ppm N/X	FP3E		E: 0ppm N/X	NP3E

Table 3. The different treatments obtained from the combination of the factors plant age, N input and inoculation at harvesting.

2.2) Applying alder-*Frankia* symbionts for the remediation and revegetation of oil sands processes affected materials (OSPM)

2.2.1) Description of the experimental area

The overburden reclamation site: Millenium Dump 5 (MD5) was created in 2008 and finished in the spring of 2009, encompassing 160 hectares. The site consisted of 1 m of saline-sodic overburden (parent material) covered with a 30 cm layer of peat-mineral mix (soil cover) and was seeded with barley on the slopes for erosion control. Although current reclamation practices by Suncor Energy Inc. include the fertilization of the soil cover once it has been added to the reclamation site, the area designated for the experiment was not fertilized.

2.2.2) Preparation of the alder-*Frankia* symbionts

2.2.2.1) Greenhouse

In January of 2009, ~1500 seedlings each of *Alnus viridis ssp. crispa* (seedlot # 8360546.3) and *Alnus incana ssp. rugosa* (subsequently referred to as *A. rugosa*) (seedlot # 8431680) were grown in a 50/50 peat-vermiculite mixture under greenhouse conditions in Bonnyville, Alberta. The seeds were obtained from the National Tree Seed Centre, Fredericton, NB, Canada.

2.2.2.2) Inoculation with *Frankia* AvcI1 (F9)

In March of 2009, when the plants were at the 4-6 leaf stage, half of the seedlings of *A. crispa* and half of *A. rugosa* were inoculated in the greenhouse with *Frankia* F9 as previously described in section 2.1.3 (Figure 4a) to generate inoculated (F9-In) and non-inoculated (Ni) plants: F9-In *A. rugosa* (RF); F9-In *A. crispa* (CF); Ni *A. rugosa* (R) and Ni *A. crispa* (C). F9-In and Ni seedlings were grown and hardened in separate greenhouses from March until June of 2009 (Figure 4b).

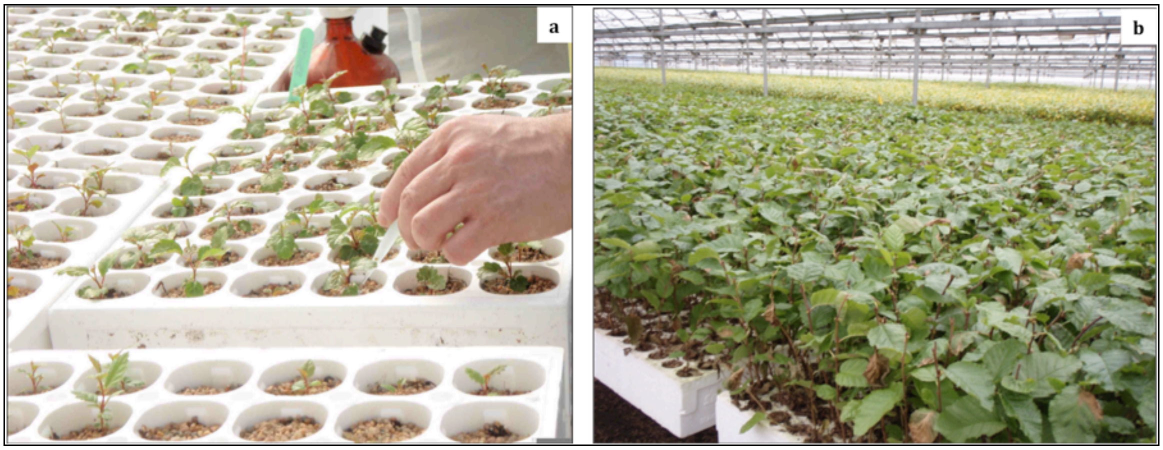

Figure 4. Inoculation and growth. (a) Seedlings of alders were inoculated with F9 and (b) growth and hardening in the greenhouse from March until June of 2009.

2.2.3) Fieldwork

2.2.3.1) First sampling

The experimental area consisted of two plots: the F9-In plot and the Ni plot, separated from each other by >300 m. Each plot was divided into 6 subplots and had an adjacent area that remained unplanted (UF9 and UNi). Before out-planting, seedlings of RF, CF, R and C (~13, each) were taken from the greenhouse and kept for weight measurement, N content analysis of their aerial parts, and separation of the rhizosphere (RHZ) from roots. A total of 6 samples of pre-planted soil (bulk soil) were obtained from the F9-In and Ni plots. The bulk soil samples (BK) were taken by combining 5 sub-samples and mixing them thoroughly to obtain a final composite sample. *Alnus rugosa* and *Alnus crispa* subplots were randomly arranged in the F9-In and Ni plots (Figure 5).

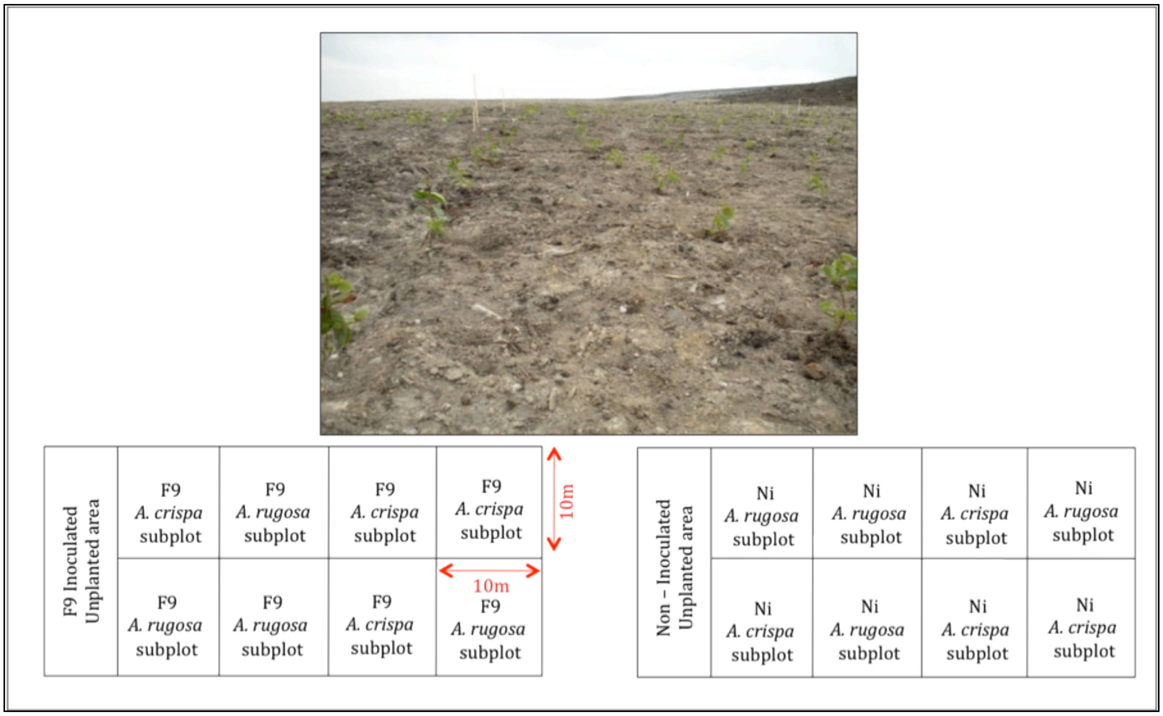

Figure 5. First sampling and out-planting of alder seedlings. Experimental area following planting; schematic arrangement of the subplots in each planting area.

2.2.3.2) Second sampling

Due to heavy rains, during the summer of 2010 three subplots from the F9-In plot were lost (Figure 6a). The second sampling was performed in September of the same year (S10). A considerable number of the remaining plants from each subplot were measured by height and root collar diameter to calculate their seedling volume index (SVI). Thirteen plants from each subplot were taken for weight and chemical measurements of their aerial parts and separation of the RHZ from their roots. BK and RHZ samples from the Ni plot were combined to balance the number of samples between subplots, as follows: C9&C13, R19&R11, R12&R16 and C15&C14 (Figure 6b).

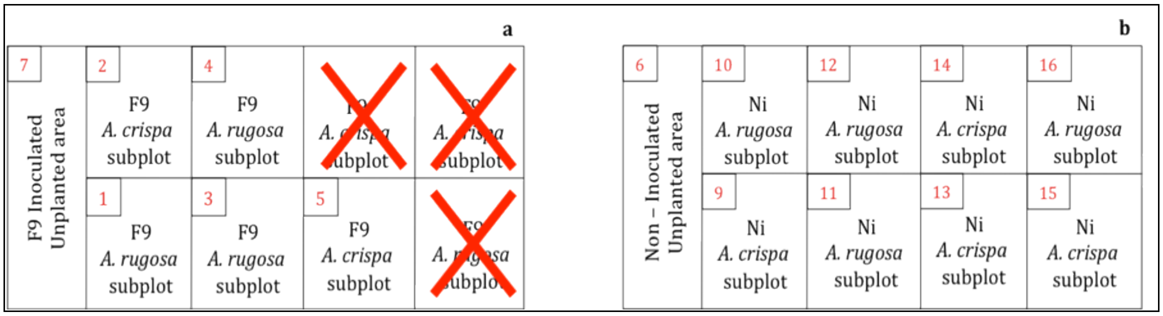

Figure 6. Experimental area in S10. (a) Three subplots from the F9-In plot were lost during the summer of 2010 due to heavy rains. (b) Subplots and unplanted areas were numerated for working purposes.

2.2.3.3) Third sampling

In September of 2011 (S11), no plants were sacrificed. Alders were measured in the field by height and root collar diameter to calculate their SVI. For the sampling of BK, four samples were obtained from each of the remaining treatments and three samples were taken from the unplanted areas

2.2.4) Laboratory work

2.2.4.1) Soil samples and nutrient content in plant tissue

Soil samples

One hundred grams dry weigh of soil from each BK sample was sent to Agridirect (Laboratoire Agridirect. Longueuil, Québec, Canada) for physicochemical analyses. The analyses included the determination of soil texture, particle size and composition, pH, organic matter content, cation exchange capacity, concentration of mineral nutrients, and total N content, amongst others.

Nutrient content in plant tissue

The aerial parts of alders from samplings S10 and S11 were separated from the roots, dried overnight, weighed and sent to Agridirect (Laboratoire Agridirect. Longueuil, Québec, Canada) for total content of the following macronutrients: N, P, Mg, Ca, and K; and trace elements: Zn, Cu, Mn, Fe, and B. seedlings from J09 were analyzed for their total N content only.

2.2.4.2) Preparation of the rhizosphere samples

In order to separate the soil attached to the roots and obtain the RHZ, plants were prepared under aseptic conditions, starting with the Ni alders to avoid contamination. For the J09 samples, roots were placed in sterilized 100 mL beakers while S10 samples were placed in 2 L beakers. Autoclaved MilliQ water was added in a volume equal to 4 the wet weigh of the roots (1 g = 1 mL) and agitated in a shaker at 115 rpm for 1 hour. Following this, roots were removed and put into a non-sterile Ziploc bag and stored at 4°C. If roots were not to be used in the subsequent week they were stored at -20°C. The remaining slurry was poured into two sterile core tubes of 250 mL (J09 samples) and 700 mL (S10 samples) and put into a Beckman centrifuge model J2-21M for 10 minutes. The supernatant was discarded while the saturated soil at the bottom was kept. This was considered to be the RHZ. This last step was repeated until the entire slurry from the beaker had been poured into the core tubes. RHZ samples were stored at 4°C and worked immediately. The samples were only kept for a maximum of 1 week under these conditions.

2.2.4.3) Sterilization of the roots and separation of the nodules

Roots stored at 4°C were used immediately; those stored at -20°C were put at 4°C overnight for thawing. Again, F9-In and Ni samples were worked separately, always starting with the controls to minimize contamination. The roots were put under running distilled water to wash off the remaining attached soil and then put into a clean beaker. In a biohood, under sterilized conditions, the samples went through a sterilization battery consisting of the following steps: 1 min in 100% ethanol, 1 min in 2.5 sodium hypochlorite (NaClO), 10 min in freshly made 2.5 NaClO under gentle shaking, and 1X of 100% ethanol. Roots were then transferred into an Erlenmeyer flask and autoclaved MilliQ water was poured and shaken thoroughly for about 1 minute; this was done 4 times. From the final wash 1 mL was kept for amplification of the bacterial 16S gene to corroborate the effectiveness of the protocol. Still under the hood, nodules (NOD) were removed with the help of sterilized scissors and tweezers, to minimize hand contact with the freshly sterilized roots. NOD were put into a sterile falcon tube and stored at -20°C.

2.2.4.4) Microbiological analyses

2.2.4.4.1) Viable bacterial counts

The Most Probable Number technique (MPN) was used to count the viable bacteria: heterotrophic bacteria (HTB) and polycyclic aromatic hydrocarbon degrading bacteria (PAHB), found in the BK and RHZ. For each test, 10 g of soil was used per sample. The Wrenn and Venosa (1996) procedure was followed with modifications added to the protocol. For both tests, a sterile 96-well plate was used and filled with a specific substrate that would target portions of the bacterial community that were of interest within the samples. For counting of the HTB, YTS_{250} medium was used. Plates were filled with the solution and a suspension of the samples wrapped in aluminum foil and immediately incubated in the dark at room temperature for two weeks. After the incubation period, p-iodonitrotetrazolium solution was added to each well, the plates were wrapped in aluminum foil once again, and incubated for another 24 h. Wells that turned violet were counted as positives. For the counting of PAHB, a solution containing four representative polycyclic aromatic hydrocarbons (PAHs): anthracene, dibenzothiophene, fluorine and phenanthrene, was used. PAH plates were wrapped in aluminum foil and incubated for 3 weeks in the dark at room temperature. No further solutions were added and positive wells developed a yellowish-brown color due to PAH degradation. Data from both tests were fed into a computer program (Klee, 1993) that allowed the calculation of the number of active bacteria found in the samples.

2.2.4.4.2) Mineralization assays

Microbial activity from the indigenous microbial community found in BK and RHZ samples was measured in the ability of microbes to mineralize three representative hydrocarbon substrates: hexadecane, phenanthrene and naphthalene. Microcosms and mini-microcosms were prepared using 20 g of BK from J09 and S10 samples, and 1 g and 10 g of RHZ from J09 and S10 samples, respectively. Samples were tested in triplicates, while one group of triplicated samples was autoclaved twice and used as the negative control. For the setting of the experiment, the protocol described by Greer et al. (2003) was followed. Samples were spiked with 100000 dpm hexadecane-1-C^{14}, 100000 dpm naphthalene-1-C^{14}, or 100000 dpm phenanthrene-9-C^{14}. Microcosms and mini-microcosms were incubated at room temperature in the dark and sampled once a week. Mineralization rate was recorded until microbial activity had reached a plateau phase.

2.2.4.5) Molecular analyses

2.2.4.5.1) Verification of root surface sterility

One milliliter of water obtained from the last step of the sterilization protocol was used to verify the effectiveness of the procedure. The water was boiled for 10 min to release DNA molecules that were possibly present in the water. After this, 1 µL of the suspension was used to perform a 20 cycle touchdown 16S PCR with the bacterial universal primers U431 and U758 (Fortin et al., 2004). PCR products were viewed by running them with a 100 bp ladder in a 1.4% agarose gel stained with SYBR® Safe DNA Gel Stain (Invitrogen Life Technologies, Carlsbad, California, United States). Quantification was performed by employing a Chemiimager (Alpha Innotech, Mississauga, Ontario, Canada) and following the instructions of the manufacturer.

2.2.4.5.2) Extraction of genomic DNA: bulk soil and rhizosphere samples

The 10 g PowerMax™ Soil DNA Isolation Kit (MoBio Laboratories, Carlsbad, California, United States) was used for bacterial genomic DNA extraction from BK and RHZ samples. The extraction was performed following the manufacturer's instructions. On the last step, DNA was re-suspended in TE pH 8.0 (1 mM Na_2EDTA, 10 mM Tris-Cl) and stored at -20°C.

2.2.4.5.3) Extraction of bacterial genomic DNA: crushed nodule-endophytic DNA

A chemical lysis protocol (Fortin et al., 1998) was performed on 5 g of frozen NOD to obtain total endophytic DNA. Frozen NOD were crushed in autoclaved MilliQ water under aseptic conditions inside a biological hood using a sterile frozen mortar and pestle to obtain a final slurry-like suspension. Plant tissue was mechanically broken down in a bead-beating step with 0.1 mm and 1.0 mm zirconium-silica beads that were added to the suspension. Proteins were precipitated with 7.5 M ammonium acetate and DNA material was precipitated with cold isopropanol in an overnight step at -20°C. DNA was then resuspended in TE pH 8.0. DNA extracted from the lysis protocol went through a purification step using PVPP/Sephacryl columns with a solution of TE pH 7.5 NaCl 0.1 M added to the matrix for a better recovery of the DNA.

2.2.4.5.4) DNA extraction effectiveness and bacterial genomic DNA quantification

DNA concentrations in the extracts were estimated by agarose gel electrophoresis of 5 μL of purified material against the λ *HindIII* DNA ladder (Amersham Biosciences, Piscataway, New Jersey, United States) standard, by densitometry using a Chemiimager (Alpha Innotech, Mississauga, Ontario, Canada) and following the manufacturer's instructions.

2.2.4.5.5) 16S rRNA gene amplicon Ion Torrent sequencing

BK, RHZ and NOD samples from J09 and S10 samplings were used to sequence 16S rRNA gene amplicons of the bacterial community and to determine how this had changed over the period of two growing seasons. No samples from S11 were included in this step. The 16S gene PCR amplicons for Ion-torrent sequencing were performed on a 25 μL PCR mix, containing a final concentration of the following reagents (loaded in the same order): 1X of PCR Buffer, 2.5 mM $MgCl_2$, 0.4 mg/mL BSA, 0.5 μM reverse primer U926R-P1, 0.2 mM dNTPs, 0.025 U/μL rTaq Polymerase, 0.5 μM forward primer E786F-MID (a different one for each sample), 1 μL of DNA template and autoclaved Milli Q H_20. The PCR program started at 95°C for 5 minutes; 25 cyles at 25°C for 30 seconds, annealing temperature at 55°C for 30 seconds and 72°C for 45 seconds; 72°C for 10 minutes and finished at 4°C. PCR products were loaded in a 2% agarose gel and run at 70V for 50 minutes, after which bands were cut and DNA was purified with the Qiagen® Qiaquick Gel Extraction Kit (Qiagen, Valencia, California, United States) according to the manufacturer's instructions. Purified PCR products were then quantified with the Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen, Burlington, Ontario, Canada), according to the manufacturer's instructions, and diluted to obtain a stock solution at $5X10^8$ molecules/ μL (~1ng/ μL). A volume of 10 μL of each stock was pooled in a sterile 1.5 mL Eppendorf tube and used for sequencing with the Ion Torrent Personal Genome Machine™ using the Ion XpressTM 186 Template Kit and the Ion 314™ 187 chip following manufacturer's instructions.

2.2.4.5.6) Detection of *Frankia* AvcI1 (F9)

A nested PCR technique was employed for the monitoring and detection of F9 in the NOD of alders. Although the main interest of the experiment was to determine if F9 was part of the indigenous microbial community found in the roots of alders, the procedure was not restricted to NOD but was also performed on BK and RHZ samples for the detection of potential indigenous *Frankia* strains that may have colonized the roots and that could also share some genetic similarities with F9. The technique consisted on the running of two PCRs; one meant to detect members of the *Frankia alni* group, and the other to specifically detect F9. The first PCR employed the primers FGDP807 and FGDK333 (Nalin et al., 1995), which amplify the intergenic spacer region (IGS) between the *nifD* and *nifK* genes of the dinitrogenase enzyme; the PCR mix had a final volume of 50 µL containing 25 ng of DNA template, 25 pmol of each primer, 1.25 mM of dNTPs and 20 mg/uL of BSA. The PCR cycling conditions were as follows: an initial 3 min at 95°C, 40 cycles of 1 min at 95°C, annealing at 63°C for 1 min and 72°C for 2 min, and a final 3 min at 72°C. The second PCR employed the F9-specific primers FRIGS-F (5′-CAG CCG CCA GCG ATC CCG TGA CCC CG-3′), and FRIGS-R (5′-CGC GGG TCC AGT CGA GGA CCC GCT GG-3′) (Greer et al., 2005), which amplify a small portion of ~300 bp from the IGS *nifD*-K. Five µL of the positive products obtained from the first PCR were used as templates. The PCR mix had a final volume of 50 μL and contained the 5 μL of DNA template, 25 pmol of each primer, 100 mM of $MgCl_2$, 1.25 mM of dNTPs and 20 mg/uL of BSA. The PCR cycle consisted of an initial 5 min at 95°C, 25 cycles of 94°C for 1 min, 68°C for 1 min and a final 5 min at 72°C. Verification of PCR products was done by agarose gel as described in section 2.2.4.5.1, without the quantification step.

2.1.7) Statistical Analysis

For the analysis of the data, Analysis of Variance (ANOVA) and Multiple Correspondence Analysis were performed, employing the software IBM SPSS 19 (SPSS Inc., Chicago, Illinois, United States.) and the software XLSTAT 2012 (Addinsoft Inc., New York, New York, United States.).

CHAPTER THREE: Results

3.1) Plant age, nitrogen input and inoculation effects on the production and development of alder-*Frankia* symbionts

3.1.1) Production of pre-inoculated alder seedlings

In the pre-inoculation stage, alder seedlings were exposed to the effects plant age and N input. The analyses showed that plant age (*p*-Value: 0.0001) and N input (*p*-Value: 0.04), significantly affected the production of pre-inoculated alder seedlings, although, independently. At the pre-inoculation stage, each planting group was comprised of 160 root trainer cells, and each N treatment had a total of 96 root trainer cells (Figure 1). Figure 7 shows the production of pre-inoculated alder seedlings, expressed as the total of root trainer cells containing plants from each planting group (Figure 7a) and each N treatment (Figure 7b). It can be observed that plant age treatment P3, yielded more seedlings, and from N input, treatment C produced the most seedlings. However, Duncan's test showed that the plant age, treatments P2 and P3 were statistically similar, while from the N treatments, A-B-D-E and C-B-D-E were statistically similar, but A and C were different.

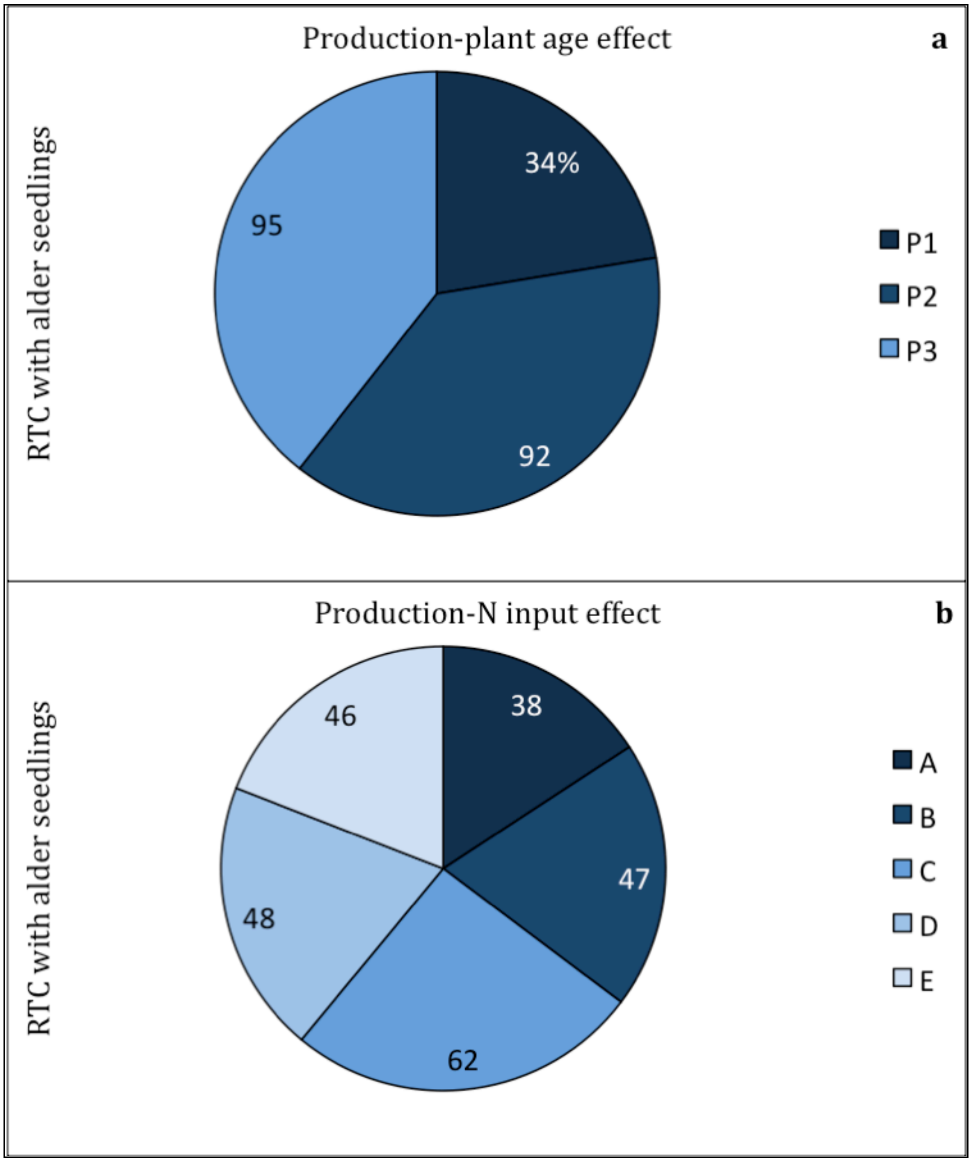

Figure 7. Production of pre-inoculated alder seedlings expressed as the number of root trainer cells (RTC) containing plants at the end of the pre-inoculation stage. (a) Plant age effect, and (b) N input effect.

3.1.2) Nodulation, shoot height and health status

The total number of seedlings analyzed at the end of the experiment and the results for nodulation, shoot height and health status are shown in Tables 6, 7 and 8, respectively.

Nodulation									
F9-In					Ni				
Plant age	N input	Nodules			Plant age	N input	Nodules		
		No. of seedlings	Nod-1	Nod-0			No. of seedlings	Nod-1	Nod-0
P1	Total	32	22	10	P1	Total	22	10	12
	A	4	4	0		A	2	0	2
	B	3	1	2		B	3	0	3
	C	12	4	8		C	6	0	6
	D	5	5	0		D	6	5	1
	E	8	8	0		E	5	5	0
P2	Total	48	30	18	P2	Total	44	26	18
	A	9	3	6		A	6	4	2
	B	11	7	4		B	11	9	2
	C	13	11	2		C	12	8	4
	D	9	6	3		D	9	3	6
	E	6	3	3		E	6	2	4
P3	Total	52	29	23	P3	Total	43	7	36
	A	9	4	5		A	8	0	8
	B	9	3	6		B	10	2	8
	C	11	6	5		C	8	1	7
	D	11	10	1		D	8	1	7
	E	12	6	6		E	8	3	6

Table 4. Nodulation efficiency. Seedlings were classified as Nod-1 for nodules present, and Nod-0 if no nodules were detected.

Shoot height							
F9-In				Ni			
Plant age	N input	No. of seedlings	Shoot height averages (cm)	Plant age	N input	No. of seedlings	Shoot height averages (cm)
P1	A	4	92±14	P1	A	2	82±18
	B	3	84±34		B	3	62±13
	C	12	47±9		C	6	50±10
	D	5	35±6		D	6	35±15
51±26	E	8	36±20	46±20	E	5	31±12
P2	A	9	74±28	P2	A	6	85±21
	B	11	59±24		B	11	55±16
	C	13	45±20		C	12	37±17
	D	9	37±11		D	9	31±14
52±24	E	6	41±9	47±23	E	6	35±6
P3	A	9	81±23	P3	A	8	68±25
	B	9	45±17		B	10	55±20
	C	11	38±13		C	8	31±11
	D	11	32±14		D	8	23±9
43±23	E	12	27±8	43±23	E	8	35±14

Table 5. Shoot height. Seedlings were measured in cm from the root collar to the tip bud.

Health status											
F9-In						Ni					
Plant age	N input	No. of seedlings	HS-1	HS-2	HS-3	Plant age	N input	No. of seedlings	HS-1	HS-2	HS-3
P1	Total	32	7	25	0	P1	Total	22	3	17	2
	A	4	0	4	0		A	2	0	2	0
	B	3	0	3	0		B	3	0	1	2
	C	12	2	10	0		C	6	3	3	0
	D	5	1	4	0		D	6	0	6	0
	E	8	4	4	0		E	5	0	5	0
P 2	Total	48	26	17	5	P2	Total	44	9	28	7
	A	9	3	5	1		A	6	1	2	3
	B	11	8	3	0		B	11	3	5	3
	C	13	10	3	0		C	12	4	8	0
	D	9	4	4	1		D	9	1	7	1
	E	6	1	2	3		E	6	0	6	0
P3	Total	52	16	33	3	P3	Total	42	17	32	1
	A	9	7	1	1		A	8	6	1	1
	B	9	4	3	2		B	10	8	5	0
	C	11	0	11	0		C	8	0	8	0
	D	11	2	9	0		D	8	1	8	0
	E	12	3	9	0		E	8	2	10	0

Table 6. Health status of the seedlings. HS-1: green and healthy seedlings; HS-2: green-yellowish seedlings; HS-3: dried or dead seedlings.

Nodulation was found to depend on plant age (*p*-Value: 0,003) and inoculation (*p*-Value: 0,001). Exposure of the seedlings to different N treatments, on the other hand, had no significant effect on nodulation (*p*-Value: 0,189). Shoot height was found to depend on plant age and N input independent effects (p>Value: 0,0001). The effect of inoculation and the interaction of the three factors were not significant. Comparing the planting groups and N treatments, using the Duncan's test, showed that P1 and P2 were statistically similar, and that both yielded taller seedlings than P3. As for the N treatments, treatment A yielded the tallest seedlings while treatment D yielded the smallest. Treatments D and E were statistically similar, and C, B and A were different. For plant health status, Figure 8 shows the results obtained using MCA. Based on the literature, the presence of nodules was expected to be superior in the F9-In group, as a result of inoculation with F9 and since symbiosis provides an advantage in growth and wellness to the inoculated seedlings, nodulation was included in this analysis to determine if the presence of nodules did have an impact on the health status of the seedlings.

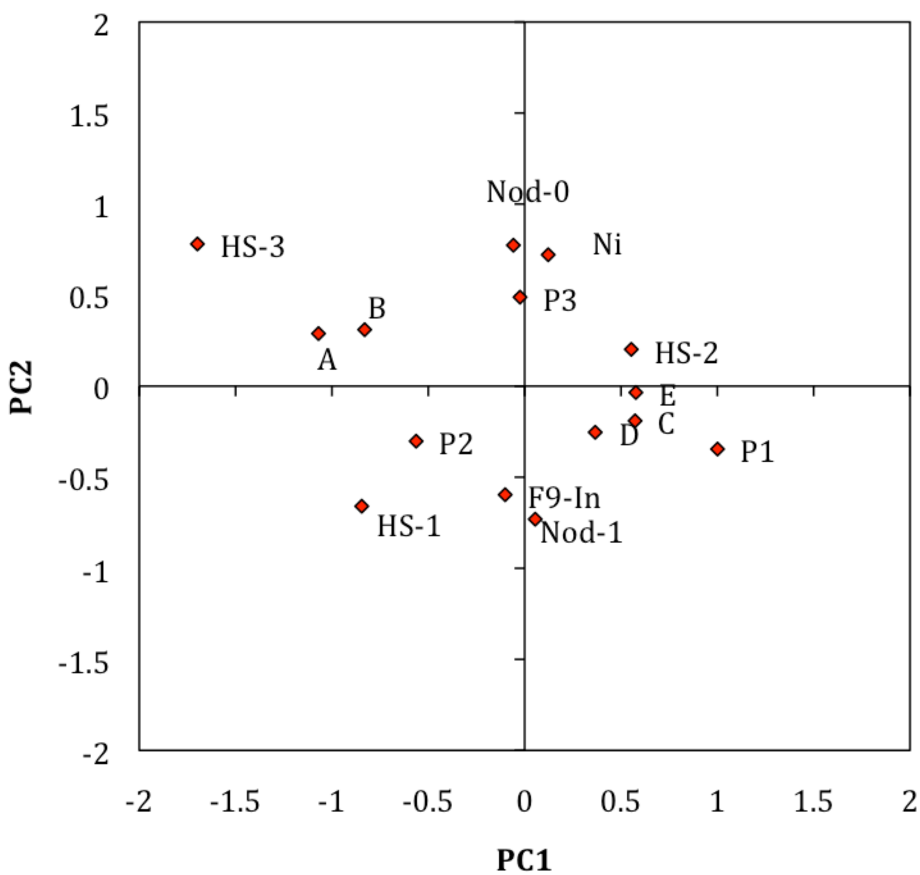

Figure 8. Health status of the seedlings. MCA results show the interaction between plant age, N input, inoculation, nodulation, and health status.

The proximity of HS-1 with the planting group P2 indicates that green-healthy seedlings were produced in greater number here than in groups P1 and P3. HS-2, on the other hand, was more related to the groups P1 and P3, as well as the N treatments D, C and E. These results indicate that young and older seedlings (P3: 7 week-old; and P1: 11 week-old) did not use the N provided through fertilization efficiently, and/or N_2 fixation in nodules was poor; for the concentration of N provided with treatments C and D, these were too low, as they acted the same way as treatment E (0ppm N). As such, it can be said that these treatments did not fulfill the requirements of N for alder seedlings. A strong correlation was observed between inoculation with F9 (F9-In) and the presence of nodules (Nod-1) meaning that inoculation was a determinant step in the production of well-nodulated seedlings. In addition, F9-In and Nod-1 are closer to the planting group P2 than P1 and P3, meaning that inoculation with *Frankia* for 9-week old seedlings was the most effective time to perform inoculation. In contrast, the proximity of the planting group P3 to the non-inoculated condition (Ni) and the absence of nodules (Nod-0) means that inoculation with *Frankia* for 7-week old seedlings do not promote the formation of nodules, therefore it is not recommended. The proximity observed between the N treatments A and B to the categories HS-1 and HS-3 suggest that addition of a high concentration of N (100ppm) either continuously (A) or during the pre-inoculation stage only (B) may promote the over-all wellness of the seedlings but could also have a toxic effect after a certain point, promoting leaf loss, dryness, and the death of seedlings. A rational alternative could be to work with concentrations of N lower than 100ppm, but higher than 10ppm.

3.2) Applying alder-*Frankia* symbionts for the remediation and revegetation of oil sands process-affected materials (OSPM)

3.2.1) Performance of alders and nutrient content in plant tissue.

Both F9-In and Ni seedlings developed NOD in the greenhouse and had NOD after two growing seasons in the field. However, F9-In alders were in better shape than Ni, having longer and thicker roots and more NOD. Figure 9 shows images of the roots of greenhouse and field alders. The seedling volume index (SVI) provided an accurate measure of the performance of alders in the field. Figure 10a shows a view of the symbionts growing in the field in S10 (after 2 growing seasons), and Figure 10b shows the SVI values calculated for samples taken in S10 and S11 (after 2 and 3 growing seasons, respectively). It was found that alder growth was highly dependent on the time of sampling (J09, S10, S11), the condition of the plant (F9-In, Ni), and the interaction effect between the time of sampling and the condition of the plant (*p*-Value: 0.0001). The growth of both alder species (*A. rugosa* and *A. crispa*) was statistically similar. These results indicate that alder-*Frankia* symbionts out performed control alders over time, and that F9 promoted the rapid growth of the plants, and ensured their survival in the field. F9-In alders, CF and RF produced more biomass by almost 3 and 5 times in S10 and S11, respectively, over their counterparts Ni, C and R. For the nutrient content in plant tissue, no significant differences between alder species or condition were observed in the nitrogen content compared to J09 alder seedlings. As for the S10 and S11 samples, it was found that the nutrient content was highly dependent on the type of plant tissue examined: leaves (L) and stem (S), and that differences found in the values were significant for all the macronutrients and trace elements analyzed, being greater in leaves. The levels of Ca, Cu and Fe were significant for the time of sampling, having increased in S11; the levels of Cu and Zn were significantly different between alder species; and only the level of Cu was significant to the condition of the plant. Results on the nutrient content of alder tissue can be observed in detail in Table 12 and Figure 11.

Figure 9. Field performance of the symbionts. (a) Root samples of F9-In, and (b) Ni alders taken from the greenhouse in J09; (c) root samples of F9-In and (d)Ni alders taken from the field in S10.

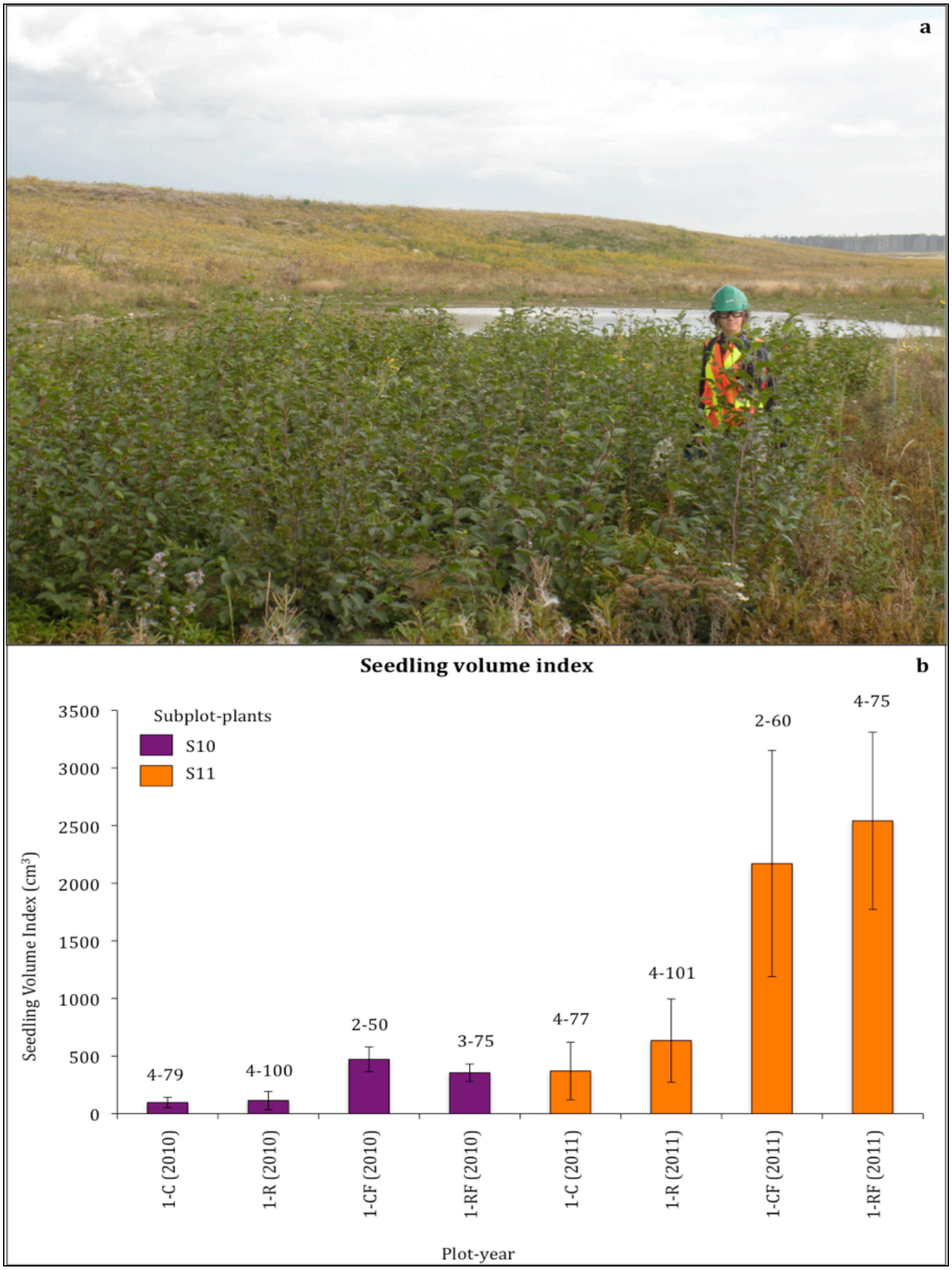

Figure 10. Field performance of F9-In verses Ni alders. (a) View of the symbionts growing in the field in S10 (after 2 growing seasons). (b) SVI: numbers at the top-left of the columns represents the number of subplots analyzed, and the number at the top-right represents the total number of plants that were measured.

Nutrients		Nutrient/element content analysis of plant tissue															
		S10								S11							
		F9-In				Ni				F9-In				Ni			
		RF		CF		R		C		RF		CF		R		C	
		L	S	L	S	L	S	L	S	L	S	L	S	L	S	L	S
MN (%)	N	3±0	1±0	3±0	1±0	3±0	1±0	2.5±0	1±0	2±0	1±0	2±1	1±0	2±0	1±0	2±0	1±0
	P	0±0	0±0	0±0	0±0	0±0	0±0	0±0	0±0	0±0	0±0	0±0	0±0	0±0	0±0	0±0	0±0
	Mg	0±0	0±0	0±0	0±0	0±0	0±0	0±0	0±0	0±0	0±0	0±0	0±0	0±0	0±0	0±0	0±0
	Ca	1±0	0±0	1±0	1±0	1±0	1±0	1.5±0	1±0	1±0	1±0	2±0	1±0	1±0	1±0	2±0	1±0
	K	0±0	0±0	1±0	0±0	1±0	0±0	1±0	0±0	1±0	0±0	0±0	0±0	1±0	0±0	1±0	0±0
TE (ppm)	Zn	36±7	21±1	53±6	28±5	46±8	19±9	46±6	29±6	26±4	16±4	41±18	25±11	34±8	18±4	41±11	29±10
	Cu	8±2	5±1	8±1	6±1	8±2	5±2	6±1	5±1	8±1	6±1	6±1	6±2	8±2	5±1	6±2	5±2
	Mn	84±10	26±4	152±22	47±10	60±31	32±27	96±41	27±8	86±25	33±20	107±94	45±35	83±42	26±11	118±96	44±28
	Fe	469±42	58±9	549±42	90±5	378±32	91±13	569±27	93±18	957±352	227±68	1082±834	321±217	684±120	129±6	992±212	224±67
	B	36±3	9±0	47±11	11±1	34±7	14±6	40±12	12±4	36±5	11±2	38±3	11±1	44±15	11±3	44±10	12±2

Table 7. Nutrient/element content analysis of plant tissue. Macronutrients (MN); trace elements (TE); time of sampling (S10, S11); condition of the plant (F9-In, Ni); alder species (*A. rugosa,* RF/R; *A. crispa*, CF/C); and type of plant tissue examined (L, S).

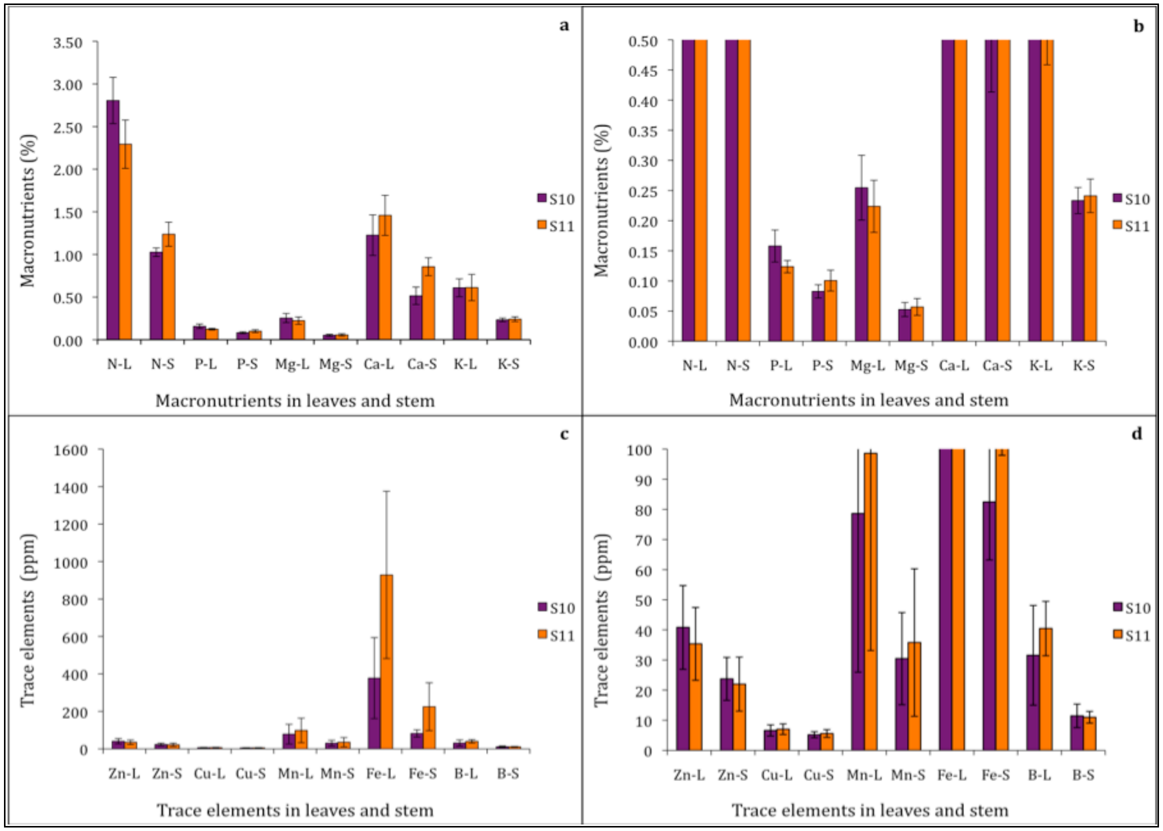

Figure 11. Nutrient content in plant tissue. Leaves (L) and stems (S) were taken for the analyses. (a,b) Macronutrients: N, P, Mg, Ca and K, expressed in percentage (%); and (c,d) trace elements: Zn, Cu, Mn, Fe and B, expressed in parts per million (ppm). The scales for figures b and d were reduced for better comprehension.

3.2.2) Soil quality

The values of the following parameters were found to depend on the soil condition and be significantly lower in soil where F9-In alders were planted, compared to soil with Ni alders and adjacent unplanted areas: CEC, pH, pH buffer, Lime requirement, Ca, Sat Ca, Mg, Zn, Cu, Mn, B and Sat K+Mg+Ca. The parameter Al was also dependent on the soil condition, although it was found to be higher in the F9-In and unplanted soil. The changes registered for the parameters: P, S, Na, electrical conductivity and chloride, were found to be dependent on the time of sampling, having increased, significantly, in S11, in contrast with the values for K and nitrate that were lower. Finally, the total N content in soil was significantly affected by the soil condition and the time of sampling, having increased in S11 and being significantly greater in soil planted with alders, both F9-In and Ni. No significant changes in the values of organic matter content, Sat K, Sat Mg and Fe were found. The soil quality results are presented in Figure 12, where values are expressed as change over time. The results were split in three and scales reduced to facilitate comparison.

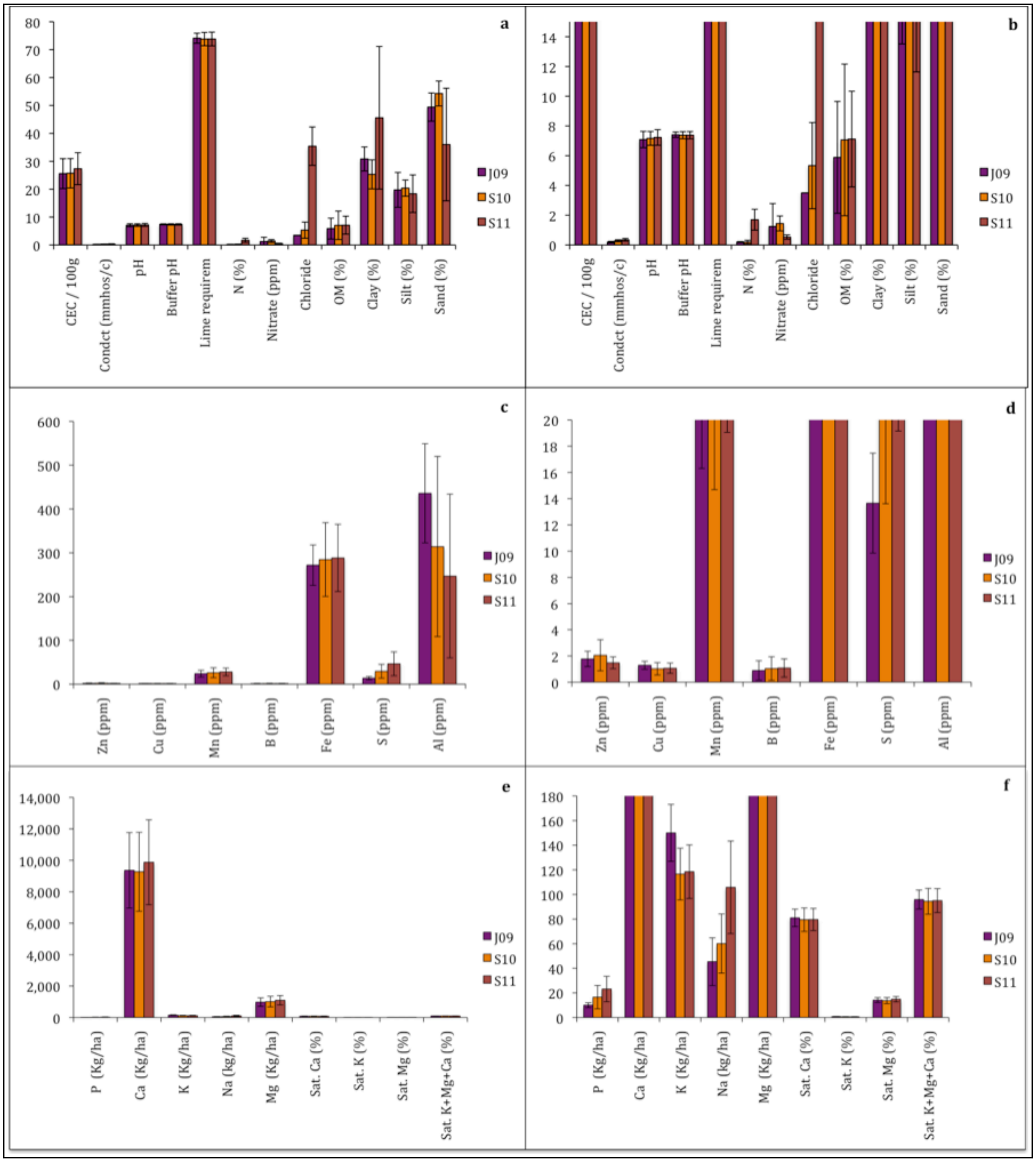

Figure 12. Soil quality results. The values of the parameters evaluated in the study are expressed as the change over time, for samplings J09, S10 and S11. (a,b) parameters: CEC, Electrical conductivity, pH, pH buffer, Lime requirement, N, Nitrate, Chloride, OM, clay, silt and sand; (c,d) parameters: Zn, Cu, Mn, B, Fe, S and Al; (e,f) parameters: P, Ca, K, Na, Mg, Sat. Ca, Sat. K, Sat Mg and Sat. K+Mg+Ca. The scales of graphs b, d and f were reduced for better comprehension. Each figure has a generalized scale (Y axis), since different parameters are shown in each figure. The units of concentration corresponding to each of the parameters are shown in parenthesis (X axis).

3.2.3) Viable bacterial counts

The number of HTB and PAHB was found to be dependent on the type of sample analyzed (BK, RHZ) ($p<0.05$), and not on the time of sampling (J09, S10), nor on the soil condition (unplanted, F9-In, Ni). MPN results are presented in Figures 13 and Figure 14. HTB and PAHB populations were higher in the RHZ of alders than in BK samples from J09 and S10. In addition, HTB and PAHB populations in BK and RHZ samples were higher compared to the populations in the unplanted areas (UF9, UNi). It should be noted that the RHZ samples from J09 represented the time zero of this study, which means that these were samples obtained directly from the greenhouse, where seedlings grew under optimal conditions in a peat-vermiculite mixture, BK samples from S10 turned out to be challenging for reading the MPN test, as by the end of the incubation period it seemed as if no chemical reaction had occurred, making interpretation of the test difficult and so these results are not included in Figure 14. This error was probably due to bad preparation of the test or the PAHB population had a very low density. Considering this, comparisons were made between the RHZ samples from S10 with BK and the RHZ samples from J09. Here, it can be seen that PAHB were found in greater number in the RHZ of alders. However, RHZ samples from J09 had more PAHB but this again, can be related to the optimal conditions alders grew in during the greenhouse period.

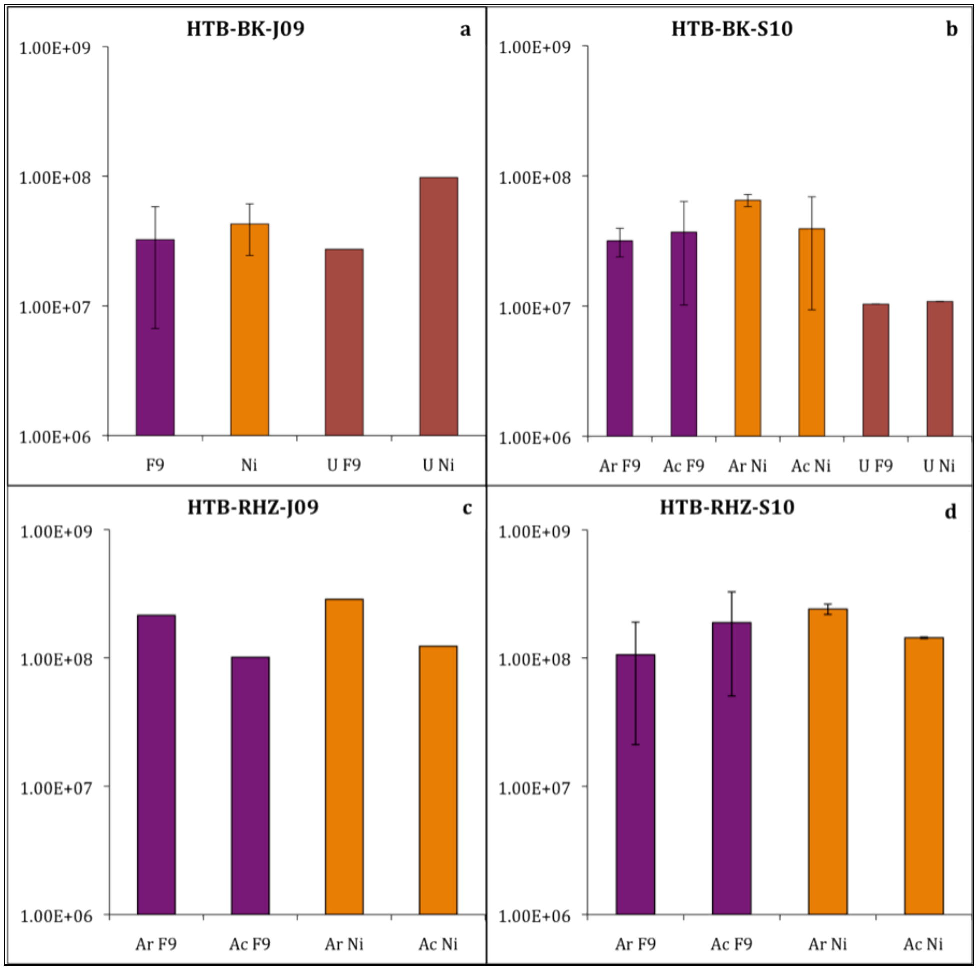

Figure 13. MPN test results for HTB. BK samples initially, J09 (a) and in S10 after 2 growing seasons (b); and RHZ samples initially J09 (c) and in S10 (d).

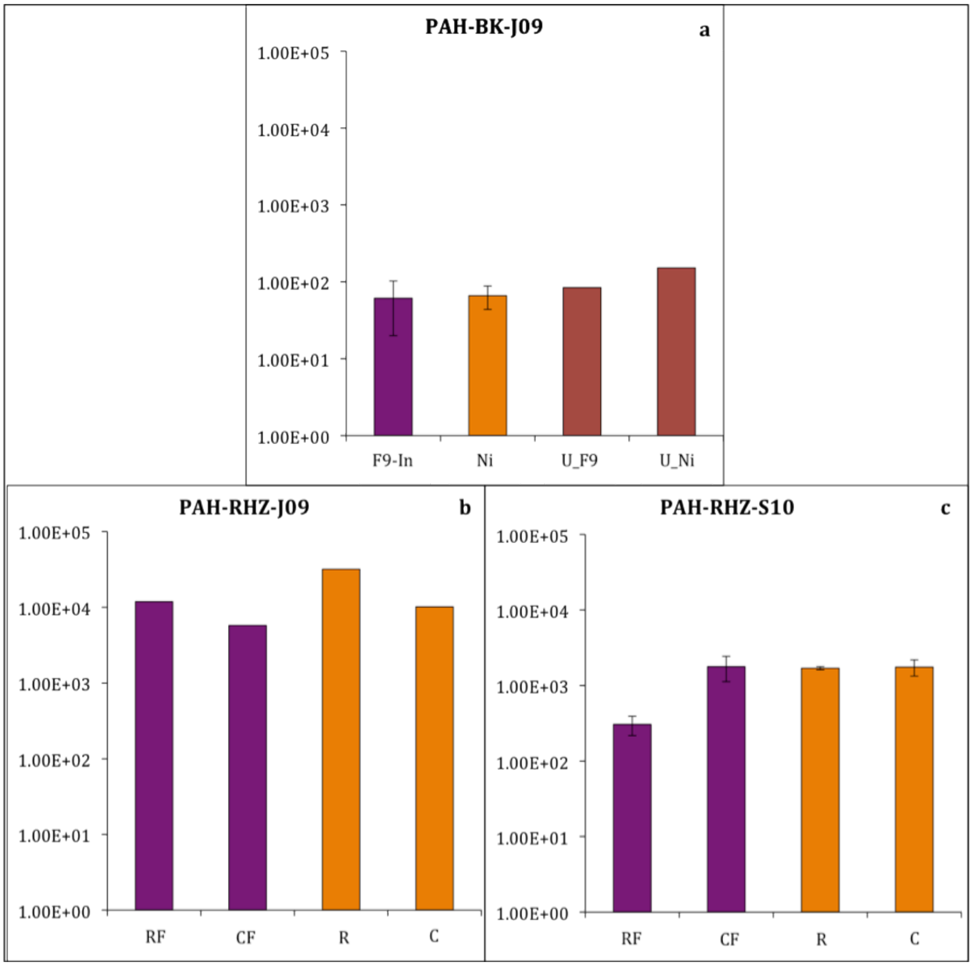

Figure 14. MPN test results for PAHB. BK samples initially, J09 (a); and RHZ samples initially J09 (b) and in S10 (c).

3.2.4) Mineralization assays

Three representative hydrocarbon substrates, hexadecane, phenanthrene and naphthalene, were used to set up microcosms and mini-microcosms containing BK and RHZ samples, respectively, to evaluate the metabolic activity of the indigenous microbial community present in the field soil. It was found that changes in the microbial mineralization activity depended, significantly, on the time of sampling, soil condition and substrate tested (p-Value: 0.0001), and not on the type of sample collected. Duncan's test results showed the mineralization rates were statistically similar in S10 and S11, and higher than in J09; the three substrates were significantly different, with hexadecane showing the highest extent of mineralization, followed by naphthalene, then phenanthrene; no statistical differences were found between soil conditions, meaning that another source of organic carbon, besides deposition through alder leaf litter, root decay and root exudates, was being used by the bacteria, most likely the peat that was included in the site capping process. Figure 15 shows the mineralization rate of each of the substrates at each sampling period.

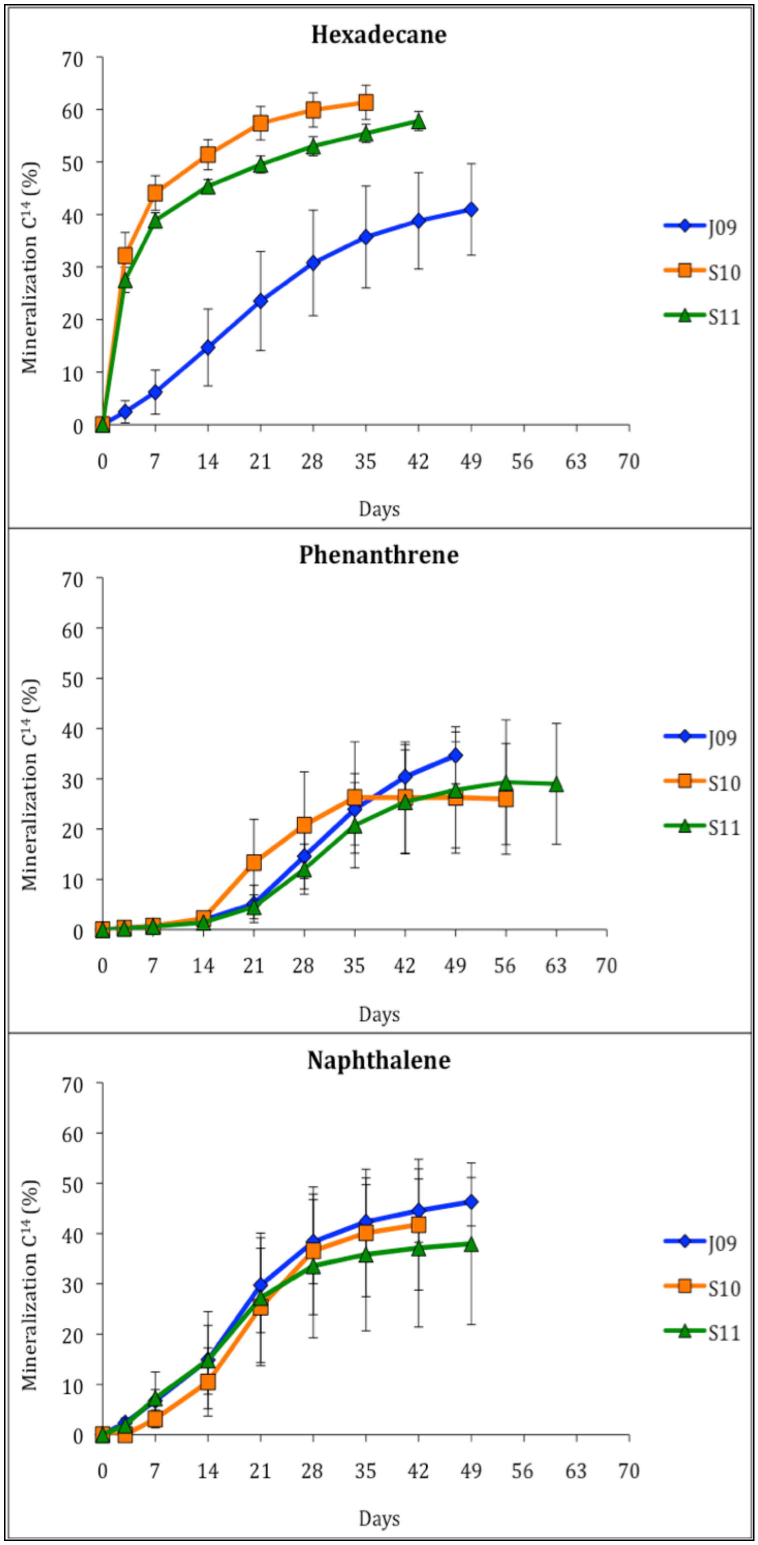

Figure 15. Mineralization of hexadecane, phenanthrene and naphthalene, initially (J09), and after 2 (S10) and 3 growing seasons (S11).

3.2.5) Microbial community structure in bulk soil, rhizosphere and nodules

Ion Torrent sequencing of 16S rRNA gene amplicons was used to examine the composition of the indigenous microbial community present in BK, RHZ and NOD samples, for a better insight into how the bacterial population had changed over time. Figure 16 shows the taxonomic classification, represented as the percentage of bacterial phyla present in each sample and Figure 17 shows the MCA results. To generate the MCA graph, the name of the samples and phyla were re-named for simplicity (Table 8). It can be seen how NOD samples S14-S17 are distant from the rest, clustering together and being proximal to the phylum *Actinobacteria* (Figure 17). This proximity to *Actinobacteria* is the result of the inoculation with F9, since these samples are from nodule tissue in the J09 samples. In contrast, and still proximal to the *Actinobacteria* but also closer to the category Uba are the NOD samples S37-S40, corresponding to nodule tissue of the S10 samples. These results could mean that, once greenhouse-nodulated alders are in the field, other bacteria start colonizing their roots, and might also compete with the inoculated symbiont. Defined clusters were not observed for the BK and RHZ, instead, these are all very proximal to one another, suggesting that over this amount of time in the field, no significant differences in the composition of the microbial community found in the soil attached to alder roots (RHZ) and the surrounding bulk soil (BK) are evident.

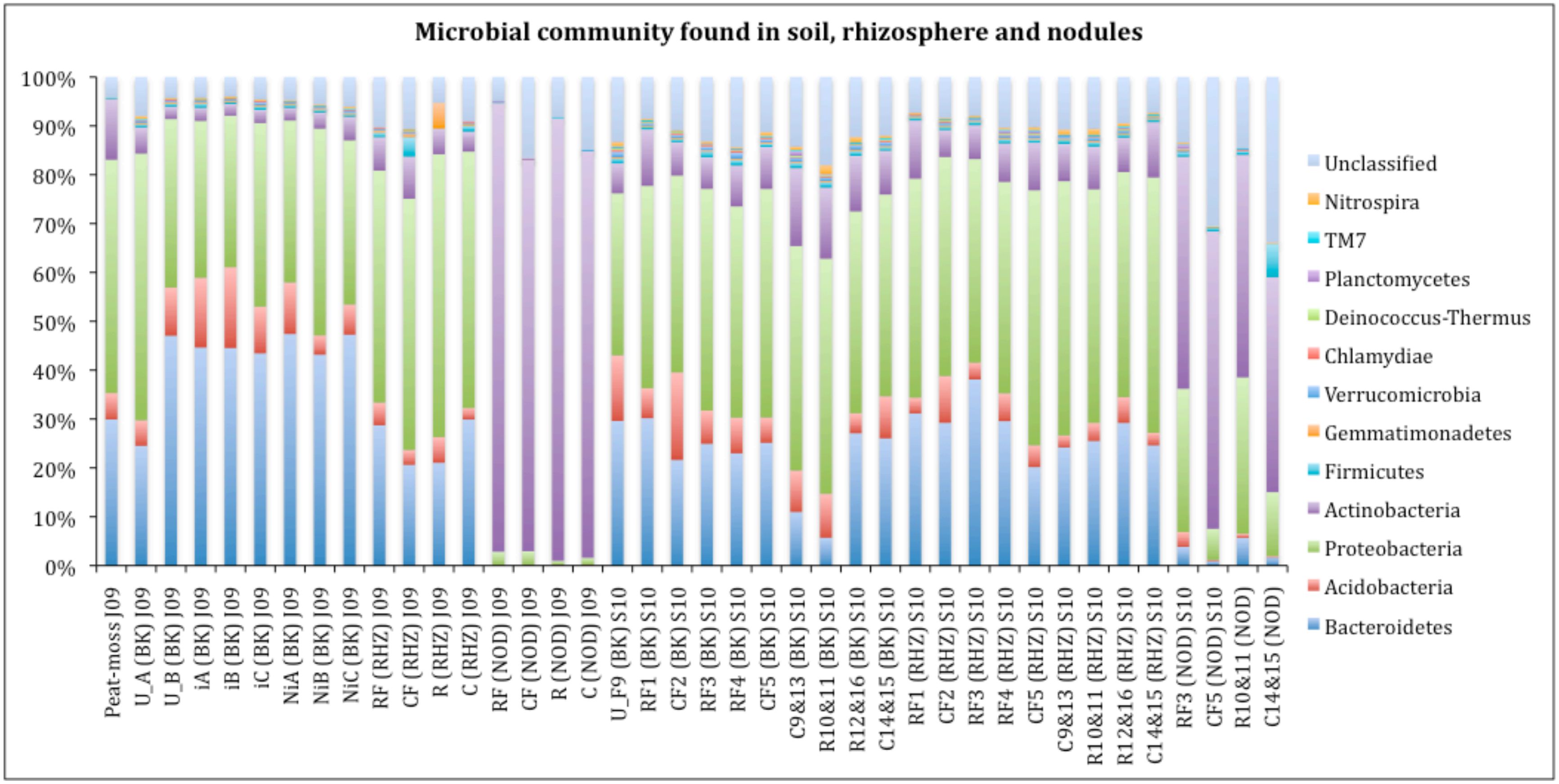

Figure 16. Bacterial community structure of greenhouse and field samples in study. Samples were analyzed Ion Torrent sequencing of 16S rRNA gene amplicons.

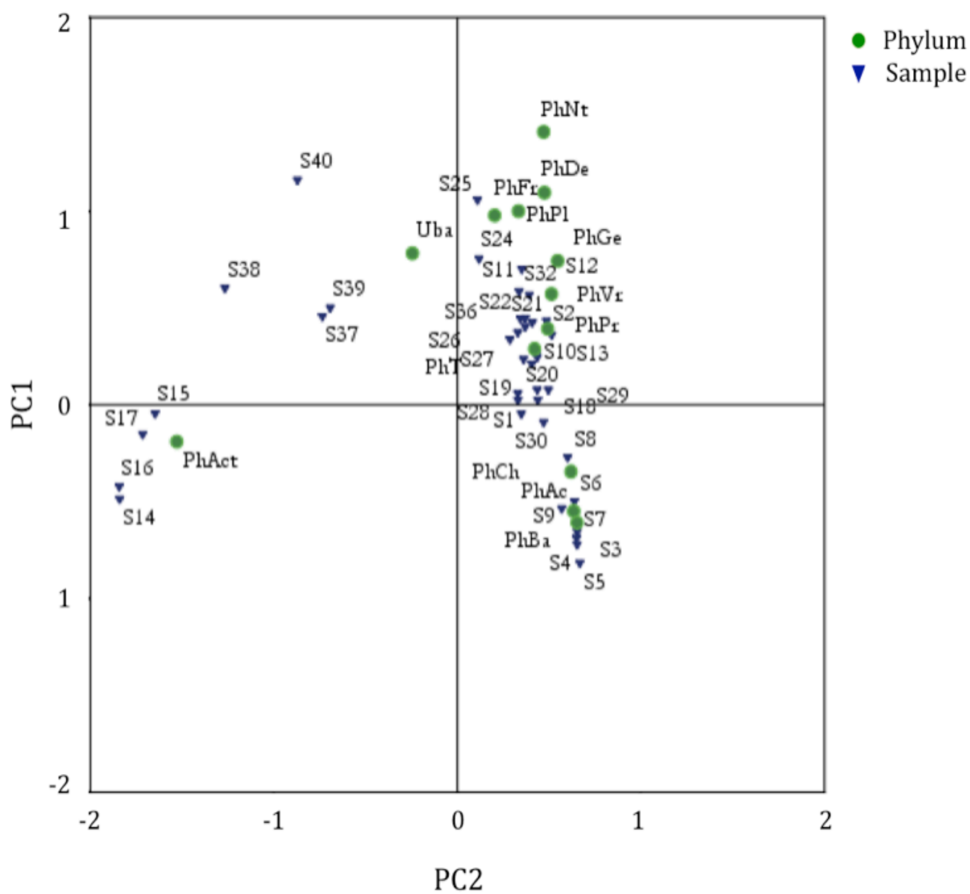

Figure 17. Relationship between the microbial communities present in BK, RHZ and NOD samples. MCA results show the interaction between the samples taken in the study and the different bacterial phyla determined by Ion Torrent sequencing 16S rRNA gene amplicons.

Key table for MCA graph							
Sample	Graph-label	Sample	Graph-label	Sample	Graph-label	Sample	Graph-label
GH J09	S1	CF (RHZ) J09	S11	RF3 (BK) S10	S21	RF4 (RHZ) S10	S31
UNi (BK) J09	S2	R (RHZ) J09	S12	RF4 (BK) S10	S22	CF5 (RHZ) S10	S32
UF9 (BK) J09	S3	C (RHZ) J09	S13	CF5 (BK) S10	S23	C9&13 (RHZ) S10	S33
iA (BK) J09	S4	RF (NOD) J09	S14	C9&13 (BK) S10	S24	R10&11 (RHZ) S10	S34
iB (BK) J09	S5	CF (NOD) J09	S15	R10&11 (BK) S10	S25	R12&16 (RHZ) S10	S35
iC (BK) J09	S6	R (NOD) J09	S16	R12&16 (BK) S10	S26	C14&15 (RHZ) S10	S36
NiA (BK) J09	S7	C (NOD) J09	S17	C14&15 (BK) S10	S27	RF3 (NOD) S10	S37
NiB (BK) J09	S8	U_F9 (BK) S10	S18	RF1 (RHZ) S10	S28	CF5 (NOD) S10	S38
NiC (BK) J09	S9	RF1 (BK) S10	S19	CF2 (RHZ) S10	S29	R10&11 (NOD) S10	S39
RF (RHZ) J09	S10	CF2 (BK) S10	S20	RF3 (RHZ) S10	S30	C14&15 (NOD) S10	S40

Table 8. Key table for MCA graph. BK, RHZ and NOD samples were re-named for simplicity, labeling them from S1-S40.

3.2.6) Detection of *Frankia* AvcI1 (F9)

A nested PCR technique was performed on NOD, BK and RHZ samples to determine the presence of *Frankia*-like and F9-like bacteria. NOD samples from the plants RF, CF, R and C gave positive results for both PCRs: *Frankia* detection (Figure 18a) and F9 detection (Figure 18b). In addition, *Frankia*-like and F9-like bacteria were detected in some of the BK and RHZ samples (Table 10). If samples were negative for *Frankia*, they were not tested further.

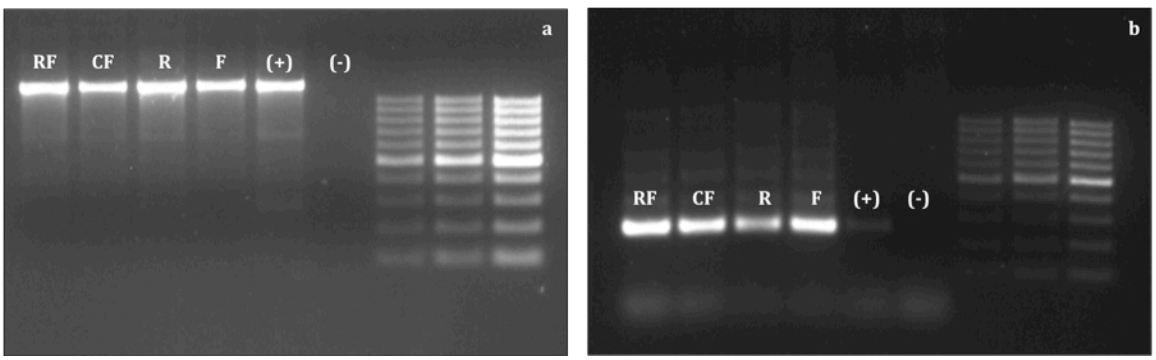

Figure 18. Nested PCR of total DNA extracted from nodules of alders. (a) *Frankia*-like bacteria, and (b) F9-like bacteria. Fragment length of the *nifD-nifK* is ~1100bp (a), and for the nested region it is ~269bp (b).

Time of sampling	Type of samples	Sample	*Frankia*-like PCR	F9-like PCR
J09	BK	UF9	×	no
		UNi	×	no
		F9-In1	×	no
		F9-In2	×	no
		F9-In3	×	no
		Ni1	×	no
		Ni2	×	no
		Ni3	×	no
	RHZ	RF	×	no
		CF	×	no
		R	×	no
		C	×	no
	NOD	RF	✓	✓
		CF	✓	✓
		R	✓	✓
		C	✓	✓
S10	BK	UF9	✓	×
		UNi	×	no
		RF1	✓	✓
		RF3	×	no
		RF4	×	no
		CF2	✓	✓
		CF5	×	no
		C9&13	✓	×
		R10&11	✓	×
		R12&16	✓	×
		C14&15	×	no
	RHZ	RF1	✓	✓
		RF3	✓	×
		RF4	✓	✓
		CF2	✓	×
		CF5	✓	✓
		C9&13	✓	×
		R10&11	✓	✓
		R12&16	✓	✓
		C14&15	✓	✓
	NOD	RF3	✓	✓
		CF5	✓	✓
		R10&11	✓	✓
		C14&15	✓	✓

Table 9. *Frankia*-like and F9-like PCR results. Positive products (✓), negative products (×), and no PCR performed (no).

CHAPTER FOUR: Discussion and Conclusions

4.1) Discussion

4.1.1) Production of containerized alder-*Frankia* symbionts

The main objective of this study was to optimize the production of nodulated alder seedlings by examining the effects of plant age, N input and inoculation. An optimum protocol would yield a high production of pre-inoculated seedlings and healthy post-inoculated well-nodulated seedlings. It was found that after seeds had germinated, seedlings could be left to grow and be fertilized for no more than 9 weeks, or production of pre-inoculated seedlings was reduced. Even though the interaction between plant age and N input was poor, seedlings from the planting group P1 were exposed for a longer period of time to the addition of fertilizer solutions and this could have had an impact on the production of seedlings from this group. If this was the case, one explanation could be that, at such an early stage, the development of alder seedlings is rather critical, where the continuing input of fertilizer can suppress seed germination and seedling growth. Since alder seeds typically have a low germination rate (Bliss, 1958), and filled seeds (containing the embryo) are difficult to manipulate and separate from empty ones, seeds that need more than one week to germinate may be negatively affected by the addition of fertilizer to the soil, suppressing their germination. In addition, exposure to fertilizers could have also affected some alder sprouts that grew out of seeds that took more than a week to germinate, the time established to start the fertilization regime. It is known that the exposure of seeds to fertilizers can have a burning effect due to the salt content in the solutions (Deibert, 1994). If the soil medium where seeds are placed is dry, chances are that water in the seed embryo moves outwards, and the process may be accelerated if fertilizer is applied, leading to dehydration of the seeds. In contrast to what has been found here, studies on the negative effect that fertilizers can have, have focused primarily on the addition of high concentrations of N and how this impacts seeds germination and seedling growth. One example is the study by Raghavan and Torrey (1964) who found that concentrations of 100ppm N and above caused browning and death of seedlings of the orchid *Cattlayea*, shortly after germination. Here, we report an inhibitory effect on seed germination, and possibly on young seedling growth, due to the application of fertilizer solutions for a period longer than 9 weeks, a time frame that seems critical for *Alnus crispa* seeds and young seedlings.

Similarities found on the effects of each of the N treatments at the pre-inoculation stage lead us to conclude that differences are not detectable at this stage, but are in the longer term. Evidence of this is how treatments A and B were correlated with the HS-3 status, meaning that not only did a continuous input of high concentrations of N have a negative effect on the over-all wellness of seedlings, but there was also an inhibitory effect due to the stored N in the plants. The effect of stored N has been reported by Thomas and Berry (1989), who found that addition of 75ppm N, and above, to seedlings of *Ceanothus griseus* var *horizontalis* applied before inoculation and continuously afterwards, was positively correlated with the growth of the plant but reduced nodule formation. In contrast, application of lower concentrations of N, 10ppm and 20ppm, promoted nodulation without compromising growth. Results on shoot height measurements and health status of the seedlings support this: that elevated concentrations of N do promote seedling growth, but do have a negative effect on the over-all wellness of the plants, promoting leaf loss, dryness and, ultimately, death of the seedlings (Mackay et al., 1987). On the other hand, and contrary to what has been established, addition of N to the soil medium did not have a significant effect on the formation of nodules, instead, inoculation with F9 and the time of inoculation (age of the plant) played a major role. By examining the MCA, it can be concluded that inoculation of 9-week-old alder seedlings (P2) with F9 was a determinant step for the production of well-nodulated seedlings, as it contributed to the over-all wellness of the plants. As for the exposure of alder seedlings to fertilizers, the high concentration of N applied in this study (100ppm) was counterproductive and, since treatments C, D and E were highly correlated, it can be assumed that 10ppm N was too low to fulfill the N demand, suggesting that concentrations higher than 10ppm and lower than 100ppm could be optimal for the growth and nodulation of *Alnus crispa* seedlings.

4.1.2) Growth and performance of alder-*Frankia* symbionts and their effect on soil quality

The pre-inoculation of alders with F9 was a determinant step in the production of alder seedlings, and the efficacy of the inoculation could be seen in the greenhouse when a well-nodulated stock was obtained, and after three growing seasons in the field where inoculated alders grew remarkably better than non-inoculated plants. It was already established by Berry and Torrey (1985) that artificial symbiosis of alder seedlings under controlled conditions is advantageous for the plants as it helps in the early development of the seedlings, yielding stronger plants that can be better established under adverse conditions. Burgess et al. (1986) reported how beneficial inoculation with *Frankia* in the nursery was for alder seedlings in a 3-year study, as the formation of effective nodules provided the plants with a growth advantage that was noticeable after one year of growing in the field and persisted until harvesting. The rapid growth of alders means more biomass production and a greater and faster accumulation of organic matter and nutrients in the soil in the long run. Since nutrient content values were higher in leaves, and more biomass was recorded from inoculated alders, deposition of nutrients would be higher from alder-*Frankia* symbionts, enriching the soil below. The growth and increased biomass productivity of alders as a result of *Frankia* inoculation was reported by Hendrickson et al. (1993) in a 5-year field trial that found that pre-inoculation of alder seedlings, before out-planting, produced an increase of 25% to 33% in biomass production over non-inoculated seedlings. In a more recent 7-year study by Kuznetsova et al. (2010) on a post-mining oil shale reclamation site in Northeast Estonia, *Alnus glutinosa* (black alder) performed remarkably better than silver birch and scot pine, potential candidates for the revegetation of post-mining lands, as determined by means of aboveground biomass production and nutrients accumulation (N, P and K). In addition to the use of alders, and emphasizing the importance of the pre-inoculation step, similar results were reported with other actinorhizal plants by Visser et al. (1991), who found that growth of inoculated silver berry was three to seven times greater that non-inoculated seedlings after just one growing season, and shoot weights of inoculated buffalo berry were three to five times greater than those of their non-inoculated counterparts. Alders were planted in soil that would ensure their establishment and survival. Pre-planted soil followed the standards established by the OSVRC (1998) for the creation of natural-like structures for further reclamation. Results obtained from the soil quality analysis reveal the importance of using inoculated-alders over non-inoculated ones, and, most important, as an effective alternative to accelerate soil formation. Inoculated-alders decreased soil pH and the saturation of the cations K, Mg and Ca, and increased the total N content in soil, one of the main purposes of their use in revegetation practices. Similar results were reported by Greer et al. (2005) and Lefrançois et al. (2010) from greenhouse experiments and small-scale field trials, However, an increase over time in the values of electrical conductivity, Na and chloride, suggest a possible salinization of the soil cover, a phenomenon reported by Kessler et al. (2010) after studying the diffusion of salt in the soil cover of an overburden reclamation site in Athabasca. Klessler et al. (2010) found that salt migrates into the soil during the initial 4-yr period following placement, and that the phenomenon is not affected by cover thickness. The increasing salt content in the soil cover has not yet affected alders, however, it is an aspect that should be monitored since the new conditions can harm the root system and eventually the overall health of the plants.

4.1.3) Effect of alder-*Frankia* symbionts on the indigenous microbial community

Greer et al. (2005) first reported the degradation of hydrocarbons through alder-assisted rhizodegradation. In their study, symbionts growing in oil sand tailings positively affected the microbial population located in the rhizosphere by increasing its diversity, its metabolic activity and number of PAHB by one to two orders of magnitude. In the current study, alders had a positive impact on the indigenous microbial community in soil as they increased the number of viable bacteria (HTB and PAHB) that were in direct contact with the roots. MPN results showed that both F9-In and Ni alders increased the population of HTB and PAHB, which surpassed the numbers obtained in bulk soil from planted and unplanted areas. It is known that the rhizosphere is a nutrient rich environment that stimulates the activity and diversity of microbial communities (Rovira, 1965), however no significant differences were observed from the MCA results with regards to the microbial community composition in bulk soil and rhizosphere samples. It is possible that after this short a period in the field, significant changes in the microbial community structure are not yet discernible. Results of the mineralization assays suggest that changes are occurring in the soil matrix that are favoring aliphatic degraders and that it is a time effect rather than an alders presence effect alone. In reclamation sites, the peat present in the soil cover is the main source of organic matter for the soil microorganisms, while root exudates can only be reached by microorganisms present in the root zone, so this must indicate that the organic matter present in the soil matrix (peat and alder deposition) is being decomposed, which is a good indicator of the progressive development of the forest floor in the first years of reclamation.

4.1.4) Detection of *Frankia* AvcI1 (F9)

Development of nodules in the roots of non-inoculated alders was a matter of time as the soil cover originated from forest lands surrounding the mining areas and species of alders are growing there naturally. Even though the soil has been highly disturbed, it has been found that *Frankia* remain present (Visser et al., 1991), regardless of the presence of a suitable host (Chaia et al., 2010; Hendrickson et al., 1993; Smolander, 1990). It was of interest to detect F9 through the employment of a simple molecular technique, in this case nested PCR that would detect, firstly, the presence of *Frankia*-like bacteria and subsequently detect F9 specifically. Results indicated that *Frankia*-like bacteria were present in the nodules of inoculated and non-inoculated alders, as well as in some of the bulk soil and rhizosphere samples. Considering these results, and by finding no significant differences in the amounts of N from inoculated and non-inoculated alder tissue, it can be concluded that non-inoculated alders became infected by *Frankia* present in the greenhouse, in the peat-vermiculite mix, or in the soil-cover, once out-planted. These natural strains can be considered competent symbionts as they permitted the survival and growth of their non-inoculated hosts. On the other hand, the detection of F9-like bacteria in the nodules and some of the soil samples indicates that not only F9 was possibly present in the nodules of alders but that the detected indigenous *Frankia* shared a high degree of similarity with F9 at the DNA level, which was also reported by Lefrançois et al. (2010). This indicates that the molecular detection technique used in this study is either not sufficiently specific or that the indigenous *Frankia* are essentially identical to F9. One option could be the use of alternative housekeeping genes for bacterial identification (Stackebrandt et al., 2002). A recent study by Bernèche-D'Amours et al. (2011), offers a new approach for the molecular detection of *Frankia alni* strains based on the *rpoB* gene, a housekeeping gene that has been shown to be a promising alternative for the differentiation and speciation of closely related strains (Danhllof et al., 2000; Mollet et al., 1997). In their study, different primer sets were developed, from which the primer set *rpoB*-2, that targets a 694 bp fragment, allowed the separation of *Frankia alni* strains known to share from 94%-100% identity at the 16S rRNA gene level; and, at the same time, reflected the host plant species they were isolated from. Another gene that has been considered for development of a similar PCR technique that could differentiate *Frankia* strains is the *cpn60* gene. The *cpn60* gene was recently tested by Verbeke et al. (2011) for the differentiation of *Thermoanaerobacter* species, based on the comparison of the *cpn60* universal target sequence (*cpn60* UT sequence), a 549-567 bp segment of the *cpn60* coding region, to other bacteria. This UT sequence is found in virtually all bacteria as a single copy; and universal primer sets and a *cpn60* database are currently available to facilitate bacterial detection (Hill et al., 2004).

4.2) Conclusions

- Seeds of *Alnus crispa*, and early seedlings, grew better over a period of no longer than 9 weeks before inoculation was performed and produced well-nodulated seedlings for subsequent out-planting. Fertilizers can be provided before inoculation only, or continuously, as long as the concentration of N is kept below 100ppm. Inoculation with *Frankia* strain AvcI1, or comparable strains, is highly recommended as it improves seedling growth and promotes nodule formation, which would facilitate more efficient N_2 fixation once out-planted.
- It is suggested to use pre-inoculated alders for the revegetation of oil sands reclamation sites in Athabasca. Symbionts are capable of survival and growth without aid of any sort, representing a cost-effective alternative to the use of fertilizers and other amendments. The presence of inoculated-alders improves soil quality, increases the N content in soil and ultimately accelerates soil floor formation.
- In this study the extent of the alder's effect on viable bacteria was limited to the rhizosphere, representing a nutrient enriched zone for the indigenous soil microbial community found in the soil matrix, that promotes their proliferation and improves their metabolic activity. PAH degraders, however, benefited not only from alder leaf litter deposition but from the ongoing changing conditions found in the soil matrix and alder roots, as the mineralization rates of representative hydrocarbon substrates increased over time.
- Detection of F9-like bacteria indicates that competent symbionts were present in the soil and successfully infected uninoculated control plants; nevertheless,

natural alders on these sites were typically not well-nodulated and their growth was much poorer in comparison to greenhouse inoculated plants.

- The F9-effect could be limited to the first stages of alder growth as results indicated that nodules are colonized over time by bacteria present in the soil. However, since there is still no accurate technique that can tell if the symbiont remains present as part of the endophytic community in nodules of alders, it is not known if it is further displaced by competent strains found in the soil after the plants have spent some time in the field. Future experiments for the development of a new PCR approach would help determine which of these alternatives is occurring.

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