Revegetation and Reclamation of Oil Sands Process-Affected Material Using *Frankia*-Inoculated Alders: Field and Greenhouse Trials

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Abstract

Canada's oil sand industry produces substantial quantities of oil sands process-affected material (OSPM) including composite tailings (CT) and tailings sands (TS) that need to be reclaimed on site. This work evaluated the establishment of a pioneer plant species, alder, inoculated with a nitrogen-fixing actinomycete, *Frankia*. The main objectives were to evaluate alder performance in OSPM and its impact on soil characteristics and microbial community structure and function. This project was divided into two phases: a field study and a greenhouse trial. The greenhouse trial tested 2 alder species, *Alnus glutinosa* and *A. crispa*, in CT and TS. In addition to *Frankia* inoculation, a tripartite association with a mycorrhizal fungus, *Glomus intraradices* was evaluated. The field study consisted of a 2 year monitoring of *Frankia*-inoculated alders (*A. crispa* (Ait.) Pursh.) planted in TS capped with overburden material and peat moss. The parameters tested were the following: plant biomass and nitrogen content; soil chemical characteristics; microbial biomass; microbial petroleum hydrocarbon mineralization capability; and microbial community diversity and composition using the molecular techniques, PCR and DGGE. Alders performed well in OSPM and *Frankia* inoculation improved biomass acquisition. *Frankia*-alders improved a number of soil quality parameters such as pH, sodium content, and CEC. *Frankia*-alders presence modified soil microbial activity and diversity. Alder rhizosphere sustained more microbial biomass than unplanted soil, whereas the tripartite association did not provide additional benefits. Overall, *Frankia*-inoculated alders are an interesting biotechnological approach for the reclamation and revegetation of oil sand process-affected materials.

Abbreviation: PCR (polymerase chain reaction), DGGE (Denaturating Gradient Gel Electrophoresis), CEC (Cation Exchange Capacity), TS (Tailings Sands), CT (Composite Tailings)

Résumé

L'exploitation des sables bitumineux d'Alberta par les compagnies pétrolières entraîne une importante production de matières résiduelles de sable bitumineux (MRSB). Ces MRSB, incluant les résidus de sable (RS) et les résidus composites ou consolidés (RC), doivent être réhabilités à même les sites d'exploitation. Ce projet a évalué l'utilisation d'une espèce pionnière, l'aulne, inoculée avec un actinomycète fixant l'azote, *Frankia,* pour la végétalisation de ces résidus. Les objectifs principaux étaient d'évaluer la performance des aulnes dans les MRSB et leurs impacts sur les caractéristiques du sol et ses communautés microbiennes. Le projet a été divisé en 2 volets : un essai sur le site minier et un essai en serre. L'essai en serre a évalué 2 espèces d'aulne, *Alnus glutinosa* et *A. crispa*, dans les RS et les RC. En plus de l'inoculation avec *Frankia,* une association tripartite avec un champignon mycorrhizien, *Glomus intraradices*, a été évaluée. L'essai sur le site minier a consisté en un suivi sur 2 ans d'aulnes (*A. crispa* (Ait.) Pursh.), inoculés avec *Frankia,* plantés dans des RS recouverts de morts-terrain et de mousse de tourbe. Les paramètres suivants ont été évalués : la biomasse et le contenu en azote des aulnes, les caractéristiques chimiques du sol, la biomasse microbienne, la capacité microbienne de minéralisation d'hydrocarbures pétroliers et, à l'aide de technique de biologie moléculaire (PCR et DGGE), la diversité et la composition des communautés microbiennes. Les aulnes ont bien performés dans les MRSB et l'inoculation avec *Frankia* a entraîné un important gain en biomasse. Les aulnes inoculés avec *Frankia* ont entraîné l'amélioration de plusieurs caractéristiques du sol, dont le pH, la CEC, et le contenu en sodium. Ils ont aussi modifié l'activité et la diversité des communautés microbiennes du sol. La rhizosphère des aulnes a maintenu une biomasse microbienne supérieure à celle du sol non-planté. L'association tripartite n'a pas fourni de bénéfice additionnel. En conclusion, les aulnes inoculés avec *Frankia* présentent un fort potentiel pour la végétalisation et la réhabilitation des matières résiduelles des sables bitumineux.

Abréviations : MRSB (Matière résiduelle des sables bitumineux); RS (résidu de sable); RC (résidus consolidés ou composites); PCR (Réactions en chaîne par polymerase); DGGE (Électrophorèse sur gel à gradient dénaturant); CEC (Capacité d'échange cationique)

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Table of Contents

Abstract i
Résumé ii
Table of Contents iv
List of Tables vi
List of Figures vii
Contributions of authors viii
List of abbreviations ix
Chapter 1 Literature Review 1
1.1 Athabasca Oil Sands Reserves 1
1.1.1 Flora 1
1.1.2 Climatic Conditions 1
1.1.3 Oil Sands Exploitation 1
1.2 Environmental Issues 2
1.2.1 Ecosystems Disturbance 2
1.2.2 Greenhouse Gases and Atmospheric Pollution 3
1.2.3 Pollution Reduction 3
1.3 Oil sands process-affected materials (OSPM) 3
1.3.1 Nature of OSPM 3
1.3.2 Reclamation of OSPM 4
1.3.2.1 Reclamation Strategies 4
1.3.2.2 Existing Reclamation Efforts 4
1.3.2.3 Natural Reclamation 5
1.3.2.4 Phytoremediation 5
1.3.2.5 CT Remediation Efforts 6
1.4 Alders 7
1.4.1 Importance of Alders 7
1.4.2 Alders in Contaminated Soil 7
1.5 Alders' Symbiotic Association 8
1.5.1 *Frankia* 8
1.5.1.2 *Frankia* Classification 8
1.5.1.2 Alder, *Frankia*, and Nitrogen 9
1.5.1.3 Importance of *Frankia* Greenhouse Inoculation 9
1.5.2 Mycorrhiza 10
1.5.2.1 *Glomus intraradices* 10
1.5.2.2 Tripartite Association 10
Chapter 2 Project Objectives 11
Chapter 3 Field Performance of Alder-*Frankia* Symbionts for the Reclamation of Oil Sands Sites 14
Abstract 15
3.1 Introduction 16
3.2 Material and Methods 18
3.2.1 Site description 18

3.2.2 Sampling 19
3.2.3 Microbial enumeration 19
3.2.4 Bulk soil and plant analyses 20
3.2.5 Mineralization assays 20
3.2.5 Molecular microbial analysis 21
3.2.5.1 Sterility check 21
3.2.5.2 DNA extractions 21
3.2.5.3 *Frankia* sp. strain AvcI1 detection 21
3.2.5.4 16S rRNA gene PCR-DGGE 22
3.3 Results 23
3.3.1 Bulk soil, rhizosphere soil and plant characterization 23
3.3.2 Mineralization Assays 24
3.3.3 Molecular analysis 24
3.4 Discussion 25
Acknowledgments 28
Chapter 4 Growth of alders in tailings sand and composite tailings: effects of inoculation with *Frankia* sp. and/or *Glomus intraradices* 38
Abstract 38
Introduction 39
Material and Methods 41
Results 44
Discussion 46
Conclusions 50
Acknowledgments 51
Conclusions 56
Bibliography 59

List of Tables

Chapter 2

Table 1. Soil analysis of capped overburden-tailings sand either unplanted, planted with non-inoculated alders (control-alders) or *Frankia*-inoculated alders (*Frankia*-alders) after 1.5 (2006) and 2.5 (2007) years of growth in the field..... 29
Table 2. Average plant biomass and nitrogen (N) content of non-inoculated alders (control-alders) and *Frankia*-inoculated alders (*Frankia*-alders) planted in capped overburden-tailings sand after 1.5 (2006) and 2.5 (2007) years of growth in the field. 30
Table 3. Closest microbial match of sequenced 16S rRNA gene DGGE bands... 31

Chapter 3

Table 1. Average plant biomass of *A. glutinosa* and *A. crispa* growing in TS or CT inoculated with *Frankia*, *G. intraradices* (mycorrhiza), both (dual inoculation), or non-inoculated..... 52
Table 2. Soil analysis of initial TS or CT (t_0) and planted with *A. glutinosa* or *A. crispa* inoculated with *Frankia*, *G. intraradices* (mycorrhiza), both (dual inoculation), or non-inoculated..... 53

List of Figures

Chapter 2

Figure 1. Mineralization of representative hydrocarbon substrates, hexadecane (A, B), naphthalene (C, D) and phenanthrene (E, F), in bulk soil of capped overburden-tailings sands either unplanted, planted with non-inoculated alders (Planted) or *Frankia*-inoculated alders after 1.5 (2006) and 2.5 (2007) years of growth in the field. 32

Figure 2. Mineralization of representative hydrocarbon substrates, hexadecane (A, B), naphthalene (C, D) and phenanthrene (E, F), in the rhizosphere of non-inoculated alders (Planted) or *Frankia*-inoculated alders after 1.5 (2006) and 2.5 (2007) years of growth in an overburden-tailings sand mixture. 33

Figure 3. DGGE of PCR amplified 16S rRNA gene fragments from total DNA extracted from roots (endophytes) of alders inoculated or not with *Frankia* strain AvcI1 after 1.5 year growth in a overburden-tailings sand mixture. Non-inoculated a and c, and *Frankia*-inoculated a and b identifies field replicates. AvcI1 is the pure culture of *Frankia* strain AvcI1, and the arrow indicates the position of its dominant band. Letters (a-i) indicate bands that were excised for subsequent nucleotide sequencing (see Table 3). 34

Figure 4. Cluster analysis, with corresponding DGGE lanes, of 16S rRNA gene DGGE banding pattern of 2006 bulk soil samples. Fa, Fb and Fc, Ca, Cb and Cc and Ub and Uc identify field replicates of *Frankia*-inoculated alders (F), non-inoculated control alders (C) and unplanted (U) treatments, respectively. 35

Figure 5. Cluster analysis, with corresponding DGGE lanes, of 16S rRNA gene DGGE banding pattern of 2007 bulk and rhizosphere soil samples. Soil and Rhizo indicate bulk soil samples and rhizosphere soil samples, respectively. F1, F2 and F3, C1, C2 and C3 and U1, U2 and U3 identify field replicates of *Frankia*-inoculated alders (F), non-inoculated control alders (C) and unplanted treatments (U), respectively. Numbers indicate bands that were excised for subsequent nucleotide sequencing (see Table 3). 36

Chapter 3

Figure 1. Mineralization of two representative hydrocarbons: hexadecane and naphthalene, in bulk (A, B) or rhizosphere (C, D) soil of CT or TS either unplanted, planted with non-inoculated *A. crispa* (Planted) or *Frankia*-inoculated *A. crispa*. 54

Figure 2. Mineralization of two representative hydrocarbons: hexadecane and naphthalene in bulk (A, B) or rhizosphere (C, D) soil of CT or TS either unplanted, planted with non-inoculated, *Frankia*-inoculated (frankia), *G. intraradices* inoculated (mycorrhiza) or dual inoculated (dual) *A.* glutinosa. 55

Contributions of authors

Elisabeth Lefrançois : wrote both manuscripts, did the laboratory and field work, and all data analyses.

Charles W. Greer : Thesis supervisor, principal investigator of the project, participated in design, planning and analysis of data, participated in manuscript revision, and preparation of figures (mineralization figures-Field study).

Lyle G. Whyte: Thesis co-supervisor, participated in the review of the manuscripts.

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Ali Quoreshi: Responsible for field site establishment and maintenance, collection and analysis of data.

Martin Fung: Industrial collaborator on the field project, responsible of environmental project management and maintenance at Syncrude Canada Ltd.

List of abbreviations

C: Carbon

CEC: Cation exchange capacity

CT: Composite or consolidated tailings

DGGE: Denaturating gradient gel electrophoresis

dpm: Disintegrations per minute

MFT: Mature fine tailings

MPN: Most probable number

N: Nitrogen

OSPM: Oil sands process-affected materials

PAH: Poly-cyclic aromatic hydrocarbon

PCR: Polymerase chain reaction

RFLP: Restriction fragment length polymorphism

TS: Tailings sands

VAM: Vesicular-arbuscular mycorrhizal fungi

Chapter 1 Literature Review

1.1 Athabasca Oil Sands Reserves

Canada has important oil reserves with the greater part found as oil sands. There are 1.7 trillion barrels of bitumen separated into 4 deposits in Alberta (Fung and Macyk, 2000). The biggest one, the Athabasca deposit, is estimated to be 700 billion barrels, and is the one that is the most extensively mined. There are many companies involved in the oil sands exploitation with the biggest being Suncor Energy inc., Syncrude Canada Ltd., and Albian Sands Energy. The principal city near the mine sites is Fort McMurray.

1.1.1 Flora

The Athabasca oil sands mining sites are located in the Boreal Forest, more precisely in the Boreal Plain ecozone, including the Wabasca Lowlands, the Slaveriver Lowlands, and the Boreal Lowlands (Gillanders et al., 2008). The principal tree species are a mix of conifers and deciduous: white spruce, black spruce, trembling aspen, balsam poplar, and white birch. Understorey vegetation is typical of acidic soils and includes berry-bearing species (such as *Vaccinium* sp.), lichens, mosses, and horsetails (Fung and Macyk, 2000).

1.1.2 Climatic Conditions

The annual mean temperature at Fort McMurray is 0.7^{o}C with a mean of –18.8^{o}C in January and 16.8^{o}C in July (NCDIA, 2009). Fort McMurray receives an average of 455.5 mm of precipitation yearly with the majority as rain between the months of May and October. There is an average of 145 days with at least 5 cm of snow cover. The Fort McMurray area has a short and cool growing season.

1.1.3 Oil Sands Exploitation

The Athabasca deposit is shallow, which allows for oil sands recovery through surface mining. Oil sands are mined and then transported to the processing plant for bitumen (a naturally occurring viscous mixture of hydrocarbons) extraction. Transport was initially performed by conveyer belt, but is now done by hauler truck (Syncrude

Canada Ltd., 2007). The current method used to recuperate bitumen from the oil sands requires hot water, steam, and caustic soda (Fung and Macyk, 2000; Quagraine et al., 2005). It is estimated that 3 cubic meters of water are used for each cubic meter of oil sands processed. Most of the water is recuperated in the extraction process, although the outflow has the consistency of a slurry, and is discharged into tailings ponds. Bitumen is refined into high quality synthetic crude oil (Syncrude Canada Ltd., 2007).

The three main mining companies, Syncrude Canada Ltd., Suncor Energy Inc., and Albian Sands Energy, are planning important investments to promote mining and oil sands processing (Scales and Weniuk, 2008). Moreover, a rising number of companies are initiating new projects in the area (NEB, 2006). Production could reach 5.8Mb/d by 2040 (Méjean and Hope, 2008). However, the rate of expansion is closely linked to oil prices and production costs (NEB, 2006). Oil sands exploitation costs are difficult to predict in the long-term because the resource is heterogeneous. The most important factor is the rate of exploitation, and this is influenced by the depth of the oil sands, and their physical properties (Méjean and Hope, 2008). The average thickness of the oil sands deposits is estimated at 38 meters; however, they are located below an overburden that needs to be removed before any processing can be performed. Where the overburden material is more than 45 m deep, open-pit mining is considered uneconomical and in-situ techniques must be used (Fung and Macyk, 2000). In-situ-mining consists of mining on site without transporting the oil sands to an industrial plant; a combination of drilling, steaming and pumping is used (OSDC, 2009). For many years, in-situ mining had been considered uneconomical but with increasing oil prices, large investments are currently being used to develop these techniques (Scales and Weniuk, 2008). The growth of this industry will lead to the increase in production of oil sands process-affected materials (OSPM): overburden, tailings and associated water.

1.2 Environmental Issues

1.2.1 Ecosystems Disturbance

Oil sands exploitation, because of the open-pit mining technique and the OSPM that are produced, results in substantial land disturbance. Dense mixed wood and dense conifer forests are the land covers principally affected by the oil sands mining industry

(Gillanders et al., 2008). In 2000, a total disturbance of 40,000 ha of land was estimated for the 2 main mining operations (Fung and Macyk, 2000). However, it was evaluated through satellite imagery that from 1984 to 2005, 30,380 ha of land (forests or wetlands) was cleared or altered for mining activities (Gillanders et al., 2008). With an increasing number of companies in operation in the Athabasca oil sands and an increase in mined areas the 40,000 ha proposed in 2000 (Fung and Macyk, 2000) will need to be scaled up. Disturbance of forest ecosystems is critical for greenhouse gas management since they are important carbon sinks (Peters et al., 2007b). It is estimated that forest ecosystems represent 80% of terrestrial above ground carbon (Jandl et al., 2007).

1.2.2 Greenhouse Gases and Atmospheric Pollution

Greenhouse gas production by two of the main producers, Syncrude Canada Ltd. and Suncor Energy Inc., reached 24,197,975.35 tonnes of CO_2 equivalents in 2007 (GGERP, 2007). This represented approximately 9% of all greenhouse gases reported for that year in Canada. Besides greenhouse gas production, oil sands exploitation is a substantial source of atmospheric pollution in the form of SO_2 and NO_x. For 2007, Syncrude Canada Ltd., Suncor Energy Inc., and Albian Sands Energy declared the release of 111,693 tons of sulphur dioxide and 27,446 tonnes of nitrogen oxides (NPRI, 2007).

1.2.3 Pollution Reduction

It must be mentioned that efforts have been made to reduce pollution resulting from the oil sands processing and so minimize its environmental impacts. There have been many investments to reduce atmospheric pollution by reducing the production of sulphur dioxide, oxides of nitrogen, and other odour causing agents. Moreover, new extraction techniques are being investigated to reduce the amount of water used (Scales and Weniuk, 2008).

1.3 Oil sands process-affected materials (OSPM)

1.3.1 Nature of OSPM

Due to the zero discharge policy, all OSPM must be kept on site, and the slurry produced by the extraction process is directed into large settling ponds (Fung and Macyk,

2000). The slurry consists mainly of sand, silt, clay, and residual hydrocarbons. The coarse material settles rapidly forming beaches and dikes, and can be reclaimed in the form of tailings sand (TS). TS consist mainly of sand particles with residual hydrocarbons. Fine tailings, mostly silt and clay materials, sediment at a much slower rate forming a 30% solid by weight substrate called "mature" fine tailings (MFT). A strategy has been developed to remix MFT and coarse tailings using gypsum ($CaSO_4 \bullet 2H_20$) yielding composite or consolidated tailings (CT) (Franklin et al., 2002; Fedorak et al., 2003; Syncrude Canada Ltd., 2007). TS and CT present many characteristics that are limiting for plant growth: high sodium content, high pH, low organic matter, low nutrient content, low CEC, and they are almost devoid of microbial activity.

1.3.2 Reclamation of OSPM

1.3.2.1 Reclamation Strategies

MFT, due to their high water content, are candidates for wet landscape reclamation. This method consists of capping tailings with water (Quagraine et al., 2005). These ponds will self clean over time and become capable of sustaining an ecosystem. However, tailings ponds raise some concerns. They have been found to harbour methanogens (Holowenko et al., 2000), and methane is known as one of the most potent greenhouse gasses. Also, divergent results concerning tailings water toxicity to fish (Quagraine et al., 2005; Peters et al., 2007a) suggest a more cautious use of the wet landscape approach for the remediation of oil sands tailings. Another option is the dry landscape approach, which consists of the establishment of trees and shrubs. This method can be applied to both TS and CT. As a reclamation strategy, TS can be capped with overburden material and peat moss (M. Fung, personal communication). This buffers the TS and provides a source of organic matter and microorganisms.

1.3.2.2 Existing Reclamation Efforts

Many efforts have been made for the reclamation of oil sands process-affected material (Syncrude Canada Ltd., 2007). Research on the remediation of mining residues has been going on for many years (Fedkenheuer, 1979; Fessenden, 1979). Satellite

imagery has shown that some land has been returned to a vegetation cover similar to what was present before mining (Gillanders et al., 2008); however, with the intensification of oil sands mining and, therefore, increasing production of OSPM, it is important to provide additional prospective options to the mining companies for the reclamation and revegetation of these materials and areas.

1.3.2.3 Natural Reclamation

Researchers have investigated the reclamation of OSPM capped with overburden, peat or forest floor but without the establishment of plants (McMillan et al., 2007). Unfortunately, this generated an incomplete remediation. Ground cover was dominated by herbaceous species. Herbaceous species have a shallow root system, and provide limited shade and protection due to their small size. Hence, soil temperature was higher and soil moisture lower. These parameters are creating sub-optimal conditions for soil microorganisms and some soil processes such as organic matter decomposition. Moreover, it is not a multi-storey vegetation providing shelter and habitat for fauna, flora, and microorganisms, as the forest was before disturbance. The prompt introduction of a vegetation cover is necessary, as a delayed plant re-establishment after disturbance can provoke soil deterioration (Perry et al., 1989), which will not facilitate the reclamation process. This outlines the limited success of natural succession in oil sands process-affected material and how important it is to introduce species that can develop well into the OSPM.

1.3.2.4 Phytoremediation

There are many advantages of establishing vegetation on contaminated land. Plants contribute to soil aggregation (Perry et al., 1989), provide ground cover, are a source of organic matter and can alter microbial population composition and activity. Many research projects demonstrate the advantages of plant establishment through their impact on microbial communities. Introduction of plants have resulted in a decrease in soil total petroleum hydrocarbons as compared to unplanted soil (Siciliano et al., 2003). The establishment of plants increased the number of PAH-degrading bacteria and improved hydrocarbon mineralization as compared to unplanted contaminated soil

(Phillips et al., 2006). This could be due to rhizodeposition (root turnover and exudates) that has been found to increase the number of PAH-degraders (Da Silva et al., 2006). Rhizodeposition is important in soil processes, representing from 10-20% of the plant net photosynthetic carbon (as reviewed by Singer et al., 2003). Mulberry (*Morus* sp.) loses up to 58% of its fine roots by the end of the growing season (Leigh et al., 2002). Root decay is an important source of rhizodeposition, a reserve for the formation of organic matter, and an energy source for microorganisms. The rhizosphere (soil-root interface) is responsible for the relative increase in the abundance of microorganisms in comparison to the bulk soil (Smalla et al., 2001). Moreover, rhizospheric microorganisms are capable of degrading naphthenic acids with a preference for low molecular weight compounds (Biryukova et al., 2007). This could lead to a reduction in toxicity of the OSPM (Armstrong et al., 2008; Frank et al., 2009).

1.3.2.5 CT Remediation Efforts

Numerous studies have evaluated the possibility of reclaiming and revegetating CT (Renault et al., 2000; Renault et al., 2001; Franklin et al., 2002; Kernaghan et al., 2002; Khasa et al., 2002; Redfield et al., 2003; Renault et al., 2003). A variety of species were tested, and all indicated significant plant damage and reduced growth. Barley was investigated as a precursor plant to stabilize CT. The addition of peat and nutrients was found to be important for optimal plant development (Renault et al., 2003). Red-osier dogwood was also identified as a good candidate for CT reclamation (Renault et al., 2001). In a long-term study, no mortality was recorded, but CT affected the ion content of the different plant parts and high proportions of necrotic tissues were observed. The high pH and salt content in CT were identified as being the major concerns for reclamation. Jack pine growth was also hypothesized to be affected by the high pH and salinity of CT (Franklin et al., 2002). As for red-osier dogwood, no mortality was recorded but significant growth reduction was observed. In addition to pH and salinity, the structure and texture of CT, because of the fine particles, can limit root penetration and affect soil oxygen content (Renault et al., 2000). This can create an important impediment for plant growth in this substrate.

1.4 Alders

Alders are members of the Betulaceae family. They grow as shrubs or small trees (up to 8 m) (Johnson et al., 1995). There are many species that are native to Canada; green alder (*Alnus crispa*), speckled alder (*A. incana* spp. r*ugosa*), and smooth alder (*A. serrulata*).

1.4.1 Importance of Alders

Alders are ecologically important plants. They are a source of food for many animals such as grouse, hares and beavers. They are early flowering trees and for that reason it is an important food source for bees in spring (Johnson et al., 1995). Also, native populations of northwestern Canada have used alders for many different applications. They have been used in the tanning process, for smoking meat, as part of dyes, and they have been used in the production of canoes and toboggans (Johnson et al., 1995; Marles et al., 2000). Different parts of the plant (roots, bark, cones) have medicinal value, and have been used as such by native populations (Marles et al., 2000; Johnson, 2008).

1.4.2 Alders in Contaminated Soil

Research has shown that alders have the capacity to perform well in stressful environments (Graves and Gallagher, 2003; Mertens et al., 2004; Vares et al., 2004; Densmore, 2005; Markham, 2005; Roy et al., 2007). Alder performance in oil shale detritus was similar to its performance in fertile soil (Vares et al., 2004). In placer gold mine spoil, alders improved the growth of companion plants, provided a N-rich abundant litter, and increased graminoid cover (Densmore, 2005). OSPM are characterized by high pH, high salt and sodium content. Alders can grow under these unfavourable conditions (Mertens et al., 2004) and can survive in highly saline environments (Graves and Gallagher, 2003). Non-inoculated alders survived in CT, but suffered some growth reduction (Khasa et al., 2002). Greenhouse inoculation of *Frankia*, a nitrogen-fixing actinomycete, could insure adequate nodule formation and help alders establish in the low nutrient conditions, typical of OSPM.

1.5 Alders' Symbiotic Association

1.5.1 *Frankia*

Frankia is a nitrogen-fixing actinomycete that forms a symbiosis with a group of plant species known as actinorhizal plants. Actinorhizal plants are separated into 8 families and 25 genera, including among others *Casuarina* spp., *Comptonia* spp., *Myrica* spp., *Elaeagnus* spp., and *Alnus* spp. (Baker and Schwintzer, 1990). Actinorhizal plants are found on all continents with the exception of Antarctica. *Frankia* spp. are found as free-living microorganisms or in symbiosis with an actinorhizal plant (Benson and Silvester, 1993). *Frankia* enters into symbiosis with alders by root hair infection, and will form perennial multiple lobe nodules (Berry and Sunell, 1990). *Frankia* spp. have also demonstrated the ability to remain infective in soil devoid of host plants (Nickel et al., 2001; Ridgway et al., 2004).

1.5.1.2 *Frankia* Classification

Frankia identification and phylogeny have proved to be quite challenging. They have a slow growth rate with doubling times of 15 h or more (Benson and Silvester, 1993). This was seen as a major obstacle for identification through isolation (Akkermans et al., 1991; Hahn et al., 1999). Another methodology, plant bioassays, is limited by *Frankia*'s infectivity and can only detect strains capable of infecting a host. Moreover, in some cases strains isolated from plant nodules were not able to re-infect the same host it was isolated from (Benson and Silvester, 1993). It has been proposed that classical microbiological methods should be supplemented or even replaced by molecular techniques (Hahn et al., 1999). Molecular methods could allow the identification of *Frankia* not only from nodules but also from soil. It could provide more information on free-living *Frankia*, and help to understand its biology. Molecular techniques have been developed that allow the differentiation of *Frankia* infectivity groups and identification to the strain level. DNA-DNA homology, PCR, restriction fragment length polymorphism techniques targeting the nitrogenase genes (*nif* genes) or 16S rRNA genes have been developed and used (Benson and Silvester, 1993; Jamann et al., 1993; Nalin et al., 1995;

Dai et al., 2004). Increasing efforts in the sequencing of *Frankia* genomes (Normand et al., 2007) will also improve the precision of molecular techniques.

1.5.1.2 Alder, *Frankia*, and Nitrogen

Frankia is an important symbiont for alders as only 1 in 600,000 alder plants have been found to be non-nodulating (Tremblay et al., 1984). At low nitrogen levels, alders rely mainly on *Frankia* for nitrogen acquisition (Markham and Zekveld, 2007). This symbiotic association makes them self-supportive for nitrogen under nutrient limited conditions (Vares et al., 2004). Nitrogen fixation by its' symbiont is an important aspect for the ability of alders to survive and grow in OSPM. Nitrogen-fixing plants have a lower foliar N resorption at senescence (Rodríguez-Barrueco et al., 1984; Stewart et al., 2008) providing soil with a N-rich litter. Over the long-term, alders can reduce the soil C:N ratio, increase soil N, and, more importantly, plant-available N (Myrold and Huss-Danell, 2003). Surrounding non-nitrogen fixing plants can benefit directly from the nitrogen assimilated by alders (Coté and Camire, 1984; Kohls et al., 2003; Densmore, 2005). OSPM are low in nitrogen, a condition that can often limit growth (Vitousek and Howarth, 1991). As anthropogenic N deposition cannot be envisioned as a significant N source (Nadelhoffer and Emmett, 1999), nitrogen fixation by *Frankia* could be an important nitrogen source for this environment.

1.5.1.3 Importance of *Frankia* Greenhouse Inoculation

For rapid growth, alders rely mainly on nitrogen-fixation by *Frankia* (Martin et al., 2003). Inoculation is necessary for adequate nodule formation (Hilger et al., 1991). As research has demonstrated the importance of well nodulated plants prior to planting in a stressful environment (Markham, 2005), greenhouse inoculation during seedling production is important. It has been shown that *Frankia* inoculation could be easily inserted into regular nursery operations without major changes (Quoreshi et al., 2007). As OSPM present challenging growth conditions, for optimal symbiont performance it is important that alders are well nodulated with a *Frankia* strain capable of surviving under the conditions present in tailings materials (Wheeler et al., 1991).

1.5.2 Mycorrhiza

Alders can also have a symbiotic relationship with both vesicular-arbuscular mycorrhizal fungi (VAM) and ectomycorrhizal fungi (Rose, 1980; Gardner et al., 1984). Mycorrhizae form a mutualistic symbiosis with their host. They are widely known to improve plant water and nutrient acquisition, especially phosphorous, by increasing the length and contact area of the root system. In return, they are dependent on the plant host for carbon acquisition (Smith and Read, 1997; Fortin et al., 2008). Mycorrhizae are important elements that contribute to the health and biological diversity of soil.

1.5.2.1 *Glomus intraradices*

Glomus intraradices is a VAM. It is a member of the Glomaceae family and the genus *Glomus*, which is characterized by spores produced singly or in loose aggregates (Smith and Read, 1997). Mycorrhizal fungi do not all possess the same level of resistance to salt (Bois et al., 2006), so to colonize and be beneficial for a plant growing in OSPM, the mycorrhizal fungi must be able to survive not only under saline conditions but also in an alkaline substrate. *G. intraradices* was able to create a symbiotic relationship with alders under such conditions . As *G. intraradices* is commercially produced, it could easily be included in nursery production for land reclamation.

1.5.2.2 Tripartite Association

There is considerable evidence that a combined inoculation consisting of a nitrogen-fixing actinomycete and a mycorrhizal fungus can improve the growth of actinorhizal plants (Rose and Youngberg, 1981; Yamanaka et al., 2003; Oliveira et al., 2005a). Dual inoculations have resulted in improved plant growth, an increase in nodule number, and improved nitrogen fixation abilities (Rose and Youngberg, 1981). As alders rely mainly on N-fixation at low soil nitrogen levels (Markham and Zekveld, 2007), these tripartite symbioses could be beneficial for alder establishment in OSPM. Numerous studies have demonstrated successful symbioses between alders, *Frankia* and *G. intraradices* (Russo et al., 1993; Orfanoudakis et al., 2004; Oliveira et al., 2005a).

Chapter 2 Project Objectives

The purpose of this work was to evaluate the performance of *Frankia*-inoculated alders in oil sands process-affected materials (OSPM). The project had three main objectives: 1) Evaluate if *Frankia* inoculation improves alder establishment in OSPM; 2) Evaluate the impact *of Frankia*-inoculated alders on microbial population in terms of diversity, activity, and biomass; 3) Evaluate if there is an enhancement in soil quality following the establishment of *Frankia*-inoculated alders. Elements of the project were conducted in both the greenhouse and in the field.

The field study was conducted at an operational scale on tailings sands capped with overburden material and peat moss on the Syncrude Canada Ltd. mine site. Green alder (*A. crispa* (Ait.) Pursh) was inoculated with *Frankia* strain Avc1I prior to transplantation to the field site. In fulfillment of the objectives, plant growth, soil chemical characteristics, bulk and rhizosphere soil microbial diversity, biomass, and activity, and *Frankia* strain Acv1I presence were monitored over 2 consecutive years. Plant growth was evaluated through biomass acquisition and plant nitrogen content. The soil chemical characteristics that were monitored were: pH, pH-buffer, cation exchange capacity (CEC), organic matter, electrical conductivity, total nitrogen content and main base cations calcium, potassium, magnesium, and sodium. These parameters influence plant growth, soil chemical or microbiological processes such as nutrient cycling, organic matter production/degradation or xenobiotic degradation. Microbial population biomass was assessed by the most probable number (MPN) technique for total heterotrophic bacteria. As the project was concerned with OSPM, aliphatic and polycyclic aromatic hydrocarbon (PAH)-degrading bacteria were also determined. The capacity of the bulk and rhizosphere soils and the endophytic microbial community to degrade hexadecane, naphthalene and phenanthrene was evaluated. These compounds are representative of the residual hydrocarbons that can be found in OSPM and the results will give an indication of the capacity of the indigenous microbial population to degrade residual hydrocarbons. Hexadecane is a 16-carbon alkane, naphthalene a 2-ring PAH, and phenanthrene a 3-ring PAH. Finally, molecular techniques were utilized to monitor microbial population composition. Denaturating Gradient Gel Electrophoresis (DGGE) of PCR amplified 16S

rRNA gene fragments was used to monitor changes in the bacterial diversity pattern resulting from the establishment of *Frankia*-inoculated alders. As *Frankia* strain Avc1I was introduced into the environment it is important to be able to monitor its presence and potential dispersal. PCR was used to identify *Frankia* strain Avc1I using specific primers targeting the intergenic region of the *nif*D-*nif*K genes.

The purpose of the greenhouse trial was to evaluate *Frankia*-inoculated alder performance directly in tailings sands (TS) and composite tailings (CT). Working directly with TS and CT will make it possible to evaluate the real potential of *Frankia*-inoculated alders to survive and grow under the harshest conditions. Two different alder species (*Alnus glutinosa*, and *A. crispa*) were tested in the greenhouse trial. An additional component was added, as compared to the field trial, by evaluating a tripartite association (alders/*Frankia*/*Glomus intraradices*) in TS. Mycorrhizae are part of a healthy soil ecosystem, and offer advantages to their plant hosts. As tailings materials have limited microbial activity it was hypothesised that mycorrhiza could provide additional benefits to alders. Similar parameters to those assessed in the field study were monitored: plant biomass; soil chemical characteristics; microbial biomass (total heterotrophic and PAH-degrading bacteria); and bulk and rhizosphere soil microbial community potential to degrade hexadecane and naphthalene. Mycorrhiza infection was also evaluated to assess inoculation and infection performance.

The overall goal of this project was to provide a valid option for the revegetation and reclamation of oil sands process-affected materials to help in the management of disturbed lands and to achieve the re-establishment of a balanced ecosystem.

Chapter 3 is a manuscript containing results obtained from the field trial. At the time of submission of this thesis it had been submitted to the *Soil Biology and Biochemistry* journal. The field trial evaluated *Frankia*-inoculated alders performance in a operational scale reclamation site in a non-controlled setting. Alders were confronted with the environmental conditions prevailing on the reclamation site (Fort McMurray, Alberta).

Chapter 3 Field Performance of Alder-*Frankia* Symbionts for the Reclamation of Oil Sands Sites

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Abstract

The Canadian province of Alberta is the world's largest producer of petroleum products from oil sands exploitation. Tailings sand produced as a result of bitumen extraction from oil sands has low fertility, low organic matter content, it is alkaline, compactable, and contains residual hydrocarbons, making it a very inhospitable growth environment. The petroleum industry is currently involved in efforts to revegetate and remediate the tailings sand. One approach used is revegetation of the reclamation sites with *Frankia*–inoculated alders. Alders are primary successor trees that have the ability to grow in nutrient poor and waterlogged environments, in part because they form a symbiotic relationship with the nitrogen-fixing actinomycete, *Frankia*. In 2005, field trials were established at Syncrude Canada Ltd. The effect of *Frankia*-inoculated alders on soil quality was evaluated by monitoring the chemical, physical and microbiological characteristics of the soil. The impact on the indigenous microbial community was also studied using hydrocarbon mineralization assays, and molecular approaches, such as denaturing gradient gel electrophoresis (DGGE). Plant parameters (biomass, nitrogen content) were measured to evaluate the impact of *Frankia* on alder health and growth. After two growth seasons, *Frankia*-inoculated and non-inoculated alders yielded comparable amounts of plant biomass and there was an increase in hydrocarbon (hexadecane, naphthalene and phenanthrene) mineralization where the reclamation site had been planted with *Frankia*-alders. The alder rhizosphere samples all had comparable hydrocarbon mineralization rates. DGGE profiles confirmed a change in the microbial communities of the bulk soil between unplanted and *Frankia*-alder treatments. Soil tests showed that *Frankia*-alders decreased soil pH and plant-available sodium content, and had a positive impact on soil organic matter content. The field results have confirmed that the alder-*Frankia* combination results in improved remediation capabilities and enhances soil quality. These improvements in soil quality of the reclamation site may allow the subsequent establishment of more sensitive species, leading ultimately to the reforestation of the site, and the re-establishment of a balanced ecosystem.

Keywords: Alders; *Frankia* sp.; tailings; oil sands; microbial community analysis; reclamation

3.1 Introduction

Canada has one of the largest oil sands reserves in the world, located mainly in central Canada. It is estimated that 1.7 trillion barrels of bitumen are contained in 4 deposits (Fung and Macyk, 2000). The largest deposit (700 billion barrels), in the Athabasca region of Alberta, is a near-surface deposit that allows recovery through surface mining. This involves the removal of the existing vegetation and an overburden layer to reach the oil sands. For Syncrude Canada Ltd. and Suncor Energy Inc., two of the largest companies operating in this area, the amount of disturbed land will reach 40,000 ha when mining is completed.

The oil is recovered through a process using hot water, NaOH and steam (Clark and Pasternack, 1932), which produces a tailing slurry as the end-product. For each cubic meter of oil sand processed, 4 m^3 of slurry is generated. Part of the water is recycled into the extraction process and the remaining slurry, consisting of solids (sands and clay), water, unrecovered bitumen and dissolved organic and inorganic compounds, is discharged into settling ponds (Quagraine et al., 2005). The solid tailings sand separated from the slurry has low nutrient content, high salinity, high pH, low or no organic matter and contains residual hydrocarbon products, including toxic naphthenic acids. The nature of the tailings sand and the extent of the disturbed area make it necessary but challenging to remediate and revegetate. Such harsh environments reduce the ability of more sensitive plant species to establish, leaving the soil almost bare.

These oil sand extraction by-products can be reclaimed either by a wet or dry landscape approach (Quagraine et al., 2005). As the names suggest, wet landscape remediation implies the use of a layer of water on top of the tailings, creating ponds. However, methanogens were found to be present in tailings ponds(Holowenko et al., 2000) , which can be a major disadvantage, as methane is an important greenhouse gas. Dry landscaping is challenging due to the low-nutrient status, high pH and high salt content of the tailings, as well as the presence of residual hydrocarbons (Fung and Macyk, 2000). These conditions reduce the ability of the vegetation to repopulate these sites naturally.

The colonization by pioneer species that can tolerate harsh conditions is essential to improve the tailings sand and allow the re-establishment of a natural forest. Increased litter and plant cover reduces erosion and increases soil water retention (Cerdà, 1997). Moreover, continuous addition of organic matter, through living plants can contribute to soil stability (Huang et al., 2005). Plants have also been found to help eliminate contaminants through their impact on soil microorganisms (Siciliano et al., 2003; Phillips et al., 2006). Plants have an impact at different levels of the ecosystem, and their establishment is a first step in the improvement of the overall biodiversity and the re-establishment of a balanced ecosystem.

Different plants have been evaluated for growth on tailings sand. Barley was studied as a pioneer plant, however the low nutrient level of the tailings sand was reducing plant performance (Renault et al., 2003). Trees with nitrogen-fixing symbionts can facilitate vegetation development through addition of nitrogen to the system (Parrotta, 1999), and could alleviate the nutrient limitation of the tailings sand. Alders are actinorhizal plants that have a symbiotic relationship with *Frankia* sp., a nitrogen-fixing actinomycete. This allows them to establish in nutrient-poor, harsh environments (Roy et al., 2007). Alders have been found to increase soil total nitrogen and carbon (Martin et al., 2003). In addition to thriving in low nitrogen conditions, alders have been reported to support growth in salt affected environments (Graves and Gallagher, 2003; Mertens et al., 2004) and in composite tailings (Khasa et al., 2002). Alder growth was monitored in oil-shale mining detritus and found to be comparable to that recorded on two reference fertile sites (Vares et al., 2004). No difference in leaf nitrogen content was detected, emphasizing that alders are self-sufficient for nitrogen acquisition in the low nitrogen conditions of oil-shale mining waste. All of these characteristics make alders and *Frankia* ideal candidates for revegetation of the reclamation sites.

There are many examples of the excellent performance of alders in harsh growth substrates. However, Markham (2005) demonstrated the importance of fully developed *Frankia* sp. nodules prior to the transplanting to a stressful environment, such as mine tailings or oil sands tailings, to improve survival rates. Greenhouse inoculation could be an approach to insure adequate nodulation.

Previous greenhouse trials have shown that *Frankia*-inoculated alders could successfully grow directly in tailings sand or composite tailings, and have a positive impact on the diversity and activity of the indigenous soil microbial populations (Greer et al., 2005). Another study previously demonstrated that alders had a positive impact on soil fertility and on the physiological activity of the soil microbial population (Selmants et al., 2005).

The objectives of this study were to evaluate the ability of *Frankia*-inoculated alders to grow on reclamation sites containing tailings sand capped with mineral soil and peat according to current practices (Fung and Macyk, 2000), and to characterize how alder establishment would impact soil quality and microbial communities. The site was monitored for the establishment and growth of alders, for changes in soil quality and indigenous soil microbial population composition and activity.

3.2 Material and Methods

3.2.1 Site description

The site is located at Syncrude Canada Ltd., Fort McMurray, Alberta, Canada (56^{o}59'N; 111^{o}47'E). Coarse tailings were capped, during the winter of 2000, with a 23 cm layer of reclaimed mineral soil and covered by 16 cm of muskeg peat. No mixing, or fertilization was performed. Green alders (*Alnus crispa* (Ait.) Pursh) seedlings were started in a greenhouse, in March 2004, and a portion of the seedlings were inoculated with *Frankia* sp. strain AvcI1 (Quoreshi et al., 2007). The seeds were collected in Fort McMurray (latitude 56.3, longitude 112.1) and were obtained through the National Tree Seed Centre (Fredericton, NB, seedlot 8360545.0). Alders were out-planted on the research site in June 2005: four plots were planted with *Frankia* (strain AvcI1)-inoculated alders and four plots with non-inoculated alders (control), with each plot having 12 plants planted at 2 m intervals. An adjacent zone was kept unplanted. Two sampling events took place, the first at the end of August 2006 after ~1.5 years of growth in the field, and the second at the beginning of September 2007 after 2.5 years of growth in the field.

3.2.2 Sampling

Triplicate alders were harvested for each treatment. Non-inoculated plants were harvested first to reduce contamination risks. Roots were shaken gently to remove excess soil, and then roots were shaken vigorously to recover bulk soil. The aerial portion of each plant was cut 1cm above the soil level. Triplicate bulk soil samples were also collected from the unplanted zones. For rhizosphere recovery, a random portion of roots was selected and shaken at 90 rev min^{-1} for 90 min in sterile water (2 to 4 volumes of the weight) to completely cover the roots. The soil slurry was centrifuged at 12,400 x *g* for 10 min. The rhizosphere soil was the remaining pellet after the supernatant was discarded. Bulk and rhizosphere soil moisture contents were determined by drying at 105^{o}C for a minimum of 24 h.

Roots and nodules were surface sterilized using a protocol modified from Seghers et al. (2004). Roots were rinsed twice with sterile Milli-Q water, shaken at 90 rev min^{-1} for 1 min with 5% bleach solution, then for 10 min with fresh 5% bleach solution, rinsed with 95% ethanol, and then rinsed 4 times with sterile Milli-Q water. A 1 ml aliquot of the final rinse was kept to check for sterility using molecular techniques (Section 3.2.5.1). Surface sterilized roots were stored at –80^{o}C. In 2007, nodules were separated from the roots, and analyzed separately (see below).

3.2.3 Microbial enumeration

Total heterotrophic bacteria and polycyclic aromatic hydrocarbon (PAH)-degrading bacteria were determined for 2007 rhizosphere and bulk soil samples using the most probable number (MPN) technique. PAH-degrader counts were determined in 96-well plates as described by Wrenn and Venosa (1996) except that four PAHs (10 g phenanthrene/l, 1 g anthracene/l, 1 g fluorene/l, and 1 g dibenzothiophene/l) were dissolved in hexane and the Bushnell-Hass medium was used without NaCl addition. After incubation for 3 weeks at room temperature, positive wells developed a yellow-brown colour due to PAH degradation. Total heterotroph counts were performed following the same methodology but with minor modifications. Plates were filled with YTS_{250} medium (250 mg each of yeast extract, bacto-tryptone, and starch per litre of water) and incubated in the dark at room temperature for two weeks. After incubation, 50

µl of a 50:50 *p*-iodonitrotetrazolium violet (6 g/l): succinate (1 M) in PBS (9.6 mM) solution was added to each well of the YTS_{250}plates. After 6 to 24 hours incubation at room temperature in the dark, positive wells developed a violet-red colour. Results were expressed as most probable number of heterotrophic or PAH-degrading bacteria (MPN)/g of dry soil.

3.2.4 Bulk soil and plant analyses

Soil and plant analyses were performed by Agridirect Inc. (Longueuil, Québec) according to their standard operating methods. Organic matter content of the 2006 soil samples was determined in our lab using the same technique used by Agridirect Inc., i.e. 16 h at 375°C (CEAEQ/MAPAQ, 2003). Electrical conductivity was determined according to the CPVQ (1988) protocol using a *Sens***ion**™5 conductivity meter (HACH Company, Loveland, CO). Plant biomass was determined by weighing plants after drying for more than 36 h at 65°C.

3.2.5 Mineralization assays

Mineralization assays were performed for three representative petroleum hydrocarbons (hexadecane, naphthalene and phenanthrene), in microcosms for bulk soil and in mini-microcosms for rhizosphere soil and endophytes (crushed, surface sterilized roots and nodules). Bulk soil microcosms (20 g soil) were set up and sampled as described by Greer et al. (2003). Samples were spiked with 100,000 dpm hexadecane-1-C^{14} (specific activity: 12mCi/mmol) in 100 mg kg^{-1} hexadecane, 100,000 dpm naphthalene-1-C^{14} (specific activity: 2.3mCi/mmol) in 10 mg kg^{-1} naphthalene, or 100,000 dpm phenanthrene-9-C^{14} (specific activity: 55.7mCi/mmol) in 10 mg kg^{-1} phenanthrene. Mini-microcosms followed the same methodology but in a smaller format: 60 ml serum bottles containing 1 g of sample, and a 1 ml tube containing 0.5 ml of 1 M KOH. Samples were spiked with 10,000 dpm hexadecane-1-C^{14} in 100 mg kg^{-1} hexadecane, 10,000 dpm naphthalene-1-C^{14} in 10 mg kg^{-1} naphthalene, or 10,000 dpm phenanthrene-9-C^{14} in 10 mg kg^{-1} phenanthrene. Microcosms and mini-microcosms were incubated at room temperature and the KOH was extracted and analyzed at regular intervals for up to 8 weeks.

3.2.5 Molecular microbial analysis

3.2.5.1 Sterility check

To insure adequate surface sterilization of the roots or nodules, 1 ml of the final rinse water was boiled for 10 min to release possible DNA. One µl was used to perform a 20 cycle touchdown PCR, using the universal bacterial primers U341 and U758 as described in Fortin et al. (2004) with the exception that Taq DNA polymerase was added prior to the initial denaturation step.

3.2.5.2 DNA extractions

Total soil DNA extraction was performed using the 10g PowerMax™ Soil DNA Isolation Kit (MoBio Laboratories, Carlsbad, CA) according to the manufacturer's instructions. DNA was stored at –20^{o}C in TE (10 mM Tris-Cl; 1 mM Na_2EDTA, pH 8.0). Total rhizosphere DNA was extracted from 5 g of soil using the PowerMax™ Soil DNA Isolation Kit as described above.

Total DNA extraction was performed on crushed nodules and/or roots using a chemical lysis approach (Fortin et al., 1998) combined with 2 x 1 min of bead beating with 0.1 mm and 1.0 mm zirconium/silica beads. Proteins and debris were precipitated using 7.5 M ammonium acetate, DNA was then precipitated overnight at –20^{o}C in cold 2-propanol, and resuspended in TE (pH 8.0). DNA crude extracts were purified using PVPP/Sephacryl spin columns as described in Jugnia et al. (2008). Before the addition of the DNA extracts, resins were washed twice with TE (pH 7.5)-NaCl (0.1 M), to improve DNA recovery.

3.2.5.3 *Frankia* sp. strain AvcI1 detection

The presence of *Frankia* sp. strain AvcI1 in nodules or roots was determined by using PCR with primers designed to be specific for this strain, 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'). The primers target a portion of the intergenic spacer region between the *nif*D and *nif*K genes. Each 50 µl PCR mix contained 25 or 50 ng of template DNA. Fragment amplification was performed under the following conditions: initial denaturation at 98 ^{o}C for 5 min, Taq DNA polymerase addition, followed by 25 cycles of

30 s at 97 °C, 1 min at 68 °C, and 1 min at 72°C. When it was not possible to detect strain AvcI1 through direct PCR, a nested strategy was utilized. The first PCR targeted the area flanking the intergenic spacer region. Primers FGDP807 and FGDK333 were used as described by Nalin et al. (1995) with the number of cycles reduced to 20. Five µl of the final product was used as template for a second PCR using primers FRIGS-F and FRIGS-R, as described previously.

3.2.5.4 16S rRNA gene PCR-DGGE

Bulk soil, rhizosphere and endophytic microbial community patterns were investigated using Denaturating Gradient Gel Electrophoresis (DGGE) of PCR amplified 16S rRNA gene fragments. PCR amplification with bacterial primers U341 (with GC clamp) and U758 was performed using a 20 cycle touchdown procedure as described previously (Section 2.5.1). From 7 to 16 PCR reactions were combined and precipitated to obtain sufficient DNA and to reduce bias associated with PCR amplification. DGGE was carried out as described by Labbé et al. (2007). For bulk soil and rhizosphere, 300 ng of DNA per sample was loaded into individual lanes of a 30-70% denaturant gradient gel (7M urea and 40% deionized formamide were 100% denaturant). For endophytes, 250 ng of DNA was loaded onto a 45-65% denaturant gradient gel. Bands of interest were excised using a sharp scalpel blade, and eluted overnight at 4°C in 60 µl of sterile Milli-Q water. Re-amplification for sequencing was performed using the same primers as above but without the GC clamp: 1 µl of eluted DNA was used in a 50 µl reaction of 25 cycles with annealing at 61 °C for 1 min and extension at 72°C for 1 min. When amplification was non-specific, the annealing temperature was increased from 61°C to 64°C. One or two PCR reactions for the same band were purified using the Illustra GFX PCR DNA purification kit (GE Healthcare, Baie d'Urfé, Québec). Sequencing was performed by the "Laboratoire de synthèse et d'analyse d'acides nucléiques" of Université Laval (Ste-Foy, Québec) with a capillary ABI Prism 3100 sequencer. Sequences were analyzed using sequence analysis software: MacVector v9.0 (Accelrys Inc., San Diego, CA) or BioEdit v7.0.5 (Ibis Bioscience, Carlsbad, CA). All sequences were checked for chimeras with Bellerophon, using Huber-Hugenholtz correction, (Huber et al., 2004) or Pintail (Ashelford et al., 2005), depending on the sequence length. The 16S rRNA gene

sequences were compared against different databases using specific algorithms: BLAST for GenBank, FASTA for EMBL, and SeqMatch for RDP II. Image analysis was performed using GelComparII (Applied Maths, Austin, TX). Cluster analysis was performed using the Dice similarity coefficient, and a UPGMA dendogram was generated.

3.3 Results

3.3.1 Bulk soil, rhizosphere soil and plant characterization

Variations were observed for many soil parameters between the *Frankia*-inoculated alders and the unplanted treatments (Table 1). For both 2006 and 2007 samples, soil planted with *Frankia*-alders were almost one full pH unit lower, there was a 3-fold decrease in plant-available sodium (Na) content, and a decrease in percent saturation of three main cations (Ca, Mg, and K) when compared to unplanted soil. Furthermore, more organic matter content was measured in *Frankia*-alder planted soil. However, soil total nitrogen content remained below detection limits (0.2%).

Plant percent nitrogen was not significantly different between inoculated and non-inoculated alders, and varied from ~3.0% in 2006 to ~2.5% in 2007 (Table 2). Plant biomass was also similar between the 2 treatments. Even with soil nitrogen being below detection limits, there was at least a five-fold increase in plant biomass from 2006 to 2007 for both treatments. Thus, total nitrogen captured in alders increased per plant from 3.5g ± 1.3 and 4.8g ± 1.0, in 2006, to 18.1g ±1.6 and 20.3g ± 7.0, in 2007, for non-inoculated alders and *Frankia*-inoculated alders, respectively.

Microbial enumeration of total heterotrophic bacteria and PAH-degraders demonstrated that population densities were higher in the rhizosphere than in the bulk soil, and that overall, heterotrophic bacteria (10^6 to 10^8 MPN/g of dry soil) were more abundant than PAH-degraders (10^3 to 10^4 MPN/g of dry soil). In the bulk soil, PAH-degrader counts were similar irrespective of treatment (10^3). However, total heterotrophic bacterial counts were slightly higher for the *Frankia*-alder (10^7) and the control-alder treatments (10^7) than the unplanted treatment (10^6).

3.3.2 Mineralization Assays

The mineralization activity of the indigenous microbial population in the bulk and rhizosphere soils was evaluated using three representative hydrocarbon substrates: hexadecane, naphthalene, and phenanthrene (Figs. 1 and 2). In 2006, mineralization increased in the bulk soil for all three test substrates in the *Frankia*-alder treatment compared to both unplanted and control-alder treatments (Fig. 1). However, after 2 y of growth in the field, mineralization rates of the non-inoculated alder bulk soil increased to rates that were comparable to the *Frankia*-alder treatment, for hexadecane and naphthalene. Unplanted bulk soil mineralization rates remained lower than both planted treatments. No significant differences between the treatments were found for mineralization in the rhizosphere soil for all three substrates tested (Fig. 2). Phenanthrene mineralization in the bulk soil took more than 4 weeks to start and rates remained low. This was not seen in the rhizosphere where at least 50% mineralization was obtained. On the other hand, mineralization of hexadecane remained low in the rhizosphere while it was high in the bulk soil (for the vegetated treatments). No mineralization was detected in the endophytic community samples (data not shown).

3.3.3 Molecular analysis

When field plants were harvested numerous nodules were present on the *Frankia*-alder roots, but also on non-inoculated plants although in smaller amounts. *Frankia* strain AvcI1 was detected in all root and/or nodule samples, whether alders had been inoculated in the greenhouse or not, and for samplings in both 2006 and 2007 (data not shown). Moreover, it was possible through 16S rRNA gene DGGE of the endophytic community (Fig. 3) to detect the presence of *Frankia* sp. (bands g to i, Table 3) in all of the samples. Analyses of nodules, in 2007, of natural alders growing on the site both adjacent to and farther away from the research plots showed that amplification of a fragment was possible with AvcI1-specific primers either through direct or nested PCR.

The banding pattern seen on the 16S rRNA gene DGGE shows that there was a high bacterial diversity in the bulk soil and in the rhizosphere. While there were many common bands between the different samples some bands were unique to each treatment. This was confirmed by cluster analysis of the DGGE band profiles, which separated

samples according to their treatments (Fig. 4 and 5). In the bulk soil, unplanted samples (Soil U) grouped separately from the *Frankia*-alder samples (Soil F) for both 2006 and 2007 analyses. Control-alder bulk soil samples (Soil C), which grouped closer to the unplanted treatment (Soil U) in 2006, clustered with *Frankia*-inoculated alder samples (Soil F) in 2007. In 2007, rhizosphere soil profiles (Rhizo F and Rhizo C) grouped separately from all bulk soil samples. However, rhizosphere samples clustered closer to planted bulk soil (*Frankia*-inoculated as well as non-inoculated) (Soil F and Soil C), than to unplanted bulk soil (Soil U).

Even though the best matches in the different databases was often from uncultured bacteria, sequencing of predominant bands allowed the identification of bacteria that were often found in contaminated or saline environments, or soils. Several dominant bands that were present in the unplanted bulk soil samples were not found in the planted bulk soil samples. These bands corresponded to bacteria previously isolated from saline environments (bands 4 to 7, Table 3).

3.4 Discussion

The establishment of *Frankia*-inoculated alders in the capped tailings sand improved general soil quality as shown in this study. The presence of alders increased the organic matter content and the cation exchange capacity of the soil. Organic matter is important for soil structure, and the soil biogeochemical balance (as reviewed by Huang et al., 2005). Also, growth of the *Frankia*-alders caused a significant decrease in the soil pH. Similar results were found with alders growing on gold mine tailings (Densmore, 2005). Van Miegroet and Cole (1985) suggested that the decrease in pH seen in alder stands could be linked to nitrification processes associated with nitrogen fixation. However, as soil total nitrogen was below the detection limit it seems unlikely that this hypothesis is valid for this study. Plants are known to be adversely affected by soil salt content (Tester and Davenport, 2003). Therefore the reduction of plant-available soil sodium seen under *Frankia*-alder treatment can result in improving the establishment of more salt-sensitive plant species. The enhancement of these soil characteristics after only 2 years shows that *Frankia*-alders can create an environment more favourable for plant growth in an otherwise harsh environment.

The percent nitrogen content of alder plant tissues found in this field trial was similar to those reported by Pérez-Corona et al. (2006) for alders growing in a non-contaminated environment. Furthermore, the increase in alder biomass between 2006 and 2007 indicates that alders are performing well in this low soil nutrient environment. Nitrogen is often considered as a major limiting factor for plant growth (Vitousek and Howarth, 1991). In this study, even though soil total nitrogen was below detection limits, total plant nitrogen increased over time, through biomass acquisition. Since there was no detectable total nitrogen in the soil, nitrogen acquisition is most likely due to atmospheric nitrogen fixation by *Frankia* sp. (Markham and Zekveld, 2007). In the long-term, this could bring a new nitrogen source into the system, with increased biomass producing a flow-on effect to eventually increase organic matter and nutrients in the surrounding soil (Pérez-Corona et al., 2006). This nitrogen could facilitate the growth of other tree species and the establishment of a multi-storey vegetation (Kohls et al., 2003; Densmore, 2005).

The presence of nodules on non-inoculated plants could explain the absence of differences in plant biomass and nitrogen content between inoculated and non-inoculated plants. Alders are known to become inoculated naturally under greenhouse and field conditions, and the differences seen after one year in the field disappear during subsequent years of growth. In order to conclusively determine the effects of greenhouse inoculation, it is necessary to ensure that greenhouse and field conditions are established to maintain clear differences between the two treatments. However, the detection of *Frankia* sp. strain AvcI1 in nodules of alders growing naturally at Syncrude Canada Ltd. could indicate that an indigenous *Frankia* sp. similar to strain AvcI1 has infected alders once in the field. *Frankia* spp. are known to survive and remain infective in soil devoid of host plants (Nickel et al., 2001; Ridgway et al., 2004).

Information on petroleum hydrocarbon degradation was obtained by mineralization assays of three representative hydrocarbons: hexadecane, naphthalene, and phenanthrene. The increase in degradation potential seen in *Frankia*-inoculated alder bulk soil compared with unplanted bulk soil for all three substrates in 2006, and both hexadecane and naphthalene in 2007, indicates that the establishment of alders had a positive impact on the hydrocarbon degradation capacity of the soil indigenous microbial population. Similar results were reported in a study that looked at phytoremediation of

PAHs when fertilizer was added to the soil (Siciliano et al., 2003). In our study, alders were able to promote microbial activity without the addition of any fertilizer. It was demonstrated that rhizodeposition (root turnover and exudates) can increase the relative abundance of genes involved in PAH degradation (Da Silva et al., 2006). In this study, microbial enumeration of total heterotrophs and PAH-degrading bacteria clearly showed that rhizosphere soil sustained a higher population density of microorganisms than bulk soil. Moreover, the differences in substrate mineralization (hexadecane and phenanthrene) between bulk and rhizosphere soils indicate differences in the composition and activity of their corresponding microbial communities. The establishment of plants and their rhizospheric microbial communities represent a major source of abundant and diverse microorganisms to improve the diversity of this system. The increase in the mineralization capacity of the bulk soil microbial population under alder treatment suggests that residual hydrocarbons present in tailings sand are more effectively degraded, and so improve soil conditions for all organisms.

The DGGE technique has been used in the characterization of microbial communities (Hadwin et al., 2006), and to monitor shifts in microbial populations during bioremediation studies (Hamamura et al., 2006; Miyasaka et al., 2006) or phytoremediation trials (Siciliano et al., 2003). This technique has proven to be useful in the study of complex microbial communities over time and space. In this study, it was used to evaluate microbial population diversity and transformation in bulk and rhizosphere soils after alder establishment. The DGGE profiles showed a high diversity of microorganisms in the bulk and rhizosphere soils. Cluster analyses of the 2006 and 2007 bulk soil banding patterns indicated that the microbial community of the *Frankia*-alder treatment differed from the unplanted treatment and that *Frankia*-inoculated alder establishment did have an impact on the soil microbial community composition. The DGGE fingerprint and the cluster analyses support the trend that was seen for the mineralization results of the control-alder bulk soil microbial populations between 2006 and 2007. It appears that a variation in microorganism dominance might be responsible for the change in mineralization capacity of the bulk soil microbial population. In 2007, rhizosphere samples grouped separately from bulk soil samples, supporting a difference in microbial population composition as observed by Smalla et al. (2001). However,

planted bulk soil samples grouped closer to rhizosphere samples than to unplanted soil samples indicating that microbial diversity patterns are more similar between planted bulk soil and rhizosphere soil than between planted and unplanted bulk soil. This is interesting since rhizosphere soil is known to be a nutrient rich environment which stimulates the activity and diversity of microbial communities (Rovira, 1965). Sequencing of selected bands emphasized the need for more research to better characterize this environment. Disappearance of bands related to microorganisms found in saline environments and the decrease of soil sodium (Na) content between the unplanted and planted samples also suggests that the initial conditions favouring the growth of saline-adapted microorganisms have changed after the establishment of alders. It confirms the positive impact of *Frankia*-inoculated alder on the reclamation site.

Overall, *Frankia*-inoculated alders grew well on the reclamation site, producing significant biomass, improving soil quality indicators and having a positive impact on indigenous soil microbial community structure and function. Moreover, the molecular analyses showed a microbial population shift between unplanted and alder-planted bulk soil. Some microorganisms that are adapted to saline conditions have faded from the microbial diversity profile. This study showed that *Frankia*-inoculated alders are capable of establishing and performing well without any fertilizer addition in this harsh and nutrient-limited environment. The improvements in the soil quality of the reclamation site may allow the subsequent establishment of more sensitive species, leading ultimately to the reforestation of the site, and the re-establishment of a balanced ecosystem.

Acknowledgments

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Table 1. Soil analysis of capped overburden-tailings sand either unplanted, planted with non-inoculated alders (control-alders) or *Frankia*-inoculated alders (*Frankia*-alders) after 1.5 (2006) and 2.5 (2007) years of growth in the field.

		Unplanted		Control-Planted		*Frankia*-inoculated	
		2006	*2007*	*2006*	*2007*	*2006*	*2007*
	pH	7.5 ± 0.3	8.2 ± 0.0	7.5 ± 0.2	7.5 ± 0.1	6.6 ± 0.4	7.2 ± 0.6
	buffer-pH	>7,5	>7.5	>7,5	>7.5	7.0 ± 0.4	7.3 ± 0.2
Mehlich-III (kg/ha)	K	211 ± 25	145 ± 10	217 ± 16	155 ± 16	116 ± 9	131 ± 39
	Mg	1533 ± 170	1843 ± 404	1697 ± 183	1937 ± 108	1637 ± 106	1400 ± 216
	Ca	7633 ± 887	8990 ± 1112	8017 ± 449	8473 ± 247	7597 ± 395	9060 ± 1115
	Na	498 ± 175	1780 ± 217	359 ± 121	87 ± 57	160 ± 23	474 ± 205
Total N	(%)	ND	ND	ND	ND	ND	ND*
Saturation (%)	K	1.1 ± 0.1	0.6 ± 0.1	1.0 ± 0.1	0.7 ± 0.1	0.5 ± 0.0	0.5 ± 0.2
	Mg	25.1 ± 3.5	25.8 ± 6.6	25.9 ± 3.1	27.4 ± 1.7	22.4 ± 1.8	18.7 ± 2.8
	Ca	73.8 ± 3.6	73.6 ± 6.6	73.1 ± 3.1	71.9 ± 1.7	62.4 ± 4.4	72.5 ± 7.7
	K+Mg+Ca	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	85.3 ± 6.3	91.7 ± 8.3
Estimated CEC	(meq/100)	23.0 ± 1.7	27.1 ± 1.2	24.5 ± 0.4	26.3 ± 0.2	27.2 ± 0.6	27.8 ± 0.6
Electrical conductivity	(µS/cm)	627 ±170	879 ± 171	441 ± 134	219 ± 15	354 ± 172	355 ± 77
Organic matter	(%)	2.7 ± 0.4 n=6	2.5 ± 0.2	2.7 ± 0.1 n=9	7.2 ± 3.2	12.2 ± 0.2 n=6	15.1 ± 11.1

Results are expressed as means with corresponding standard error; n=3, unless specified

ND: not detectable (detection limit: 0.2%)

* 2 out of 3 replicates had non-detectable levels; the third one was just above detection limits

Table 2. Average plant biomass and nitrogen (N) content of non-inoculated alders (control-alders) and *Frankia*-inoculated alders (*Frankia*-alders) planted in capped overburden-tailings sand after 1.5 (2006) and 2.5 (2007) years of growth in the field.

	Control-alders		*Frankia*-alders	
	2006 n=3	2007 n=3	2006 n=5	2007 n=3
Dry weight (g)	111.8 ± 37.8	689.0 ± 69.3	154.1 ± 32.8	800.2 ± 281.3
Total N (%)	3.0 ± 0.2	2.6 ± 0.1	3.1 ± 0.0	2.5 ± 0.1
Total N (g) /plant	3.5 ± 1.3	18.1 ± 1.6	4.8 ± 1.0	20.3 ± 7.0

Results are expressed as means with corresponding standard error

Table 3. Closest microbial match of sequenced 16S rRNA gene DGGE bands

Band	Accession number	Closest match	BLAST % similarity	FASTA % similarity	RDP II % similarity	Characteristics
r,s,t, u,v,w		Plant DNA	99	99	99-100	unspecific amplification associated with plant DNA
x,y,z	CT573213	*Frankia alni* str. ACN14A	99	99.4	99.7	
4	Eu196300	*Gillisia* sp. NP8	98	98.0	98.0	cold saline (7.5%) sulfidic spring
5	EU196340	*Gillisia* sp. NP17	99	99.1	99.1	cold saline (7.5%) sulfidic spring
6	AY913287	uncultured forest soil bacterium	99	99.1	99.5	mixed forest
7	EU196340	*Gillisia* sp. NP17	98	98.6	99.5	cold saline (7.5%) sulfidic spring
	AY259507	*Gelidibacter* sp.BSD S1 19	97	97.0	96.7	salt marsh sediment
8	DQ004377	uncultured *Bacteroidetes* bacterium	100	100	NP	soil
	AJ626894	*Adhaeribacter aquaticus*	NP	NP	84.9	freshwater biofilm
10	EF540531	uncultured soil bacterium	95	95.4	NP	semi-coke
	DQ448698	*Modestobacter* sp. CNJ793	NP	NP	88.5	marine sediment
17	EF020181	uncultured *Bacteroidetes* bacterium	99	99.3	99.3	rhizosphere
	AB192296	*Terrimonas lutea*	NP	NP	96.0	
23	EF662647	uncultured flavobacteria	98	98.6	NP	cropland
	AB329629	*Niastella* sp.KP03	NP	NP	94.2	soil
26	AM934629	*Flavobacterium* sp. WB1.2-3	100	100	100	hard water creek
29	DQ640006	*Flavobacterium* sp. PRO1	99	99.5	99.5	Cd/Pb/Zn contaminated soil
32	AY310305	*Arthrobacter* sp. CPA2	100	100	NP	lead mine tailings
	AY177360	*Arthrobacter* sp. M4	NP	NP	100	phenanthrene-degrading
35	EF020181	uncultured *Bacteroidetes* bacterium	98	99	98.9	rhizosphere
37	AM230490	*Flavobacterium pectinovorum*	100	100	100	

NP: match not present in the database

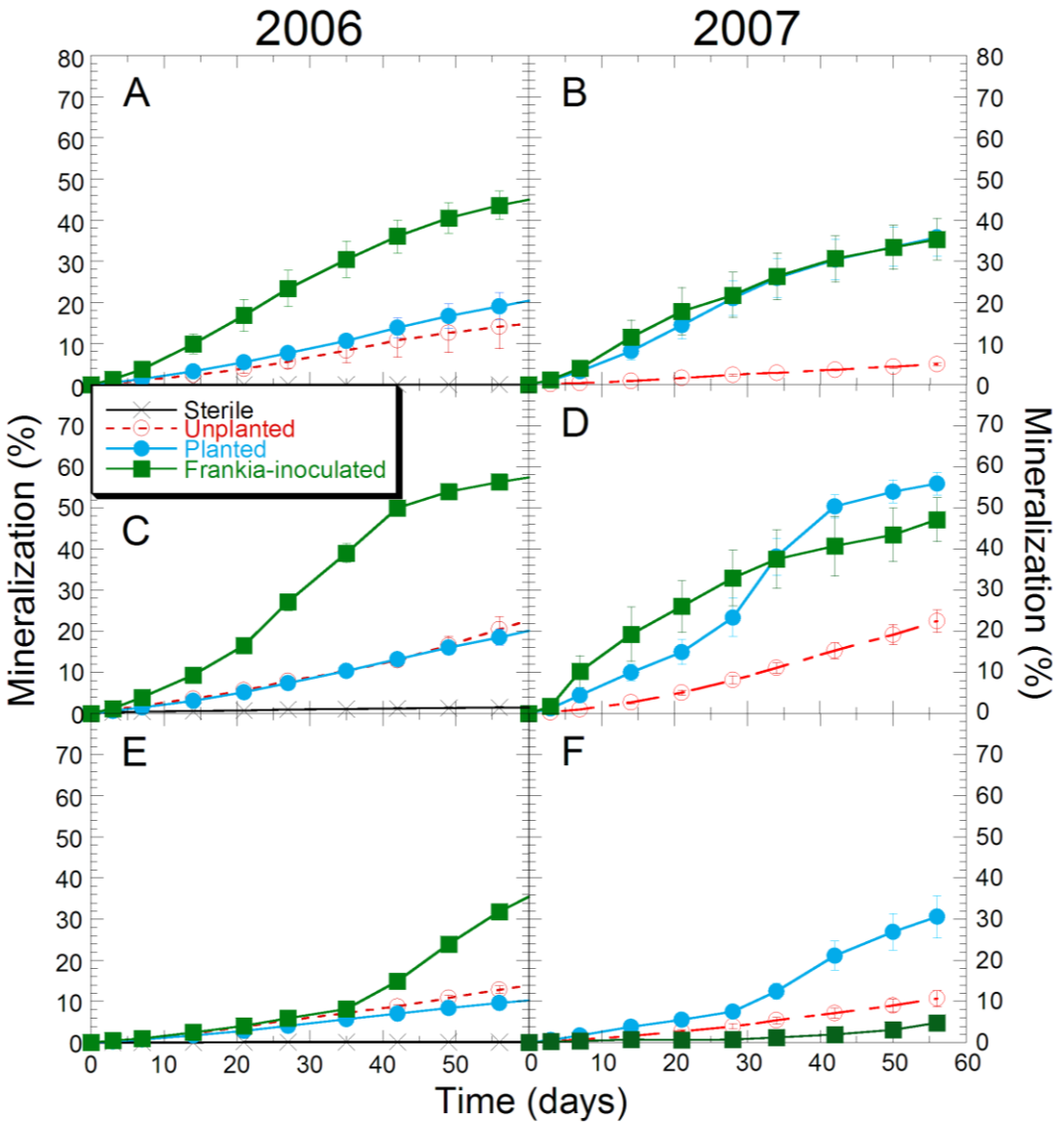

Figure 1. Mineralization of representative hydrocarbon substrates, hexadecane (A, B), naphthalene (C, D) and phenanthrene (E, F), in bulk soil of capped overburden-tailings sands either unplanted, planted with non-inoculated alders (Planted) or *Frankia*-inoculated alders after 1.5 (2006) and 2.5 (2007) years of growth in the field.

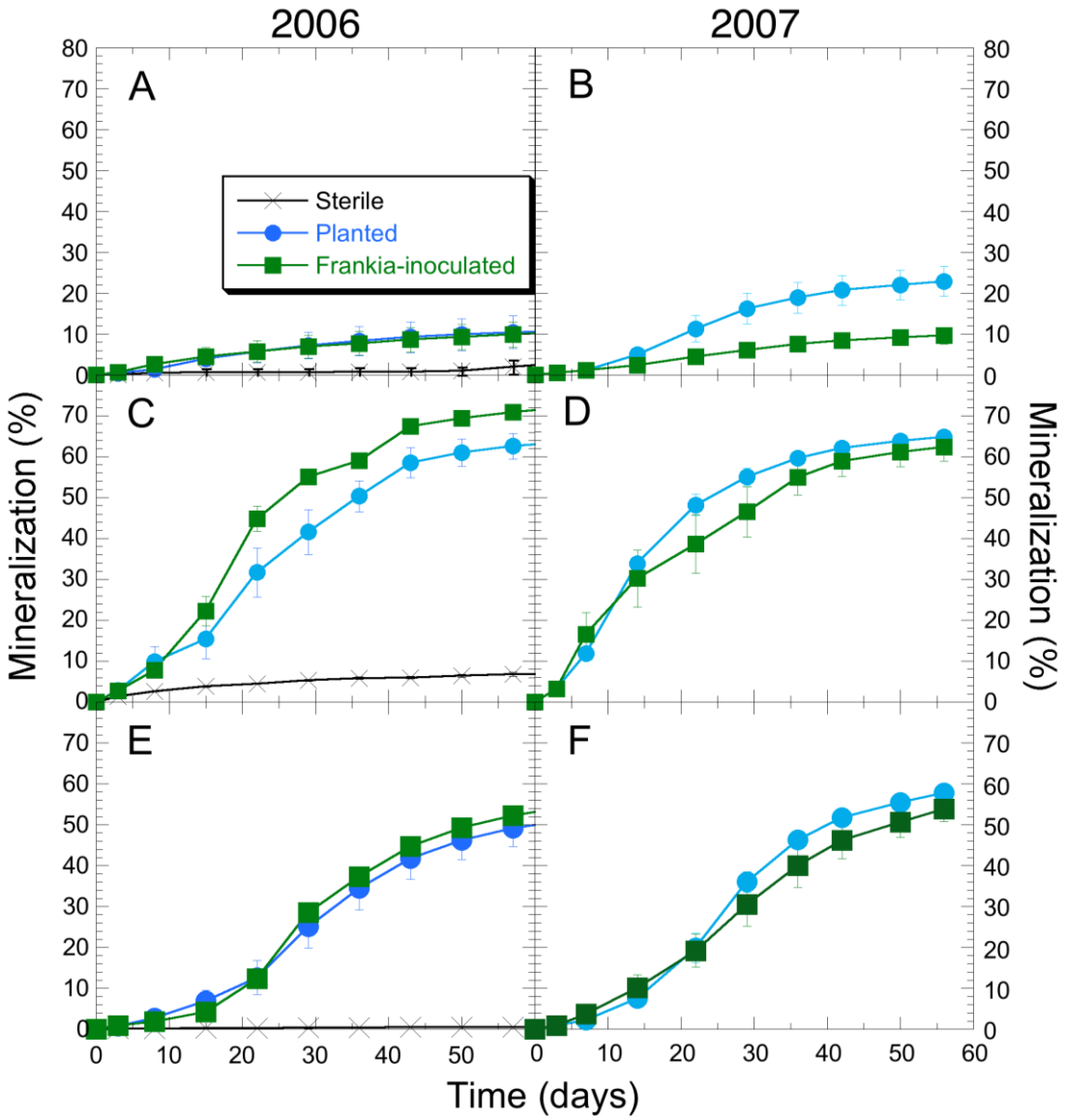

Figure 2. Mineralization of representative hydrocarbon substrates, hexadecane (A, B), naphthalene (C, D) and phenanthrene (E, F), in the rhizosphere of non-inoculated alders (Planted) or *Frankia*-inoculated alders after 1.5 (2006) and 2.5 (2007) years of growth in an overburden-tailings sand mixture.

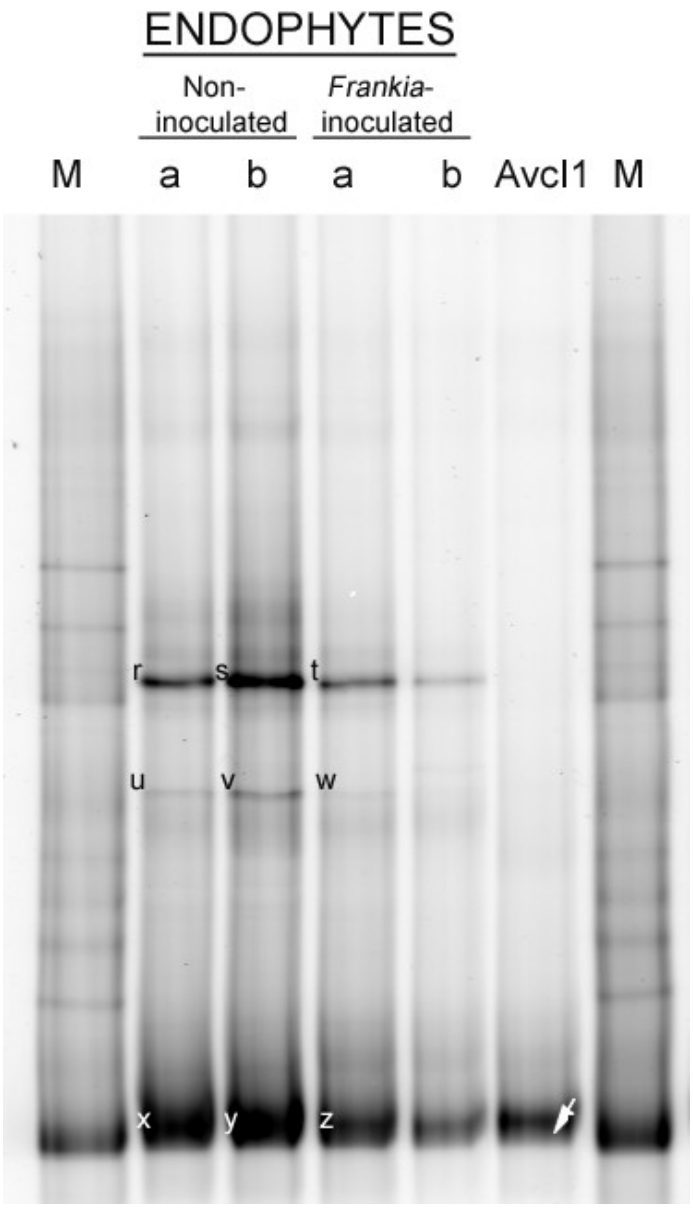

Figure 3. DGGE of PCR amplified 16S rRNA gene fragments from total DNA extracted from roots (endophytes) of alders inoculated or not with *Frankia* strain AvcI1 after 1.5 year growth in a overburden-tailings sand mixture. Non-inoculated a and c, and *Frankia*-inoculated a and b identifies field replicates. AvcI1 is the pure culture of *Frankia* strain AvcI1, and the arrow indicates the position of its dominant band. Letters (a-i) indicate bands that were excised for subsequent nucleotide sequencing (see Table 3). M is a marker lane composed of pure strains with known migration patterns.

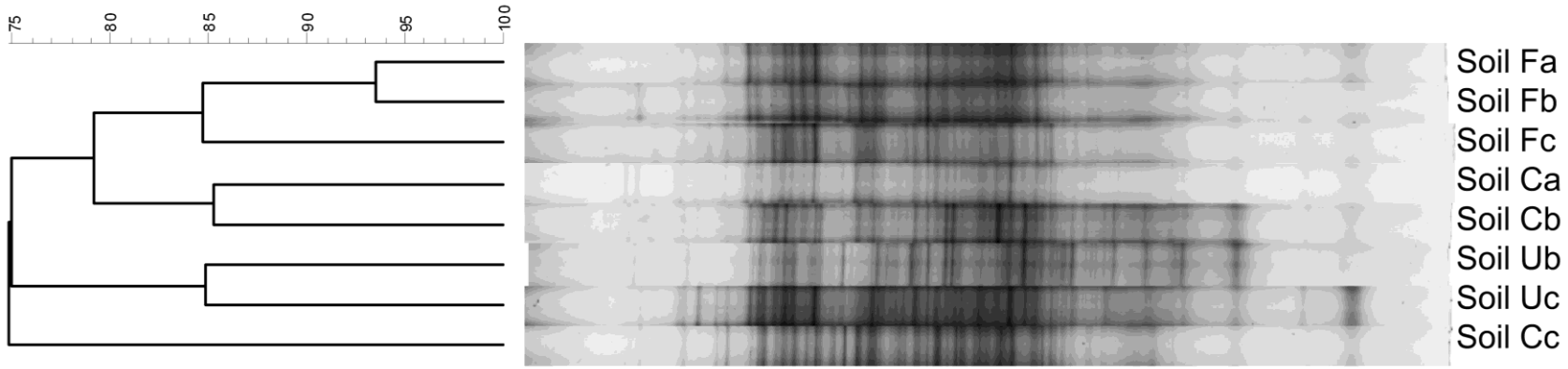

Figure 4. Cluster analysis, with corresponding DGGE lanes, of 16S rRNA gene DGGE banding pattern of 2006 bulk soil samples. Fa, Fb and Fc, Ca, Cb and Cc and Ub and Uc identify field replicates of *Frankia*-inoculated alders (F), non-inoculated control alders (C) and unplanted (U) treatments, respectively.

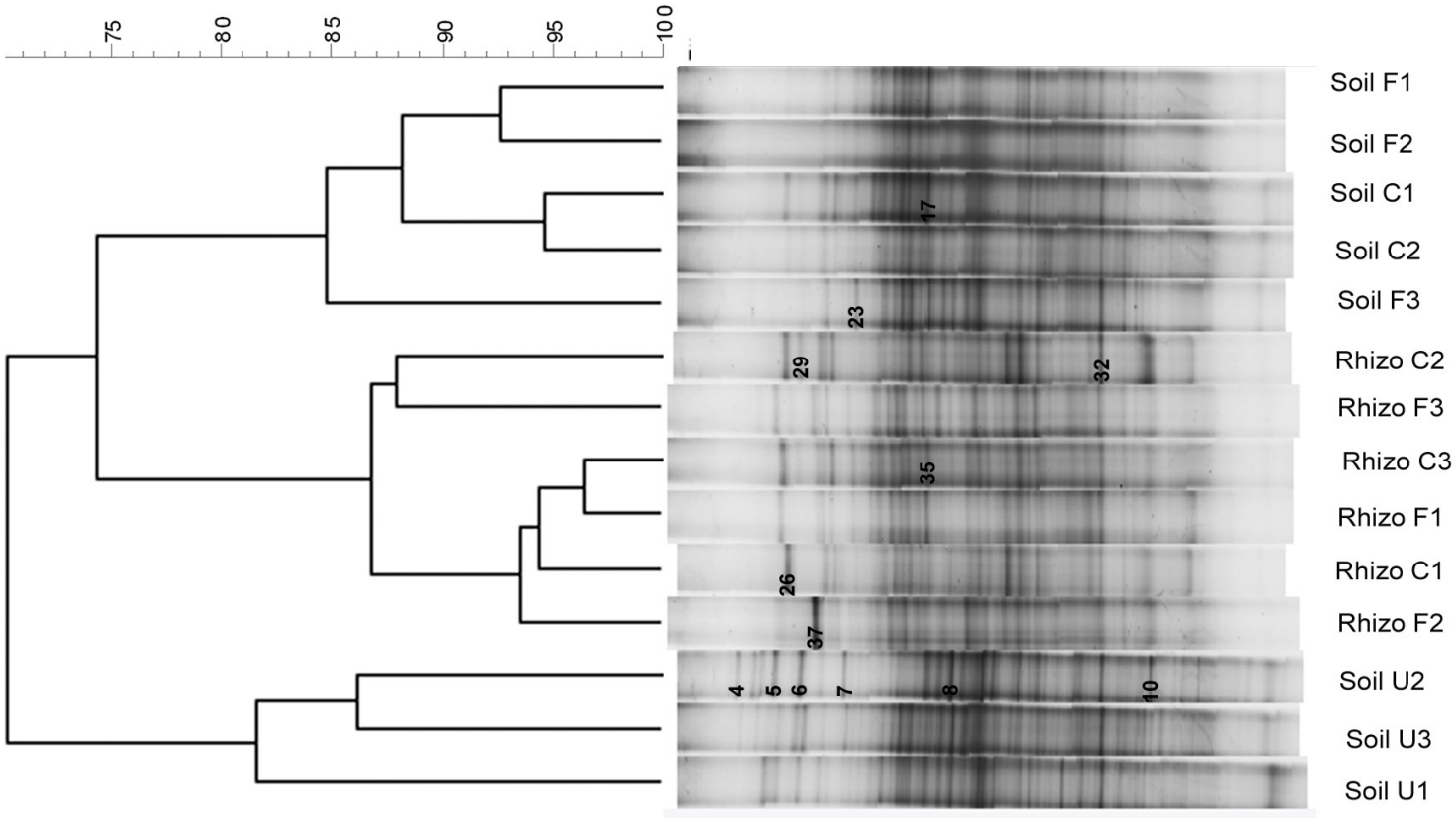

Figure 5. Cluster analysis, with corresponding DGGE lanes, of 16S rRNA gene DGGE banding pattern of 2007 bulk and rhizosphere soil samples. Soil and Rhizo indicate bulk soil samples and rhizosphere soil samples, respectively. F1, F2 and F3, C1, C2 and C3 and U1, U2 and U3 identify field replicates of *Frankia*-inoculated alders (F), non-inoculated control alders (C) and unplanted treatments (U), respectively. Numbers indicate bands that were excised for subsequent nucleotide sequencing (see Table 3).

Chapter 4 is a manuscript describing the greenhouse study results. The greenhouse trial allowed to evaluate *Frankia*-inoculated alder performance directly in tailings sands (TS) and composite tailings (CT). This part of the project outlined the real potential of *Frankia*-inoculated alders to survive and grow under the harshest soil conditions. Two different alder species (*Alnus glutinosa*, and *A. crispa*) were tested in the greenhouse trial. An additional component was added, as compared to the field trial, by evaluating a tripartite association (alders/*Frankia*/*Glomus intraradices*) in TS. This part of the project provides additional data that can serve to plan future reclamation strategies. At the time of submission of this thesis this manuscript had been submitted to the journal *Plant and Soil*.

Chapter 4 Growth of alders in tailings sand and composite tailings: effects of inoculation with *Frankia* sp. and/or *Glomus intraradices*

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Abstract

The extraction of bitumen from oil sands generates process-affected materials that need to be reclaimed; two of these are tailings sands (TS) and composite or consolidated tailings (CT). These materials are poor in nutrients and in organic matter, have a high salinity and alkalinity, and contain residual hydrocarbons, characteristics that make reclamation challenging. We investigated the use of alders (*Alnus crispa* and *A. glutinosa*), pioneer species, to revegetate these materials. Alders can grow in harsh environments as they form a symbiotic relationship with *Frankia* spp., nitrogen-fixing actinomycetes, and fungi, such as *Glomus intraradices*, a vesicular-arbuscular mycorrhizal fungus. In this study we evaluated the impact of *Frankia* strain AcvI1 and/or *G. intraradices* inoculation on alder growth, and on the chemical and microbial population biomass and hydrocarbon mineralization potential in TS and CT. Plant growth was reduced in CT and more signs of injury were visible than in TS. *A. glutinosa* growth performance surpassed that of *A. crispa*, in TS and in CT. *Frankia* inoculation greatly improved *A. glutinosa* growth in TS, with growth equal to or superior to that in general

greenhouse growth substrate soil. Alders modified the petroleum hydrocarbon degradation potential of the CT and TS microbial populations. They altered TS chemical characteristics while CT was more recalcitrant to change; the pH of TS was decreased and the CEC increased, improvements that can facilitate the establishment of other plants. Dual inoculation (*Frankia* and *G. intraradices*) of *A. glutinosa* in TS did not significantly enhance plant growth, nor change soil chemical characteristics or microbial biomass and activity.

Introduction

The extraction process used in the production of crude oil from the Athabasca oil sands (Alberta, Canada) generates large quantities of residues. Up to three cubic meters of water per cubic meter of oil sand are used for bitumen extraction (Quagraine et al., 2005). Even though part of the water is recycled in the extraction process, the oil sand process-affected materials (OSPM) produced are in the form of a slurry, containing coarse sand, fine silty clay and residual hydrocarbons (Syncrude Canada Ltd., 2007). The OSPM are discharged into large sedimentation ponds where the coarse material separates from the fine tails and water and can be recovered, yielding tailings sand (TS) (Fung and Macyk, 2000). It is estimated that 125 to 150 years are necessary for the sedimentation of the fine tailings (Fedorak et al., 2003). It is a slow process and management of these residues is challenging. Gypsum ($CaSO_4 \bullet 2H_20$) addition is an avenue that has been evaluated to allow for the fine and coarse tailings to be remixed, generating composite or consolidated tailings (CT) (Franklin et al., 2002; Fedorak et al., 2003; Syncrude Canada Ltd., 2007). This process accelerates the handling of the residues and enables the material to be reclaimed faster. However, due to their characteristics, CT and associated CT water are difficult to revegetate (Renault et al., 2003) causing plant growth reduction and increased signs of injury (Renault et al., 2001; Franklin et al., 2002). CT and TS are poor in nutrients, have a high salinity, are alkaline and contain residual hydrocarbons (Fung and Macyk, 2000). Although both are challenging materials for plant growth, it is important to revegetate these materials to re-establish a balanced ecosystem on land that has been disturbed by the mining operation.

Frankia sp. is an actinomycete that forms a symbiotic relationship with actinorhizal plants such as *Casuarina* sp., *Ceanothus* sp. and *Alnus* sp. (Schwintzer and Tjepkema, 1990). When associated with its hosts, *Frankia* fixes atmospheric nitrogen, allowing its host to grow in nutrient limited environments. Alders (*Alnus* spp.) are pioneer species and this characteristic, as well as the advantages derived from its symbiosis with *Frankia*, have been utilized in the reclamation and revegetation of anthropogenic sediments or contaminated lands (Fedkenheuer, 1979; Fessenden, 1979; Khasa et al., 2002; Mertens et al., 2004; Vares et al., 2004; Densmore, 2005; Roy et al., 2007). Alders were part of the boreal forest that covered the land before it was mined (Fedkenheuer, 1979) and with many native Canadian species, are interesting plants for the reclamation of OSPM. The formation of fully functional root nodules in the alders before transplantation into a stressed environment is necessary to ensure adequate plant establishment and performance (Markham, 2005), emphasizing the importance of greenhouse inoculation with *Frankia* prior to outplanting in CT or TS.

In addition to their association with nitrogen-fixing bacteria, alders have a symbiotic relationship with both vesicular-arbuscular mycorrhizal fungi (VAM) and ectomycorrhizal fungi (Rose, 1980; Gardner et al., 1984). Mycorrhizal fungi are widely known to improve water and nutrient (especially phosphorous) acquisition by plants by increasing the root system length and contact with the soil (Fortin et al., 2008). There is solid evidence that a combined inoculation of a nitrogen-fixing actinomycete and a mycorrhizal fungus can improve the growth of actinorhizal plants (Rose and Youngberg, 1981; Yamanaka et al., 2003; Oliveira et al., 2005a). Dual inoculation has resulted in improved plant growth, increased nodule numbers and improved acetylene reduction, a parameter used to evaluate nitrogen fixation (Rose and Youngberg, 1981). As alders rely mainly on N-fixation in low nitrogen soils (Markham and Zekveld, 2007), such tripartite symbiosis could be beneficial for their establishment in TS or CT by increasing N-fixation capability.

Mycorrhizal fungi do not all have the same resistance to high salt concentrations (Bois et al., 2006). In order to colonize and be beneficial for a plant growing in TS or CT, mycorrhizal fungi must be able to survive under saline conditions in an alkaline growth substrate. *Glomus intraradices*, a VAM, is able to develop a symbiotic relationship with

alders under such conditions (Oliveira et al., 2005a). Many studies demonstrate successful symbiosis between alder, *Frankia* sp. and *Glomus intraradices* (Russo et al., 1993; Orfanoudakis et al., 2004; Oliveira et al., 2005a). As *Glomus intraradices* is commercially produced, it could easily be included in nursery production for land reclamation.

In this study, the performance of *Frankia*-inoculated alders in CT and a tripartite association (alder/*Frankia* sp strain AvcI1/*Glomus intraradices*) in TS was evaluated. Two different species of alders were tested, one native to western Canada, *Alnus crispa*; the other foreign in western Canada, *Alnus glutinosa*. At the end of growth trial, plant performance and soil chemical characteristics were evaluated. Moreover, the impact on the soil microbial community was assessed using the Most Probable Number (MPN) technique and petroleum hydrocarbon mineralization assays. The main hypotheses were that *Frankia* would ensure that plants produce more biomass, and that the presence of *Frankia*-alders in oil sand tailings will improve soil quality and increase microbial community biomass and residual hydrocarbon degradation ability. Dual inoculation should further improve plant and microorganism performance, thereby more rapidly improving soil quality characteristics and further colonization by more sensitive plant species.

Material and Methods

Alder seeds (obtained from the National Tree Seed Centre: *Alnus glutinosa* #7984680, France, and *A. crispa* #8360546, Obed summit, Alberta) were stratified 48 hrs at 4^{o}C, and then germinated on a moist filter paper in petri dishes in a greenhouse. At the cotyledon stage, plantlets were transferred to a Rootrainer™ filled with a mixture of peat moss:vermiculite (4:1; v/v). Plants were kept in the greenhouse with supplementary neon lighting (16h light/8h dark schedule) and watering was performed as needed. Plantlets were fertilized twice with N-free Crone's solution. At the 4 leaf stage, half of the seedlings were inoculated with *Frankia* sp. strain AvcI1. Inoculum was prepared as follows: a pure culture grown in liquid Qmod media (provided by S. Roy, Université de Sherbrooke) was sonicated at 1 sec intervals for 12 secs, the tube was then centrifuged, the supernatant removed, and the pellet transferred to a graduated sterile tube containing a small portion of the supernatant. The tube was centrifuged and the number of µl of

packed cell volume (pcv) was estimated. 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.

Three months after germination, alders were transplanted to TS, CT or greenhouse-type substrate (peat moss:vermiculite:perlite; 3:1:1; v/v). Plants were transplanted to 1 gallon pots, 1 seedling per pot. Tailings sands and composite tailings were obtained from Syncrude Canada Ltd. TS had the appearance of a fine golden beach sand, and CT, a mixture of sand and finer material, was a dark-grey colour. *G. intraradices* inoculation was performed at the time of transplantation: 100 g of MykePro AN1® (lot 688010), a commercial inoculum containing 1 propagule/g, was added below the seedling plugs. Each pot had an individual saucer to reduce contamination through watering. Pots were placed in a random fashion in the greenhouse, each species being kept separated from the other, and pots were placed taking into account a possible light gradient. No fertilization was done after transplanting.

The different treatments were as follows. For *A. glutinosa* in TS and greenhouse-type substrate: *Frankia*-inoculated, *G. intraradices*-inoculated, dual-inoculated (*Frankia* and *G. intraradices*) and non-inoculated. For *A. glutinosa* in CT and *A. crispa* in TS, CT and greenhouse-type substrate: *Frankia*-inoculated and non-inoculated. *A. glutinosa* had a total of 10 treatments with 3 replicates each and *A. crispa* had a total of 6 treatments with 4 replicates each. The greenhouse-type substrate was used to monitor alder growth and inoculation success in a substrate that is commonly used in greenhouses.

Plant height was measured monthly. Harvesting was performed 7 months after transplanting. Plants were removed from the pots, first shaken gently to remove loose soil, and then shaken more vigorously in a sterile bag to recover the bulk soil. The aerial portion was cut 1 cm above root collar. Roots and the remaining attached soil were shaken (90 rpm; 90 min) in sterile Milli-Q water to recover rhizosphere soil. The generated slurry was centrifuged (12,400 x g, 10 min) to remove excess water. Bulk and rhizosphere soil moisture content was evaluated after drying more than 16 h at 105^{o}C. Plant biomass was measured after drying for a minimum of 24 h at 65^{o}C. Soil chemical analyses were performed by Agri-Direct Inc. (Longueuil, Québec).

Bulk and rhizosphere soil microbial biomass was evaluated using the most probable number technique (MPN). Total heterotrophic bacteria and polycyclic aromatic hydrocarbon (PAH)-degrading bacteria were measured using the protocols described in Lefrançois et al. (2009). Results were expressed as most probable number of heterotrophic or PAH-degrading bacteria (MPN)/g of dry soil. Bulk and rhizosphere soil microbial populations were also evaluated for their mineralization potential of 2 petroleum hydrocarbons: hexadecane, a 16 carbon straight-chain alkane, and naphthalene, a 2-ring PAH, as previously described in Lefrançois et al. (2009).

Glomus intraradices infection assessment was performed using the grid/intercept method (Giovannetti and Mosse, 1980), and mycorrhizae staining was done with ink and vinegar according to Vierheilig et al. (1998). Preliminary trials were conducted to establish the optimal clearing and staining times for alder roots. Clean roots, cut in small sections of 1-3 cm, were cleared by boiling in a 10% (wt/vol) KOH solution for 10 min. The roots were then rinsed several times in tap water. Cleared roots were then stained for 3 min in a boiling solution of 5% ink (Shaeffer black)-household white vinegar (5% acetic acid). Destaining was done by rinsing for 20 min in tap water slightly acidified by a few drops of vinegar (solution was replaced once). The grid/intercept method was used as follows to assess mycorrhizal infection rate (Giovannetti and Mosse, 1980): roots were placed in a petri dish with a 0.5 cm square grid; 100 roots/gridline intersect were examined under the binocular microscope at 50X magnification and each root segment that contained a mycorrhizal structure was counted as positive. Percent infected roots were calculated.

This study followed a completely randomized experimental design. Plant and soil characteristics were analysed using the general linear model in SAS 9.1 (SAS Institute Inc., Cary, NC). An ANOVA was performed to test the model. Data normality and homogeneity of the variance were tested to ensure they met the ANOVA assumptions. Differences between means were assessed by performing Scheffe multiple comparisons.

Results

Frankia-inoculated plants were all well nodulated. Some nodules were also present on non-inoculated plants, but they were minute and not present in significant numbers. Alders growing in CT showed much more sign of injury (leaf chlorosis, necrotic margins) and growth reduction than those growing in TS. *Frankia*-inoculated *A. glutinosa* (alone or dual inoculum) growing in TS showed no sign of injury. They had a significant ($p<0.05$) increase in plant biomass as compared to alders inoculated with *G. intraradices* only or non-inoculated, and had equal or superior biomass to alders growing in greenhouse-type substrate (Table 1). *Frankia*-inoculated *A. glutinosa* growing in CT gained slightly more biomass than the non-inoculated alder, but the difference was not statistically significant. For *A. crispa* grown in TS or CT, there was an increase in biomass with *Frankia* inoculation compared to the non-inoculated alders, but it was not statistically significant.

Mycorrhizal evaluation was done using the grid/intercept method (Giovannetti and Mosse, 1980). *A. glutinosa* growing in TS and inoculated with *G. intraradices* alone had an average of 66.7% mycorrhizal root length colonization. Structures seen were mainly vesicles, with a few hyphae. When *G. intraradices* was inoculated in combination with *Frankia* the average root length colonized decreased to 13.6%, and only hyphae were observed. Alders growing in TS not inoculated with mycorrhizal fungi had an average observed root length colonization below 10%. In greenhouse-type substrate (peat moss:vermiculite:perlite; 4:1:1; v/v), plants inoculated with *G. intraradices* alone or with *Frankia* had an average of 64.6% and 67.6% respectively, and non-inoculated plants had an average of 57.2% root length colonization. Only hyphae were observed in these cases.

Treatment impacts on soil quality were assessed by soil chemical analysis (Table 2). The cation exchange capacity (CEC) of initial TS and CT was very low. This is a reflection of the high proportion of sand in both materials, low organic matter and 100% base cation saturation. When planted with *A. glutinosa* or *A. crispa*, the CEC TS increased and % base cation saturation decreased in general. However, due to high variability between replicates, the differences observed are not statistically significant. TS planted with alders showed a significant ($p<0.05$) decrease in pH, with the exception of

non-inoculated *A. glutinosa*. The decrease in pH was even more marked in treatments with *Frankia*. Alders did not significantly change the chemical characteristics of CT.

Total heterotrophic bacterial counts for bulk and rhizosphere soils were 10^6 and 10^7(MPN/g of dry soil), respectively. No differences were seen between TS or CT, or different treatments, and similar results were obtained for *A. glutinosa* and *A. crispa*, and for unplanted soil. PAH-degrading bacterial counts in *A. crispa* bulk soil were one order of magnitude higher in CT (10^3) than in TS (10^2). The same trend was seen for the rhizosphere of *A. crispa*, but there was also a treatment response, as counts were higher for *Frankia*-inoculated treatments than non-inoculated treatments. In *Frankia*-inoculated CT, and non-inoculated CT, the PAH-degrader counts were 10^4 and 10^3, and in *Frankia*-inoculated TS and non-inoculated TS, PAH-degrader counts were $5x10^3$ and $5x10^2$. For *A. glutinosa* bulk and rhizosphere soils all PAH-degrader counts were within one order of magnitude between 10^2 and 10^3. In unplanted-TS there was no detection of PAH-degraders, and in unplanted-CT the population size was comparable to planted samples at 10^3.

The petroleum hydrocarbon degradation potential of the rhizosphere and bulk soil microbial communities was assessed by mineralization assays using hexadecane and naphthalene as representative substrates. Hexadecane mineralization in rhizosphere samples was below 10% for both *A. crispa* (Figure 1C) and *A. glutinosa* (Figure 2C) and no significant differences were seen between treatments. Similar results were obtained for all of the bulk soil samples of *A. crispa* (CT and TS) (Figure 1A) and for CT (all treatments), TS-non-inoculated and TS-mycorrhiza of *A. glutinosa* with mineralization rates below 20% (Figure 2A). Higher mineralization rates were obtained by the unplanted-TS microbial population at ~60% and for the TS-*Frankia*-inoculated and TS-dual inoculated treatments of *A. glutinosa* with close to 40% mineralization.

Much higher mineralization rates were obtained for naphthalene. For *A. crispa* rhizosphere samples (Figure 1D), although CT began mineralizing faster, after 49 days all treatments in both TS and CT had similar degradation extents (~65-70%). For bulk soil of *A. crispa* (Figure 1B) in CT, *Frankia*-inoculated and non-inoculated samples had slightly higher mineralization rates than unplanted-CT. In sharp contrast, unplanted-TS achieved the highest mineralization. For *A. glutinosa* rhizosphere (Figure 2D), no significant

differences were seen between the treatments in CT. However, in TS, non-inoculated and *G. intraradices*-alders achieved higher mineralization rates than *Frankia*-alders (Figure 2B). The same pattern observed for *A. crispa* was seen in the bulk-TS of *A. glutinosa*: the unplanted-TS achieved even higher extents of mineralization. In the CT-bulk soil of *A. glutinosa*, the microbial population of the *Frankia*-alder treatment had higher mineralization rates than the non-inoculated treatments and the unplanted-CT.

Discussion

A. glutinosa and *A. crispa* were able to establish and grow in TS and CT and no mortality was observed during the experiment. *A. glutinosa* growth in TS was clearly enhanced by *Frankia* inoculation. There was a large increase in plant biomass from less than 1g for non-inoculated plants to more than 12 g for *Frankia*-inoculated plants. Moreover, the biomass of *Frankia*-inoculated *A. glutinosa* growing in TS was equal to or superior to plants that were growing in greenhouse-type substrate. *Frankia* inoculation allowed *A. glutinosa* to overcome the difficult growth conditions of TS. Dual inoculation did not give additional benefits for *A. glutinosa* growth in TS. While there are many examples of improved growth with dual inoculation (Rose and Youngberg, 1981; Yamanaka et al., 2003; Oliveira et al., 2005a), there are also studies that observed no effect (Russo et al., 1993; Orfanoudakis et al., 2004). In this study, because percent root colonization by mycorrhizal fungi was very low in the dual inoculation treatments, it seems logical that it did not provide additional benefits. In CT, the effect of *Frankia* inoculation was not as marked, but still slightly improved *A. glutinosa* growth. CT is a challenging substrate for plant growth (Franklin et al., 2002; Redfield et al., 2003; Renault et al., 2003), and both alder species showed signs of injury when growing in CT. Fine tailings present in CT, and not TS, could be responsible for increased plant damage, through their impact on soil chemistry, texture and structure (Renault et al., 2000). The biomass improvement for *Frankia*-inoculated *A. glutinosa* demonstrates the importance of *Frankia* inoculation on the development of plants in oil sand tailings, a nutrient poor environment. Biomass production of *A. crispa* was greatly reduced in TS and CT as compared to greenhouse-type soil, and inoculation treatments did not give significant advantages to plants, with plant dry weights remaining below 2 g. Biomass associated

with roots was not evaluated in this study (Markham and Zekveld, 2007). It could have been an interesting experiment to further assess the effect of *Frankia* inoculation on root growth. While both *A. glutinosa* and *A. crispa* were able to grow in oil sand tailings and while no mortality was recorded for both species, *A. glutinosa* demonstrated much more vigorous growth. *A. crispa* showed significantly more sign of injury, even in TS in which *A. glutinosa,* in contrast, showed none. This illustrates the species-specific growth response to a substrate (Khasa et al., 2002).

Although contaminated sites have been found to harbour *Frankia* sp. (Ridgway et al., 2004), limited nodulation was found in non-inoculated plants. Our results emphasize the importance of *Frankia* inoculation for adequate nodule formation (Hilger et al., 1991). For both alder species, no reduction in growth was associated with inoculation treatments, as was previously observed by Markham (2005). Inoculation should be done prior to the transplantation in tailings so that nodules benefit the plant instead of requiring energy for their development.

Inoculation of *A. glutinosa* with *Glomus intraradices* had a limited impact on plant performance and on soil microorganisms. While the level of infection in plants growing in TS was comparable to those growing in a greenhouse-type substrate, the main structures seen were vesicles. Vesicles can form as a protection mechanism in stressful conditions (Cooke et al., 1993). TS, by its nature, provide difficult growth conditions for plants and microorganisms and there are various studies that have demonstrated the difficulty of mycorrhizal growth in mine tailings (Kernaghan et al., 2002; Markham, 2005). The low nutrient status of oil sand tailings might not be optimal for mycorrhizal development (Russo et al., 1993; Yamanaka et al., 2003). Dual inoculation resulted in marginally higher infection rates than in non-inoculated plants. Due to the difficult growth conditions, the plants might have invested all of their energy towards nodule formation. Mycorrhizal infection rates observed in the greenhouse-type substrate (~60%) were in the range of what has been obtained in other studies (Lopez Aguillon and Garbaye, 1989; Oliveira et al., 2005a; Oliveira et al., 2005b). To mimic normal nursery conditions, the greenhouse-type substrate was not sterilized, and this could explain the high infection rate of non-inoculated alders. The mycorrhizal evaluation used did not allow identification to the species level, and the observed hyphae may not have been due

to *G. intraradices*. Many studies report that dual inoculation increases mycorrhizal infection (Gardner et al., 1984; Yamanaka et al., 2003; Orfanoudakis et al., 2004), but this was neither observed in TS nor in the greenhouse-type substrate in this study.

The impact of *A. glutinosa* and *A. crispa* growth on soil chemical characteristics was similar. The presence of the alders mainly influenced TS for pH, CEC and percent saturation of three main cations K, Ca, and Mg. CEC is influenced by soil organic matter and clay content, and is a measure of a soil's potential to hold nutrients. A sandy soil, such as TS, will always tend to have a lower CEC than a clay soil. The initial CEC of CT was higher than that of TS (2.9 vs. 0.8), probably due to the fine tailings segregated with the coarse tailings by the gypsum. CT was more refractory to change than TS. At a high pH, soils are highly buffered by carbonates (Brady and Weil, 1999) and soil chemical characteristics, such as pH and CEC, will be more recalcitrant to change. In conjunction with a limited growth of alders in CT, reducing the root system impact on soil, this might explain why there was less variation in CT than TS. TS that had been planted with alders saw an increase in CEC, and therefore its capacity to hold nutrients. Differences in CEC and % saturation were not statistically significant in this study due to the limited number of replicates and the high variability between plants, but with a larger experimental design these differences would likely be significant. When TS was planted with alders, the pH decreased significantly to slightly acidic levels, a pH level that improves the availability of plant-nutrients (Brady and Weil, 1999). Soil N levels were below detection limits even when *Frankia*, a nitrogen-fixer, was inoculated and clearly promoted growth. N transfer from N-fixation to plant and then to soil is a long-term process (Kohls et al., 2003; Martin et al., 2003), that would not necessarily be observed in a short-term greenhouse study. However, over a period of 20 years, alders were found to reduce soil's C:N ratio (Myrold and Huss-Danell, 2003). An increase in CEC, and decrease in % base saturation and pH under planted treatments will be beneficial to more sensitive plant species and help the establishment of an increased plant diversity in TS.

Rhizosphere total heterotroph counts were one order of magnitude higher than unplanted soil (CT and TS) as was seen by Phillips et al. (2008) in hydrocarbon contaminated land and also one order of magnitude higher than bulk soil counts (Da Silva et al., 2006; Lõhmus et al., 2006). However, planted bulk soil (CT or TS) did not yield an

increase in total heterotrophic bacterial biomass as compared to unplanted CT or TS (Siciliano et al., 2003; Phillips et al., 2006). Even though there was no treatment response associated with *A. crispa* in terms of mineralization, there was one for the biomass. *Frankia* inoculation resulted in an increase of one order of magnitude for PAH-degrading bacteria in the rhizosphere, for CT and TS, as compared to non-inoculated plants. The opposite was seen for *A. glutinosa*: the impact of *Frankia* inoculation observed for hexadecane and naphthalene mineralization in TS was not reflected in PAH-degrader counts as no difference was seen between treatments.

The microbial populations in TS and CT had different capacities to degrade the two petroleum hydrocarbons tested, hexadecane and naphthalene, and varied in their response to the different treatments. CT microorganisms had a lower ability to degrade hexadecane than naphthalene. Almost no hexadecane degradation occurred in unplanted-CT (~10%) and regardless of treatment, alders (*A. crispa* or *A. glutinosa*) were not able to increase its degradation. However, naphthalene mineralization in unplanted CT reached 60%. Moreover, alder inoculated with *Frankia* consistently, for both alder species tested, increased naphthalene mineralization in the bulk soil as compared to unplanted-CT. *Frankia*-alders had a positive impact on the naphthalene degradation potential of the CT microbial communities. Completely different results were obtained for TS. Even though no PAH-degrading bacteria were detected by MPN counts, unplanted TS microbial communities had the capacity to degrade close to 60% of initial hexadecane and naphthalene. This confirms the presence of microorganisms capable of degrading petroleum hydrocarbons in the tailings sand (Herman et al., 1994). For both alder species and both petroleum substrates, the presence of alders lead to a decrease in mineralization in the bulk soil (TS), in contrast to what was hypothesized and what was found in a related field trial (Lefrançois et al., 2009). Also, there was an alder species-specific response to the treatments. For *A. crispa*, inoculation treatments did not have different impacts on mineralization, but for *A. glutinosa*, inoculation treatments influenced the degradation rates differently. For naphthalene mineralization in TS, the decrease in mineralization was even more pronounced for the *Frankia*-alder. This is in sharp contrast to what was observed in CT. For hexadecane mineralization, even though alders showed decreased rates in TS as compared to the unplanted-TS, the *Frankia*-inoculated alders had

less reduction in their mineralization, which was the opposite to what was seen for naphthalene. These results suggest that different microorganisms are involved in hexadecane and naphthalene degradation and that even if TS and CT have the same origin, they support different microbial activities. No generalization is possible and different soil-plant combinations have been shown to lead to different responses by the microorganisms (Siciliano et al., 2003).

Our results illustrate that two different species of the same genus (*Alnus*) can have both similar and different impacts on the biomass and activity of soil microbial populations. Microbial population composition and activity can vary depending on their associated plant species (Smalla et al., 2001; Phillips et al., 2006). Differences have been seen at the plant genus level (Haichar et al., 2008; Weisskopf et al., 2008) but also at the species level (Yan et al., 2008). The diverse and specific root exudates produced by each plant species and their utilization by microorganisms can exert a selective pressure on microbial communities.

Conclusions

Overall, CT, TS and their associated microbial populations did not react similarly to alder establishment. CT is a much more challenging substrate for plant growth than TS, and its chemical characteristics, such as pH and CEC, are more recalcitrant to change. CT could benefit from the addition of a substrate such as peat moss (Renault et al., 2003) or forest floor (McMillan et al., 2007) to facilitate plant growth. In pure tailings, *A. glutinosa* growth surpassed *A. crispa*, whose growth was highly reduced by both TS and CT. *Frankia*-inoculated *A. glutinosa* performance in TS was equal to or superior to its growth in a greenhouse-type substrate, and *A. glutinosa* had fewer signs of injury in CT than *A. crispa*. Alders had a negative impact in TS on the capacity of the microbial population to degrade both hexadecane and naphthalene. Nonetheless, inoculation of *A. glutinosa* with *Frankia* promoted naphthalene mineralization in CT. The inoculation of a VAM, *Glomus intraradices*, in addition to a nitrogen-fixer, *Frankia*, did not significantly enhance plant growth, soil characteristics or affect microbial population biomass or activity. Even though *G. intraradices* has been found to enhance alder growth in alkaline anthropogenic sediments (Oliveira et al., 2005a), it might not be the best

candidate for tailings sands. *Frankia*-inoculated alders, which grew much better than non-inoculated plants, do have the potential of revegetating and reclaiming oil sand tailings materials.

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Table 1. Average plant biomass of *A. glutinosa* and *A. crispa* growing in TS or CT inoculated with *Frankia*, *G. intraradices* (mycorrhiza), both (dual inoculation), or non-inoculated.

	Non-inoculated		*Frankia*-inoculated		Mycorrhiza		Dual inoculation	
	(g)		(g)		(g)		(g)	
A. glutinosa								
TS	0.30	(0.03)	12.20	(1.57)	1.37	(0.53)	13.86	(0.93)
CT	0.30	(0.04)	1.19	(0.09)	ND		ND	
Greenhouse-type substrate	9.96	(1.08)	8.17	(0.93)	16.49	(0.40)	13.99	(0.76)
A. crispa								
TS	0.35	(0.04)	1.48	(0.49)	ND		ND	
CT	0.10	(0.02)	0.66	(0.18)	ND		ND	
Greenhouse-type substrate	4.41	(0.40)	7.58	(1.74)	ND		ND	

Data presented are means (*A. glutinosa* n=3; *A. crispa* n=4) with corresponding standard errors in parantheses
ND: Not Determined

Table 2. Soil analysis of initial TS or CT (t_0) and planted with *A. glutinosa* or *A. crispa* inoculated with *Frankia*, *G. intraradices* (mycorrhiza), both (dual inoculation), or non-inoculated.

					Mehlich-III (kg/ha)			Saturation (%)				Estimated CEC	Organic matter
		pH	pH-buffer	K	Mg	Ca	K	Mg	Ca	K+Mg+Ca		(meq/100)	(%)
	TS t_0	7.6	>7.5	15	43	259	1.9	21.2	77.0	100		0.8	0.2
	CT t_0	7.7	>7.5	50	166	984	2.0	21.5	76.5	100		2.9	1.9
A. glutinosa													
TS	Unplanted	8.5 ***a***	>7.5	32	106	391	2.8	30.2	67.0	100	***ab***	1.3 ***a***	0.1 ***a***
	Frankia-inoculated	5.7(0.1) ***b***	7.3(0.0)	16(1)	109(17)	209(71)	0.7(0.1)	17.1(1.2)	19.1(4.8)	37(6)	***a***	2.4(0.2) ***a***	0.3(0.0) ***a***
	Mycorrhiza	7.0(0.1) ***c***	7.4(0.1)	40(3)	204(18)	689(49)	1.3(0.3)	22.5(5.2)	45.0(9.2)	69(14)	***ab***	3.7(0.7) ***ab***	0.8(0.1) ***a***
	Dual inoculation	5.9(0.2) ***b***	7.3(0.0)	20(3)	136(10)	470(181)	0.7(0.1)	15.3(1.4)	30.6(10.2)	47(10)	***a***	3.3(0.2) ***a***	0.4(0.1) ***a***
	Non-inoculated	7.8(0.0) ***a***	>7.5(0.0)	83(2)	431(17)	1753(73)	1.7(0.0)	28.6(0.1)	69.8(0.1)	100(0)	***b***	5.6(0.2) ***b***	4.4(0.2) ***b***
CT	Unplanted	8.0 ***a***	>7.5	62	252	1220	1.9	25.2	72.9	100	***a***	3.7 ***a***	1.7 ***a***
	Frankia-inoculated	7.7(0.0) ***a***	>7.5(0.0)	48(3)	219(17)	823(178)	2.1(0.3)	31.0(3.0)	66.8(3.3)	100(0)	***a***	2.7(0.4) ***a***	1.8(0.0) ***a***
	Non-inoculated	7.3(0.1) ***b***	>7.5(0.0)	32(1)	171(23)	577(69)	1.9(0.2)	32.3(0.4)	65.7(0.1)	100(0)	***a***	2.0(0.2) ***a***	0.6(0.0) ***b***
A. crispa													
TS	Unplanted	8.5 ***a***	>7.5	32	106	391	2.8	30.2	67.0	100	***a***	1.3 ***a***	0.1 ***a***
	Frankia-inoculated	6.9(0.1) ***b***	7.4(0.1)	118(74)	204(18)	1405(1002)	2.3(0.6)	21.3(6.1)	46.5(14.6)	70(14)	***a***	5.2(2.2) ***a***	0.6(0.1) ***a***
	Non-inoculated	7.1(0.1) ***b***	7.3(0.0)	45(2)	163(12)	348(36)	2.6(0.5)	29.9(6.6)	38.8(9.5)	71(17)	***a***	2.3(0.4) ***a***	0.4(0.0) ***a***
CT	Unplanted	8.0 ***a***	>7.5	62	252	1220	1.9	25.2	72.9	100	***a***	3.7 ***a***	1.7 ***a***
	Frankia-inoculated	7.7(0.0) ***b***	>7.5(0.0)	61(4)	222(11)	669(56)	3.0(0.1)	34.8(1.3)	62.3(1.4)	100(0)	***a***	2.4(0.2) ***b***	2.3(0.0) ***ab***
	Non-inoculated	7.8(0.0) ***ab***	>7.5(0.0)	71(6)	280(16)	898(62)	2.6(0.2)	33.4(0.8)	64.1(1.0)	100(0)	***a***	3.1(0.2) ***ab***	2.7(0.1) ***b***

Data presented are means (*A. glutinosa* n=3; *A. crispa* n=4) with the exception of TS t_0. CT t_0. unplanted TS and unplanted CT; standard errors are in parentheses; data with the same letter are not statistically different ($p<0.05$); t_0: Sample taken at transplantation

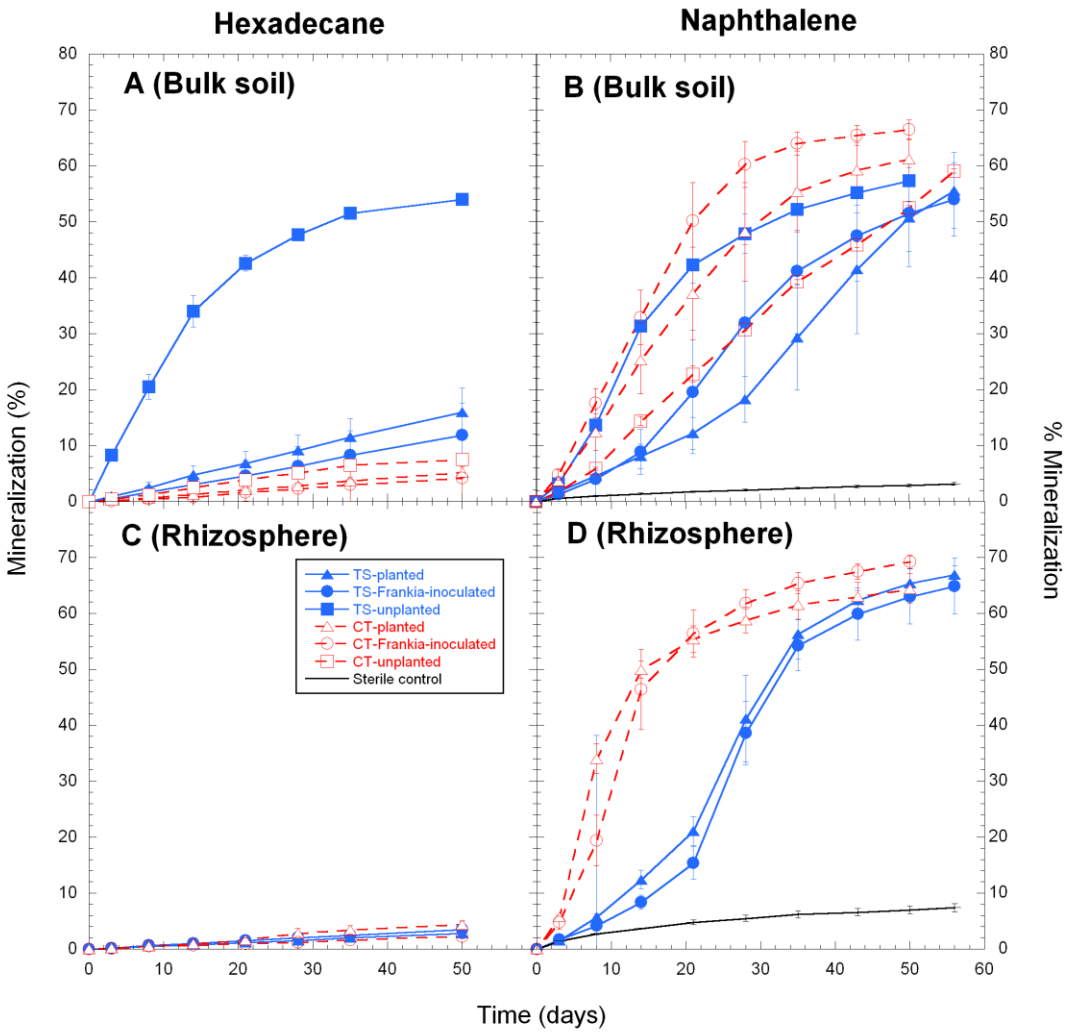

Figure 1. Mineralization of two representative hydrocarbons: hexadecane and naphthalene, in bulk (A, B) or rhizosphere (C, D) soil of CT or TS either unplanted, planted with non-inoculated *A. crispa* (Planted) or *Frankia*-inoculated *A. crispa*.

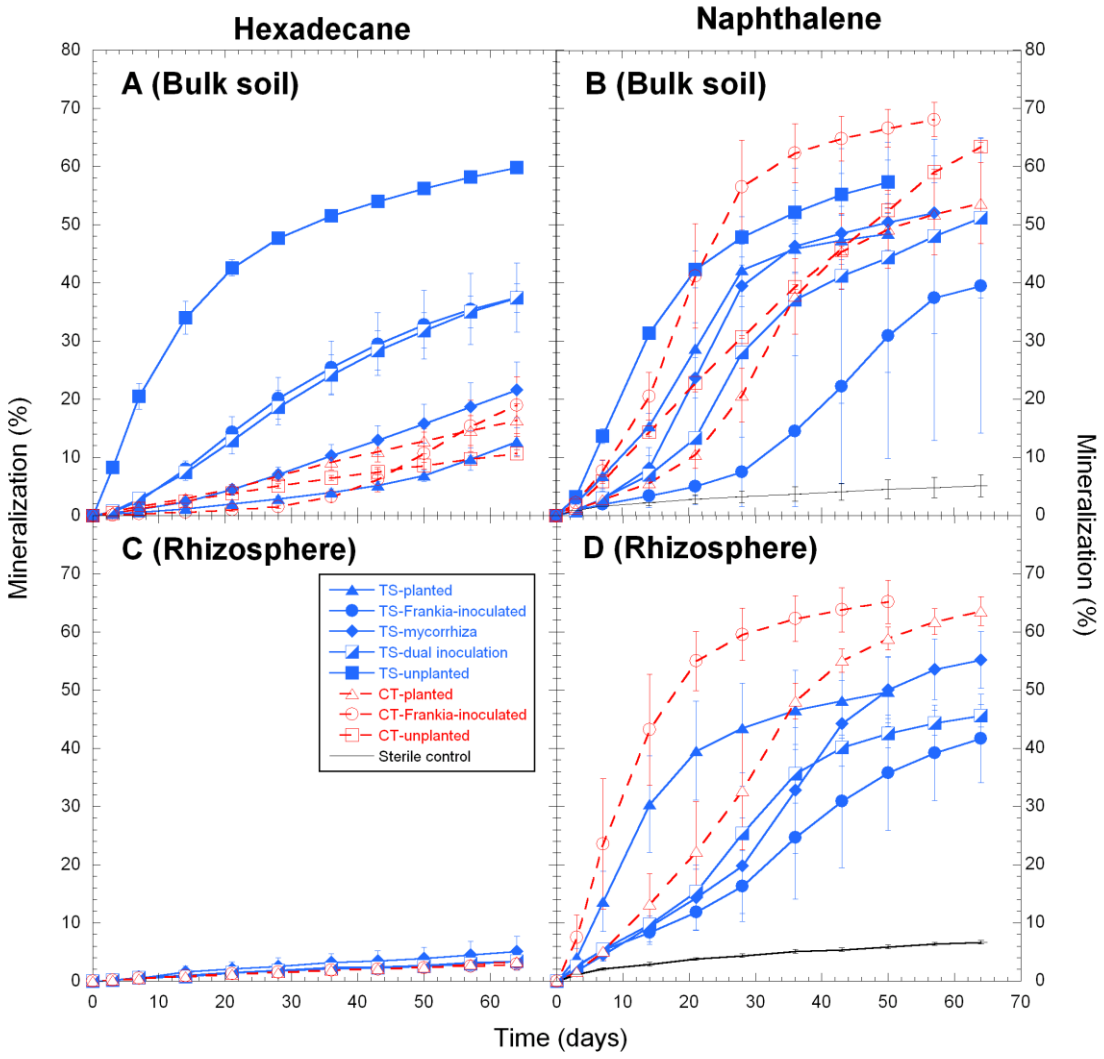

Figure 2. Mineralization of two representative hydrocarbons: hexadecane and naphthalene in bulk (A, B) or rhizosphere (C, D) soil of CT or TS either unplanted, planted with non-inoculated, *Frankia*-inoculated (frankia), *G. intraradices* inoculated (mycorrhiza) or dual inoculated (dual) *A.* glutinosa.

Conclusions

The greenhouse and field trials conducted in this research project provided evidence that alders are a very interesting biotechnological approach for the reclamation and revegetation of oil sands process-affected material (OSPM). Alders performed very well in OSPM when considering plant survival and growth. In the field trial, there was a substantial biomass gain each year. Even under nutrient limiting conditions, *Frankia*-inoculated alder nitrogen levels were comparable to alders growing under nitrogen non-limiting conditions. The greenhouse study also demonstrated a substantial biomass increase when alders were inoculated with *Frankia*. This emphasizes the importance of nitrogen fixation by *Frankia* in the overall performance of alders. Alders have shown that they can grow in pure tailings material (TS and CT) under greenhouse controlled conditions and that they can perform well in OSPM in the cool, relatively dry and short growing season of northeastern Alberta. The dual alder inoculation (*Frankia*/*Glomus intraradices*) did not demonstrate any additional benefits as compared to *Frankia* inoculation alone.

The establishment of alders in the OSPM resulted in the improvement of several different soil quality parameters. *Frankia*-inoculated alder growth resulted in a decrease in soil pH, a significant decrease in plant-available sodium and an increase in soil CEC. These soil quality improvements create favourable conditions for the growth of more sensitive species, leading to the overall desired goal in the reclamation of these residues, the re-establishment of the boreal forest.

The establishment of alders in the OSPM provided an important soil-root interface: the rhizosphere. The root exudates highly influence the rhizosphere, and help to create a rich environment with increased microbial diversity and activity (Rovira, 1965). Microbial population enumeration indicated an increase in microbial biomass in the rhizosphere as compared to the bulk soil. This can be seen a source of microorganisms for the surrounding soil. As OSPM are known to have limited microbial activity (Fung and Macyk, 2000), this is an appreciable gain. Also, DGGE, a molecular tool, revealed a modification in the microbial community composition when planted bulk soil was compared to unplanted soil. The planted soil community structure was closer to that of

rhizosphere soil, known to be a rich growth environment. Mineralization assays demonstrated that petroleum degradation in the tailings sands capped with overburden material and peat moss was enhanced by the presence of *Frankia*-inoculated alders. Moreover, it was observed that the mineralization activity in the rhizosphere differed from that in the bulk soil. Again, the rhizosphere can be seen as a source of microbiological diversity in the soil ecosystem. Alders are supporting a highly diversified and active microbial population.

The sequencing of bands excised from DGGE provided limited information on the microorganisms present in the oil sands tailings. A more detailed knowledge of individual microorganisms found in the tailings sands could help understand on-going processes and help provide a better assessment of the impact of certain management practices.

The introduction of a foreign microorganism into an ecosystem should always be monitored carefully. The successful PCR amplification of DNA fragments from nodules of alders growing naturally on site with primers designed specifically for *Frankia* strain AvcI1 clearly shows the importance of having an effective identification tool. Alders are present naturally on the mining site and even though they showed reduced growth compared to greenhouse inoculated alders, their nodules could serve as an inoculum for other alders, whether natural or from greenhouse production. The use of a *Frankia* strain isolated from the mining site could be an interesting option, as it would provide a strain native to the area that is adapted to the prevailing growth and climatic conditions. It would be important to future studies to have a technique that would allow the clear differentiation of introduced *Frankia* from indigenous *Frankia*. The utilization of *nif* gene restriction fragment length polymorphism (RFLP) (Benson and Silvester, 1993; Dai et al., 2004) could be investigated to evaluate its ability to specifically detect *Frankia* strain Avc1I.

The production of CT for the management of the mature fine tailings presents many technical challenges. The greenhouse trial demonstrated that it is a far more challenging material for plant growth than TS. McMillan et al. (2007) proposed the use of forest floor material instead of peat moss to improve the potential for tailings remediation. Forest floor material is hypothesized to serve as a source of microorganisms,

organic matter and indigenous plant propagules. This would be an interesting aspect to investigate for CT revegetation if the production of CT is continued in the future.

Reclamation and revegetation of oil sands process-affected materials (OSPM) is a very important issue, and this research project has contributed to the increase of knowledge in this area. Rehabilitation of OSPM is essential for reducing the amount of disturbed land and for the re-establishment of a balanced ecosystem for all levels of organisms. Moreover, the reforestation of disturbed land is valuable for greenhouse gas management, since forests support higher carbon densities than other ecosystems (Jandl et al., 2007). As shown in this research project, *Frankia*-inoculated alders are promising for the remediation and revegetation of OSPM. The results support the idea that this approach should be incorporated into the reclamation plans of the oils sands mining companies.

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