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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. Oil sands process-affected materials (OSPM), such as tailings sand, produced as a result of bitumen extraction, 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 succession 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 *Actinobacteria*, *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 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 alder-*Frankia*. 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 alder-*Frankia* treatments. Soil tests showed that alder-*Frankia* decreased soil pH (from 7.5 to 6.6, in 2006, and from 8.2 to 7.2 in 2007) and plant-available sodium content (70% reduction), and had a positive impact on soil organic matter content (increase in up to 6 times in alder-*Frankia* plots). 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 provide evidence of the potential of alder-*Frankia* symbionts to be part of a reclamation strategy for the reforestation of the site, and the re-establishment of a balanced ecosystem.

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 removal of the existing vegetation and overburden 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 crude oil is recovered through a process using hot water, NaOH and steam (Clark and Pasternack, 1932), which produces a tailings 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

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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.

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 (*Hordeum vulgare*) was studied as a pioneer plant; however the low nutrient level of the tailings sand reduced 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 (*Alnus* sp.) are actinorhizal plants that have a symbiotic relationship with *Frankia* sp., a nitrogen-fixing *Actinobacteria*. 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 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); there was an increase in total viable microbial populations, and an increase in hydrocarbon degradation capacity. Another study previously demonstrated that alders had a positive impact on soil fertility and on the physiological activity of the soil microbial population by enhancing enzymatic activities (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.

2. Materials and methods

2.1. Site description

The site is located at Syncrude Canada Ltd., Fort McMurray, Alberta, Canada (56°59′N; 111°47′E). A large area of coarse tailings was 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 alder (*Alnus crispa* (Ait.) Pursh) seeds, collected in Fort McMurray (latitude 56.3, longitude 112.1), were obtained through the National Tree Seed Centre (Fredericton, NB, seedlot 8360545.0). The seedlings were started in a greenhouse in March 2004, and a portion of the seedlings was inoculated with *Frankia* sp. strain AvcI1, as previously described (Quoreshi et al., 2007). 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 (36 m^2). An adjacent area was kept unplanted (76 m^2). A buffer zone of 2 m separated each plot.

2.2. Sampling

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. Triplicate alders were harvested for each treatment. Non-inoculated plants were harvested first to reduce contamination risks. As much as possible the complete root system was recovered and at first, 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 1 cm above the soil level. Triplicate bulk soil samples were also collected from the unplanted zones. Samples were collected at a depth comparable to the root depth (15–45 cm). For rhizosphere recovery, a portion of the roots was selected randomly (from all the root system collected) and shaken at 90 rev min^{-1} for 90 min in sterile water (2–4 volumes of the weight) to completely cover the roots. The soil slurry was centrifuged at 12,400 × *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 °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 (see below). Surface sterilized roots were stored at −80 °C. In 2007, nodules were separated from the roots, and analyzed separately (see below).

2.3. Microbial enumeration

Total heterotrophic bacteria and polycyclic aromatic hydrocarbon (PAH)-degrading bacteria were determined for 2007 rhizosphere and bulk soil samples (field triplicates) 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–Haas 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, bactotryptone, 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 6–24 h incubation at room temperature in the dark, positive wells developed a violet-red colour. For both PAH-degrader and total heterotroph counts, the number of positive wells per dilution was recorded and using a computer program developed by Klee (1993), the results were expressed as most probable number of heterotrophic or PAH-degrading bacteria (MPN)/g of dry soil.

2.4. Bulk soil and plant analyses

Soil and plant analyses were performed by Agridirect Inc. (Longueuil, Québec) according to their standard operating methods (http://agridirect.ca/systeme/nosmethodes.asp#sol). 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 Sension™5 conductivity meter (HACH Company, Loveland, CO). Plant biomass was determined by weighing plants after drying for more than 36 h at 65 °C.

2.5. Mineralization assays

Mineralization assays were performed for three representative petroleum hydrocarbon substrates (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 of 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: 12 mCi/mmol) in 100 mg kg^{-1} hexadecane, 100,000 dpm naphthalene-1-C^{14} (specific activity: 2.3 mCi/mmol) in 10 mg kg^{-1} naphthalene, or 100,000 dpm phenanthrene-9-C^{14} (specific activity: 55.7 mCi/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, sampled and analyzed as previously described (Chénier et al., 2003). The results are expressed as the cumulative percentage of $^{14}CO_2$ recovered relative to the amount initially introduced into the microcosm.

2.6. Molecular microbial analysis

2.6.1. 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.

2.6.2. DNA extraction

Total soil DNA extraction was performed using the 10 g PowerMax™ Soil DNA Isolation Kit (MoBio Laboratories, Carlsbad, CA) according to the manufacturer's instructions. DNA was stored at −20 °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.

Total DNA extraction was performed on crushed nodules and/or roots using a chemical lysis approach (Fortin et al., 1998) combined with 2 × 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 °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. (2009). Before the addition of the DNA extracts, resins were washed twice with TE (pH 7.5)–NaCl (0.1 M), to improve DNA recovery.

2.6.3. Frankia sp. strain AvcI1 detection

The presence of *Frankia* sp. strain AvcI1 in nodules or roots was determined 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 *nifD* and the *nifK* 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 °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.

2.6.4. 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 a GC clamp, Fortin et al., 2004) and U758 was performed using a 20 cycle touchdown procedure as described previously. 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 (7 M 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 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 GelCom-

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-alders		*Frankia*-alders	
		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[a]
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	2.5 ± 0.2	2.7 ± 0.1	7.2 ± 3.2	12.2 ± 0.2	15.1 ± 11.1
		n = 6		*n* = 9		*n* = 6	

Results are expressed as means with corresponding standard error; *n* = 3, unless specified; CEC: Cation Exchange Capacity; ND: not detectable (detection limit: 0.2%).
[a] 2 out of 3 replicates had non-detectable levels; the third one was just above detection limits.

parII (Applied Maths, Austin, TX). Cluster analysis was performed using the Dice similarity coefficient, and a UPGMA dendrogram was generated.

2.7. Statistical analyses

Statistical analyses were carried out in R (v 2.7.1; The R Foundation for Statistical Computing). When necessary, data were log transformed to meet the assumptions of parametric analysis of variance (ANOVA). Normality was tested using the "shapiro.test" function. ANOVA and subsequent Tukey's honestly significantly different (HSD) tests were carried out using the "aov" and "TukeyHSD" functions, respectively.

3. Results

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 the 2006 and the 2007 samples, soil planted with *Frankia*-alders had a lower pH, had approximately a 70% reduction in plant-available sodium (Na), and there was a decrease in percent saturation of three main cations (Ca, Mg, and K) when compared to unplanted soil. In 2006, the pH and Mehlich K were significantly different ($P < 0.05$) in *Frankia*-inoculated treatments compared to unplanted and planted treatments. In 2007, the soil Na and organic matter were significantly different ($P < 0.01$) between the *Frankia*-inoculated and the other two treatments, with more organic matter and less available Na being detected in the *Frankia*-alder planted soil. However, soil total nitrogen content remained below detection limits (0.2%).

Plant percent nitrogen was not notably 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. Therefore, there was a corresponding increase in total nitrogen captured in the above-ground portion of the alders, both for inoculated and for non-inoculated alders.

Microbial enumeration of total heterotrophic bacteria and PAH-degraders demonstrated that population densities were higher in the rhizosphere (10^8 and 10^4 MPN/g of dry soil, for heterotrophic bacteria and PAH-degraders, respectively) than in the bulk soil (10^6–10^7 and 10^3 MPN/g of dry soil, for heterotrophic bacteria and PAH-degraders, respectively), and that overall, heterotrophic bacteria (10^6–10^8 MPN/g of dry soil) were more abundant than PAH-degraders (10^3–10^4 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).

3.2. 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 hydrocarbons tested in the *Frankia*-alder treatment compared to both unplanted and control-alder treatments (Fig. 1). However, after 2 years 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 and higher for phenanthrene. Unplanted bulk soil mineralization rates remained lower than both planted treatments. No substantial differences between the treatments were found for mineralization in the rhizosphere soil for all three hydrocarbons 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

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 mass (g)	112 ± 38	689 ± 69	154 ± 33	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.

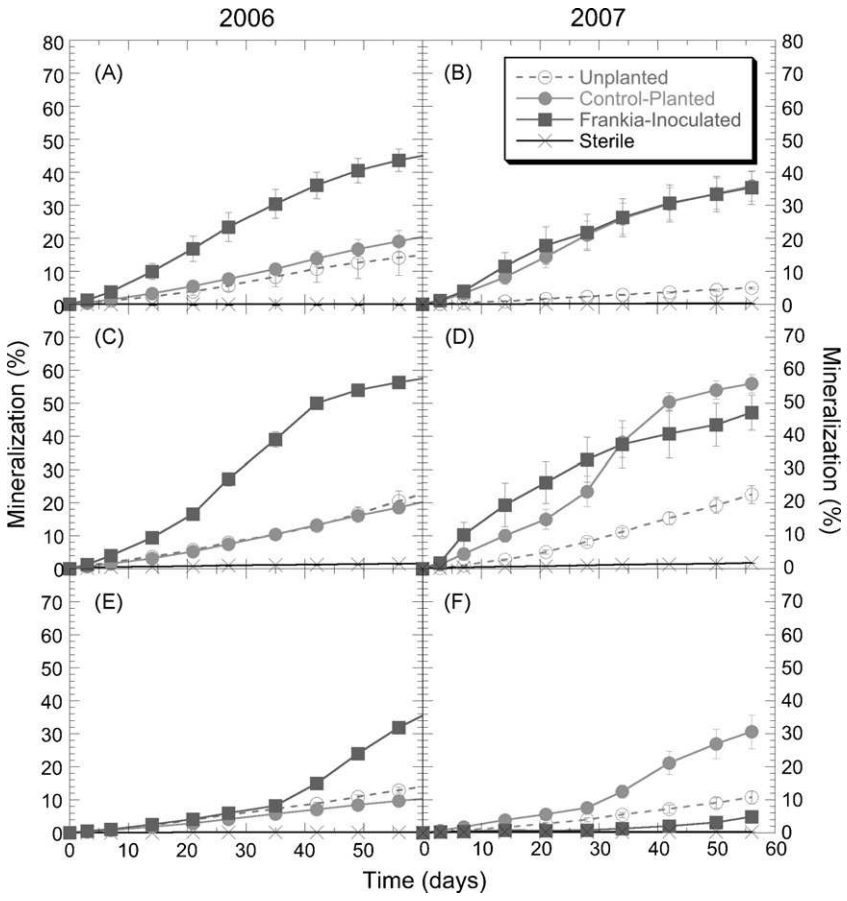

Fig. 1. Mineralization of representative hydrocarbon substrates: hexadecane (A and B), naphthalene (C and D) and phenanthrene (E and F), in bulk soil of capped overburden-tailings sand either unplanted, planted with non-inoculated alders (Planted) or *Frankia*-inoculated alders after 1.5 (2006) and 2.5 (2007) years of growth in the field. Sterile represents bulk soil samples that were sterilized to serve as negative controls. Data are means with corresponding standard errors.

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. 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). Markham (2005) suggests that nodule development constitutes a stress that could limit plant growth, especially in reclamation sites. Since the growth of some of the non-inoculated alders was similar to that of inoculated alders, it appears that natural inoculation with *Frankia* sp. occurred in the greenhouse. This was confirmed by16S rRNA gene DGGE of the endophytic community (Fig. 3), whereby *Frankia* sp. (bands g–i, Table 3) was detected in all of the samples. Analyses of nodules (in 2007) from natural alders growing on the site both adjacent to and farther from the research plots showed that amplification of a fragment was possible with *Frankia* sp. AvcI1 primers either by direct or nested PCR. These results make it difficult to determine whether non-inoculated alders were colonized by *Frankia* sp. in the greenhouse or in the field. Current studies are being performed to address this issue.

The banding pattern seen on the 16S rRNA gene DGGE showed 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. 4A and B). 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 were often from uncultured bacteria, sequencing of predominant bands indicated a relatedness to bacteria that were often found in contaminated or saline environments, or soils. Several dominant bands (bands 4–7, Table 3) 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.

4. Discussion

The establishment of *Frankia*-nodulated alders in the capped tailings sand improved general soil quality as compared to

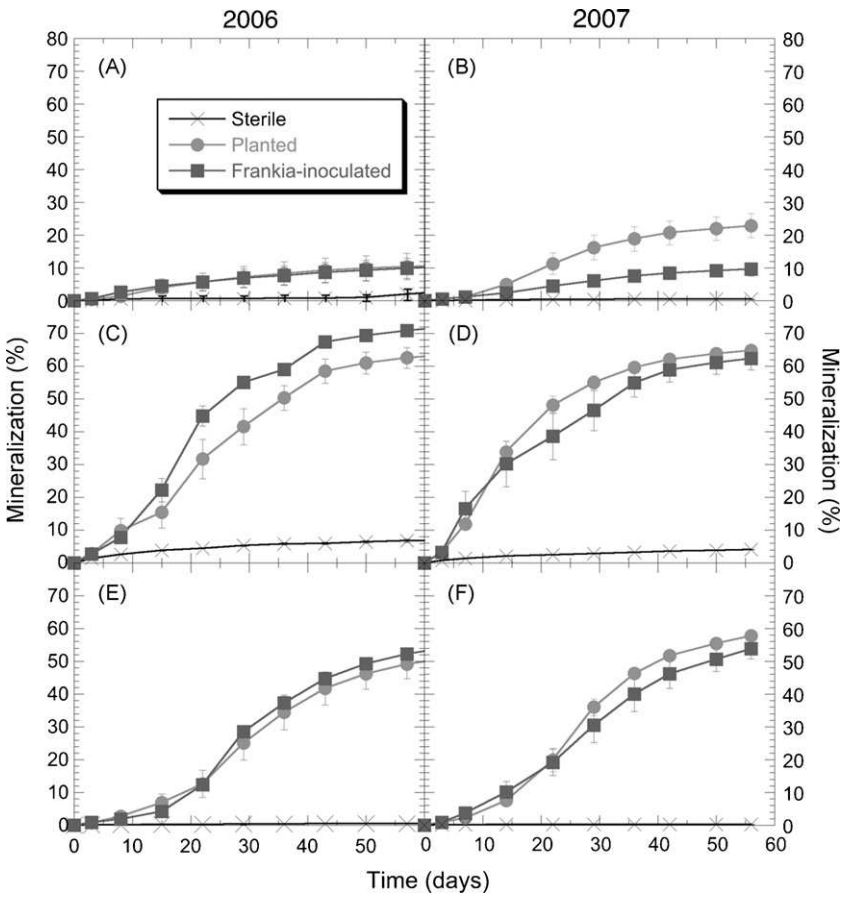

Fig. 2. Mineralization of representative hydrocarbon substrates: hexadecane (A and B), naphthalene (C and D) and phenanthrene (E and F), in the rhizosphere of non-inoculated alders (Planted) or *Frankia*-inoculated alders after 1.5 (2006) and 2.5 (2007) years of growth in an overburden-tailings sand mixture. Sterile represents rhizosphere samples that were sterilized to serve as negative control. Data are means with corresponding standard errors.

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
a, b, c, d, e, f		Plant DNA	99	99	99-100	Unspecific amplification associated with plant DNA
g, h, i	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 flavobacterium	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.

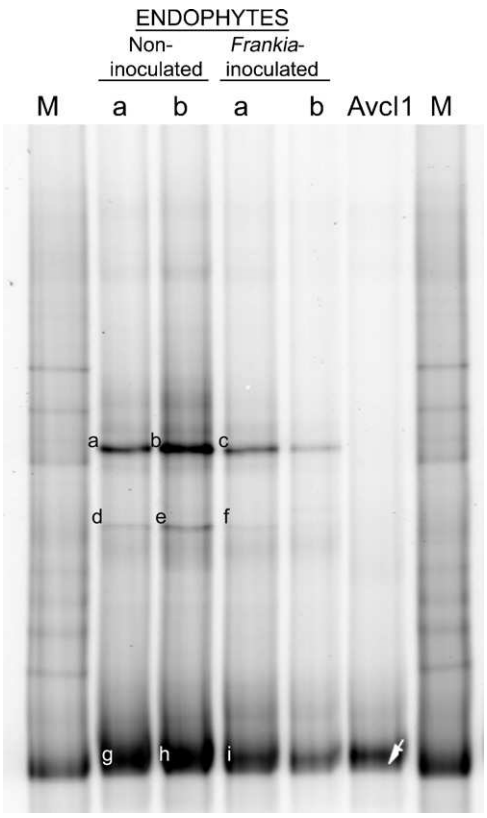

Fig. 3. DGGE of PCR amplified 16S rRNA gene fragments from total DNA extracted from roots (endophytes) of alders inoculated or not with *Frankia* strain AvcI1 after 1.5 years growth in an overburden-tailings sand mixture. Non-inoculated a and b, and *Frankia*-inoculated a and b identifies field replicates. 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.

unplanted soil 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 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. Plants are known to be adversely affected by soil salt content (Tester and Davenport, 2003). Therefore the decrease of plant-available soil sodium seen under *Frankia*-alder treatment could 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 were similar to those reported by Pérez-Corona et al. (2006) for alders growing in a non-contaminated environment (2.36%). Furthermore, the increase in alder biomass between 2006 and 2007 indicates that alders are performing well in this low 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). Both inoculated and non-inoculated alders had *Frankia*-nodules where nitrogen fixation could have occurred. 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 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. 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 colonized 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). Nonetheless, these results indicate good performance of *Frankia*-nodulated alders in OSPM and that planting alders to improve soil characteristics is a viable approach to enhance revegetation.

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 plant 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 extensively 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 (Phillips et al., 2006; 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

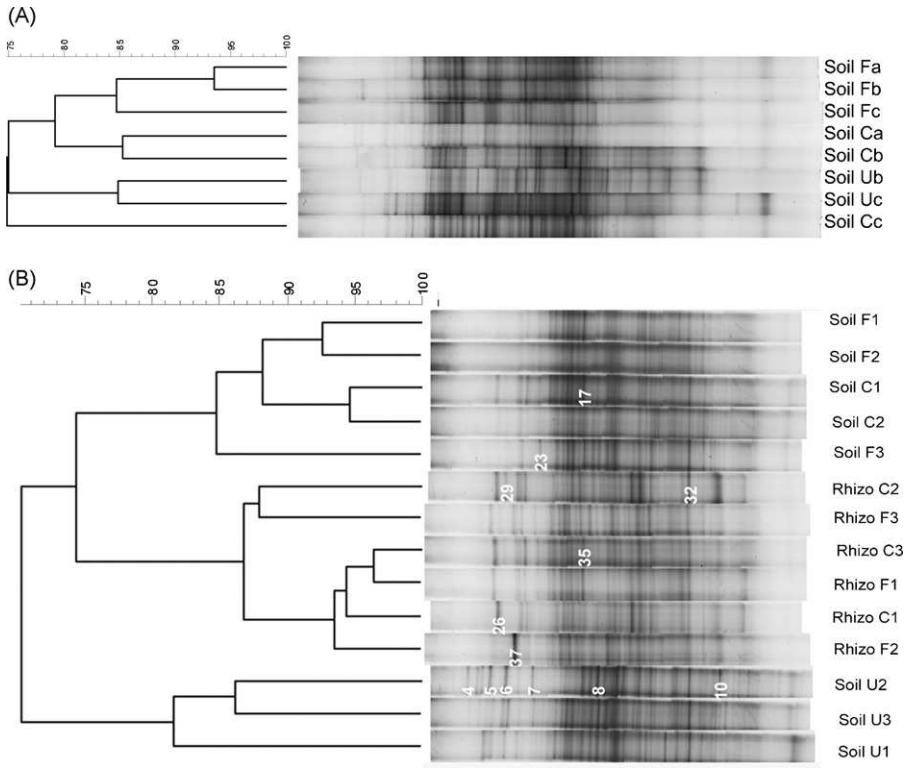

Fig. 4. Cluster analysis, with corresponding DGGE lanes, of 16S rRNA gene DGGE banding pattern of 2006 bulk soil samples (A) and 2007 bulk and rhizosphere soil samples (B). Fa, Fb and Fc, Ca, Cb and Cc and Ub and Uc (2006) and F1, F2 and F3, C1, C2 and C3 and U1, U2 and U3 (2007) identify field replicates of *Frankia*-inoculated alders (F), non-inoculated control alders (C) and unplanted (U) treatments, respectively. Numbers in B indicate bands that were excised for subsequent nucleotide sequencing (see Table 3).

impact on the soil microbial community composition. DGGE fingerprints and their corresponding cluster analyses supported the trend that was seen for the mineralization results of the control-alder bulk soil microbial populations between 2006 and 2007. It would be interesting to evaluate whether this variation in microorganism dominance is 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 the 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 the 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 as compared to unplanted soil. 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 and they have the potential to be part of a reclamation strategy for these types of sites. The improvements in the soil quality of the reclamation site should help in the subsequent establishment of more sensitive species, leading ultimately to the reforestation of the site, and the re-establishment of a balanced ecosystem.

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