Evaluating the Potential of Alder-*Frankia* Symbionts for the Remediation and Revegetation of Oil Sands Tailings

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Abstract

Tailings are the waste produced as a result of the extraction of oil from the tar sands in northern Alberta. Many avenues for the reclamation of tailings are being researched, but one area that has received little attention is phytoremediation. The Alder-*Frankia* symbiotic relationship in the tailings was investigated for its potential in revegetation and remediation of the tailings. Two species of alders were examined *Alnus glutinosa* and *A. rugosa*. The impact of the alders was monitored through the investigation of the differences in the microbial community present in the oil sands tailings and composite tailings (CT) with and without alders. For our investigation we used culture dependent techniques (plate counts and mineralization assays) and culture independent techniques (16S rRNA gene PCR, catabolic PCR and DGGE). The alders lowered the pH of the tailings, increased rates of mineralization, increased the general microbial population in the tailings by one to two orders of magnitude and increased the microbial diversity .

A. rugosa however, had a greater impact on the mineralization of poly aromatic hydrocarbons (PAHs) and, being native to Alberta, was chosen for further experimentation, using only composite tailings. The aim of the experiments was to determine the effect of a *Frankia* inoculum on the growth of *A. rugosa* in (CT) and the associated microbial community. The microflora in the bulk soil, rhizosphere and inside the root of inoculated and non-inoculated *A. rugosa* were compared through microbial enumerations of the community, with general and selective media and mineralization assays. *A. rugosa* inoculated with *Frankia* was taller and the roots were more developed and the endophytic community of inoculated *A. rugosa* had greater rates of naphthalene mineralization.

The results indicate that *A. rugosa* inoculated with *Frankia* could be used for the phytoremediation of tailings and for the re-establishment of a forest ecosystem.

Résumé

L'extraction du pétrole à partir des sables bitumineux dans le nord de l'Alberta génère une grande quantité de résidus. Bien que plusieurs avenues aient été envisagées dans le but de restaurer ces résidus, très peu d'études se sont intéressées à la phytoremédiation. L'objectif de ce projet de recherche était donc d'évaluer le potentiel d'utilisation de la relation symbiotique entre l'aulne et un actinomycète du genre *Frankia* dans le processus de restauration et de végétalisation des résidus de sables bitumineux. Dans une première expérience, deux espèces d'aulnes, *Alnus glutinosa* et *A. rugosa*, ont été utilisées. L'impact des aulnes sur l'activité biologique des résidus et des résidus composites a été déterminé en évaluant les populations microbiennes en présence ou en l'absence de la plante. Des techniques nécessitant la culture des micro-organismes (dénombrements bactériens, essais de minéralisation) et d'autre techniques indépendantes de la culture des micro-organismes (PCR de l'ADNr 16S, PCR de gènes cataboliques, DGGE) ont été utilisées. L'implantation des aulnes a diminué le pH des résidus, augmenté le taux de minéralisation et augmenté les populations microbiennes totales d'un à deux ordres de magnitude. De plus, une augmentation de la diversité microbienne dans les résidus a été observée en présence des aulnes.

A. rugosa, une espèce indigène de l'Alberta, s'est avérée être plus efficace pour favoriser la dégradation des hydrocarbones polycycliques aromatiques et a donc été sélectionnée pour poursuivre l'expérimentation dans les résidus composites seulement. Le but de cette deuxième expérience était d'évaluer l'impact de l'inoculation d'une souche de *Frankia* sur la croissance de l'aulne et sur les populations microbiennes des résidus composites. La microflore du sol, de la rhizosphère et de l'intérieur des racines de plants d'*A. rugosa* inoculés ou non avec *Frankia* ont été comparées à l'aide de la technique MPN (Most Probable Number) sur milieu général ou sélectif, de même que par des essais de minéralisation. La hauteur des plants, le développement des racines et la dégradation du naphtalène par les populations microbiennes endophytes se sont avérés supérieurs chez les plants inoculés avec *Frankia*.

Les résultats obtenus indiquent que les plants d'*A. rugosa* inoculés avec *Frankia* pourraient être utilisés pour la phytoremédiation des résidus de sables bitumineux et semblent d'excellents candidats dans l'établissement d'un couvert forestier.

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1. Introduction

One of the world's largest reserves of hydrocarbons is found in the Athabasca Basin in northeastern Alberta, Canada (Fedorak et al., 2002). The Athabasca oil sands, an area greater than 42 000 km^2 (Madill et al., 2001), is exploited by three oil sand mining companies, Syncrude Canada Limited, Suncor Energy and Albian Sands. There is a fourth company, Canadian Natural Resources Limited, which should be operating in the oil sands by 2008 (Wikipedia, 2005). Together Suncor and Syncrude meet approximately 20% of Canada's crude oil demand (Madill et al., 2001). The oil sands industry produces over 120 million barrels of crude oil per year and this is expected to increase to 400 million barrels by 2010 (Holowenko et al., 2000). Impurities in crude oil, such as naphthenic acids, can promote the corrosion of the high temperature distillation units involved in the oil extraction process.

To avoid these problems, the oil is extracted using a procedure based on the Clarke caustic hot water extraction method (Quagraine et al., 2005). The naphthenic acids are removed in the water fraction, as naphthenates, which are surfactants. The naphthenates reduce the surface tension of the water and help to detach bitumen from the sand particles. The bitumen floats to the top, where it is collected and the sand sinks to the bottom of the vessel.

The amount of water needed for the extraction process is 3 m^3/m^3 of oil sand, which produces 4 m^3 of sand - slurry-waste (Fedorak et al., 2002). The slurry, referred to as tailings, is transported by pipeline to Mildred Lake Settling Basin at Syncrude, or 1 of 4 settling ponds at Suncor (MacKinnon and Sethi, 1993). Reclamation of the tailings by the industry is a concern, and an active area of research since it is estimated that more than 1 billion m^3 of tailings will have accumulated by 2025, given the current rate of oil sands extraction and processing (Herman et al., 1994).

Few plant species are able to tolerate the stringent conditions of the tailings, and as a result revegetation for reclamation of the tailings has been a challenge. Alders are successful pioneer species, which have been planted for decades for

erosion control, reclaiming strip mine areas and revegetating road sides (Oliveira et al., 2005). They are preferred for strip-mine reclamation because of their ready establishment, rapid growth, abundant litter production and their symbiotic relationship with nitrogen fixing *Frankia*, all of which contribute to an increase in soil fertility in preparation for the establishment and for the promotion of growth of other plant species (Pregent et al., 1987, Nickel et al., 2001). An increase in nitrogen and other nutrients, resulting from leaf litter and root exudates, in the tailings could lead to an increase in the indigenous microbial population and activity. Examining the tailings microbial diversity, ecology and enumerations before and after the growth of alders would serve as an indicator of environmental change, restoration progress and would evaluate the potential of alders for remediation and revegetation of oil tar sand tailings.

1.1. Tailings

The extraction tailings are fluid wastes that are collected and retained on site in settling ponds because both oil extraction companies have a zero discharge policy. They are committed to the eventual reclamation of the tailings, in a safe, economical, and environmentally acceptable manner (MacKinnon and Sethi, 1993). The tailings are transported as slurries, which contain extraction processing water, sand and unrecovered hydrocarbons (50:50:1) to the tailings retention pond (Herman et al.,1994). The bitumen content of the tailings after the extraction process is relatively low, ranging from 1.5% - 4%. In tailing ponds, the coarser solids and fine particulates (clay and silt) settle out of the water, and the extraction process waters are then re-used in the oil extraction process.

1.1.1. Composite Tailings (CT)

Different methods of tailings management are being explored since consolidation of clays and silts would require 125 -150 years (Fedorak et al., 2002). Filtration of the extraction process waters is an inefficient process for clarification of the tailings, since the filter pores would clog quickly due to the large amount of fine

particulate matter. The settling rate can be accelerated with chemical treatments (MacKinnon and Sethi, 1993). Gypsum (calcium sulfate) is added at dosages, ranging from 800-1200 g/m^3 of extraction tailings, sufficient to initiate the coagulation of the fine particulates into larger flocs that settle out of the water relatively quickly (MacKinnon and Boerger, 1986). Tailings that contain gypsum are called composite or consolidated tailings (CT). When the CT slurry is allowed to settle, 62% - 70% of the particulates have precipitated and consolidated within a few hours (Fedorak et al., 2002). Alum is another flocculant that has been shown to perform comparably to gypsum at doses of about 1000 g/m^3. Chemical analysis has shown that as a result of adding gypsum, CT and CT water have a high pH and a high concentration of ions such as SO_4^{2-}, Na^+, Cl^- and Ca^{2+} in the water and sand (Renault et al., 2003). Using alum reduces the salinity and pH of CT. Alum is a more effective treatment for flocculation, but it is not as readily available as gypsum, it has a strong acidic reaction with water and when used for an extended period of time at high concentrations, as would be required in the tailings, is toxic to fish. Gypsum however, has a neutral reaction with water and does not have any toxic effects on animals (Ohio Department of Natural Resources 2005, Williams 2000). However, as long as inorganic chemicals are being used in the tailings, it will reduce the number of options for reclamation (Redfield et al., 2003). Using gypsum or alum for tailings management is known as the dry landscape approach. This approach aims to reduce the water content of the fine tailings and form a solid deposit, which may be revegetated (Fedorak et al., 2002).

1.2. The Physical and Chemical Properties of Oil Sand Tailings

At both Syncrude and Suncor the tailings ponds provide an area for the disposal and containment of both solid and fluid wastes generated from the extraction of bitumen. Without the large holding capacity and water residence times provided in the settling basins, the quality of the water would be reduced and more water would be required from the Athabasca river (MacKinnon and Sethi, 1993). A summary of the physical aspects of Suncor and Syncrude settling basins are presented in Table 1.

There is no such thing as a single representative oil sand, whose properties could be considered indicative of what tailings or wastes components to expect. On the Athabasca Oil Sands Deposit, oil sand properties change with location as a result of differences in origin, facies and depositional and post-depositional environments (MacKinnon and Sethi, 1993). Water soluble (dissolved or ionic) components in the tailings include both organic and inorganic species. The primary source of dissolved components in the tailings pond water is from the release of leachable material from the oil sands during bitumen extraction. Some of the main sources and factors affecting dissolved components include leachable ions released from the oil sand ore, ions added from drainage and import of water, process chemicals, rates at which the water is recycled and chemical and biological interactions in the water (MacKinnon and Sethi, 1993). There are differences in the oil sand ore processed at the 2 plants, as well as some operational factors. Suncor uses only about 30 - 40% of the caustic NaOH used by Syncrude, which would be reflected in lower concentrations of sodium ions in the waste water (MacKinnon and Sethi, 1993). The major ions are Na^+, HCO_3^-, SO_4^{2-} and Cl^-. Bicarbonate is the dominant anion in the Suncor ponds. At Syncrude, chloride and sulphate are present at much higher concentrations than at Suncor. This difference results from the higher salt content in the Syncrude ore. Differences in the ore type (marine, estuarine and fluvial) and properties (salt content, mineralogy) are found throughout the McMurray formation. It is predicted that even greater differences can be expected in the future. However sodium is the dominant cation in both the Syncude and Suncor settling basins (MacKinnon and Sethi, 1993). A summary of the chemical properties of Suncor and Syncrude settling basins are presented in Table 2.

Table 1. Physical Properties of the Suncor and Syncrude Settling Basins (adapted from MacKinnon and Sethi, 1993)

Settling Basin	Total Area (km^2)	Water Surface (km^2)	Total Depth (m)	Free Water Zone (10^6m^3)	Fine Tails Zone (10^6m^3)
Suncor					
Pond 1	4.5-5	1.7	35	2.4	24
Pond 1A	0.5-1	0.5	50	1.7	12
Pond 2	3-3.5	1.8	40	3.5	20
Pond 3	3.5-4	2.7	60	12	32
Pond 4	2.5-3	0.5		7	
Syncrude					
Mildred Lake Settling Basin	20-25	12	50	55	233

Table 2. Chemical Properties of Composite Tailings Sands and Composite Tailings Water (adapted from Fedorak et al., 2002)

	Composite Tailings Sands		Composite Tailings Water	
Parameter	Syncrude	Suncor	Syncrude	Suncor
pH	8.0	7.5	7.8	7.8
Solids (%)	28.7	30.0	77.9	72.9
Bitumen (%)	2.7	9	0.1	0.1
Naphtha (%)	0.21	0.6	ND	ND
Na^+ (mg/L)*	870	410	1000	650
Mg^{2+} (mg/L)*	10	23	14	23
Ca^{2+} (mg/L)*	14	45	61	78
Fe (mg/L)*	ND	0.9	1.7	0.7
Cl^- (mg/L)*	560	53	670	69
HCO_3^- (mg/L)*	1530	1800	1200	1050
SO_4^{2-} (mg/L)*	19	34	1290	960
NO_3^- (mg/L)*	6†	ND	ND	ND
NH_4^+ (mg/L)*	10	64	1.4	4
Kjehdahl N (mg/L)*	12	96	5	6
Total P (mg/L)*	0.2	0.4	ND	ND
Dissolved Organic Carbon (mg/L)*	70	68	100	110
Naphthenic acids (mg/L)*	58	48	96	62

Note: *- indicates that Pore water was analyzed, not detected = ND.

1.2.1. Salinity and Plants

Renault et al. (1998) demonstrated that sodium concentrations are in excess of 400 mg/L and sulfate was present at 800 mg/L in CT water. The aim is the eventual revegetation of the tailings, but the concentration of salt and phytotoxic ions, such as boron, fluoride, aluminum and strontium makes the tailings a very inhospitable environment. The effect of salt on plants varies with the plant. Uptake and accumulation of sodium in plant tissues can lead to enzyme inactivation, inhibition of protein synthesis and modification of membrane permeability. An excess of salt can affect germination and decrease available photosynthates, N_2 fixation and nitrification. It has previously been reported that salinity also produces membrane lesions that results in leakage of solutes from cells (Renault et al., 1998).

As mentioned earlier, the addition of gypsum results in the flocculation of sand and fine particulates (clay and silt). The CT mixture contains 55% - 65% solids by weight, 15% - 30% of which are fine particulates. CT sands have a high concentration of sodium. Sodium can affect the physical structure of soil due to the swelling of clays, which reduces its permeability to water, oxygen and roots (Renault et al., 2003).

1.3. Naphthenic Acids

Naphthenic acids (NA) are a mixture of compounds of varying sizes that are naturally present in tar sands (Fig.1). Naphthenic acids are predominantly saturated mono and poly-cycloalkane carboxylic acids with aliphatic side chains of various lengths (Herman et al., 1994). Naphthenic acids constitute about 50% by weight of the total acidic compounds in crude oil (CEATAG, 1998). In nature, NA may form by diagenesis of sedimentary organic materials and become a part of the crude oil reserves and may also form by direct conversion of petroleum compounds by microbes and plants. NA have been isolated, refined and used as an antimicrobial agents in industrial applications such as wood preservation (CEATAG 1998). Since the tailings water is recycled numerous times, NA are concentrated in the tailing ponds. Typical concentrations range from 40 -120 mg/L of NA (Clemente et al.,

2004). These compounds are highly soluble and have an extremely low volatility. NA are liberated in the extraction process, solubilized and are present in the tailings as sodium salts of NA. As mentioned earlier, NA have surfactant properties, which are important in the oil extraction procedure but are also considered to be one of the factors responsible for the low settling rates of the fine particulates in the tailings (CEATAG 1998).

The partitioning of NA from water to the sediment depends on the pH, residence times, temperature, the presence of other compounds in the water and the structure of the individual NA. NA adsorb to clays through hydrogen bonding, electrostatic - dipoles and Van der Waals interactions and are thought to concentrate at the interface between water and sediment. Soil studies performed on single ring model NA, at concentrations ranging from 0-100 mg/L, have revealed that inorganic salts such as $CaCl_2$ and a low pH can enhance the adsorption of NA to soil and sediment (Peng et al., 2002). At present the relative partitioning of NA from water to clays and sand is unknown, but this information is essential in predicting the fate of the NA and their bioavailability (Quagraine et al, 2005).

1.3.1. Toxicity

The acute and chronic toxicity of the fluid wastes from the tailings on many aquatic organisms, plants and mammals is often associated with the surfactant properties of NA. As surfactants, NA can easily penetrate the cell wall. Some molecular toxicity studies with *Escherichia coli* showed that stress inducible genes were implicated in membrane disruption and cytotoxicity, and osmotic stress was mainly responsible for the toxicity response upon exposure to NA (Quagraine et al., 2005).

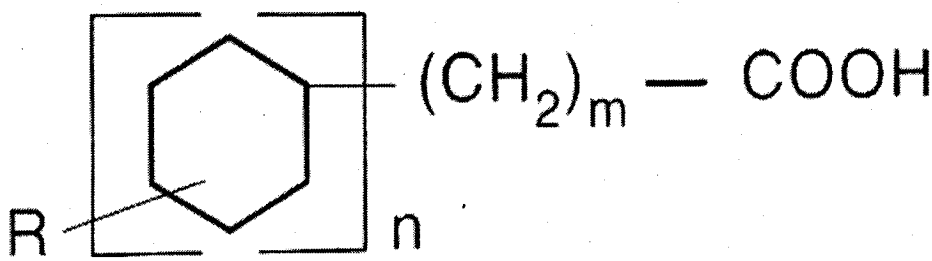


Figure 1. The typical structure of naphthenic acids is a saturated mono or poly-cycloalkane carboxylic acid with aliphatic side chains of various lengths (Herman et al., 1994). The structural formula of naphthenic acid shows the carboxylic acid group (COOH) at the end of the aliphatic side chain where "m" is greater than one. "R" is a small aliphatic group, such as methyl, and "n" equals one or more. Previous studies have shown that compounds of 2n predominate in oil sands tailings pond wastewater (CEATAG, 1998).

The extent of sorption of NA to fine particulates influences their bioavailabilty, toxicity and therefore the degradation activity of microorganisms. It is often assumed that the contaminants are only available for biodegradation in the aqueous phase and when adsorbed to the sediment they are not available to the bacteria. However microbes are also able to degrade adsorbed compounds. This was demonstrated in an experiment using granular activated carbon columns inoculated with bacteria. However, degradation of the adsorbed substrate depends on the microorganisms present (Speital and DiGiano, 1987).

1.3.2. Acute and Chronic Toxicity

Acute toxicity is often expressed as the lethal concentration (LC_{50}) or the lethal dose (LD_{50}) which kills 50% of the test population. The LD_{50} is generally expressed as the dose, in milligrams of chemical per kilogram of body weight, while LC_{50} is often expressed as milligrams of chemical per volume of medium (i.e., air or water) that the organism is exposed to (CCOHS, 2005). A 96 h static bioassay with trout and *Daphnia* found an LC_{50} of less than 10% CT water for these organisms (MacKinnon and Boerger, 1986). Snails were exposed to sodium naphthenate solutions during a 96 h bioassay when the LC_{50} was from 6.6 -7.5 mg/L. The LD_{50} of naphthenic acids in young, male, white mice was determined to be 3550 mg/kg body weight for orally exposed mice and 860 mg/kg body weight for intraperitonally exposed mice. Symptoms of toxicity included central nervous system depression, convulsions, diarrhea and respiratory arrest leading to death (CEATAG 1998). Acute toxicity symptoms in rats included both cardiovascular and hepatic effects.

The chronic effects of NA have also been studied. The response of sturgeon fish exposed to sodium naphthenate, at concentrations ranging from 0.5 -10 mg/L over a 15 day period, was monitored on the basis of carbohydrate metabolism, and more specifically blood sugar content and the glycogen level in the liver. The greatest changes in blood sugar and glycogen levels were observed at a concentration of 5 mg/L of naphthenic acid. The sugar content decreased to 50% of the control fish and remained at this level until the end of the experiment (CEATAG

1998). Interestingly, rats that consumed naphthenic acids in their drinking water also demonstrated hepatic effects (Rogers et al., 2002).

The results of toxicity are often related back to the NA concentration in the samples. There is also the possibility that other compounds, such as olefinic, hydroxy and dibasic acids, which are present as minor components of NA, may have initiated or played a part in the biological response. However, it is difficult to analyze and isolate the individual NA from the mixture, so the main compound(s) responsible for toxicity are not known (Quagraine et al., 2005).

1.3.3. Toxicity in Plants

Exposing aspen seedlings to NA affects their physiology and morphology. Treatment with solutions of 75, 150 and 300 mg of NA per liter of nutrient solution (Hoagland's solution) brought about a decrease in root water flux, root respiration, leaf stomatal conductance, photosynthesis, chlorophyll concentration and leaf size. The authors (Kamaluddin and Zwiazek, 2002) hypothesized that the surfactant properties of NA had altered the uptake and transport of minerals, nutrients and water from soil. The lack of nutrients and water produced secondary responses in plants and seem to have altered gas exchange and leaf growth. NA entrance into the xylem of aspen may have also interfered with other metabolic processes of aspen. Since NA are in saline tailings and salinity is also known to lead to a decrease in leaf stomatal conductance, NA may have aggravated the effects of salt on plants (Kamaluddin and Zwiazek, 2002).

In contrast, potassium naphthenate had a stimulatory effect on plant growth when sprayed on shoots of kidney beans. The effect may have resulted from increasing respiration rates and photosynthetic efficiency. It was shown that plant growth was stimulated by leaf applied – cyclohexane carboxylic acids but plants were not affected by other NA but it is possible that individual NA may exert different effects on plants (Kamaluddin and Zwiazek, 2002).

1.3.4. Detoxification of Tailings

Storage of process waters for one year, as demonstrated in a 96 hour bioassay with trout and *Daphnia*, resulted in a LC_{50} value of 100% (ie. undiluted) (MacKinnon and Boerger, 1986), indicating that degradation of NA by the indigenous microbial community can contribute to a decrease in toxicity of oil sand tailings. The variability of the detoxification rates is mainly due to temperature, which is naturally higher in the summer. However, a decrease in toxicity is accompanied by an increase in the proportion of NA that have greater than 22 carbons. It is not known which components are the most toxic but, a decrease in the proportion of lower weight NA contributes to a reduction in acute toxicity (Clemente et al., 2004).

Treatment of the tailings pond water also includes clarification. Physical methods such as settling, centrifugation and filtration are slow and don't result in detoxification. However, when H_2SO_4 was added to the pond water, which was acidified to a pH of less than 5, there was a reduction in toxicity, due to the precipitation of the NA. This was demonstrated by a 96 hour acute toxicity test with trout and daphnia. When the pond water was acidified, the LC_{50} was over 90% and approached 100% when a flocculent, like gypsum, was added (MacKinnon and Boerger, 1986).

1.4. Remediation

The dry and wet landscape approaches are the two main bioremediation technologies being considered by Syncrude and Suncor. The dry landscape approach aims to reduce the water content of the fine tailings (sand, silt and clay). There are two types of wet landscape approaches; the self sustaining wetland and the managed wetland. The self sustaining wetland, also called the capping method, does not involve any external influences. The constructed or managed wetland involves continuous manipulation to enhance wastewater treatment.

The capping method involves the transfer of fine tailings into an abandoned mined out pit, over which a layer of water is placed to establish a water cap over the fine tailings base (Holowenko et al., 2000). To asses the potential effects, both

petroleum extraction companies have constructed experimental pits, with those at Syncrude ranging in size from 0.25 - 3 ha in area and up to 7 m in depth. The pits contain mature fine tailings (sand, silt and clay) and are capped with either water from the settling basins or with boreal forest surface runoff water. Computer generated models indicated that after 1-2 years of capping the concentrations of NA would be below the levels acutely toxic to fish and other aquatic organisms (Gulley and MacKinnon, 1993). NA concentrations of 6 mg/L were measured in water samples collected from a depth of 2.5 m in a mesocosm, containing fine tailings capped with local surface runoff, after eight years of natural exposure. The water had little or no effect on the phytoplankton community, compared to reference water. But a mesocosm capped with Mildred Lake Settling Basin water, after 8 years of natural exposure, had NA concentrations greater than 19 mg/L and did produce effects on the phytoplankton community (Leung et al., 2001). It has been shown that natural aging does reduce the toxicity and NA concentrations in the water, as mentioned earlier. However, the capping method can be amended to increase the efficiency and rate of NA degradation.

One suggestion includes aeration of the ponds. Oxygen is poorly soluble in water. Its transfer requires intensive air bubbling and mechanical stirring which can be extremely expensive. Algal photosynthesis provides a cheaper alternative. With light, algae produce the oxygen needed for degradation processes and use the CO_2 released by the bacteria. This approach is already used to treat some wastewaters (Mara and Pearson 1986).

Other suggestions include the addition of nutrients, such as phosphorus, nitrogen as ammonia, and supplemental organic materials to enhance degradation activity. The addition of organic materials has been demonstrated, in the laboratory, to promote the degradation of individual NA by co-metabolism. However, the addition of organic compounds must be evaluated to ensure the microbes don't preferentially degrade the added compounds and leave the target NA untouched (Quagraine et al., 2005).

Harnessing natural microbial processes to clean up NA contaminated tailings is possible but challenging. NA vary in size, structure and degree of

biodegradability. Some tailings pond waters have been stored for more that 10 years and it appears that the remaining high molecular weight NA are refractory to natural biodegradation. Currently, no single reclamation option has been developed. An integrated approach is needed to detoxify the water first, so it becomes capable of sustaining aquatic life, and then the fine tailings can be dealt with (Quagraine et al., 2005).

1.4.1. Phytoremediation

The clean-up of contaminated soils may involve the removal and replacement of soil and incineration of the contaminants, which are expensive and invasive procedures. However, the exploitation of plants to remediate contaminated soils is a relatively inexpensive and a more environmentally friendly approach. The conditions in the oil sand tailings and composite tailings are stringent and the choice of the plant for remediation and reclamation will be influenced by their tolerance to pH, naphthenic acids, salinity and phytotoxic ions in these tailings (Renault et al., 2003).

Plants can have extensive root systems that are in contact with large amounts of soil. Hydrophobic compounds, such as high molecular weight naphthenic acids, often adhere to suspended solids. The immobilized material may serve to concentrate the contaminant and the degrading microbial population (Muratova et al., 2003).

Plant roots are capable of supporting, changing and increasing the activity of the indigenous microflora by secreting of compounds such as organic acids, polyphenolic compounds, and nutrients. The success of phytoremediation of contaminated soils is connected to the plant's capacity to enhance microbial activity, which may arise from the release of plant lysates and root exudates or an increase in the soil microbial population, or both (Siciliano et al., 2003).

The main detoxifying agent is the microflora while plants provide favorable conditions such as aeration, nutrients and biosorption, which stimulate degradation and mineralizarion of organic compounds (Muratova et al., 2003), such as naphthenic acids.

A comparative analysis was made between the microbial population from unplanted bitumen contaminated soil and the rhizosphere of alfalfa in bitumen contaminated soils. The planted soil had a greater microbial diversity than the unplanted soil. It was also found that over 27 months, bitumen contamination reduced the total number of bacteria in the unplanted soil. However in the planted soil, a significant reduction in the bacterial population was not observed, demonstrating that the conditions were more stable and favorable for the microbial community in the planted soil than the unplanted soil (Muratova et al., 2003).

To date there are no reports specifically addressing NA and phytoremediation, but various bacteria isolated from the tailings have been shown to be able to degrade mixtures of NA. It is possible that the "rich rhizobacterial" population in the vicinity of the plant roots can contribute to the degradation of NA (Quagraine et al., 2005).

1.5. Indigenous Microbial Populations

The tailing ponds are inhospitable to plants and aquatic organisms, but they do harbour bacteria. NA are subject to biodegradation by a variety of microorganisms, which are indigenous to the tailings. The biodegradation of NA is expected to begin with the β-oxidation of the aliphatic side chain leading to the oxidation of the cycloalkane ring (CEATAG 1998).

Enrichment cultures have revealed an aerobic population of bacteria, consisting of *Acinetobacter*, *Pseudomonas*, *Alcaligenes* and *Xanthomonas* species (Herman et al., 1993, 1994). Enrichment cultures were found to be able to degrade compounds representative of naphthenic acids such as cyclopentyl carboxylic acid, cyclohexyl carboxylic acid and a commercial preparation of naphthenic acids (Herman et al., 1993). A diverse microbial population was also isolated from bitumen coated filters left on the bottom of a river for 8 weeks, and included *Pseudomonas*, *Xanthomonas* and *Rhodococcus* species (Wyndham and Costerton, 1981).

The tailings ponds also contain an anaerobic population of methane generating bacteria. Since the early 1990s, visible bubbling activity has been

observed on the surface of the Mildred Lake Settling Basin at Syncrude Canada Ltd. Methanogens were previously below the level of detection but as of 1999, about 40% - 60% of the 12 km^2 water surface area is considered an active bubbling zone and has an estimated daily flux of 12 g CH_4/ m^2 (Holowenko et al., 2000). Methane is an important greenhouse gas and methanogenesis may pose a problem for the remediation of the tailings using the wet landscape approach. Methane percolating into the overlying water could decrease oxygen levels in the water cap and as a result may prevent the establishment of a lake ecosystem (Holowenko et al., 2000). It is well established that methanogenesis is inhibited by the presence of other terminal electron acceptors such as oxygen, nitrate and sulfate (Holowenko et al., 2000). It was observed that the numbers of methanogens were lower where the sulfate concentrations in the Mildred Lake Settling Basin were high (Holowenko et al., 2000). Further experiments demonstrated that the addition of sulfate significantly inhibited the production of methane. This inhibition arose from the competition for substrates, such as H_2 and acetate between sulfate reducing bacteria present in the tailings, and methanogens. Although sulfate does inhibit methanogenesis, the addition of sulfate to the tailings is not feasible due to the volume and insufficient mixing of the tailings. However the addition of gypsum, which contains sulfate, is thought to help control methane production (Holowenko et al., 2000).

1.6. Revegetation

Terrestrial plants are rarely exposed to tailings and as a result, the phytotoxicty and effects of salinity of those wastes to plants have not been thoroughly investigated. There have been some studies performed where plants have been examined for their tolerance to CT and CT water (Renault et al., 1998). Prior to oil extraction mining activities, the oils sands development area supported a mixed wood boreal forest. Seeds of several boreal forest species including Jack pine, white spruce, dogwood, black spruce, lodgepole pine, hybrid poplar, aspen, peachleaf willow, strawberry and raspberry were tested for their response to 25%, 50% and 100% CT water. Strawberry and raspberries were very susceptible to CT

water and had a low survival rate. Leaf tip necrosis, a typical symptom of sodium and fluoride toxicity, was observed in the berry plants, white spruce, lodgepole pine, aspen and willow. White spruce, aspen and lodge pole pine all had lower water potential when receiving CT water than uncontaminated water. Hybrid poplar showed a reduction in transpiration rates. However dogwood showed a high transpiration rate and a very high tolerance to CT waters with no visible injury (Renault et al., 1998). Dogwood was further tested for its response to CT and CT water. It was found that although the seedlings survived in the CT treatment, most growth was completely inhibited. Examination of the plant tissue indicated that there were increased concentrations of sodium in the shoots (Redfield et al., 2003).

Other types of vegetation, such as grasses, have also been examined for their potential in reclamation of the tailings. Initial CT deposits are soft, and grasses can be easily established on this soft substrate. The planting of grasses can also modify substrate properties and aid in dewatering CT. Barley was selected for its relative tolerance to salinity and it has been used in tailings sands as a soil stabilizer (Renault et al., 2003). The CT substrate reduced germination rates, plant survival, caused leaf injury and a reduction in growth. Renault et al., 2003, suggested that barley could be used for phytoremediation due to its high accumulation of ions in its above-ground biomass, but an amendment of peat would be required (Renault et al., 2003). Barley could be used to remove some salts from the ecosystem and aid in the eventual establishment of more salt - sensitive plant species.

1.6.1. Alders

The objective of reclamation is to produce areas that are stable, self sustaining and productive with no long term toxicity (Renault et al., 2003). Using actinomycete-nodulated woody plants for the reclamation of mine-land soils, roadsides and landscape sites is not common. Plants that form a symbiotic relationship with actinomycetes can supply nitrogen to the entire plant community (Carpenter et al., 1979). Alders are pioneer species, which are among the first species to become established naturally in many denuded areas, and grow very

well in dry sandy soil or gravelly terrain. Seedlings have been planted successfully for reforestation of such areas and soil fertility was improved through nitrogen fixation, as a result of their symbiotic relationship with the nitrogen fixing bacterium, *Frankia*.

Over 6000 ha of land in the James Bay territory has been denuded due to the construction of roads, dykes and dams and an extensive program to revegetate this site was initiated. In this project the green alder, *Alnus crispa*, and 2 other plants were chosen. At the end of the third growing season, green alder had the best survival rate compared to the other two trees. The green alder appeared to be well suited for the revegetation of impoverished sites due to its capacity for dinitrogen fixation, rapid early growth, high survival rate and high juvenile fruiting capacity (Pregent et al., 1987).

Alders contribute to soil formation by producing litter that is decomposed and incorporated into the soil organic matter. The organic matter improves soil structure, water holding capacity, cation-exchange capacity and soil fertility. When effectively nodulated with *Frankia*, alders grow well on sand or soils with low nitrogen availability. It has been noted that one of the first trees to appear on burned and logged areas is the red alder (Pregent et al., 1987).

Reforestation of mines and sand dune wastelands have been performed in East Germany using the black alder, *Alnus glutinosa*, which able to thrive in acidic soils and its dense foliage provides shade during times of maximal evaporation (Kohnke 1941).

There is very little information regarding the response of alders to tailings. Several different trees, including the red alder, *A. rubra*, were tested for their response to salt stress and CT water, in order to determine their potential for revegation of tailings (Khasa et al., 2002). Red alders had a good survival rate when receiving the CT water and the salt treatment. Similarly, the speckled alder, *A. rugosa*, a species indigenous to Alberta, that was collected at the Syncrude site, survived the salt treatments very well but its germination rate was low (Khasa et al., 2002). The authors hypothesized that, if the germination problems are overcome,

seed lots of this family of plants could be inoculated with specific *Frankia* strains for CT revegetation.

1.7. *Frankia*

Actinorhizal plants, such as alders, serve as pioneer species. These plants have a symbiotic relationship with, and are nodulated by *Frankia*, which are actinomycetes. *Frankia* are known for their ability to fix N_2 in root nodules on non-leguminous species. All *Frankia* strains that have been isolated are obligate heterotrophic aerobes that grow very slowly, with doubling times of 15 hours or more. They are recognized by their extensive hyphae and sporangiospores in liquid culture (Benson and Silvester, 1993).

Frankia use a variety of nitrogen and carbon sources for growth, including amino acids, urea, nitrate, ammonia and N_2. The major factor for promoting and maintaining *Frankia* populations is the host plant. However *Frankia* can fix nitrogen outside the root nodule and have a free living saprophytic existence in the soil. *Frankia* have been observed in soils well outside the normal geographic range of appropriate host plants or after the host plants have disappeared (Benson and Silvester, 1993).

Nitrogenase is a highly conserved enzyme complex in *Frankia* that converts nitrogen to ammonia, and is O_2 sensitive. Vesicles are sites of nitrogenase activity and provide resistance against O_2 diffusion. Vesicles are lipid encapsulated, sphere like structures, commonly produced in culture (Benson and Silvester, 1993).

Frankia cells enter into a symbiotic relationship with *Alnus* spp. by root hair infection. Root hair infection is characterized by root hair branching and curling. Only one root hair is required for infection and nodulation. *Frankia* proliferate primarily in the root nodule of the host plant. At present, the carbon substrate(s) from the plants, which benefit *Frankia* are not known. The optimum pH for nodulation is near neutral but nodulation can occur in soils where the pH is as low as 3.5 (Benson and Silvester, 1993) and as high as 12 (Oliveira et al., 2005).

In actinorhizal symbiosis, it has been shown that high salinity could affect nodule development, N_2 fixation and the growth of the host plant. Plants of the

genus *Casuarina* are known to grow in saline environments and, like alders, also form a symbiotic relationship with *Frankia*. It was shown that at all levels of NaCl tested (0-500 mM NaCl), inoculated plants were nodulated, which shows that nodule development was not inhibited by salinity. It was also noted that N_2 fixation remained fairly constant up to 200 mM NaCl but decreased at 500 mM to 40% of the control (Ng 1987).

1.8. Investigating the Microbial Diversity

Soil microorganisms are very sensitive to environmental change, and significant changes of the microbial community can occur following disturbance, both in terms of the total biomass and species composition. Measures of the microbial community following the initiation of reclamation efforts could be used as an indicator of restoration progress and may give insights into potential ways to accelerate restoration (Hinojosa et al., 2005). The methods traditionally applied for the analysis of diversity and soil microbial ecology are based on cultivation and isolation of bacteria. Many commercial identification systems rely on pH based reactions, enzyme based reactions, utilization of carbon sources, and visual detection of bacterial growth (O'Hara 2005). A wide variety of culture media have also been designed to maximize the recovery of diverse microbial groups. Culture based techniques are also useful for understanding the physiological and functional potential of isolated organisms (Orphan et al., 2000). Unfortunately, these techniques are biased towards copiotrophic organisms and the resulting metabolic fingerprints are unlikely to accurately represent the diversity of most natural microbial communities (Garbeva et al., 2004) and don't necessarily provide information on the composition of the microbial community (Orphan et al., 2000). In addition, culture - based methods are limited to the 0.1% - 10% of soil microorganisms that can be cultivated (Ranjard et al., 2000).

Recent advances in the field of molecular biology (extraction of nucleic acids, polymerase chain reaction (PCR) amplification, DNA cloning, DNA sequencing) have made it possible to develop techniques which no longer require the isolation and culture of bacteria and thus reduce the associated bias

(Ranjard et al., 2000). The identification of dominant species and the microbial diversity in TS and CT and changes in the microbial diversity of TS and CT resulting from environmental change can be examined with the *cpn60* gene and 16S rRNA gene.

1.8.1. 16S rRNA gene vs *cpn60* gene

The 16S ribosomal RNA (16S rRNA) gene, recovered from environmental samples has revealed previously unrecognized microbial species in a variety of habitats and has become the standard for the assessment of microbial diversity. The 16S rRNA genes are universally distributed in all microorganisms, are key elements of the protein synthesis machinery, and are weakly affected by horizontal or lateral gene transfer (Olsen et al., 1986). A comparison of nucleotide sequences has shown that there are regions of rRNA sequences that are highly conserved between all organisms and that there are other regions that vary to different degrees. The variability in these regions increases as the evolutionary distance between two microorganisms increase, which provides a means to determine the phylogenetic relationships and to distinguish microorganisms from one another (Nakatsu et al., 2000).

Chaperonin 60 (Cpn60) is a bacterial chaperonin involved in many cellular processes but mainly in the heat shock response. Cpn60 with its co-chaperonin Cpn10, using ATP, have been shown to encapsulate and unfold a partially folded or a misfolded protein, and give it another opportunity to fold (Nagradova, 2004).

The *cpn60* gene can also be used to study microbial diversity because it is ubiquitous (Hill et al., 2002) and highly conserved, but with sufficient interspecies DNA sequence variation which can be utilized for species identification (Goh et al., 2000). Molecular methods based on protein-encoding genes, like *cpn60,* may be more discriminating for closely related organisms, since the divergence of protein-encoding nucleotide sequences is more than that of genes coding for 16S rRNA (Brousseau et al., 2001).

A concern when amplifying DNA from microbial communities is the occurrence of chimeras, which is a PCR product that results from two the

amplification of two or more parent sequences. The generation of chimeric PCR products through template switching is facilitated by the multiple copies of 16S rRNA genes within a single genome. Chimeric *cpn60* PCR products are less likely than 16S rRNA chimeras, since in general there is a single copy of *cpn60* in most prokaryotic genomes and the amplified sequence is shorter (552 to 558 base pairs versus the approximately 1.6 kb amplified from bacterial 16S rRNA gene), providing fewer opportunities for template switching (Hill et al., 2002).

The main draw back with using the *cpn60* gene is that the database is limited. The 16S rRNA gene sequence database is significantly larger. Work is currently being done to build the *cpn60* database (Hill et al., 2002).

1.9. Objectives

The extraction of bitumen from the tar sands produces tailings that are saline, have a high pH and a high concentration of residual naphthenic acids. Two species of alders, *A. rugosa* and *A. glutinosa,* were explored for their potential in the revegetation and the bioremediation of oil sands tailings and composite tailings. Their growth was monitored and measures of the microbial community were made and used as an indicator of restoration progress.

The microbial community in the samples with and without alders were compared and characterized. Culture dependent techniques such as plate counts with general and selective media, were used to obtain a quantitative and qualitative measure of organisms in TS and CT with and without alders. ^{14}C-labeled substrates were used to examine the influence of *A. rugosa* and *A. glutinosa* on the rates of contaminant mineralization. The diversity of the microbial community was further examined using culture independent techniques such as PCR amplification with primers for the *alkB* gene, *ndoB* gene and 16S rRNA gene followed by denaturant gradient gel electrophoresis (DGGE) and sequencing analysis.

Further experiments, were aimed at demonstrating the influence of a *Frankia* inoculum on the remediation and revegetation of CT using *A. rugosa*. The impact of inoculated *A. rugosa* on CT was examined by monitoring the microbial community in the bulk soil, rhizosphere and inside the root through enrichment cultures,

mineralization assays and MPN analysis with general (YTS) and selective media (MSM with naphthenic acids and Bushnell Haas with PAHs).

2. Materials and Methods

2.1. Alders

Two species of alders, *Alnus rugosa* and *A.glutinosa,* were planted in the tailings and composite tailings from Syncrude Canada Ltd. When the alders were planted they were inoculated with *Frankia*, strain F9. (Table 1).

Seeds of *A. glutinosa* and *A. rugosa* were put in regular petri dishes, between two layers of Whatman qualitative filter papers (3 mm chromatography, cat. # 3030917, Whatman Inc., Maidstone, Eng.) and submersed in non-sterile tap water 48 h, at 4°C for "stratification" (Normand and Lalonde, 1984).

Seeds were then transferred to soil in large petri dishes (140 mm x 15 mm, Anachemia, Montreal, Can.) and covered with 16-mesh sand and incubated overnight (with the petri lid closed and the petri dish wrapped in aluminum foil) in the dark at 23°C, 100% humidity in an environmental chamber. The Environmental chamber used was from Conviron (Winnipeg, MN). After the overnight incubation, the foil and petri lid were removed, and the petri dishes were placed individually in air-filled plastic bags (plain poly bags, 10 inches x 16 inches, Fisher Sci., Whitby, ON.) in the environmental chamber.

2.2. Environmental Chamber conditions used for Alder Growth

Day and night temperatures were 23°C and 18°C, respectively (Prégent and Camiré, 1985). The humidity of the chamber was set at 100%. The initial temperature of 18°C increased by 1°C every 15 minutes from 8:00 am until 9:00 am, reaching a maximum of 23°C. The temperature stayed at 23°C until 11:00 pm and decreased by 1°C every 15 minutes reaching 18°C at 12 pm. In the chamber there were two types of lighting, incandescence with light bulbs and fluorescence with fluorescent tubes. There were 12 bulbs and 16 tubes. Starting at 8:15 am, 3 bulbs and 4 tubes would turn on. Every 15 minutes another 3 bulbs and 4 tubes would turn on, and by 9:00 am all the lights were on. Starting at 11:15 pm, 3 bulbs and 4 tubes would turn off and every 15 minutes 3 more bulbs and 4 more tubes

would turn off, and by 12:00 pm all the lights were off. These conditions were maintained while the plants were grown in the chamber.

2.3. *A. rugosa* and *A. glutinosa* Growth Trials

2.3.1. Tailings and Composite Tailings

The alders were grown in root trainers (Spencer- Lemaire, Edmonton, AB). Root trainers are openable, hinged plant-growing cells. The treatment was composed of an organic soil mixture (Berger Pro-mix) and sand (1:1 vol/vol), which was adjusted to 85% of its water holding capacity and added to the root trainers. Three to five seeds were placed in each cell, sown directly in the soil and then covered with 3 – 5 mm of 8-mesh sand (Hydro-Québec, 1993). Nutrients (Crone's w/o nitrogen, 10 ml per root trainer – cell, Appendix section 5.1.1) were added every 10 -15 days until the alders were inoculated (Périnet *et al.* 1985).

2.4. Inoculation of *A. rugosa* and *A. glutinosa* with *Frankia* F9

The protocol was adopted from Berry et al. 1985, and Périnet et al. 1985. The plants were inoculated with a 10 weeks old culture of *Frankia* F9 (ATCC# 33255) grown in Qmod (Appendix, section 5.1.2) supplemented with 5 g/L succinate (disodium salt). The alders were inoculated at the 6-leaf stage. To prepare the inoculum many culture tubes were pooled inside a 50 ml conical, polypropylene Falcon tube (Becton Dickenson, Franklin Lanes, N.J.) and sonicated at the lowest setting, for 1-second intervals, to break up the aggregates. Following sonication, microscopy was used to verify if the *Frankia* had disaggregated. The tubes were centrifuged at 430 x g, at 4°C for 10 min. The supernatant was decanted and discarded. The pellet was then resuspended in 500 µl -1 ml of Qmod and transferred to two 1.5 ml Eppendorf tubes. The tubes were centrifuged at 200 x g, at 4 °C, for 3 min and the supernatant was decanted.

Table 3. Characteristics of Composite tailings (CT) and Oil Sands tailings (TS) treatments with and without *A. rugosa* and *A. glutinosa.*

Samples	Characteristics
CT	Composite Tailings at time 0 of the experiment
TS	Tailings at time 0 of the experiment
CTNP	Composite Tailings incubated in the greenhouse for 6 months without a plant
TSNP	Tailings incubated in the greenhouse for 6 months without a plant
CTAR	Composite Tailings with *A. rugosa* grown in the green house for 6 Months
TSAR	Tailings with *A. rugosa* grown in the green house for 6 months
CTAG	Composite Tailings with *A. glutinosa* grown in the green house for 6 months
TSAG	Tailings with *A. glutinosa* grown in the green house for 6 months
CSAR	Control soils with *A. rugosa* grown in the green house for 6 months
CSAG	Control soils with *A. glutinosa* grown in the green house for 6 months

The packed cell volume was estimated by eye and the pellet was transferred to a 50 ml falcon tube and resuspended in an appropriate amount of 0.85% NaCl solution to deliver 0.3 µl of a packed cell volume / 5 ml. This represents a standard inoculum, but the exact concentration of *Frankia* is not known because the cells form hyphae and cannot de disaggregated. Due to the growth media constituents, it is also not possible to obtain an accurate dry weight. The inoculum was kept uniform by stirring or by agitation and delivered at the base of the plantlet above the soil, using a 25 ml sterile pipet. To avoid flushing the *Frankia* out of the soil column, no water was added to the plants on the day of inoculation. One and a half months after the alders were inoculated they were planted in CT and TS and grown in the greenhouse. The alders were also planted in control, uncontaminated soils (CSAR and CSAG treatments).

2.5. CT and TS Treatments

Samples of CT and TS were taken at time 0 and frozen at –80°C. After 6 months the alders were harvested and the entire bulk soil content from each pot was mixed and stored at –20°C for subsequent microbial and molecular analyses.

2.6. Conductivity and pH of CT and TS

The conductivity of CT and TS was taken using a HACH Sension 5 conductivity meter (Loveland, Colorado). The machine was calibrated using the NaCl calibration solution provided. The protocol used to take the conductivity of the samples was taken from the Conseil des productions végétales du Québec (1988). For conductivity measurements, 3 g of sand was suspended in 30 ml of milliQ water. Thirty milliliters was the minimum volume needed to completely immerse the conductivity probe, which was needed for a proper measurement. The tubes were vortexed for 45 seconds at maximum speed and then shaken at room temperature for 30 min. The tubes were centrifuged at 1746 x g for 10 min. The supernatant was decanted and used for the measurement of conductivity. The analyses were performed in duplicate. The conductivity meter was used as described by the manufacturer. A standard curve was prepared using NaCl solutions, made using

milliQ water, at the following concentrations: 0, 500 mg/1000 ml (8.55 mM NaCl), 1000 mg/1000 ml (17.1 mM NaCl) and 1500 mg/1000 ml (25.66 mM NaCl).

The pH of the sands was taken using 5 g of sand suspended in 5 ml of deionized water. The protocol was adopted from McLean (1982). The samples were vortexed for 1 min. at maximum speed and centrifuged at 1300 x g for 10 min. The pH of the samples was taken using the supernatant, using an Orion pH meter (model 720A, Boston, M.A.), as described by the manufacturer.

2.7. Microcosms of CT and TS Bulk Soil Samples

The bulk soil samples of CT and TS treatments were all collected when the plants were harvested. They were frozen at -20°C and thawed for these trials. One gram of each sample was added to a 25 ml serum bottle, sealed with teflon / rubber septa, and spiked with ^{14}C-labelled substrate. The samples were spiked with a methanol solution containing either ^{14}C-naphthalene (2.3 mCi/ mM), ^{14}C-phenanthrene (46.9 mCi/ mM) or ^{14}C hexadecane (12 mCi/ mM), mixed with cold substrate, to provide a final concentration of 10 000 dpm and 100 ppm of hexadecane and 1 ppm of phenanthrene or naphthalene. The radioactive and cold substrates were purchased from Sigma-Aldrich Canada Ltd. These bottles contained a tube which had 0.5 mL of 1.0 N KOH as a CO_2 trap. All microcosms were incubated statically, in the dark, at room temperature. The microcosms were sampled by aspirating the KOH with a syringe. The tube was also rinsed by adding 0.5 ml of 1 N KOH. The former and latter KOH were combined with 9 ml of scintillation cocktail (Aqueous Counting Scintillant NACS 104, Amersham Biosciences, Baie d'Urfe, Que.). The KOH- scintillation cocktail mix was added into a scintillation vial (Fisher Sci., Nepean, Ont.). The $^{14}CO_2$ was quantified by a Canberra –Packard Scintillation counter (TRI CARB 2100 TR, Canberra – Packard Canada Ltd, Mississauga, Ont.). The mineralization results were expressed as a percentage of $^{14}CO_2$ produced from the ^{14}C- substrate initially added. No nutrients or water were added to the microcosms, and all analyses were conducted in triplicate.

One set of sterile controls was prepared for each substrate. The sterile controls were prepared by autoclaving the microcosms with CT and TS samples and allowing them to incubate overnight at room temperature to allow the spores to germinate and autoclaved again (Roy et al., 2005).

2.8. Aerobic, Heterotrophic, and Halophilic Microbial Enumerations of Oil Sands Tailings and Composite Tailings

Viable microbial populations in the tailings sands were quantified by spread plating on YTS-MSM (MSM composition in Appendix section 5.1.3.). Suspensions of tailings and composite tailings were prepared by taking a sample, weighing it and adding three times the volume of dilution solution, 0.1% tetrasodium pyrophosphate, giving a 1 in 4 dilution. Once the sample was in contact with the solution, the sample was put on ice. A serial dilution in sterile dilution solution was prepared from 10^{-1} to 10^{-4}. Dilutions 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4} were plated. Plates were incubated inverted at room temperature. The colonies that grew on the plates were then counted after one and two weeks. The microorganisms were tested for their ability to grow at 5 different salt (NaCl) concentrations, 0.5 M (29.22 g/L NaCl), 1 M (58.44 g/L NaCl), 1.5 M (87.66 g/L NaCl), 2 M (116.88 g/L NaCl)and 2.5 M (145.10 g/L NaCl) (Fig 3). All the samples with the exception of CSAG and CSAR were plated on YTS-MSM supplemented with NaCl.

2.9. Total community DNA extraction from Tailings and Composite Tailings with and without alders

Five grams of CT or TS was aseptically transferred into a 50 ml Falcon tube and washed using 10 ml of three different buffers: 1st buffer, 2nd buffer, 3rd buffer, individually, as described by Fortin et al. (2004) (details of solution compositions in section 5.2 of Appendix). In order to remove humic and fulvic acids and other organic pollutants, which interfere with the enzymes used for cell lysis and subsequent DNA amplification reactions. In the first buffer, 1% polyvinylpyrrolidone (PVP) 40 and 1% PVP 10 were added. Optimization tests showed that their addition increased the PCR amplification yield. The 1st buffer was added into the

falcon tube containing 5 g of soil, then vortexed for 30 sec and then mixed by inversion for 1.5 min. The samples were centrifuged at 4°C for 3 min. at 2900 x g and the supernatant discarded. The procedure was repeated for the 2nd and 3rd buffers. After the samples were washed with the 3rd buffer and the supernatant discarded, the samples were resuspended in 9 ml of sterile water.

Cell lysis was performed by enzymatic treatment, as described in Fortin et al. (1998). Five-hundred microlitersTris-HCl pH 8.0 containing 50 mg of lysozyme (Roche Diagnostics, Indianapolis, IN) was added and incubated with agitation at 30°C, for 30 min and then at 37°C for 30 min, mixing by inversion every 10 min. Fifty microliters of proteinase K [20 mg/ml] (Sigma Aldrich, Oakville, Ont.) were added and incubated for one hour at 37°C, mixing by inversion every 10 min. To complete the lysis treatment 500 µl of 20% sodium dodecyl sulfate (SDS) was added and incubated for 30 min., at 85°C, mixing gently by inversion every 10 min. Samples were centrifuged for 15 min. at 4 500 x g. The supernatant was transferred to a 30 ml centrifuge tube (Nalgene Nunc Int., Rochester, N.Y.) and 0.5 vol. of 7.5 M ammonium acetate was added and incubated on ice for 15 min. Samples were centrifuged for 7 min. at 9 500 x g and the supernatant was transferred into 30 ml corex tubes (Fisher Scientific, Nepean, Ont.). The DNA was precipitated overnight at –20°C by adding one volume of cold 2-propanol. The samples were centrifuged for 30 min at 4°C at 12 100 x g. The supernatant was discarded and the pellet was washed with 2 ml of 70% ethanol to remove salts. Once washed, samples were centrifuged for 5 min., at 4°C, at 12 100 x g. The supernatant was discarded, and the pellet washed with 2 ml of 95% ethanol, centrifuged for 5 min. at 4°C at 12 100 x g and the supernatant was discarded. The pellet was air dried and resuspended in 200 µl Tris-HCl - EDTA (TE) pH 8.0. Fifty microliters of crude DNA extract was purified using a microspin column (GE Health Care Baie d'Urfé, Que.) containing equal volumes of two resins: Polyvinylpolypyrrolidone (PVPP) (Berthelet et al., 1996) and Sephacryl S-400 (Nathalie Fortin, personal communication, composition in Appendix). The Sephacryl was added first, the PVPP was added on top. The column was placed inside a collection tube and spun for 3 min, at 735 x g, at room temperature. Fifty microliters

of crude DNA extract was loaded onto the centre of the column. The column was placed inside a sterile, clean Eppendorf tube and centrifuged again for 3 min. at 735 x g at room temperature. The purified DNA extract was stored at –20°C. The crude extract was also stored at -20°C, in an Eppendorf tube.

The concentrations of the purified extracts, representing total community DNA was obtained by loading 5 µl of DNA extract and a dilution series of high mass DNA ladder (#10496-016, Gibco/BRL, Life Technologies, Burlington, Ont.) onto a 0.7% agarose, made using TAE buffer (Sambrook and Russel, 2001). The gel was stained using 0.01% ethidium bromide in 1x TAE for 5 min. and detained for 30 min. using a solution of 0.1% 1 M $MgCl_2$ in water. The picture was taken using Polaroid 57 black and white film, with a 1 second exposure to UV light.

2.10. The 16S rRNA gene

2.11. 16S rRNA gene PCR Amplification

Purified extracts were added to a final volume of 50 µl of PCR mix that contained 25 pmol of each oligonucleotide primer, U341-GC2 and U758 (Table 2). The primers used targeted the conserved regions of 16S rRNA gene and amplified a 418 base pair fragment that corresponds to regions 341 to 758 in the *Escherichia coli* sequence (Fortin et al., 2004). The sequence for the forward primer contained a GC clamp, to stabilize the melting behavior of the amplified fragment (Fortin et al., 2004) when doing DGGE analysis. The PCR mix had final concentration of 1 mM $MgCl_2$, 125 µg/ml BSA, 200 µM of each dNTP, 2.5 units of Taq polymerase (Amersham Biosciences, Baie d'Urfe, Que.) in a 10x Taq polymerase buffer (100 mM Tris-HCl pH 9.0, 500 mM KCl, 15 mM $MgCl_2$). For PCR the samples were denatured for 5 min at 96°C. To increase the specificity of amplification, a touch down PCR was used in which the annealing temperatures were set to 65°C and decreased by 1°C every cycle until 55°C was reached. The samples were subjected to 30 cycles where the denaturation step was at 94°C for 1 min, the annealing time was 1 min and primer extension was carried out at 72°C for 3 min,

in a Techgene PCR machine (model FTGENE2D, Princeton, N.J., USA). The postive control used was *Pseudomonas putida,* ATCC 17484.

Five microliters of PCR product was loaded onto a 1.4% agarose gel, made with TAE buffer, with a dilution series of a 100 base pairs ladder (Fermentas Gene ruler # SM0241, Burlington, Ont.) and visualized with ethidium bromide (Sambrook and Russel, 2001).

2.11.1. DGGE of 16S gene fragments

The 16S rRNA gene fragment amplified by PCR was concentrated by precipitating with 1/10 volume 3.5 M sodium acetate pH 5.2 and 2.5 volumes ethanol. The centrifuged pellet was resupended in 15 μl of TE buffer (explained in Appendix section 5.2). DNA (700 – 800 ng) of each sample was used for DGGE analysis, as performed by Fortin et al. (2004), on a gel with a linear gradient of 45% - 65% denaturant. Denaturant consisted of 7 M urea and 40% formamide. The DGGE was performed with the BioRad DCODE Universal Mutation Detection System (Hercules, California). The DGGE gel electrophoresis was at 80V for 16 h at 60°C. The DGGE gel was stained gel for 0.5 hour in 1:10000 dilution of VistraGreen (25 μl of VistraGreen in 250 ml of 1X TAE*) with gentle shaking. The gel destained for 0.5 hours in 1X TAE with gentle shaking and viewed using a fluorImager system (Molecular Dynamics, Sunnyvale, CA). Recipe of solutions are in section 5.3 of Appendix.

2.11.2. Nucleotide Sequencing of DGGE bands

Selected bands from the 16S rRNA gene DGGE were cut and the DNA was eluted in 60 μl of sterile milli Q water at 37°C overnight or the band was stored at –20°C for later use. After the overnight incubation, the tubes were spun at 1608 x g for 1 min. at room temperature and the supernatant was used for reamplification of the DNA for subsequent sequence analysis.

Table 4. The primer sequences used for the PCR analysis

Gene	Primer sequences	Fragment size (base pairs)	Reference strain	Reference
alkB	$5'_{495}$- CIGIICACGAIITIGGICACAAGAAGG-3' $5'_{1018}$ –IGCITGITGATCIIIGTGICGCTGIAG-3'	549	*Rhodococcus sp. Strain Q15*	Chenier et al. (2003)
ndoB	$5'_{126}$-CACTCATGATAGCCTGATTCCTGCCCCCGGCG-3' $5'_{767}$-CCGTCCCACAACACACCCATGCCGCTGCCG-3'	642	*Pseudomonas putida* ATCC 17484	Kurkela et al. (1988)
16S rRNA Gene	$5'_{341}$- GC clamp* CCTACGGGAGGCAGCAG-3' $5'_{758}$-CTACCAGGGTATCTAATC-3'	418	*Escherichia coli*	(U341) Muyzer et al. (1993) (U758) Van de Peer et al. (1996)
cpn60	$5'_{H279}$- GC clamp* GAIIIIGCIGGIGA(T/C)GGIACIACIAC-3' $5'_{280}$-(T/C)(T/G)I(T/C)(T/C)ITCICC(A/G)AAICCIGGIGC(T/C)TT-3'	590	*Escherichia coli* Acc.# X07850	Hill et al. (2002)

*GC clamp: CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGG

2.11.3. PCR program for sequencing of DGGE Bands

One microliter of non-diluted eluted DNA was added to a final volume of 50 µl of PCR mix that contained 25 pmol of primer, U341 or U758, 200 µM of each dNTP,1 mM $MgCl_2$, and 2.5 U of Amersham Biosciences (Baie d'Urfe, Que.) Taq in a 10 X Taq polymerase buffer, as used for the amplification of 16S rDNA. The samples were denatured at 96°C for 5 min prior to the addition of the Taq polymerase. The PCR program was 25 cycles in length, and consisted of 1 min at 94°C, 1 min at 64°C, and 1 min at 72°C, in the Techgene PCR machine.

The PCR amplified DNA was pooled and precipitated overnight at –20°C with 1/10 vol. of 3 M ammonium acetate pH 5.2 and 2.5 vol. ethanol. The samples were centrifuged at maximum speed (16 000 x g) for 15 min at 4°C and the supernatant discarded. The pellet was washed with 70% ethanol and centrifuged for 5 min at room temperature at maximum speed and the supernatant was discarded. The DNA was dried in a speedvac. (Savant, Farmingdale, N.Y.) and resuspended in 10 µl deionized water. The 10 µl was run on a 1.4% agarose gel, made with TAE buffer, and visualized with ethidium bromide. The DNA-band was cut and purified with the Gene Clean II kit (QBiogene, Montreal, Que., lot # 1001-400-116348). The DNA was quantified by loading 0.5 µl on a 1.4% agarose gel with a dilution series of a 100 base pair ladder (Fermantas). Twenty nanograms of DNA was needed for sequencing at a concentration of 2 ng/µl. The DNA was diluted accordingly and sent to the Laboratoire de synthese et d'analyse d'acides nucleiques at Laval University (Ste-Foy, Que.) for sequencing.

2.12. *cpn60* gene PCR Amplification of Total Community DNA from Bulk CT and TS samples

For *cpn60* gene amplification, DNA was rextracted using 10 g of CT and TS samples, since the biomass in the samples are low and amplification using the DNA extracted from 5 g of CT and TS gave negative results. As a result, the volume of the reagents used in DNA extraction protocol, mentioned earlier, doubled. However the DNA was still resuspended in 200 µl of Tris- EDTA.

One microliter of pure DNA extract was added to a final volume of 50 µl of PCR mix that contained 120 pmol of each primer, H280 and H279-GC (Table 2). Primers were designed to amplify the region between amino acid codons 92 and 277 based on the *E. coli cpn60* sequence. The forward primer, H279, had a GC clamp to control the melting behavior of the amplified fragment.

The PCR mix had 2.5 mM $MgCl_2$, 125 µg/ml BSA, 200 µM of each NTP, 2.5 units of Invitrogen Taq and a home made buffer (10 mM $MgCl_2$, 100 mM KCl, 100 mM Tris-Cl pH 8.3, 5 x 10^{-6} g of gelatin). The samples were denatured for 5 min at 96°C, prior to the addition of Taq and were subject to 40 cycles of; denaturation at 94°C for 1 min., annealing at 46°C for 1 min., and elongation at 72°C for 1 min. were peformed using the Eppendorf Master Cycler (Eppendorf Scientific Inc., Westbury, N.Y.).

2.12.1. *cpn60* DGGE

DGGE analysis was performed, as described in section 2.11.1, on *cpn60* amplified fragments. Seven hundred nanograms of DNA per sample were loaded onto each lane. Several different denaturant gradients were tried (40% - 80% denaturant, 45% - 65% denaturant and 45% - 55% denaturant).

Selected bands from the DGGE were cut and the DNA was eluted in 60 µl of sterile milliQ water at 37°C overnight or otherwise stored at –20°C for later use. After the overnight incubation the tubes were spun at 1608 x g for 1 min. at room temperature and the supernatant was transferred to a clean sterile tube. The eluted DNA was further purified using Qiaquick PCR Purification Kit (Qiagen, Mississauga, ONT.). Lab tests indicated that the DNA lasted longer when purified with this kit. The protocol was done as described by the manufacturer except for some minor adjustments: At the beginning 1-2 µl of 3 M ammonium acetate pH 5.2 is added, to create a low pH and high salt environment. At the fourth step of their the flowthrough was added back into the column and the tubes were centrifuged, in doing this the binding step would be repeated. Prior to the 5th step, the elution buffer was warmed up for 5 min. at 50°C. At the 8^{th} step once the elution buffer was added, the column was incubated at room temperature for 5 min. before

centrifuging. All centrifugations were for 1 min. at 1608 x g. The DNA eluted from the column was then re-amplified for sequence analysis.

2.12.2. *cpn60* - PCR for sequencing

One microliter of the diluted (1/10) eluted DNA was added to a final volume of 50 µl of PCR mix that contained 120 pmol of each primer, H279 and H280, 200 µM of each dNTP, 2.5 mM $MgCl_2$ and 2.5 U of Taq (Amersham Biosciences, Baie d'Urfe, Que.) in a buffer made for *cpn60* amplification (see section 2.12). Five microliters of PCR product was run on a 1.4% agarose gel, made with TAE buffer, with dilution series of a 100 base pairs ladder and visualized with ethidium bromide. The PCR program was denaturation at 96°C for 5 min and then 25 cycles, and consisted of 1 min. at 94°C, 1 min. at 64°C and 1 min. at 72°C.

The *cpn60* gene fragments were pooled and precipitated overnight at –20°C with 1/10 volume of 3 M ammonium acetate pH 5.2 and 2.5 volumes ethanol. The samples were then centrifuged for 15 min at 4°C and the supernatant discarded. The pellet was washed with 70% ethanol and spun for 5 min at room temperature and the supernatant was discarded. The DNA was dried and resuspended in 10 µl, which was run on a 1.4% agarose gel and visualized with ethidium bromide. The bands were cut from the gel and were processed using the Q-Biogene gene clean kit (see section 2.11.3). The DNA was quantified by loading 0.5 µl of DNA on a 1.4% agarose gel (made with TAE buffer) with a dilution series of the 100 base pair marker from Fermentas.

2.12.3. Sequencing

Twenty nanograms of purified DNA, at a concentration of 2 ng/µl, was sent to University of Laval for Sequencing. The reaction was done as described by the sequencing facility, but with a couple of modifications. The modifications were made due to the hairpin structure of the 16S rRNA gene fragment being sequenced, but these conditions were also used for the *cpn60* gene fragment. The amplification conditions were as follows: 10 sec at 96°C, 4 min at 64°C for 25 cycles.

2.13. Catabolic PCR – *alkB, ndoB*

For the catabolic PCR, DNA from the 10 g of soil extraction was used. Two microliters of template was used in a final volume of 50 µl that contained 120 pmol of each primer, alkH1F and alkH3R (Table 2), 125 µg/ml BSA, 200 µM of each NTP, 1.5 mM $MgCl_2$, 2.5 units of Invitrogen Taq and 10x PCR buffer (200 mM Tris-HCl pH 8.4 and 500 mM KCl). Prior to the addition of Taq, the samples were denatured for 5 min at 96°C. The samples were subject to 30 cycles of denaturation at 94°C for 1 min, primer annealing at 62°C for 1 min and elongation at 72°C for 1 min. The samples were precipitated overnight at –20°C with 1/10 vol. 3 M sodium acetate pH 5.2 and 2.5 vol. ethanol. The samples were centrifuged for 15 min. at 4°C and the supernatants were discarded. The pellets were washed with 70% ethanol and air dried. The pellets were resuspended in 5 µl of water and loaded onto a 1.4% agarose gel, with a dilution series of a 100 base pairs ladder from Fermentas (# SM0241) and visualized with ethidium bromide. The conditions for PCR were as used with *alkB* except that the primers used were *ndoBb* and *ndoBe* (Table 2) and the annealing temperatures ranged from 60°C - 68°C. The Microarray lab at the NRC prepared the primers for all of the above PCR reactions.

2.14. Inoculated and Non - Inoculated *A. rugosa* in CT

A. rugosa was started out in potting media and grown in the Bonnyville nursery, in Alberta and inoculated with *Frankia*- strain F9, using the methods explained earlier, and then shipped to us. The *A. rugosa* was planted in CT, grown in the greenhouse for 9 months and harvested. The microbial population was analyzed in the bulk soil, the rhizosphere and at the endophytic level.

2.14.1. Inoculated and Non-Inoculated Bulk and Rhizosphere CT with *A. rugosa*

When *A.rugosa* was harvested, their root systems were removed and the soil left over was the bulk soil of the sample. The roots with their remaining attached soil were suspended in excess sterile distilled water, in a sterile Erlenmeyer flask. The flasks were shaken for one hour at moderate agitation and

the resulting soil slurry was poured into falcon tubes, ensuring that no roots were included in the slurry. The roots were rinsed with fresh sterile water and the rinse water was added into 250 ml bottles. The bottles were centrifuged for 10 min., at 12 400 x g and the supernatant was decanted. The sediment left over was the rhizosphere of the samples (modified from Seghers et al., 2004).

2.14.2. Endophytes of Inoculated and Non-Inoculated *A. rugosa*

The roots of inoculated and non-inoculated alders were first surface sterilized before the endophytes were extracted. Any adhering sediment was removed by rinsing soil in distilled water. The roots were placed in Erlenmeyer flasks and washed for one min with 95% ethanol, shaking by hand. The ethanol was replaced with 5% bleach solution and shaken again for one min. The bleach solution was replaced with fresh bleach solution and shaken for 10 min in a mechanical shaker. The roots were transferred to a sterile Erlenmeyer flask and rinsed once with 95% ethanol and then four times with sterile distilled water. At each step the roots were submersed in the solution. A 1 ml aliquot of the final, 4^{th}, wash was taken and stored at –20°C for a sterility check.

The roots were transferred into a sterile blender using two volumes of sodium pyrophosphate and mashed at maximum speed for 15 seconds. The root mash was transferred to a 50 ml falcon tube and centrifuged 12 400 x g for 15 min. at room temperature. The supernatant was decanted and the resulting mash was used for microbial and molecular analyses.

A sterility check was performed on the roots, to confirm the surface sterilization process. A 1 ml aliquot of the final, 4^{th} wash was taken and a boiling lysis was performed. The aliquot was boiled for 10 min to lyse cells, releasing DNA and a 16S rRNA gene PCR was performed to verify if there was any contamination. The final wash was also plated on 1/10 TSA (Difco) plates, then incubated at room temperature and inspected for colonies after one week (modified from Seghers et al., 2004).

2.15. Viable, Aerobic Microbial Enumerations of Bulk, Rhizosphere and Endophytic inoculated and non-inoculated *A.rugosa* in general and selective media

Five grams of CT or TS sample was diluted by adding 3x the volume of sodium pyrophosphate. The 96 well plates had 180 μl of media per well. The tubes were vortexed for 2 min and 20 μl of sample was added to each well in the first column of the plate. Three different media were used YTS, Bushnell Haas media with PAHs and MSM with naphthenic acids. The plates were incubated at room temperature and in the dark. The plates with YTS were incubated for 2 weeks and the plates with PAHs or naphthenic acids were incubated for three weeks.

2.15.1. PAHs in Bushnell Haas medium

Ten microliters of a hexane solution containing, 10 mg of phenanthrene, 1 mg of anthracene, 1 mg flourene and 1 mg dibenzothiophene per 1 ml of hexane, was added to the 96 well plates prior to the addition of Bushnell Haas medium (Difco). The hexane was evaporated and 180 μl of Bushnell Haas was added, per well to the plates. The medium was prepared by adding 3.27 g of the Bushnell Haas-powder into 1 L of distilled water, mixed and autoclaved.

2.15.2. MSM with Naphthenic acids

The naphthenic acids sodium salts comercial mix (Acros/ Fisher cat: NoAC415291000, Pittsburgh, PA, USA) was added to MSM at a concentration of 50 ppm using a Stock solution of naphthenic acids (2000 mg/L) were prepared, in 0.1 M NaOH, filter sterilized using 0.22 μm polyethersulfone vaccuum driven filter (Fisher, Whitby, Ont.) and kept at room temperature in the dark.

2.16. Microcosms of CT Bulk, Rhizosphere and Endophytic samples from Inoculated and Non-Inoculated *A.rugosa*

Microcosms were performed ^{14}C- substrates: hexadecane (12 mCi/ mM), phenanthrene (46.9 mCi/ mM) and naphthalene (2.3 mCi/ mM) dissolved in methanol. Twenty grams of bulk soil added to a 120 ml serum bottle and sealed with teflon /rubber septa. The samples were spiked with 100 000 dpm of ^{14}C -

hexadecane mixed with cold hexadecane (for a final concentration of 100 ppm) or with 100 000 dpm of ^{14}C -naphthalene or phenanthrene mixed with cold substrate (for a final concentration of 10 ppm). These bottles contained a tube which had 1 mL of 0.5 N KOH (CO_2 trap). The microcosms were sampled weekly by aspirating the KOH with a syringe and rinsed by adding 1 ml of 0.5 N KOH. The former and latter KOH were combined with 18 ml of scintillation cocktail and added into a scintillation vial. The radioactivity was quantified as described in section 2.7. One gram of the rhizosphere sample and endophytic residue were used for mineralization studies. The 1 g microcosms were prepared and sampled as previously described (section 2.7). All the microcosms were incubated at room temperature, statically and in the dark. Sterile controls were prepared for the bulk, rhizosphere and endophytic samples, for each substrate used.

2.17. Enrichment Cultures of Inoculated and Non-Inoculated Bulk CT Samples with *A. rugosa*

Enrichment cultures were started from cultures originally grown during MPN analysis, which were prepared from a bulk CT slurry. The medium used was MSM (Appendix in section 5.1.3.) and a commercial mix of naphthenic acids (same as mentioned earlier), at a concentration of 50 ppm as a sole carbon source. Cultures from the MPNs were taken and started out at the same concentration of naphthenic acid, 50 ppm, but in a larger volume. Since the MPN cultures contained a low biomass, instead of having a 10% inoculum, a 20% inoculum was added to start the cultures. The cultures were grown in glass Erlenmeyer flasks. After two weeks, a 10% inoculum was transferred to fresh MSM, containing 75 ppm of naphthenic acid. After a month of incubation, a 10% inoculum was transferred to MSM containing 100 ppm of naphthenic acid. The cultures grown at 100 ppm were used for HPLC analysis.

A single naphthenic acid compound, Trans-1, 4–pentylcyclohexane carboxylic acid (Aldrich Chem. Co., 26,160-2), was also used as carbon source. A 10% inoculum was transferred from a 75 ppm culture mentioned above, to MSM with 100 ppm Trans-1, 4–pentylcyclohexane carboxylic acid.

Stock solutions of NA and Trans-1, 4–pentylcyclohexane carboxylic acid were prepared in 0.1 M NaOH at a concentration of 2000 mg/L as described by Clemente et al. (2003), filter sterilized and stored in the dark at room temperature.

2.18. HPLC analysis of Naphthenic Acid concentration

HPLC analysis was performed weekly to detect degradation of the NA mixture and TCPA. Samples (500 µl –1 ml) were centrifuged at maximum speed for 10 min. to remove cells and other particulates that would interfere with HPLC anaylsis, and frozen at -20 °C for subsequent analysis.

2.18.1. HPLC derivatization step

Two hundred microliters of sample and standards (at 100, 75, 50, 25, and 10 ppm), and negative controls (autoclaved enrichment culture) were derivatized. Standards and samples were done in triplicate. The derivatization reaction was performed as indicated by Yen et al. (2004) using 80 µl of 2-nitrophenylhydrazine (2-NPH, MP Biomedicals, INC., Aurora, OH, lot # 1586a) and 80 µl of 1- Ethyl -3-(3-(Dimethylamino) Propyl) carbodiimide Monohydrochloride (1-EDC-HCl, Degussa Fine Organics, Sjöflyvägen, Sweden Cas no. 25952-53-8). These chemicals were added to the 200 µl of sample and incubated at 60°C for 20 min. Forty microliters of 140 mM KOH (prepared in 80% v/v HPLC grade methanol in Milli Q water) was subsequently added to the samples, incubated for 15 min at 60°C, cooled to room temperature and analyzed by HPLC. The 2-NPH solution was prepared by dissolving 60 mg of 2-NPH in 15 ml of 95% ethanol and 5 ml of 0.4 M HCl. 1-EDC-HCl solution was prepared by dissolving 480 mg of 1-EDC-HCl in 10ml of 95% ethanol and 10 ml of 3% pyridine in 95% ethanol.

2.18.2. HPLC Analysis System

The HPLC was a Waters system (Milford, Mass.) with a 717 plus autosampler, thermostated column compartment, 2996 UV photodiode array detector and a degasser. Empower Pro software was used for the analysis. The HPLC had a guard column and an analytical column. The guard column was

packed with 5 µm RP C18 Supelco Discovery column (Sigma- Aldrich, Oakville, Ont.) and the analytical column was a RP C18 Discovery Supelco column (5 µm particle size, 250 mm × 4.6 mm). The analytical column was kept at 40°C and the sample injection volume was 60 µl. The mobile phase was a programmed mix of HPLC grade methanol (Fisher Sci., Whitby, Ont.) and MilliQ water. The mobile phase was run on a gradient from 70:30 methanol:water at the time zero, to 100% methanol at 8 min, with a flow rate of 0.7 ml/ min. The total run time was 30 min. The detector was set at 400 nm (Yen et al. 2004).

2.18.3. Naphthenic acid extraction from CT residue

In order to detect the starting levels of naphthenic acids in CT, a naphthenic acid extraction was done. The extraction procedure is based on a naphthenic acid extraction on low solids release water from extraction tailings (Holowenko et al., 2001). One hundred grams of CT was used and distributed in falcon tubes. Milli Q water was added in sufficient quantities such that CT could shake well and they were shaken overnight. In the morning, they were left standing for 30 min. to allow the sand to settle and the water was decanted and pooled together in a beaker. The pH of the water was taken and lowered to pH 2 - 3 using H_2SO_4. The sample was left for 5 days to allow the naphthenic acids to precipitate. The samples were processed as indicated in the reference (Holowenko et al., 2001). They were centrifuged for 15 min. at 25 000 x g, using 250 ml, polycarbonate bottles and the supernatant was decanted and discarded. The pellets were then washed with 0.1 M NaOH to dissolve the naphthenic acids and shaken for 15 min., centrifuged at 25 000 x g for 15 min. and the supernatant was collected. The centrifugation was repeated to extract the maximum amount of naphthenic acids. The extract was subsequently analyzed for naphthenic acids concentrations by HPLC analysis, as indicated above.

3. Results

3.1. pH and Conductivity of TS and CT

The pH and the conductivity of CT were taken at time 0. The CT and TS samples had a pH of 8.20 and 7.82, respectively. CT had a conductivity of 38.1 µs/cm (corresponding to 183 mg/L or 3.1 mM NaCl) and TS had a conductivity of 17.56 µs/cm (corresponding to 68 mg/L or 1.2 mM NaCl), which is lower than conductivity measurements found in the literature. Renault et al. (2003) indicate that there is 1000 mg/L of Na^+ and 586 mg/L of Cl^- in CT , the pH is 8.1 and the conductivity is 4500 µs/cm (corresponding to 2467.9 mg/L or 47.77 mM NaCl)

3.2. The Growth of *A. glutinosa* and *A. rugosa* in CT and TS

Two species of alders were used for this project, *A. glutinosa* (Ag) and *A. rugosa* (Ar). Both plants grew better in TS than they did in CT (Fig. 2). A. *glutinosa* was 200% taller in CT and 45% taller in TS and 106% taller in CS, than *A. rugosa.* The root system of *A. glutinosa* had a greater biomass than *A. rugosa. A. glutinosa* also had more leaves and was generally healthier than *A. rugosa* in both TS and CT.

A. glutinosa in CS was 240% taller than in TS and 330% taller than in CT. *A. rugosa* in CS was 330% taller than in TS and 975% taller than in CT. Both alders in CS had large root systems that occupied and penetrated more soil than both alders in CT and TS. The alders in CS had greener and more leaves and branches than the alders in TS and CT.

3.3. Aerobic, Heterotrophic and Halophilic Microbial Enumeration in CT and TS With and Without Alders

The total viable microbial population varied significantly between the planted and unplanted TS and CT. In unsalted media, planted TS and CT had a population that was approximately 0.5 and 1.5 orders of magnitude greater than non-planted samples, respectively (Fig. 3). At 0.5 M NaCl (29.44 g/L) planted CT had a population that was 3 orders of magnitude higher than the non-planted CT. At 1 M

NaCl (58.44 g/L), there was no growth in non- planted CT. At 0.5 M and 1 M NaCl, planted TS had a population that was 0.5 – 1 order of magnitude greater than non-planted TS. However there was no growth in non-planted TS at 1.5 M NaCl (87.66 g/L) (Fig. 3). There was growth at 1.5 M NaCl for TSAR (not shown in graph) but the number of colonies at the dilution available was too high to count. The viable microbial population in CT and TS with alders, in unsalted and salted media was quite comparable. TSAG had the lowest CFU/g relative to the other samples with alders (Fig 3).

3.4. Mineralization

The indigenous microbial populations in the bulk CT and TS samples were tested for their ability to mineralize phenanthrene, hexadecane and naphthalene using ^{14}C-labeled substrates. Phenanthrene was degraded in all samples, and had the highest rates of mineralization of the three compounds tested (Fig. 4). TS, TSNP and CTNP reached a plateau at 10% phenanthrene mineralization. Phenanthrene mineralization by CTAR, TSAR, CTAG, and TSAG ranged from 30% - 90%. CT mineralized 60% of the phenanthrene, which is significantly greater than the other non-planted samples and the rate of mineralization came close to the rate of TSAR. The highest rates of mineralization activity were found in sample CTAR, which mineralized 90% of the phenanthrene (Fig. 4).

Hexadecane was not as readily mineralized in samples without alders. CTAG and TSAG had higher rates of mineralization relative to the non-planted samples. The highest rates of mineralization were observed in samples planted with *A. rugosa*. CTAR and TSAR mineralized 15% - 20% of the hexadecane in comparison to CTAG and TSAG, which mineralized 5% of the hexadecane (Fig. 4).

Naphthalene was not readily mineralized, and there was less than 10% mineralization, in all samples (Fig. 4).

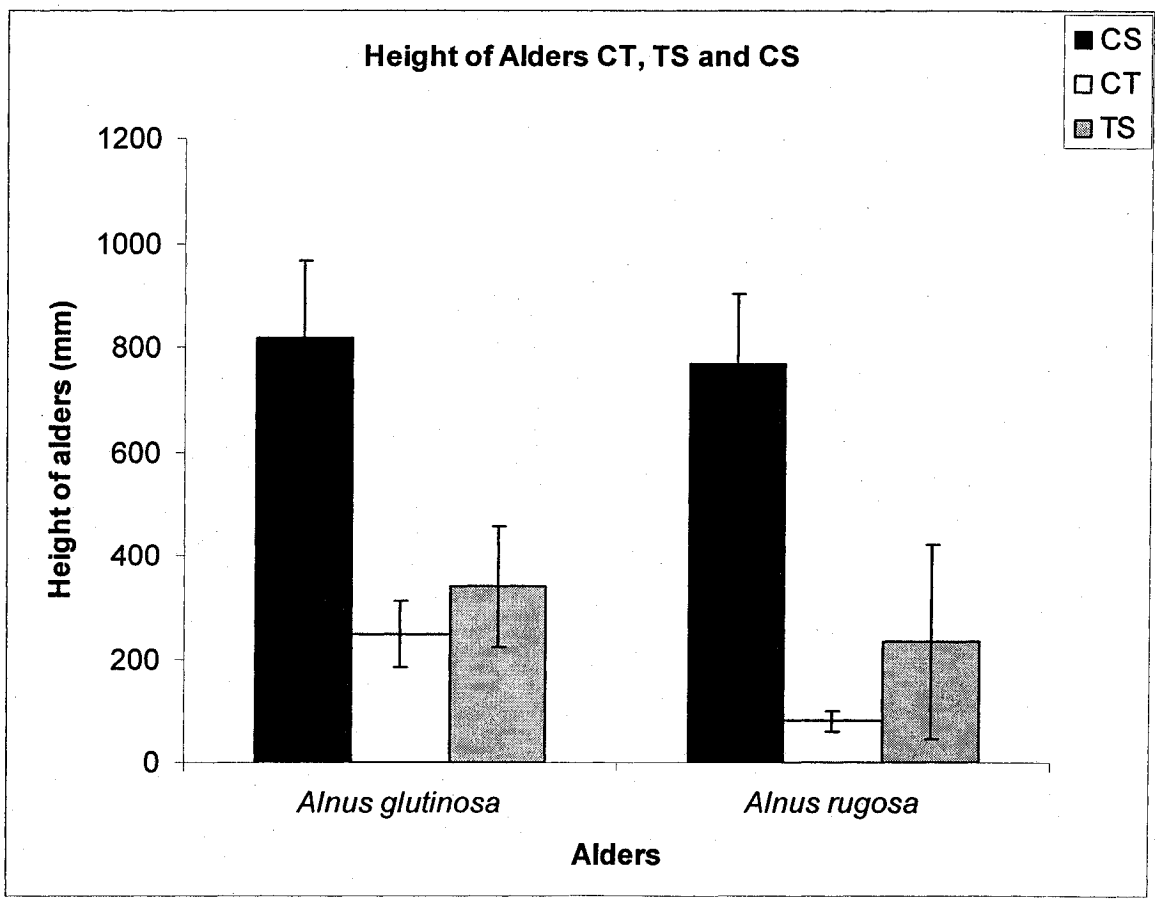


Figure 2. Height of two species of alders, *A. rugosa* and *A. glutinosa*, after six months growth in composite tailings (CT) tailings sands (TS) and control treatments (CS). The error bars represent the standard deviation of the mean of triplicate plants.

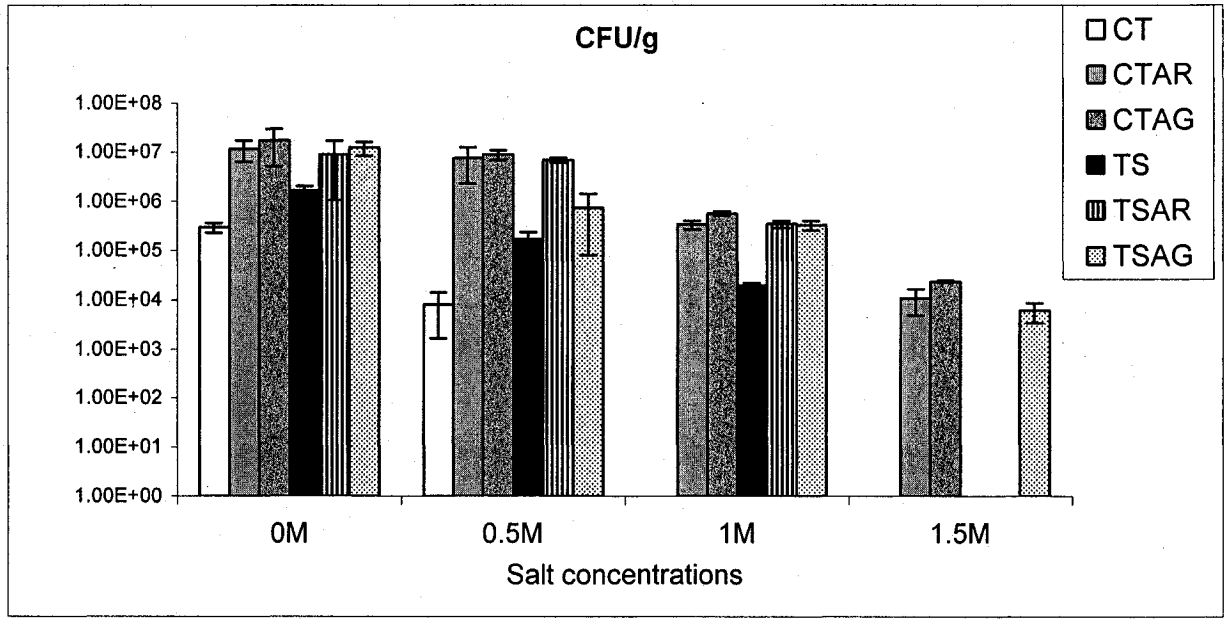


Figure 3. Total viable bacterial population in composite tailings (CT), or tailings sands (TS), and planted CT and TS with *A. rugosa* (CTAR, TSAR) or *A. glutinosa* (CTAG, TSAG). The bacteria were grown on YTS-MSM or NaCl supplemented YTS-MSM. Error bars represent one standard deviation of the triplicate assays

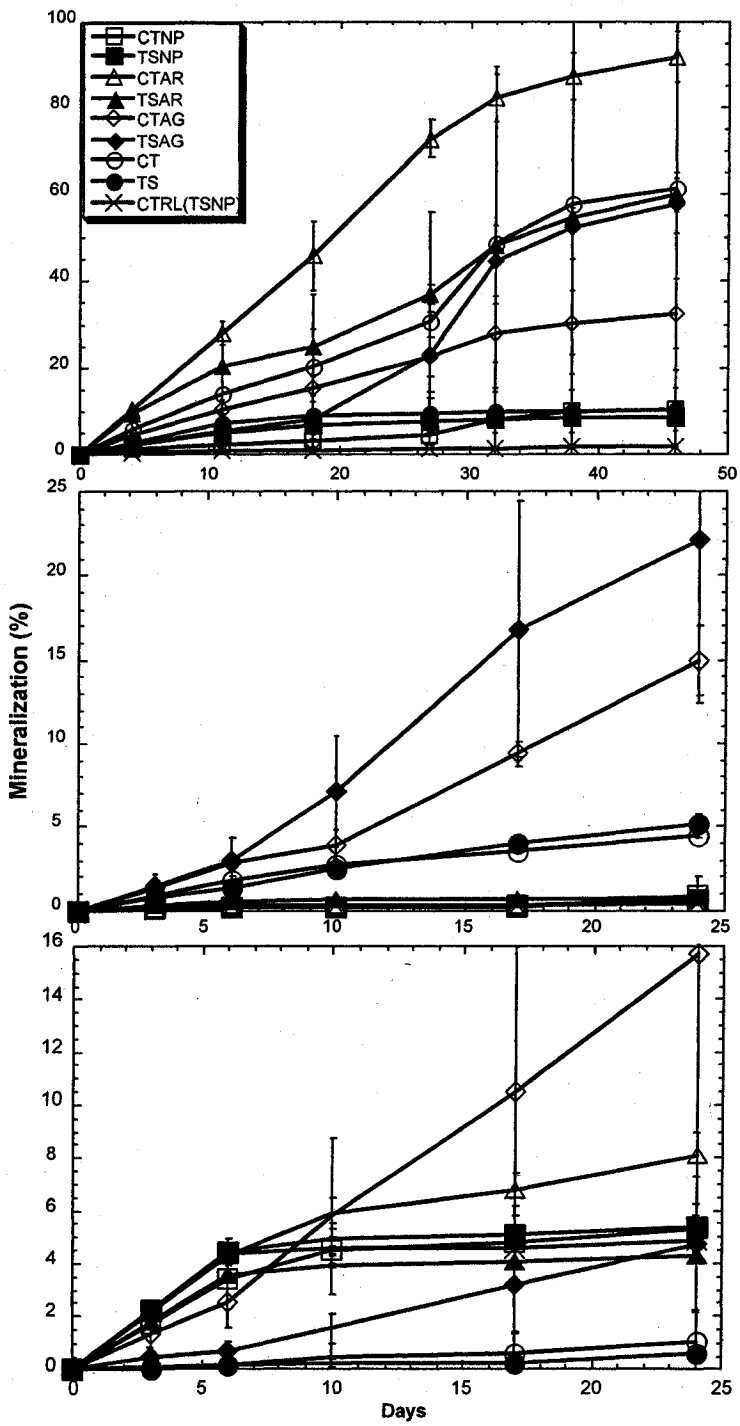


Figure 4. ^{14}C - Phenanthrene (top panel), hexadecane (middle panel) and naphthalene (bottom panel) mineralization activity in TS and CT microcosms; the tailings without plants, TSNP and CTNP; and tailings with plants; *A. rugosa* (CTAR, TSAR) and *A. glutinosa* (TSAG, CTAG). TSNP was also used as a negative control. The error bars indicate the standard deviation of triplicates.

3.5. 16S rRNA gene DGGE

Following DNA extraction, purification and subsequent PCR amplification, the 16S RNA gene fragments were subject to DGGE analysis. The banding pattern on the DGGE gel (Fig. 5) shows the difference in the bacterial diversity between all the samples. When comparing TS with TSAR and TSAG, there is an increase in the prevalence of certain bands, indicative of an enrichment mediated by alders. In samples CTAR and CTAG there is an increase in the number of bands, which suggests an increase in diversity. When comparing CT and TS lanes, a difference in diversity can also be seen. There are fewer and more dominant bands in CT lane than there are in the TS lane.

CTPB is CT that was extracted using phosphate buffer (PB) instead of water. It was put on the gel to see whether phosphate buffer had an impact on the recovery of DNA. When comparing the CT and CTPB lanes on the DGGE gel, the banding patterns were essentially the same. The bands indicated in the figure were cut out, re-amplified and sequenced. The sequences were compared to all entries in GenBank using the BLAST (Altschul et al., 1997) and Fasta (Pearson, 2000) search programs (Table 5).

3.6. *cpn60* DGGE

The purpose of this experiment was to attempt a *cpn60* DGGE and to complement the 16S rRNA gene DGGE analysis. Three different denaturant gradients were tried, 40% - 80% (not shown), 45% - 65% and 45% - 55%. The tightest denaturant gradient 45% - 55%, had a relatively better band-separation (Fig.6). Bands were cut from the gels using the latter 2 gradients, purified, reamplified and sequenced (data not shown). The sequencing results obtained with the H279 primer were significantly better than those obtained with the H280 primer. The sequences generated with the H279 primer were therefore used for searching the *cpn60* database, cpnDb (Hill et al., 2004). In most cases, the matches from the *cpn60* database were between 60% - 70%, except for Band P in lane CTAG (Fig. 6) showed 84% similarity to *Acinetobacter johnsonii*.

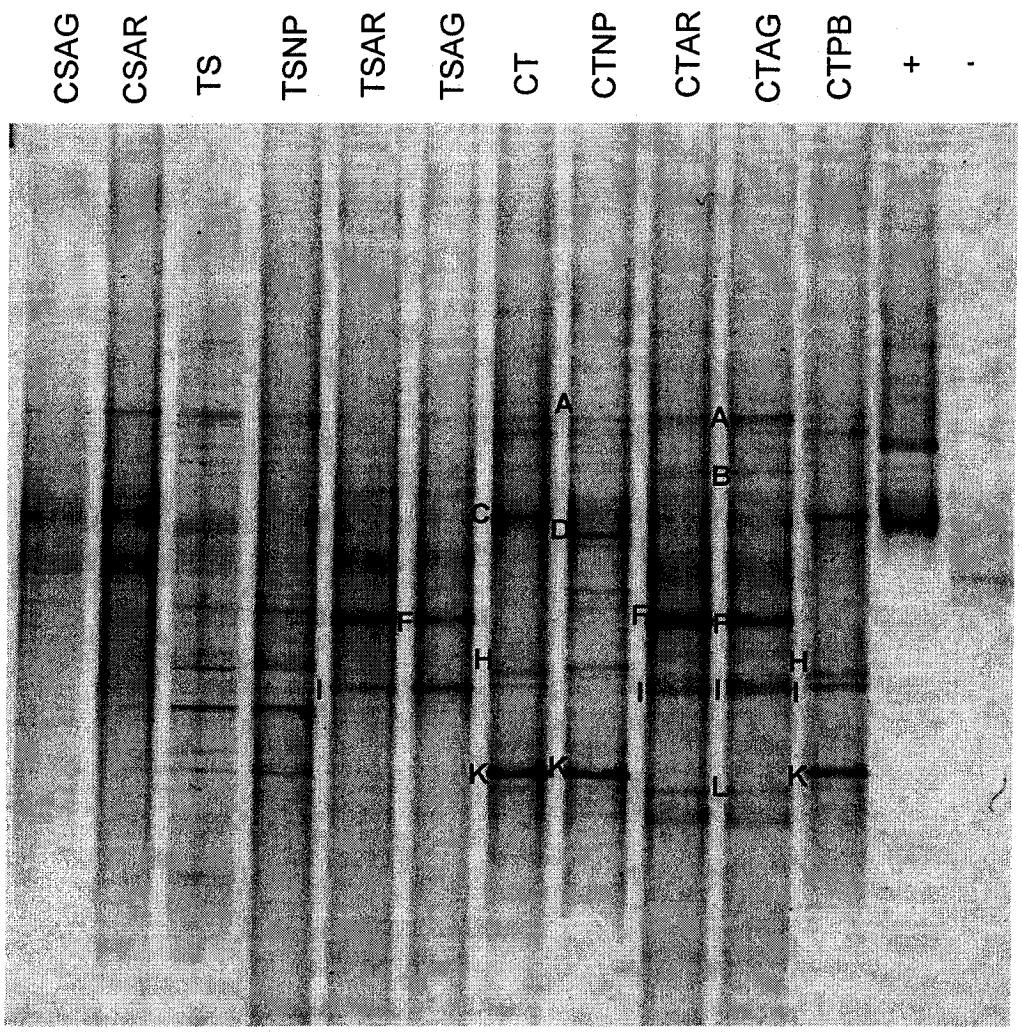


Figure 5. Denaturing Gradient Gel Electrophoresis (DGGE) of the 16S rRNA gene amplified from the total DNA extracted from the tailings (TS); composite tailings (CT); the tailings without plants, CTNP and TSNP; and tailings with *A. rugosa* (CTAR, TSAR) or *A. glutinosa* (CTAG, TSAG). CTPB is the CT sample used in order to make a comparison between the conventional method for DNA extraction and a second method involving phosphate buffer (PB). Pos is the positive DGGE control, *Pseudomonas putida* (ATCC 17484). The gradient of denaturant used was 45%-65%. The letters in the lanes identify bands that were extracted for subsequent nucleotide sequencing.

Table 5. Sequence analysis of the selected DGGE fragments. An astericks indicates only one strand was sequenced.

DGGE Band	Nearest Relative (Blast)	% Match (Blast)	Nearest relative (Fasta)	% Match (Fasta)
CTAG-A	Uncultured bacterium	95	Bacterium Ellin5247	92
*CTNP-A	Uncultured Sphingomonadaceae bacterium	92	Sphingomonadaceae bacterium	89
*CTAG-B	Unidentified bacterium	94	*Hyphomicrobium zavarzinii*	93
CT-C	Uncultured bacterium	94	Beta proteobacterium	94
*CTNP-D	Uncultured bacterium	89	Gamma proteobacterium	76
CTAG-F	Uncultured alpha proteobacterium	99	*Pedimicrobium americanum*	96
CTAR-F	Uncultured alpha proteobacterium	99	*P. americanum*	96
TSAG-F	Uncultured alpha proteobacterium	99	*P. americanum*	95
*CTPB-H	*Arthrobacter sp.*	89	*Arthrobacter sp.*	84
CT-H	*Arthrobacter sp.*	88	*Arthrobacter sp.*	97
TSAR-I	Uncultured bacterium	95	*Mesorhizobium loti*	94
*CTAR-I	Uncultured alpha proteobacterium	96	*Sinorhizobium meliloti*	81
CTPB-I	Uncultured gamma proteobacterium	98	*Marinobacter sp.*	91
CTAG-I	Unidentified bacterium	94	*Mesorhizobium loti*	91
CTPB-K	Uncultured *Actinobacterium*	98	Bacterium Ellin5247	92
CTNP-K	Uncultured *Actinobacterium*	98	Bacterium Ellin5247	92
*CT-K	Uncultured *Actinobacterium*	97	Bacterium Ellin5247	90
CTAG-L	Uncultured gamma proteobacterium	95	*Halochromatium sp*	87

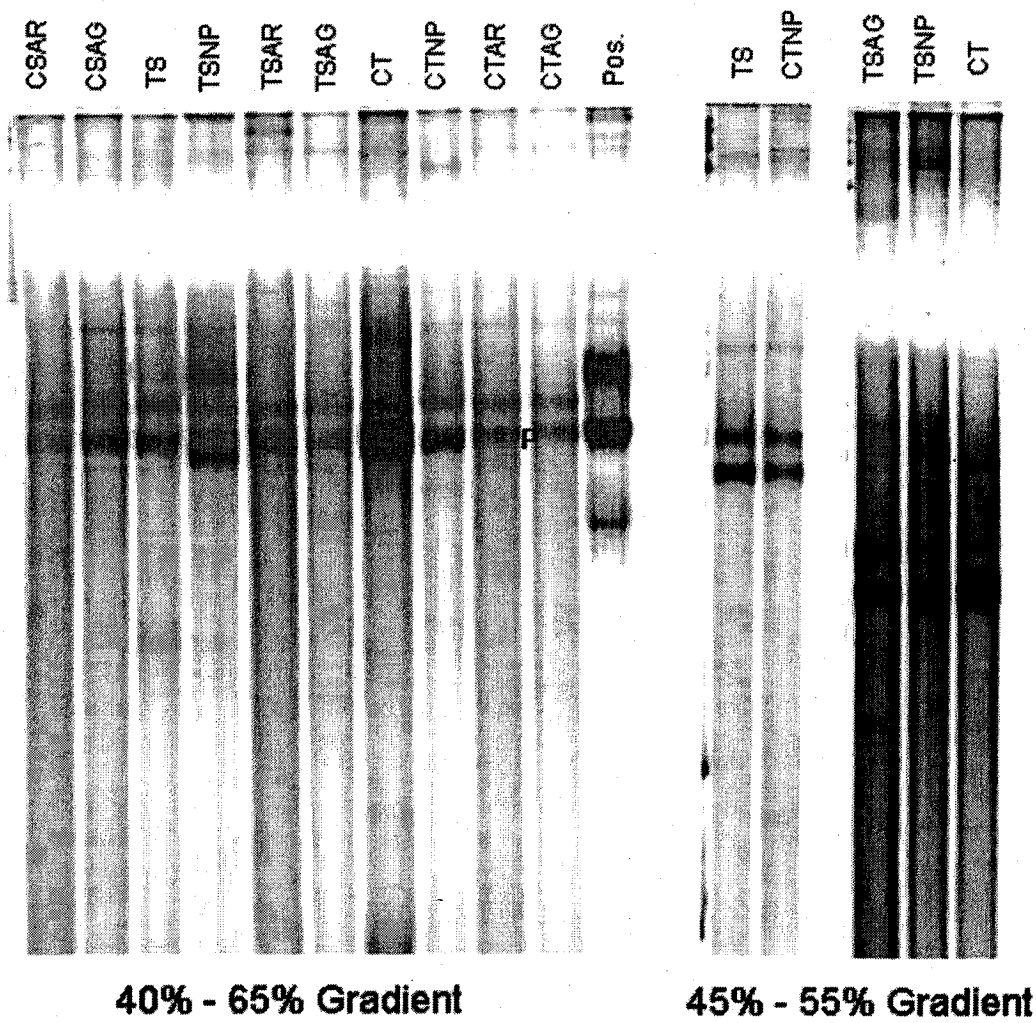


Figure 6. Denaturing Gradient Gel Electrophoresis (DGGE), using the PCR amplified *cpn60* gene fragment from the total DNA extracted from tailings (TS); composite tailings (CT); the tailings without plants, CTNP and TSNP; and tailings with a plant with *A. rugosa* (CTAR, TSAR) or *A. glutinosa* (CTAG, TSAG). Pos. is the DGGE positive control, *Pseudomonas putida* (ATCC 17484).

3.7. Catabolic PCR using the *alkB* and *ndoB* gene primers

The *alkB* gene, involved in alkane degradation, was amplified from the total DNA extracted using the *alkB* consensus primers (Chenier et al., 2003) and detected in all samples except for TSNP and CTNP (Fig. 7). The *ndoB* gene was not detected in CT andTS (data not shown).

3.8. Inoculated vs. Non – Inoculated *A. rugosa* in CT

Our objective was to study whether *Frankia* (strain F9) could increase alder survival and positively influence the indigenous CT microflora. For these experiments one alder, *A.rugosa,* and one substrate, CT, was chosen. *A. rugosa* was chosen because it is native to Alberta, and is also known as the river alder, which would tolerate water logged conditions, as are present in CT. The control samples were grown in uncontaminated potting media. There were four non-inoculated samples: A, B, C and D and four inoculated samples: E, F, G and H. Samples D and H were controls.

3.9. Growth of Inoculated and Non – Inoculated *A. rugosa* in CT

After 9 months the inoculated alders were taller than the non-inoculated plants. The average height of the non-inoculated samples was 54 cm (Standard deviation of triplicates: 22 cm). The average height of the inoculated samples was 84 cm (Standard deviation of the triplicates: 26 cm).

The pH of bulk CT was measured in triplicate at the end of the experiment, and an average was taken. The non-inoculated CT samples had an average pH of 8.11 (Standard deviation of the triplicates: 0.09). The inoculated CT samples had an average pH of 7.89 (Standard deviation of the triplicates: 0.18).

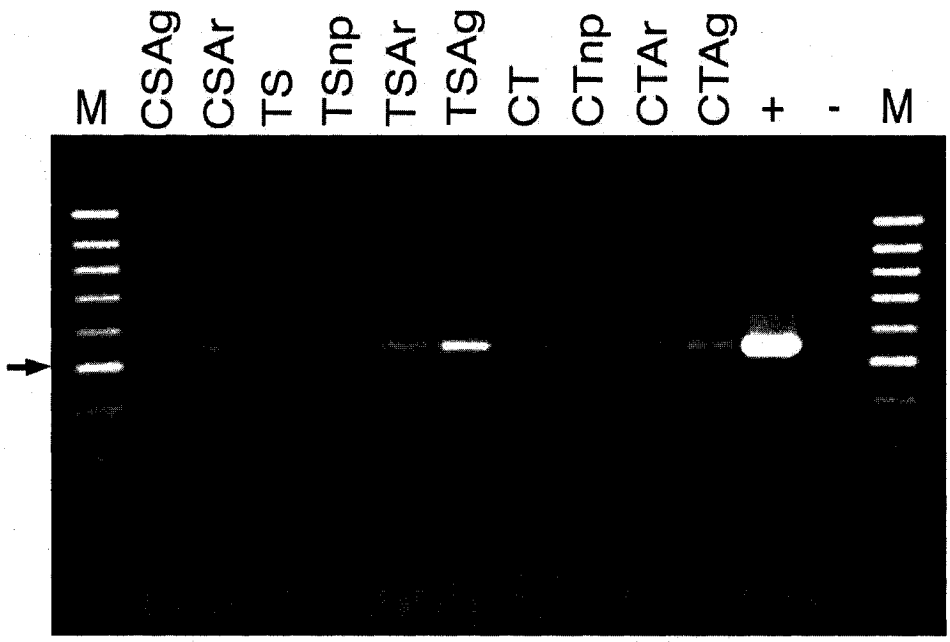


Figure 7. The *alkB* gene was amplified from all the samples using the *alkB* consensus primers, alkH1F and alkH3R. The 100 base pair ladder, designated as M, was added along both sides of the gel. The arrow shows the 500 base pair band of the ladder. The amplified fragment has a size of 549 base pairs.

3.10. Mineralization of ^{14}C – Phenanthrene, Hexadecane, and Naphthalene

Phenanthrene mineralization by the microbial community in the bulk CT and TS ranged between 50% and 60% (Fig. 8). The mineralization rates of inoculated samples. Phenanthrene was not mineralized by the endophytic (inside the root) microbial community.

Hexadecane was not mineralized very well in most cases (Fig.9). In bulk CT-samples there was 10% mineralization. There was 50% mineralization observed in the control inoculated and non inoculated samples, D and H. There was very little hexadecane mineralization in the rhizosphere and in the endophytic samples, in most cases, less than 10% mineralization.

Naphthalene mineralization in the bulk and rhizosphere CT ranged between 42% and 60%. The non-inoculated and inoculated samples had rates that were close together and in some cases identical (Fig. 10).

The endophytic inoculated samples mineralized naphthalene to a greater extent than non-inoculated samples. The rates of mineralization ranged between 10% and 46%.

and non-inoculated samples were very similar to one another. In the rhizosphere, phenanthrene mineralization ranged between 58% and 74%. There was a lag phase at the beginning of the trial in all samples which was not seen in bulk CT

3.11. Viable, Aerobic Microbial Enumerations in Bulk, Rhizosphere and Endophytic Inoculated and Non-inoculated *A. rugosa* in CT

The purpose of performing MPNs was to determine if the inoculation of *Frankia* had an affect on the total viable population in CT bulk, rhizosphere and endophytic samples. In all three media tested there was not a significant difference in the bulk, rhizosphere and endophytic microbial communities arising from the inoculation with *Frankia* (Fig. 11).

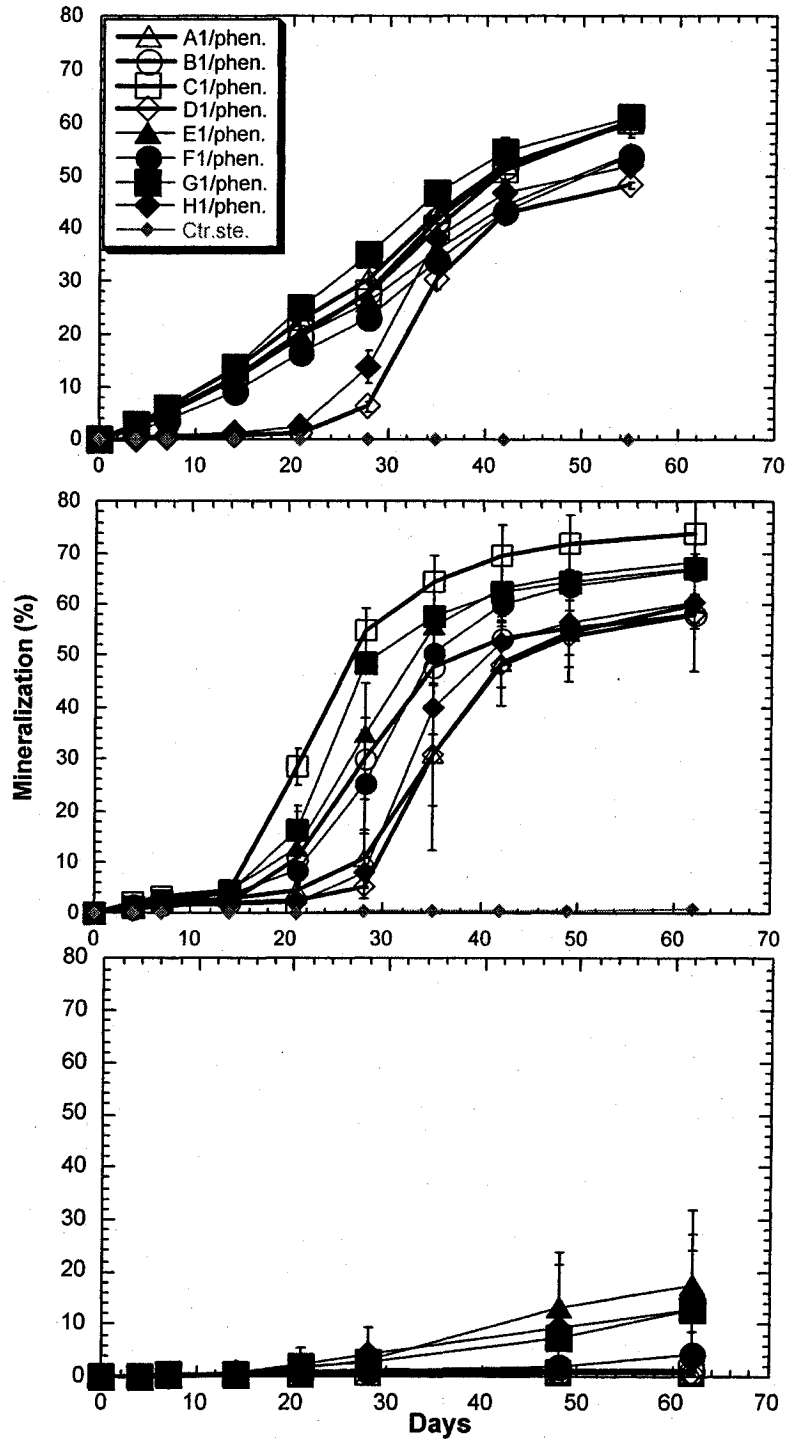


Figure 8. ^{14}C - Phenanthrene mineralization by the microbial community in bulk (top), rhizosphere (middle) and endophytic (bottom) samples of inoculated (A, B, C, and D) and non-inoculated (E, F, G, and H) *A. rugosa*. The error bars indicate the standard deviation of the mean.

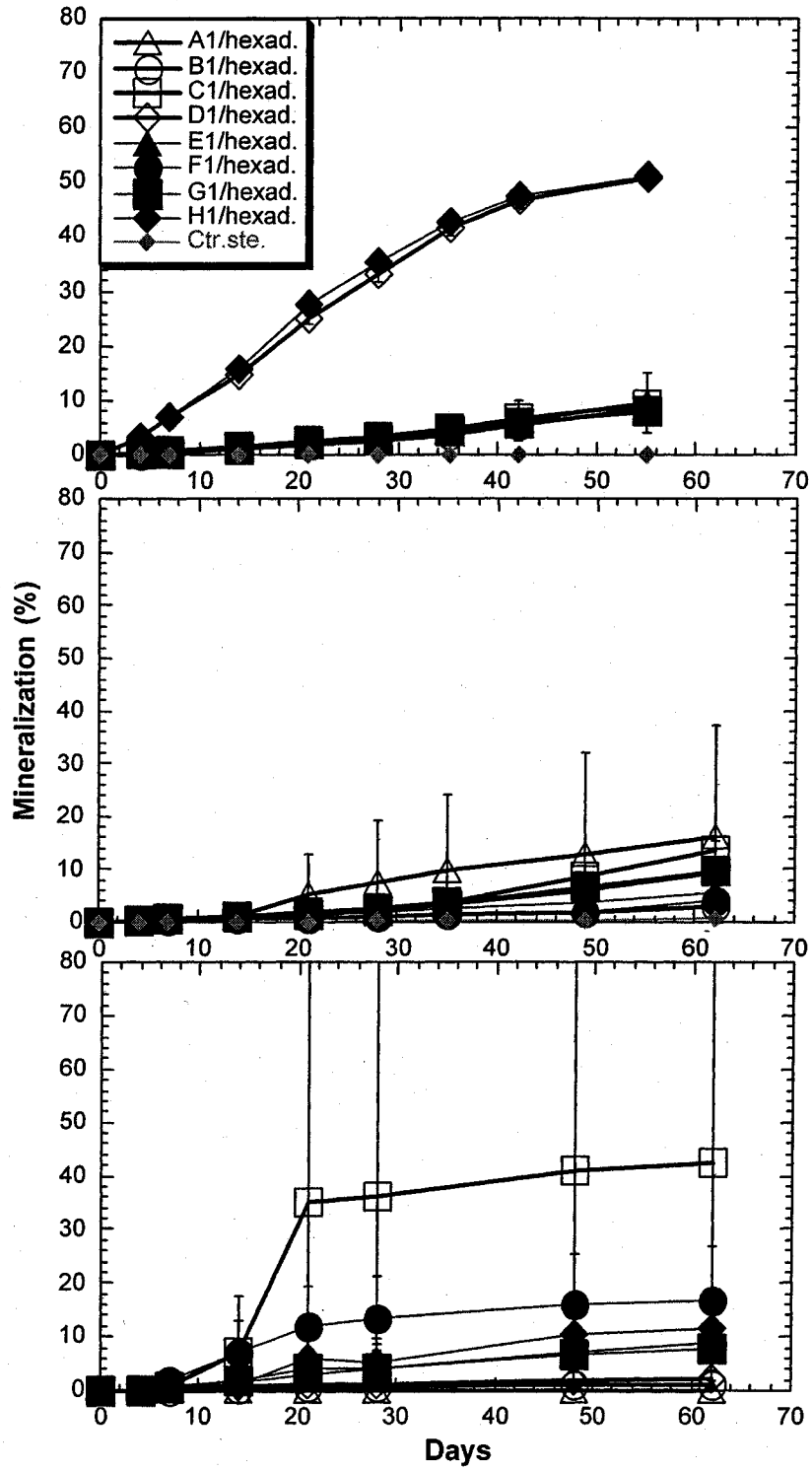


Figure 9. ^{14}C- Hexadecane mineralization by the microbial community in bulk (top), rhizosphere (middle) and endophytic (bottom) samples of inoculated (A, B, C, and D) and non-inoculated (E, F, G, and H) *A. rugosa*. The error bars indicate the standard deviation of the means.

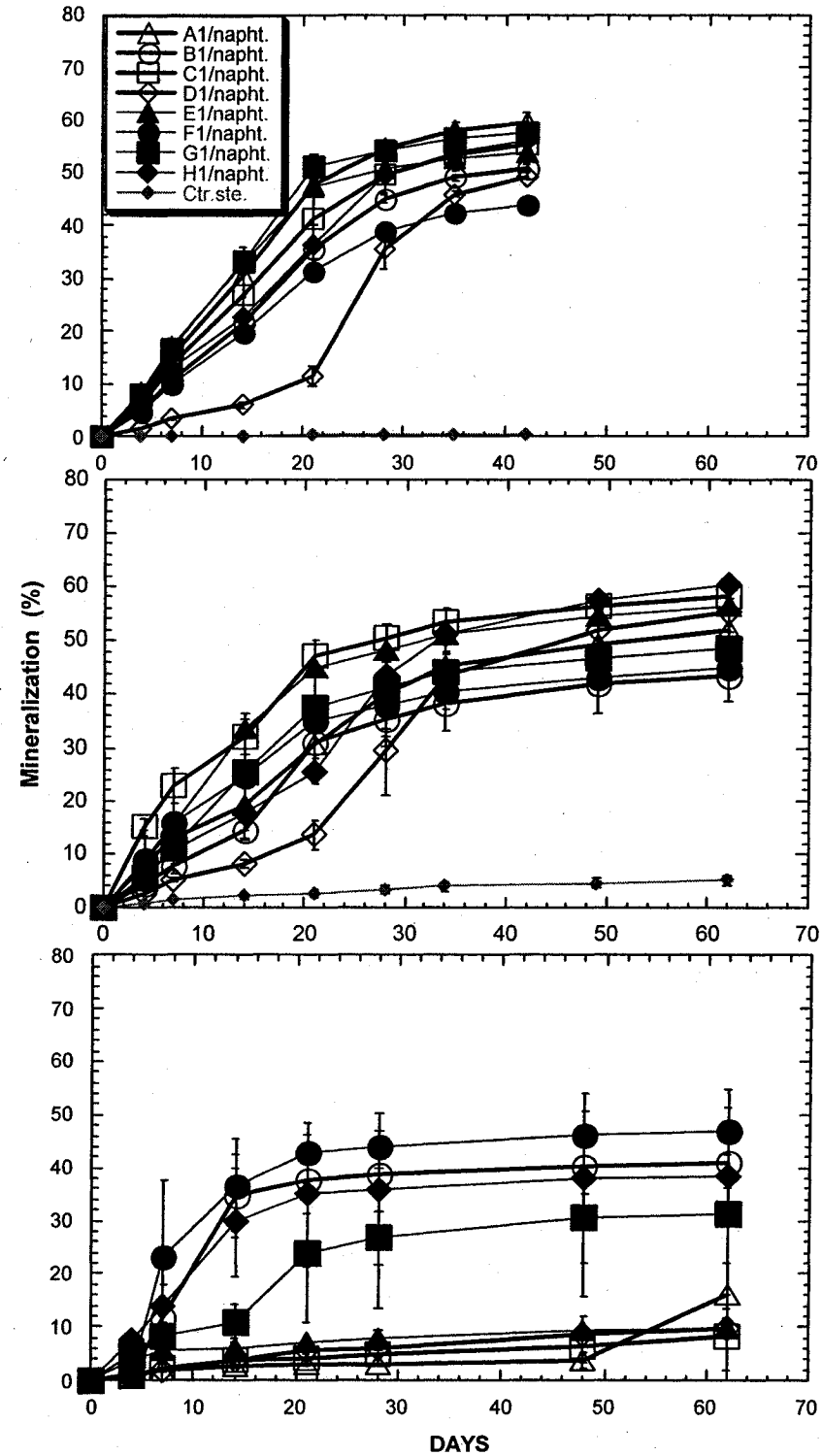


Figure 10. ^{14}C Naphthalene mineralization in bulk (top), rhizosphere (middle) and endophytic (bottom) samples of inoculated (A, B, C, and D) and non-inoculated (E, F, G, and H) *A. rugosa*. The error bars indicate the standard deviation of the means.

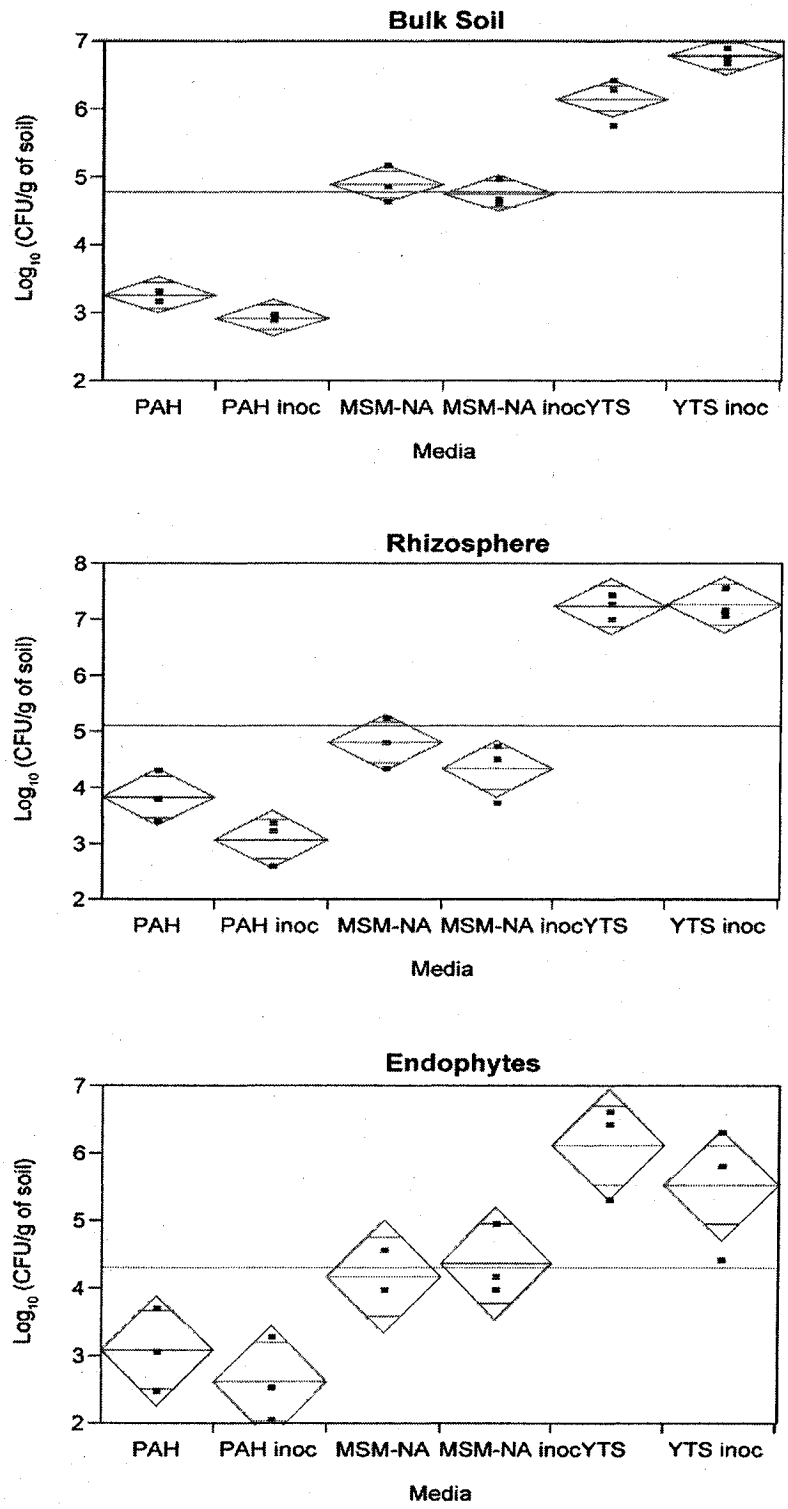


Figure 11. ANOVA statistical test for significant differences resulting from inoculation with *Frankia* strain F9, in bulk, rhizosphere and endophytic microbial communities in three different substrates.

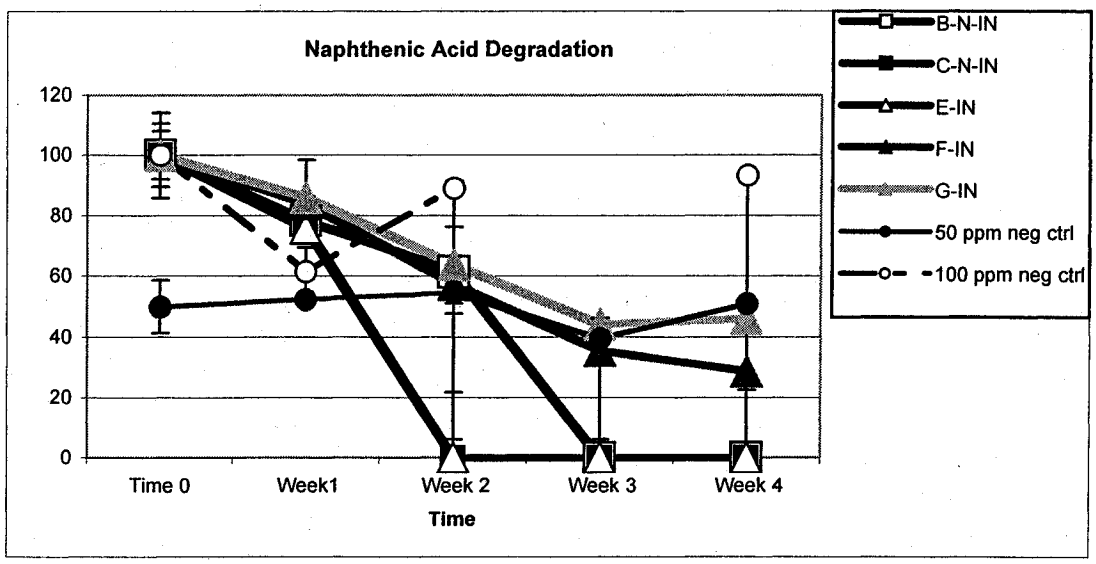
3.12. HPLC with enrichment cultures

3.12.1. HPLC analysis

Standard solution of NA and trans -1,4 -pentylcyclohexane need to be prepared fresh. The Standard curve was linear over the range from 10 to 100 ppm. The naphthenic acid concentrations were monitored by HPLC until the compounds were below the levels of detection (< 10 ppm). The cultures of inoculated and non-inoculated CT degraded the single naphthenic acid, trans -1,4 -pentylcyclohexane carboxylic acid equally well. However, when degrading the commercial mix of naphthenic acids, the non-inoculated samples had better degrading activity than the inoculated samples. Naphthenic acid degradation approached a steady state when the residual concentration was 50 ppm in sample G and 30 ppm in sample F (Fig. 12).

3.12.2. Naphthenic Acid Extraction

The purpose of this experiment was to determine the initial (at time 0) and final concentrations of naphthenic acids in CT and TS with alders. The naphthenic acid extraction was attempted on CT several times. However despite the many efforts to optimize the naphthenic acid extraction technique the results by HPLC analysis were negative. The naphthenic acid concentrations in our samples or in the extracts were perhaps below the levels of HPLC – detection (10 ppm). The extraction procedure was attempted on CT, in which 100 ppm of naphthenic acids were added. Prior to the extraction, HPLC analysis confirmed the presence of naphthenic acids but the naphthenic acids were not detected in the extracts.



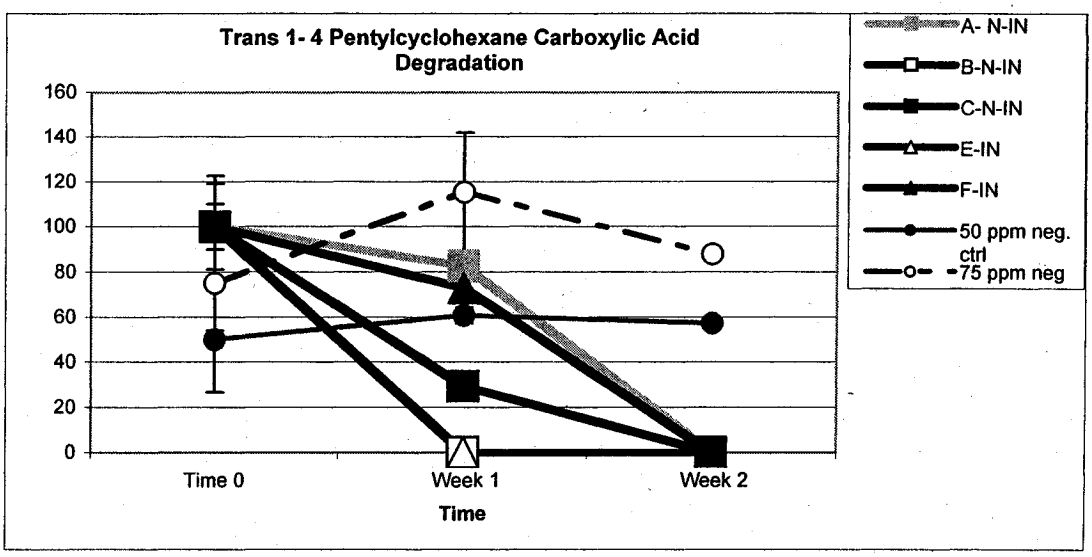


Figure 12. Naphthenic acid degradation from inoculated (IN) and non-inoculated (N-IN) bulk CT samples. The compounds used were, trans–1, 4 - pentylcyclohexane carboxylic acid, and a commercial naphthenic acid mixture. For each compound there were two negative controls (neg ctrl.).

4. Discussion

Alders are pioneer species and their planting has been successfully used to initiate the revegetation of denuded sites, accelerate reforestation and stabilize soil against wind and water erosion. Alders grow rapidly and are good nurse crops which provide nitrogen and shade. Two alder species, *A. glutinosa* and *A. rugosa* inoculated with the symbiotic nitrogen-fixing actinomycete, *Frankia,* were studied for their potential in revegetation and remediation of oil sands tailings. Their influence was evaluated through the examination of the microbial diversity and activity in the tailings. Significant changes in the microbial community can occur following reclamation efforts and could be used as an indicator of restoration progress. To harness the microbial metabolism for tailings detoxification, it is vital to know the types of microorganisms present and their response to external factors such as, pH, salinity, contaminants and the presence of plants.

4.1. Alders

In this project there were two trials, the first one in 2003 and the second one in 2004. The trials of 2003 were set up to find out if the alders could survive in the tailings, which alder was a better candidate for revegetation, and based on the microbial and molecular analysis, which alder was best suited for remediation. *A. glutinosa* generally grew better than *A. rugosa* in CT, TS and in control treatments. *A. rugosa* frequently grows along the boarders of streams, lakes and is also common in swamp and bog areas (USDA Forest Service, 2006). *A. rugosa* may have been smaller than *A. glutinosa* due to the relatively dryer conditions present in TS and CT. Neither of the alders grew in the tailings as well as they did in the control treatment, but they did survive for six months in an environmental chamber. There were problems with the growth chamber in which the plants were started, which may have affected their health.

In 2004, *A. rugosa* was planted again in CT. The purpose of the second trial was to determine the effect of a *Frankia* inoculum on the growth of alders and on the microbial community in CT. These alders grew more extensively in comparison to *A. rugosa* and *A. glutinosa* in 2003 trial.

A major difference between the trials was the way the alders were grown. *A.rugosa,* in 2004 was started at the Bonnyville nursery in Alberta, where the conditions were significantly better than in the growth chamber and the alders were started in a peat-vermiculite mixture.

Alders have a low salt tolerance since they are not capable of tolerating a salt solution having an electrical conductivity more than 2 dS/m (dS/m = decisiemens per metre, 1080 mg/L of NaCl) (Amacher et al., 1999). Despite their low tolerance to salt, the alders were able to survive in TS and CT, as demonstrated in the trial of 2003. In the second trial the alders were tall, had lots of leaves and a large root system. Most plants are affected by salinity as seedlings and are particularly vulnerable to salinity damage during their emergence and early development. After the plants are established, they generally become increasingly tolerant of salinity in later growth stages (Warrence et al., 2005). In 2004, *A. rugosa* plants were transferred into CT after they had become established in peat and they were very healthy. In 2003, the alders were transferred into the tailings when they were younger and were not as healthy. Perhaps the alders of 2003, at that stage of growth and in their condition were more sensitive to the CT environment.

One plant that is being investigated for revegetation of the composite tailings is Red - osier dogwood, which also has a low salt tolerance (Warrence et al., 2005). Studies by Renault et al. (2001) suggest that Red - osier dogwood restricts the transport of sodium from the roots to the shoots. Perhaps alders also have developed similar mechanisms of tolerance, but it remains to be investigated. When the Red - osier dogwood was planted in CT, there was a 60% survival rate (Redfield et al., 2003). The first time the alders were planted they did not grow well but all survived. Further experimentation conducted by Redfield et al. (2003) showed that the dogwood plants grew to a height of 100 mm in CT. The alders that were planted in 2003 grew to approximately the same size and the alders from the trial of 2004 were much taller.

According to the literature, CT sands have higher concentrations of sodium than TS, which reduces the permeability to water due to the swelling of clays (Renault et al. 2003). The reduction in permeability was noted when watering the

alders. According to Renault et al. (2000), soils that are compact may result in roots that are shorter and spread wider and therefore are less likely to penetrate the soil. According to Redfield et al. (2003) the roots of Red - osier dogwood would most likely not penetrate from the peat layer into the CT layer. However, although the roots of the alders were thin, they did penetrate into the CT layer and were exposed to CT sand. However the compact nature of CT may have been the reason the nodules only formed close to the surface of the root just below the ground level.

4.1.1. Inoculation of *A. rugosa* with *Frankia* F9

It is clear from the results that the inoculated alders grew better in CT and control treatments than the non-inoculated alders. Similar results have been observed in the growth of Casuarinacaea (Bulloch, 1994). Plants of the genus *Casuarina* also form a symbiotic relationship with *Frankia*, and colonize low fertility soils. Studies have demonstrated that their nodulation also resulted in increased growth for *Casuarina* plants. *Casuarina* plants don't form nodules without artificial inoculation, but in this study the uninoculated alders also had nodules. It has been shown previously that alders acquire nodules naturally (Bulloch, 1994).

The alders were inoculated with the *Frankia* strain F9. DGGE analysis on the bulk, endophytes and rhizosphere samples indicated that strain F9 was only found in the endophytic inoculated samples. This suggested that the nodules on the non-inoculated alders are formed by another organism or another strain of *Frankia* and that *Frankia* strains are a part of the tailings indigenous microbial community. Endophytic communities have been implicated in the promotion of plant growth and protection against pathogens (Seghers et al., 2004). Studies Nickel et al. (2001) and Oliveira et al. (2005) have also demonstrated that *A. glutinosa* inoculated with *Frankia* had increased plant growth. Nickel et al. (2001) hypothesize that this results from an increase in nitrogen availability. The results strongly indicate that it is due to the endophytic – *Frankia* community that the inoculated alders had larger root systems and were taller than the non-inoculated alders.

The pH of CT at the beginning of the trial was 8.2. At the end of the trial, the pH of bulk CT in the non-inoculated and inoculated samples had decreased slightly

to pH 8.11 and 7.89, respectively. Alders are known to decrease soil pH. Nitrogen fixation increases the concentration of ammonium, which influences soil properties such as pH. The fixed N_2 is released to the soil by decomposition of tree litter and sloughed-off roots (Wang et al., 2005). Oliveira et al. (2005) also observed a decrease in the pH of soil in *Frankia* – inoculated samples, which they hypothesize resulted from an increase in the production of root exudates.

4.2. Mineralization of PAHs by the indigenous microbial community

Three hydrocarbons were investigated in this study: phenanthrene, naphthalene and hexadecane. The microflora of bulk CT and TS samples from 2003 were able to mineralize phenanthrene and had low levels of hexadecane and naphthalene mineralization. The alders increased the rates of mineralization in the case of phenanthrene and hexadecane. It has previously been seen by Siciliano et al. (2003) that the rates of mineralization of phenanthrene and hexadecane doubled in planted bulk soil when compared to non-planted soil, demonstrating the beneficial and stimulatory effects of plants on PAH – degrading microbes. The influence of alders is shown particularly in the case of hexadecane. Despite less overall growth, *A. rugosa* had a greater influence on the rates of mineralization of hexadecane and phenanthrene than *A. glutinosa* in both CT and TS.

The results of the mineralization studies were confirmed by catabolic PCR performed with *alkB* and *ndoB.* PCR reactions with *ndoB* were negative, which indicates why the rates of naphthalene mineralization were very low. The *alkB* gene was detected in all samples with the exception of TSNP and CTNP. It seems that 6 months of incubation in the greenhouse negatively affected the *alkB* population. Catabolic PCR is not quantitative but the intensity of the bands imply that the *alkB* population is higher in the tailings with alders, perhaps simply due to nutrients derived from the roots, an enrichment mediated by the plant, a lower pH or a combined effect of all of the above. The TS and CT samples also were positive for the *alkB* gene but the mineralization tests showed negligible amounts of mineralization. The microorganisms with the *alkB* gene in these samples may be very low in number. When Siciliano et al. (2003) performed their field trial, they

were able to show that the indigenous microbial population in PAH contaminated sites often had the potential to degrade hydrocarbons but required nutrients for this potential to be realized. Microcosm studies were performed comparing planted fertile soil, fertilized with the commercial fertilizer 20-20-20, and planted non - fertile soil. Relative to the fertilized soil, the non-fertilized soil had reduced mineralization rates, and when the soil was fertilized, the mineralization rates increased to the same extent as the fertilized soil (Siciliano et al., 2003). The CT and TS sands contained very little organic matter. Based on the catabolic PCR results, TS and CT microflora have the potential for degrading hexadecane but may lack nutrients, such as nitrogen and phosphorus which may impede their degrading ability.

Previous studies have shown that hexadecane mineralization within tailings was nutrient limited and when amended with phosphorus the mineralization rates increased (Herman et al., 1993). Hexadecane mineralization was also tested using microorganisms from an enrichment culture developed from a naphthenic acid commercial mix. Microorganisms from the enrichment culture were unable to mineralize hexadecane. Further investigation showed that replacement of the methyl group on hexadecane with a carboxylic group, as in the structure of palmitic acid, resulted in hexadecane mineralization (Herman et al., 1994).

4.2.1. Mineralization of PAHs in CT Bulk, Rhizosphere and Endophytic samples of inoculated and non - inoculated *A. rugosa*

The inoculation of *Frankia* did not affect the rates of phenanthrene and hexadecane mineralization. Hexadecane mineralization rates in bulk CT, rhizosphere and endophytic microbial communities were very low. It was mineralized only in the bulk -control treatments and not in the rhizosphere of the control treatments, where the plants have a greater influence (Siciliano et al., 2001), suggesting that the *alkB* gene has been selected against by the alder. Herman et al. (1993) demonstrated that hexadecane mineralization was stimulated by the addition of phosphorus. Mikkelsen and Vesho (2000) demonstrated that alders nourish the microbial population through root exudates, but following the addition of nitrogen phosphorus availability was decreased. Alders have high

phosphorus requirements and as a result the microflora may have to compete for the available phosphorus in the environment (Slemants et al., 2005). The alders from 2004 were tall and their roots were well developed. These alders would have absorbed many nutrients including phosphorus, from CT. It is possible that phosphorus concentrations as a result were low in CT and consequently hexadecane mineralization was limited.

In the trial of 2003, there was less than 10% naphthalene mineralization. However, with the alders of 2004, there was 60% naphthalene mineralization in bulk CT and similar rates were observed in the rhizosphere of inoculated and non-inoculated samples. The inoculated endophytic community gave the highest rates of naphthalene mineralization.

Siciliano et al. (2003), hypothesized that if bacteria do play a role in the plant's ability to tolerate contaminants, it would be in the endophytic zone. In their investigation the plants were exposed to petroleum hydrocarbons and nitroaromatics. The results of their investigation suggested that the enrichment that occurs in the root interior is dependent on the plant species and the contaminant to which the plant is exposed. The process by which this occurs is not yet known but based on their experiments; it seems that the plants control the composition of the endophytic microbial community. On a petroleum contaminated site, *Festuca arundinacaea* had a significantly greater prevalence of *ndoB* in its root interior than *Trifolium fragiferum*, which had a higher incidence of *alkB* in its root interior. The predominance of the different genes in the endophytic communities demonstrated the influence of the plant. Some plants, such as *Scirpus pungens,* were exposed to crude oil and had an increase in *ndoB* positive endophytes (Siciliano et al. 2003). The CT sediments were formerly mixed with crude oil. Perhaps the contaminants which led to an *ndoB* positive population in *S. pungens* may have lead to an increase in *ndoB* positive endophytes in the alders. The contaminants present in CT may have stimulated and enriched the endophytic community for *ndoB*, which would explain why hexadecane and phenanthrene were not degraded by the endophytic community.

4.3. The effect of salt (NaCl) on the bacterial population

Chemical analysis of CT by Renault et al. (2003) demonstrated that the Na^+ and Cl^- ions had concentrations of 1000 mg/L and 586 mg/L respectively. In the present study, CT had lower concentrations of these ions.

Investigation of TS and CT revealed an indigenous halophilic microbial population, with a greater viable CFU/g in the TS than in CT. The higher pH in CT may not have allowed for the halophiles to survive. The viable halophilic population of TS and CT with alders had a higher tolerance to NaCl than TS and CT without alders, suggesting that the environment may have become more favourable following the addition of nitrogen and root exudates from the growing alders.

Pseudomonas, *Acinetobacter* and *Arthrobacter* species have been observed to tolerate moderate salt concentrations (0.5 M – 2.5 M NaCl) (Ventosa et al., 1998) and were found, as indicated by the 16S rRNA gene and *cpn60* sequences, in CT and CTAG. Some halophiles also have the ability to degrade PAHs, such as hexadecane and phenanthrene (Ventosa et al., 1998).

4.4. Total viable population in inoculated and non - inoculated *A. rugosa*

The ANOVA statistical analysis of the MPN results indicated that there was no significant difference in the total viable population resulting from the inoculation of *Frankia* in the media tested.

In CT bulk, rhizosphere and endophytic samples, YTS always gave the highest CFU/g of CT, followed by the MSM with 50ppm of NA, then by Bushnell Haas with PAHs. MSM with NA and Bushnell Haas medium with the PAHs quantified the viable microbial population capable of using NA and PAHs as a sole carbon source for growth respectively. The concentration of NA in the tailings is higher than the concentration of PAHs. The NA in the tailings may have selected for a microbial population which use NA as a sole carbon source, which would explain their higher population.

Microorganisms that use NA as their sole carbons sources have been revealed through enrichment cultures from the Mildred Lake Settling Basin, (Herman et al. 1993 and Clemente et al. 2004). The microorgansisms were able to

degrade compounds representative of naphthenic acids such as cyclopentyl carboxylic acid, cyclohexyl carboxylic acid and a commercial preparation of naphthenic acids.

4.5. Phylogenetic characterization using the 16S rRNA gene

The DGGE band profiles showed a decrease in microbial diversity as the treatments became more stringent. The lanes of the control treatments did not show any strong bands, but when amplified always had the largest amount of DNA accumulated per reaction, indicating a high biomass and high diversity. When looking at CT and TS on the gel, there were fewer bands, but the bands were more distinct, or intense. Genetic characterization of other contaminated sites has produced similar patterns on a DGGE gel, where the presence of contaminants selected against many organisms and resulted in a decrease in diversity (Juck et al., 2000). The pH and salt in CT may produce a harsher environment than in TS and may have selected for a certain group of organisms, which would produce a greater number of distinct bands in CT.

The alders changed the diversity and the microbial population present in CT. The change in the microbial diversity was perhaps as a result of an increase in nutrients delivered through root exudates and fixed nitrogen.

The band CTNP-A gave a 92% match to the sequence of an uncultured *Sphingomonas* found as part of a soil microbial community associated with poplar trees (Graff and Conrad, 2005). Leys et al. (2005) indicated that the *Sphingomonas* species associated with poplar trees was also found in PAH contaminated soils, and was related to *Sphingomonas* EPA 505, which degrades PAH. Poplar (*Poplus sp.*) is a fast growing soft deciduous tree, where it and the microbial population associated with it, can adapt to anoxic soil conditions caused by flooding (Graff and Conrad, 2005). Under flooded conditions, soil resembles an anaerobic freshwater habitat where the main pathways for mineralization of organic matter depend on, denitrification, iron reduction, sulfate reduction or methanogenensis (Graff and Conrad, 2005). The anoxic conditions described imitate the situation in the tailings settling ponds where methanogenesis and sulfate reduction can occur. Poplars

were a part of the boreal forest, which is what was overlying the tar sands before they were exploited. The microbial population associated with the poplar, may still constitute a fraction of the indigenous microflora present in the tailings.

The results from BLAST indicated the presence of uncultured *Actinobacteria* in CT. The Fasta search gave a 92% match to bacterium Ellin 5247- which is in the class of *Acintobacteria. Acintobacteria* are a diverse class of organisms that are widely distributed in terrestrial and marine environments and form nitrogen fixing associations with non-leguminous plants. These microorganisms have DNA with a high G-C content (Stach et al., 2003). The class of *Actinobacteria* also includes *Arthrobacter* species which were found in CT. *Arthrobacter* species have previously been found to be able to degrade NA (Quagraine et al., 2005). The BLAST searches made reference to an *Arthrobacter* species from a phenanthrene contaminated site. There was a 97% match to *Arthrobacter globiformis*, which is a species that has an anaerobic metabolism, which is something that may be needed for survival in the settling ponds (Wipple et al., 2005).

The BLAST results for CTPB-I indicated a 98% match to an uncultured gamma proteobacterium, a *Pseudomonas* sp., found in an uranium contaminated aquifer (Reardon et al., 2004). *Pseudomonas* sp. have been previously isolated from enrichment cultures developed from tailings ponds (Herman et al., 1994).

The BLAST results for TSAG, CTAG and CTAR suggested the presence of *Pedomicrobium americanum,* isolated from an aquatic environment and can live in water rich in dissolved minerals (Gebers and Beese, 1988).

4.5.1. Phylogenetic characterization using the *cpn60* gene

Sequencing analysis of the bands from the 40% - 65% *cpn60* DGGE gel was difficult. In some cases the complimentary strand could not be aligned. DGGE separates the DNA based on the G-C content. When the organisms are closely related, the G-C content may be very similar, and as a result the bands can migrate closely together and may even migrate together. When re-amplifying the DNA it is possible that the DNA from two organisms was used as a template. The primers may have preferentially amplified a strand from two DNA molecules. This would

explain why the strands did not align. In most cases the forward strand, using the H279 primer, sequenced better than the reverse strand, using H280 as the primer. Perhaps it is due to the inosines in the primer sequence which don't allow for the primers to anneal properly, which decreases the efficiency of primer annealing and extension resulting in sequences of poor quality.

The H279 sequences were used to do searches using the *cpn60* database, cpnDB. The matches were low, indicating that the *cpn60* database still needs to be further developed and that the system that we are exploring is new and needs further investigation. However, there was one band, CTAG-P, which gave an 84% match to *Acinetobacter johnsonii*. The match was not very high but the species in our sample is perhaps within the same genus. *Acinetobacter* species have previously been found from enrichment cultures developed from tailing ponds and these organisms have also been found to degrade naphthenic acids (Herman et al., 1993). In the studies conducted by Herman et al. (1993) *Acinetobacter* was found to rapidly degrade cycloalkane carboxylic acids. *Acinetobacter* species are widespread in nature, and some strains are known to be involved in the biodegradation of pollutants that are found in crude oil, such as PAHs and alkanes and have also been found to degrade PAHs in alpine contaminated environments (Margesin et al., 2003) indicating that they are adapted to cold conditions, which are typical of the conditions found in the tar sands regions.

4.6. HPLC analysis of inoculated and non-inoculated *A. rugosa*

The inoculation of *Frankia* did not seem to affect the rates of NA degrading activity. The rate of disappearance of the specific NA compound, pentyl cyclohexane carboxylic acid, was the same for inoculated and non-inoculated samples. In two of the samples, F and G, the degrading activity of the naphthenic acid commercial mix reached a steady state. However it does not seem that this was due to an effect of the inoculum. Cultures F and G were two weeks older than the other cultures, were denser and may have reached the stationary phase when they were transferred. The cultures may have stopped degrading because they had

exhausted other essential nutrients, or perhaps there were waste products that inhibited their ability to use NA.

Clemente et al. (2004) investigated the degrading activity of an enrichment culture, developed from the Mildred Lake Settling Basin, using a NA commercial mix. According to their data, after 12 days, more than 90% of the NA had been consumed, which is close to the rates produced by our enrichment cultures. Studies by Herman et al. (1994) also tested the ability of an enrichment culture, developed from Mildred Lake Settling Basin, to degrade a variety of compounds, including pentyl cyclohexane carboxylic acid. Their cultures were not able to degrade pentyl cyclohexane carboxylic acid, nor was another enrichment culture that was developed from an organic acid extract from the tailings. Pentyl cyclohexane carboxylic acid was considered to be recalcitrant to degradation due to its aliphatic side chain (Herman et al., 1994). However in our studies pentyl cyclohexane carboxylic acid was below detection limits in one or two weeks. The difference between the cultures of Herman et al. (1994) and our cultures may reflect the population difference in the CT sediments vs. the CT water. The difference may also be due to the presence of alders, which may have enriched and changed the microbial population in CT.

4.6.1. Naphthenic Acid (NA) extraction

The aim of this experiment was to determine the concentrations of NA in the tailings. Unfortunately the extraction procedure did not work. The protocol used was developed by Holowenko et al. (2001), for the extraction of NA from water. Numerous attempts were made to adapt this protocol to extract the NA from CT sediment, but were not successful.

Holowenko et al. (2001) used 160 L of water from Mildred Lake Settling Basin, whereas 100 g of CT sediment was used in our experiments. There was very little initial CT sediment in comparison and the NA in CT might have been below the levels of HPLC detection (< 10 ppm). The most commonly used method for the quantification of NA in an aqueous sample is FTIR spectroscopy. Perhaps

FTIR spectroscopy would have allowed for a more accurate measure of the NA in the extracts.

4.7. Conclusions

The revegetation and remediation of CT and TS using alder-*Frankia* symbionts was evaluated by examining alder growth and influence on the composition and activity of the indigenous microbial community. The alders lowered the pH of the tailings, increased rates of PAH-mineralization, increased the general microbial population and also increased the microbial diversity. *A. rugosa* however, had a greater impact than *A. glutinosa*, on the mineralization of PAHs and being native to Alberta, *A. rugosa* was chosen for further investigation, using only CT. The results demonstrated that *Frankia* strain F9 does enhance the growth of the *A. rugosa* in CT and, as demonstrated by the increased rates of naphthalene mineralization, the inoculation of *Frankia* did affect the mineralization activity of the endophytic community. Based on the results *A. rugosa* inoculated with *Frankia* could be used for the remediation and revegetation of CT and TS.

Further investigation of CT bulk, rhizosphere and endophytic samples through molecular biology, such as PCR, DGGE and sequencing would increase our understanding of the influence of *A. rugosa* on tailings microbiology. The true test of the *Frankia* – alder relationship and alder survival would be demonstrated through field trials. The development of a method for the extraction of naphthenic acids from sediments along with alder - tissue studies would also indicate to what extent the alders help in detoxification of the tailings.

5. Appendix

5.1. Microbial Media

5.1.1. Crones solution without nitrogen.

Crones solution is a modified form of that used by Périnet et al. (1985).

The recipe of microelements is from the reference by Dixon and Wheeler (1983)

Base	g/liter	
FeNaEDTA 1%	1 ml	----> complement A
Trace elements	100 µl	----> complement B
KCl	5 ml	----> complement C
$MgSO_4$-$7H_2O$	5 ml	----> complement D
KH_2PO_4	5 ml	----> complement E

Take note of the pH and autoclave. A pH for this solution was not found, so a pH between 5.5 and 7 was decided as reasonable.

"Complement A"	Take 1 ml/1000 ml
FeNaEDTA 1,0%	Stock 1000x
	g/100 ml
$FeSO_4$-$7H_2O$	0.697
Na_2EDTA-$2H_2O$	0.951

Sterilize by filtration (Millipore filter unit), and conserve at 4°C in the dark.

Complement B		Take 1 µl / 1000 ml
Microelements		Stock 1000 000x
	µg/L (final)	g/ 100 ml
$MnCl_2$-$4H_2O$	400	40
H_3BO_3	620	62
Na_2O-$4SiO_2$	430	43
$KMnO_4$	400	40
$CuSO_4$-$5H_2O$	55	5.5
$ZnSO_4$-$7H_2O$	55	5.5
$Al_2(SO_4)_3$-$16H_2O$	55	5.5
$NiSO_4$-$6H_2O$	55	5.5
$CoCl_2$-$6H_2O$	55	5.5
$TiCl_4$	55	5.5
Li_2SO_4-H_2O	35	3.5
$SnCl_2$-$2H_2O$	35	3.5
KI	35	3.5
KBr	35	3.5
Na_2MoO_4-$2H_2O$	30	3

Sterilize and conserve at 4°C.

Complement C	prend 5 ml/1000 ml
KCl 9.0%	Stock 200x
	g/1000 ml
KCl	90

Autoclave.

Complement D	prend 5 ml/1000 ml
$MgSO_4$ 6.0%	Stock 200x
	g/1000 ml
MgSO4-7H2O	60

Autoclave.

Complement E	prend 5 ml/1000 ml
KH_2PO_4 1.2%	Stock 200x
	g/1000 ml
KH_2PO_4	12

Autoclave.

5.1.2. Qmod media (Schwencke, 1998).

Base	g/litre	
Yeast extract	0.5	
Bactopeptone	5	
Glucose	10	
$MgSO_4$-$7H_2O$	0.2	
KCl	0.2	
Lécithine 1000x	1 ml	----> complement A
Tampon QMod	10 ml	----> complement B
Fe-EDTA	1 ml	----> complement C
Microelements	1 ml	----> complement D

Complement A	mg/L (final)	take 1 ml / 1000 ml (Stock 1000x)
Lecithin	5	0.5 g /100 ml

Dissolve 500 mg of lecithin in 50 ml EtOH 100% (sonicate if necessary) and add 50 ml H_2O. Sterilize by filtration and conserve at 4°C.

Complement B	mg/L (final)	take 10 ml / 1000 ml (Stock 100x)
Tampon QMod -1 M KH_2PO_4	1360.9	68.045 g / 500 ml

Adjust the pH to 6.5 with solid KOH, complete the solution to 1000 ml, and adjust the pH to 6.7 with NaOH 10 N. Sterilize by filtration (Millipore filter unit), and conserve at 4°C

Complement C	mg/L (final)	take 1 ml / 1000 ml Stock 1000x
FeNaEDTA 0,7%	$FeSO_4$-$7H_2O$	0.488 g/100 ml
	Na_2EDTA-$2H_2O$	0.666 g/100 ml

Sterilize by filtration, and conserve at 4°C in the dark.

Complement D Microelements-part 1	mg/L (final)	take 1 ml / 1000 ml Stock 1000x
H_3BO_3	2.86	0.286 g/ 100 ml
$MnCl_2$-$4H_2O$	1.81	0.181 g/100 ml
$ZnSO_4$-$7H_2O$	0.22	0.022 g/100 ml

Keep 90 ml of this solution.

Complement D Microelements-part 2	mg/L (final)	Stock 10 000x
$CuSO_4$-$5H_2O$	0.008	0.08 g/ 100 ml
Na_2MoO_4-$2H_2O$	0.0025	0.025 g/ 100 ml
$CoSO_4$-$7H_2O$	0.0001	0.001 g/ 100 ml

Make this stock of 100 ml, and add 10 ml to 90 ml of the former solution to make the solution of microelements. Sterilize the microelements by filtration and conserve in the dark at 4°C.

5.1.3. MSM - Minimal Salt Medium

All the components are added into distilled water. For plates, 15 g/L of agar were added.

Stock Solution	Compound	g/L	Final concentratio	Volume (ml/L) of stock solution added To dH_2O
1	1M NaH_2PO_4	0.871	6.4 mM	6.40
2	1M K_2HPO_4	2.263	12.99 mM	12.99
3	1M$(NH_4)SO_4$	1.1	8.33 mM	8.33
4	0.5M $MgSO_4 \cdot 7H_2O$	0.0974	0.395 mM	0.79
5	Trace Metals	-	-	1.0

Trace Metals

Compound	Final concentration	mg/100 ml
$CO(NO_3)$	1 μ M	29.11
$AlK(SO_4)_2.12H_2O$	1 μM	47.44
$CuSO_4$	1 μM	15.96
$ZnSO_4.7H_2O$	1 μM	28.75
$FeSO_4.7H_2O$	10 μM	278.02
$MnSO_4.H_2O$	10 μM	169.01
$Na_2MoO_4.2H_2O$	2 μM	48.2
$Ca(NO_3)_2.4H_2O$	10 μM	236.15

Using the volumes indicated mix stock solutions 1,3,4,5 in 750 ml of dH_2O and autoclave. Mix stock solution of trace metals into 250 ml dH_2O, with 15 g of agar (if desired), and 250 mg of YTS (if desired) and autoclave. Once autoclaved, combine the solutions.

5.2. DNA extraction Solutions

Solutions for Buffers

a) 1 M Tris – Cl, pH 8.3 (500 ml)

Add 60.57 g Tris (Ultra Pure) to 400 ml distilled water. Bring the pH to to 8.3 with concentrated HCl (approximately 20 ml of HCl)

Let the solution cool down to room temperature before doing the final pH adjustment, bring the volume to 500 ml, Aliquot and autoclave.

b) 5 M NaCl

Add 73.05 g of sodium chloride to 220 ml of distilled water. Adjust the volume to 250 ml and autoclave.

c) 0.5 M EDTA pH 8.0 (500 ml)

Add 93.06 g of disodium Ethylenediamine tetra acetate to 400 ml of distilled water. Adjust the pH with NaOH pellets (approximately 10 g). Aliquot and autoclave.

d) 1% Triton X-100 (w/v) (250 ml)

Take 2.5 g of Triton X-100 and add it to distilled water, such that the final volume is 250 ml. Filter sterilize.

Buffers

1st Buffer (Tris-Cl 50 mM pH 8.3 + 200 mM NaCl + 5 mM EDTA + 0.05% Triton X-100) (For 500 ml)

Mix 25 ml 1 M Tris-Cl pH 8.3 , 20 ml 5 M NaCl, 5 ml 0.5 M EDTA pH 8.0 and 25 ml 1% Triton X-100.

Complete the volume to 500 ml using distilled water and Autoclave

2nd Buffer (Tris-Cl 50 mM pH 8.3 + 200 mM NaCl + 5 mM EDTA) (For 500 ml)

Mix 25 ml 1 M Tris-Cl pH 8.3, 20 ml 5 M NaCl and 5 ml 0.5 M EDTA pH 8.0.

Complete the volume to 500 ml using distilled water and Autoclave

3rd buffer (Tris-Cl 10 mM pH 8.3 + 0.1 mM EDTA) (For 500 ml)

Mix 5 ml 1 M Tris-Cl pH 8.3 and 0.1 ml 0.5 M EDTA pH 8.0.

Complete the volume to 500 ml using distilled water and Autoclave

Other DNA extraction solutions

20% SDS (For 100 ml)

Combine 20 g of sodium lauryl sulfate (Molecular Biology Grade), and 70 ml of sterile water. Heat at 65°C for approximately 5 min. Bring the volume to 100 ml using distilled water.

TE pH 8.0 (For 250 ml)

Combine 2.5 ml 1 M Tris-Cl pH 8.0, 0.5 ml 0.5 M EDTA pH 8.0 and bring the volume to 250 ml with distilled water. Autoclave.

PVPP

For 200 mM potassium phosphate buffer

Add 81.5 mL 1 M K_2HPO_4 and 38.5 mL 1 M KH_2PO_4 and complete the volume to 600 ml with to water (pH 7.0)

For 20 mM potassium phosphate buffer:
Dilute 350 mL of 200 mM buffer to 3500 mL with water for 3.5 L of 20 mM buffer at pH 7.0 and verify pH of the buffer.

Acid washing of PVPP (modified from Holben et al., 1988)
To wash PVPP with 3 M HCl:
Pour 1034 mL conc. HCl (11.6 M) slowly with stirring into 2966 mL MilliQ water (= 4 L of 3 M).
Add 150 g PVPP and suspend with stirring at room temperature for 12-16 hours. Leave the suspension to settle for 30 – 60 min., then aspirate or decant the supernatant. Resuspend the PVPP in approx. 3.5 L of 200 mM potassium phosphate buffer (pH 7.0) and stir 1 – 2 hours. Repeat the aspirating/decanting and resuspension twice more until the supernatant pH is close to 7.0 (check aliquot with pH meter). Then repeat the aspirating/decanting and resupension 2 more times with approx. 3.5 L of 20 mM potassium phosphate buffer (pH 7.0). Aliquot the final suspension into small bottles and autoclave (15 - 20 min, 121°C). Store at 4°C

Sephacryl S-400 Preparation (for 25 ml)
TE pH 7.6 preparation (1 L)

Add 1.212 g of Tris Base and 0.372 g of EDTA into 1 L deionized water
Stir for a few minutes (5 min) to allow EDTA to dissolve. Verify the pH, it should be close to 9.1. Adjust the pH to 7.6 by using 1 N HCl +/- 1.2 ml until pH is 7.8 - 7.7 and then 0.1 N HCL: Adjustment the pH to 7.6 and Filter sterilize.

Pipet 25 ml of resin into a Falcon tube and mix the resin by shaking and inverting. Centrifuge for 1 min at 570 x g in the Centra IEC centrifuge.
The resin is still very loose, even after centrifugation, so manipulate the tube very gently. Remove and discard the supernatant. Add 25 ml of TE pH 7.6, and resuspend the resin by shaking / inverting and centrifuge 1 min at 570 x g. Remove

the supernatant as described above. Repeat the wash procedure 4 times, for a total of 5 washes with 2 ml TE. After the fifth 25 ml TE is removed, bring the total volume back to 25 ml total, i.e. add 5 ml of TE, so that there is about 5 ml of buffer above the settled resin, as it was after the initial centrifugation of 25 ml of unwashed resin. Store at 4°C in the Falcon tube.

5.3. DGGE solutions

1 - Ammonium Persulfate - APS (10%)

Add 100 mg of dry APS to 1 ml of distilled water, vortex to dissolve. This is used immediately and then discarded.

2 - Acrylamide - Denaturant Solutions

The acrylamide solutions are only stable for one month. All glassware should be rinsed with ultrapure water.

3 - 8% acrylamide - 0% denaturant: to make 100 ml of solution

20 ml 40% Acrylamide/Bisacrylamide (37.5:1) (BioRad)

2 ml 50X TAE prepared with ultrapure H_2O and ultrapure reagents

78 ml ultrapure H_2O

Mix and degas for 10-15 min. Filter through a 0.22 µm filter. Store at 4°C in a brown bottle for approximately 1 month.

4 - 8% acrylamide - 80% denaturant: to make 100 ml of solution

20 ml 40% Acrylamide/Bisacrylamide (37.5:1) (BioRad)

2 ml 50X TAE prepared with ultrapure H_2O and ultrapure reagents

32 ml deionized Formamide

33.6 g ultrapure urea

Adjust volume to 100 ml

Mix and degas for 10-15 min. Filter through a 0.22 µm filter. Store at 4°C in a brown bottle for approximately 1 month.

5- Gel Loading Dye 2X (BioRad's recipe, final concentration)

0.05% Bromophenol blue

0.05% Xylene cyanol

70% Glycerol

Prepare a 2% Bromophenol blue and a 2% Xylene cyanol solution. Mix 0.25 ml of each one with 7.0 ml of 100% glycerol, add 2.5 ml of dH_2O to make volume up to 10.0 ml. Store at room temperature.

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