Microbial community development and function at a newly reclaimed oil sands site

by

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Abstract

Assessment of microbial community development is required to determine the success of reclamation process on disturbed land after mining. Peat (PMM) or LFH mineral soil mix (LFH) is used as capping material in reclamation. Application of coarse woody debris (CWD) also facilitates reclamation by developing microsites for biogeochemical processes. To investigate the effect of reclamation material on microbial activities and functions, a study was conducted over a 3-year period at a reclaimed site in Fort MacMurray, Canada. Soil samples were collected bi-annually from LFH and PMM plots amended with or without CWD. Results revealed an increasing trend over time in microbial biomass carbon and nitrogen, respiration rate, β-glucosidase and N-acetylglucosaminidase enzyme activities, and abundances of functional genes (*amoA*, *nirS* and *nifH*) involved in nitrogen cycle. LFH showed more pronounced effect than PMM due to its higher decomposed organic matter content. Application of CWD and addition of fresh labile carbon inputs in fall enhanced microbial growth and function.

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List of Abbreviations

AMO	Ammonia monooxygenase enzyme
amoA	Gene encoding ammonia monooxygenase
Anammox	Anaerobic ammonia oxidation
AOA	Ammonia-oxidizing archaea
AOB	Ammonia-oxidizing bacteria
AOSR	Athabasca Oil Sands Region
BGLU	β-glucosidase
CT	Consolidated tailings
CFE	Chloroform fumigation-extraction method
C:N	Carbon to nitrogen ratio
CWD	Coarse Woody Debris
DNRA	Dissimilatory nitrate reduction to ammonia
DOC	Dissolved organic carbon
DON	Dissolved organic nitrogen
HAO	Hydroxylamine oxidoreductase
hao	Gene encoding hydroxylamine oxidoreductase
hzo	Gene encoding hydrazine oxidizing enzyme
LFH	LFH mineral soil mix

MANOVA	Multivariate ANOVA
MBC	Microbial biomass carbon
MBN	Microbial biomass nitrogen
OB	Overburden
OM	Organic matter
OSPM	Oil sands process-affected materials
P	Phosphorus
PCR	Polymerase chain reaction
PMM	Peat mineral soil mix
qPCR	Quantitative real time Polymerase Chain Reaction
N	Nitrogen
N_2	Dinitrogen
NAGase	N-acetyl-β-D-glucosaminidase
napA	Gene encoding periplasmic nitrate reductase
narG	Gene encoding nitrate reductase
nasA	Gene encoding nitrate reductase
NFGs	Nitrogen functional genes
NH_4^+	Ammonium
NH_2OH	Hydroxylamine

nifH	Gene encoding nitrogenase reductase
Nir	Gene encoding nitrite reductase
nirK	Gene encoding cu-type nitrite reductase
NirK	Cu-type nitrite reductase
nirS	Gene encoding cd1-type nitrite reductase
NirS	Cd1-type nitrite reductase
NO	Nitric oxide
NO_2^-	Nitrite
N_2O	Nitrous oxide
NO_3^-	Nitrate
NOB	Nitrite-oxidizing bacteria
norB	Gene encoding nitric oxide reductase
NOR	Nitric oxide reductase
nosZ	Gene encoding nitrous oxide reductase
NOS	Nitrous oxide reductase enzyme
nrfA	Gene encoding cytochrome cd1 nitrite reductase
NXR	Nitrite oxidoreductase enzyme
SAGD	Steam assisted gravity drainage
SMB	Soil microbial biomass

TMB	Total microbial biomass
TS	Tailings sands

Chapter 1. Overview of the oil sands mining disturbance, reclamation and microbial communities

1.1 Introduction

1.1.1 Rationale

Alberta's oil sands are recognized as the third-largest crude oil deposits in the world with an estimated amount of 170 billion barrels in the northeastern Alberta, Canada (Alberta Energy, 2015). These deposits are found in three regions in northeastern Alberta Peace River, Cold Lake and Athabasca River regions oil deposits underlying 142, 200 km^2. Athabasca oil sands region (AOSR) deposits are the single largest oil sands deposits in the world (Alberta Canada, 2015). The oil sands are mixture of bitumen combined with water, sand, heavy metals and clay. Oil sands deposits are extracted by two techniques, including surface mining and *in-situ* mining. Approximately 20% of AOSR deposits are surface mineable because these deposits are closer to surface at depth of less than 75 m (Gosselin et al. 2010). Current oil sands extraction is focused on surface mining in AOSR where deposits are conducive to surface mining. During this technique, vegetation cover, surface, subsurface soil layers and geologic material (overburden- OB) are removed and stockpiled for future utilization in oil sands reclamation (Giesy et al. 2010). Currently about 767 km^2 of the total surface mineable 4800 km^2 area has been disturbed due to surface mining and it has created one of the largest anthropogenic disturbances (Alberta Government, 2015). The oil sands companies are bound to reclaim the land to equivalent land capability (Cummulative Environmental Management Association, 2006) in which the reclaimed land must be a self-sustaining ecosystem with diverse natural communities to the same degree as the surrounding area or the reference ecosystem (Oil Sands Vegetation Reclamation Committee, 1998).

Oil sands reclamation involves the reconstruction of functioning ecosystem through creating novel soil profiles by applying organic materials as

cover soils such as LFH mineral soil mix (LFH), peat mineral soil mix (PMM), subsoil or both, above substrates such as overburden (OB), tailings sands (TS), clear water shale or lean oil sands (Rowland et al. 2009; Naeth et al. 2013). Practices like stockpiling of soil, mixing topsoil and subsoils (Dimitri et al. 2010), and type of capping materials (Jamro et al. 2014), transport of reclamation materials with heavy equipment (Lazarko and Van ree 2011) all can have effect on soil properties such as organic matter (OM) content and microbial biomass (Mummey et al. 2002). For instance, analysis of the microbial community in stored soil during the stage of stockpiling showed a significant decrease in total microbial biomass and the quantity of fungal propagules (Harris et al. 1993). Mining activities may also severely decrease soil organic carbon (C) and nitrogen (N) due to removal of topsoil and disturbance caused by mixing organic capping materials and soil horizons such as A with B and C soil horizon (Shrestha and Lal, 2011). The type of reclamation treatment also can affect microbial communities' response, especially in the early stages of reclamation, even more obviously than that of plants (Machulla et al. 2005). For instance, type of capping materials such as LFH and PMM affect microbial activities like enzyme activities and microbial biomass carbon (MBC) and nitrogen (MBN) due to diffrences in substrate and N availabilities (Jamro et al. 2014).

The fact that disturbance and reclamation have a significant adverse effect on the microbial community is very important. Microorganisms indirectly have roles in plant diversity and soil productivity by their impacts on nutrient availability through decomposition of OM in biogeochemical cycles (Paul and Clark, 1996). Generally, since microorganisms are critical in the re-establishment of a self-sustaining ecosystem, they can be considered as appropriate markers for the soil degradation level and reclamation success assessment. Hence, evaluating soil microbial populations through the identification and analysis of microbial communities (e.g. species diversity) and functions (e.g. specific processes, such as the N cycle, that influence ecosystem performance) is of considerable importance. On broad regional scales, having information about the microbial diversity, structure and function in response to biotic or abiotic changes will help us to

know how to manage and how to recover a disturbed area especially in oil sands mining sites which are undergoing anthropogenic disturbance. Clearly, the response and reestablishment of microorganisms to disturbance is regionally specific and more studies are needed to understand microbial dynamics and development in reclaimed post mining areas (Clayton et al. 2009). This study will comprise some microbiological and biochemical measurements, and community functions with a focus on the microbial communities involved in the N cycle to test the development of the microbial communities over time. The following sections provide background information on several key elements of the study including oil sands overview, disturbance, oil sands mining and ecosystem disturbance, soil disturbance and microorganisms, oil sands mining processes and land reclamation.

1.1.2 Oil sands overview

The vast oil sands deposit in Northern Alberta is currently undergoing surface mining activity for bitumen extraction. Oil sands are located in approximately 142,200 km^2 of Alberta regions such as Athabasca, Cold Lake and Peace River (Alberta Government, 2013). Among all Alberta regions, AOSR, located in northern Alberta, near Fort McMurray (57 °00' N, 111 °28' W), holds the largest hydrocarbon reserves in the world. The oil sands deposits in this region cover an area greater than 42 000 km^2 which contain approximately 869 billion barrels of bitumen (FTFC, 1995). Extraction of oil sands using new technologies was started by the Great Canadian Oil sands Company, "Suncor Energy Inc", in 1967 (Canadian Center for Energy, 2003), and then Syncrude Canada Ltd in 1978 started its operation after constructing a larger mine than Suncor Energy Inc. Due to increase in demand of oil and its products, several other companies have been involved in oil sands extraction. Among those companies, Shell Canada Limited and Canadian Natural Resources Limited have started their projects in 2003 and 2008 respectively in AOSR (ERCB, 2011). According to a recent report, it is estimated that Alberta oil sand industrial production is almost 1.9 million barrels of crude oil per day (Alberta Government, 2015).

1.1.3 Disturbance

There are many and various definitions of "disturbance". However, the most complete definition has been suggested by Pickett and White (1985): "Disturbance is a relatively discrete event in time that disrupts the ecosystem, community or population structure and changes the resources, substrate availability or physical environment". Based on this definition, disturbance alters both the structure and function of the ecosystem. It can also have a significant effect on the ecosystem-level processes such as nutrient cycling (Sousa, 1984).

The severity and effect of disturbance on population, communities and ecosystem depends on size, frequency and intensity of the disturbance (Oliver, 1980). According to Turner et al. (1998), disturbance size is defined as the mean area undergoing the disturbance; disturbance frequency is defined as "the average number of events occurring at an average period of time at a given location" and disturbance intensity is "the physical energy of a disturbance event". All three of these factors can affect both the biotic and abiotic components of the ecosystem and subsequently identify initial structure and composition of communities arriving or surviving after disturbance and re-establishment conditions (Turner et al. 1998).

Typically, disturbance can be mediated by natural or anthropogenic processes. Anthropogenic (human-induced) disturbances are caused by human activities and can be considered as major and severe disturbances (Pickett and White, 1985). The concept of severe disturbance has many definitions, but generally a severely disturbed area is considered as an ecosystem which through some harsh activities such as excavation or mining most of its native vegetations, animals, and microscopic organisms have been removed and a large amount of its topsoil is lost, changed, or buried (Box, 1978).

1.1.3.1 Oil sands mining and ecosystem disturbance

Oil sands development causes substantial damage and disturbance to the ecosystem due to the open-pit surface mining technique and the production of oil sands process-affected materials (OSPM) such as OB, tailings and associated water. In 2000, it was estimated that approximately 40,000 ha of land was disturbed just by two mining operations including open-pit and *in-situ* minings (Fung and Macyk, 2000). With the increase in the number of oil sands companies, the amount of disturbed area by mining is vastly increasing.

Oil sands surface mining is one of the common mining applications in Canadian boreal forests, having one of the largest oil reserves in the world; it is considered as the major anthropogenic disturbance to these areas (Purdy et al. 2005). By mining, not only is a large area of land degraded but also after the mining process, the existing ecosystem is replaced by OB and waste materials such as mine dumps and tailings sands; all of these materials change the biological and physical nature of the mined site. The most severe disturbance to the oil sands area occurs before mining when many ecological layers are removed in order to have access to the oil sands layer. In fact, by removing those layers, the integrity of the soil is changed. After removing the surface plant cover, and all ecological layers, topsoil and subsoil are removed and stored in order to be used later in the reclamation process. The lower OB which is composed of rock, clay and sand is also removed and in this stage, the underlying oil sands layer is exposed. All these processes result in severe soil disturbance and degradation and have negative effects on the growth of seedlings and revegetation during reclamation (National Energy Board, 2000). Due to this severe disturbance, reclamation of oil sands post mining areas always has been considered as a big issue for oil sands industry (Renault et al. 2003) and regarding this issue, it is thought that returning the post mining disturbed soil to its previous condition by reclamation, especially to have the similar plant cover is not possible (Woynillowicz et al. 2005).

1.1.3.2 Soil disturbance and microorganisms

Generally, soil disturbance results in many and various changes in the physical, chemical, and biological properties of soil that all affect soil productivity. The effect of soil disturbance on the biological properties of an ecosystem, especially on microorganisms, is considerable; and since they are the basis of an ecosystem, their loss, or reduction or shifts result in the disruption of normal ecosystem functioning. Indeed, microorganisms are highly sensitive to every change in the ecosystem (Turco et al. 1994), so because of soil disturbance, severe and significant changes can occur in both their structural and functional diversity (Buckley and Schmidt, 2001; Ponder and Tadros, 2002), and most of the time the consequences of soil disturbance remain for a long period of time (Mummey et al. 2002a).

In the case of oil sands mining, by removing and stockpiling the soil, the microbial communities which are inhabiting in the upper soil horizons are moved to the bottom of the stockpile, and during the first year, a shift in microbial activity takes place, especially in the upper layer, due to oxygen exposure (Williamson and Johson, 1990). Harris et al. (1989) showed that as mining operations continue, there is a significant decline in soil microbial activity because of soil stockpiling and storing. About two years after soil storage, there is not much change in the numbers of microorganisms at the surface, but the numbers of microorganisms at greater depths significantly decrease. Microbial activity also significantly decreases within a few months. Visser et al. (1984) showed that during three years of stockpiling, there is a decrease in microbial activity and metabolic rates.

Although many efforts may be made to restore the disturbed site to its natural or pre-disturbed conditions, in most cases, returning the degraded site to its original level does take many years. The reason is that microorganisms which have the major responsibilities for most of the essential activities and functions in soil (SER, 2004) including nutrient cycling and soil structure (Mummey et al.

2002b), are usually highly affected by disturbance. These natural functions of microorganisms are crucial since they support the growth of plants; thus for a successful revegetation during the reclamation process, their roles are very important. In sum, after every disturbance, in order to have a successful restoration and reclamation in disturbed areas, microbial community diversity and function and any changes in them must be considered. Microorganisms are the most important biotic elements in the soil; they have strong effects on the ecosystem, and considering their roles in order to have a sustainable ecosystem, is of paramount importance.

1.1.4 Oil sands mining processes

Several techniques are employed to extract bitumen from the deposits. Two major methods include surface or open-pit mining and *in-situ* mining are employed to access the oil sands deposits (The Oil Sands Developers Group, 2009). Of the two methods, the first is the most common in AOSR since approximately 60% of bitumen is extracted by this method. When the thickness of OB materials or surface materials is less than 75 m, the oil sands is extracted and recovered by surface mining (Gosselin et al. 2010). In surface mining, in order to have access to oil sands, all trees and vegetation covers are harvested and topsoil, subsoil and OB are removed. After removing these materials, topsoil and subsoil are either applied directly in reclamation site or put in stockpile for future use in reclamation (Lazorko, 2008). Typically OB is defined as surface material below the cover soil layer and above the minable oil sands. This layer (OB material) is removed and used to fill the open mined out pit, and will eventually be reclaimed using salvaged peat and mineral soil (Lazorko, 2008). Once oil sands deposits are accessible, they will be mined by giant shovels and trucks. However, when the deposits are at greater depths, generally more than 100 m, they are removed in situ by steam assisted gravity drainage (SAGD) (Oil Sands InfoMine, 2012). In 2010, it was estimated that approximately 89,000 m^3 (~ 0.75 million bbl/d) of crude bitumen was produced by *in-situ* mining (ECRB, 2010).

Regarding the effects of the two methods of oil sands mining, it is important to note that using *in-situ* methods like SAGD causes minimal land disturbance in the site (Gosselin et al. 2010). The probable reason is that by using this method, the ecological layers of soil in the site remain largely untouched while in surface mining many soil layers must be removed in order to access oil sands deposits.

1.1.5 Land reclamation

According to the Society for Ecological Restoration (SER), the word "reclamation" has many definitions and broad applications. However, the exact definition of reclamation is ''the process of reconverting disturbed land to its former or other productive uses'' (Powter, 2002). Today, the word is frequently used regarding mined lands. Based on the definition of "reclamation", after extraction of resources such as oil sands, the disturbed post mining site must be returned to its pre-disturbance conditions with all or most of its environmental values; and the process of reclamation is required to contain all of the steps including reconstructing and reshaping the disturbed land to its previous shape, replacing topsoil, and planting vegetation, grasses and trees. That is why during reclamation in order to return the disturbed ecosystem to its previous conditions and to have a self- sustaining environment, in addition to revegetation, significant soil factors especially soil structure, microbial communities and nutrient cycling must be addressed (Galajda, 1999).

1.1.5.1 Revegetation

Revegetation, as one of the most important steps in the reclamation process, decreases soil surface from erosion and causes accumulation of fine soil particles (Conesa et al. 2007). It is the most significant aim of the land reclamation process, involving the establishment of vegetation on the surface of disturbed or degraded land. Revegetation can be done either by seeding and transplanting, or spontaneously through which plants are reestablished naturally without the introduction of seeds. The type of vegetation covers that are chosen is

very important; they can be native or non-native plant species depending on the characteristics of the soil and the disturbed site. However, since the attempt of the reclamation process is to restore the site to its previous state, it has been strongly suggested to use indigenous or native species in revegetation processes (SER, 2004). The common woody plant species which are used in reclamation in AOSR include white spruce (*Picea glauca*), jackpine (*Pinus banksiana*), trembling aspen (*Populus tremuloides*), and paper birch (*Betula papyrifera*); and also some woody shrub species: red-osier dogwood (*Cornus stolonifera*), green alder (*Alnus crispa*), low-bush cranberry (*Viburnum edule*) and blueberry (*Vaccinium myrtilloides*) (Fung and Macyk, 2000; Yarmuch, 2003).

1.1.5.2 Re-establishing soil microbial communities

Soil microbial communities are other soil components that must be addressed during reclamation strategies. In fact, as a result of the disruption of soil layers through mining, microbial activity decreases. Since soil microorganisms have roles in the decomposition of plant material and also produce polysaccharides for soil aggregation, declining soil microbial activity decreases soil aggregation (Visser et al. 1984; Williamson and Johson, 1990). During mining, when topsoil and underlying soil become mixed, and because the stockpile is stored for a long time, the amount of soil organic carbon significantly decreases (Visser et al. 1984); this decrease in C availability to microorganisms results in a decrease in microbial activity (Visser et al. 1984; Williamson and Johnson, 1990). The use of organic amendments and fertilizers can increase the limited amount of OM and stimulate microbial activity (Williamson and Johnson, 1990). Generally, due to having extreme and adverse physical and chemical conditions in mineland soil, the development of the microbial community is limited (Tate and Klein, 1985). For instance, in a mining area in North Dakota, due to low OM content, lack of N and phosphorus (P), salinity, fine soil structure and a low rate of soil formation, the reclamation process took several years to complete (Cundell, 1977).

All of these limitations can be improved in part by amendment of soil with OM (Tate and Klein, 1985). Nutrient deficiencies such as lack of N and P can be solved temporarily with organic amendments. However, to solve this problem permanently, development or reestablishment of indigenous microbial communities contributing to the establishment of appropriate biogeochemical cycles is needed (Tate and Klein, 1985). This was supported in a study by Dancer et al. (1977), which found that in the absence of revegetation of mineland using woody leguminous plants, N accumulation and macrophyte development in reclaimed soil was retarded. In order to have high plant biomass in reclaimed soil, adequate fixed N must be supplied by nutrient amendments. However, these nutrient amendments are impractical and uneconomical, so stimulation of a stable and sustained N cycle must be addressed at mined reclaimed sites. This requires reducing extreme physical and chemical conditions and developing a constant C supply within the soil in mineland (Tate and Klein, 1985).

1.1.6 Re-establishing nutrient cycles

Since there is a direct relationship between soil microbial activity and nutrient cycling, mining procedures negatively affect nutrient cycles especially N and C cycles due to the decrease in soil microbial communities and activities (Cundell, 1977). The major objectives of mineland reclamation are to minimize soil degradation, and improve the reestablishment of functional plant-soil interactions and the stability of the newly established ecosystem. Achieving all of these objectives and success of stability of the reclaimed site rely upon development of microbial communities. The reason is that soil microbial communities contribute to soil structural development through synthesis of plant nutrients by performing biogeochemical cycles. These major processes, mediated by soil microorganisms, support the reestablishment of reclaimed sites. However, due to the abiotic properties of mineland, most microbial processes are severely limited (Tate and Klein, 1985). Biogeochemical processes such as C mineralization, N transformation and C-N interactions all are of importance in mineland reclamation. Of these two cycles, N metabolisms seem to be the most

sensitive to environmental disturbance in disturbed soils. The reason is that the number of microorganisms capable of catalyzing some specific key reactions of the N cycle is limited (Tate and Klein, 1985).

1.1.7 Reclamation materials used in post-mining oil sands area

Several types of materials are used as reclamation materials to aid restablishment of the soil ecosystem. Typically, these materials are either salvaged from the area or are by-products of mining processes. These reclamation materials are as follows (Alberta Environment and Water, 2012):

(i) Local seed is an important reclamation material collected from pre-mined areas for revegatation process. These plant species are adapted to local climate conditions and can conserve species' genetic diversity in a pre-disturbed region.

(ii) Coarse woody debris (CWD), dead woody materials include standing dead trees, above ground logs, large branches, twigs, dead coarse roots in a forest ecosystem (Pyle and Brown, 1999), and are valuable reclamation materials having several advantages. These benefits are: forming microsites that can increase seed germination, vegetation establishment, microbial presence and activity and nutrient cycling; creating a habitat for soil fauna and small invertebrates which improve soil quality; adding organic matter (OM) to soil and decreasing erosion.

(iii) Upland surface soil, known as LFH mineral soil mix (LFH) (identifiable litter, fragmented and fermented litter and humus), the most valuable reclamation material, is used as cover soil. It is salvaged from uplands and is a rich source of OM, plant nutrients, seeds and soil biota.

(iv) Transitional soil, which contains high levels of plant and microbial communities, a proper ratio of OM and mineral mix and rich source of OM, is a valuable reclamation material which is salvaged from pre-mined areas. It can increase the success of re-establishing plant and microbial species.

(v) Peat mineral soil mix (PMM), a cover soil that is an important reclamation material, is used extensively in the AOSR. It is a mixture of peat (organic horizon) and mineral soil. In most cases, it is applied as cover soil when the volumes of upland surface soil are limited.

(vi) Subsoil (coarse and fine) and suitable OB are valuable reclamation materials; suitable OB is situated below the subsoil and above the unsuitable OB layer. Subsoil/suitable OB improves and completes the reclamation processes by providing the plant with a zone which stores water and nutrients and is like a barrier between plant roots and harmful tailing sands substrates.

(vii) Underlying substrates such as tailings sands (TS), lean oil sands, coke, and consolidated tailings (CT) are also used as reclamation materials that are typically placed under subsoil and suitable overburden. Typically, LFH, transitional soil, peat or PMM, subsoil are used as soil cover in oil sands reclamation.

1.1.8 Oil sands reclamation practices in Alberta

Since the impact of disturbances in oil sand regions is intensive, various amendments must be applied to these areas via reclamation processes, and the major goal of these amendments is to improve the chemical, physical and biological properties of the OB substrate and contribute to plant establishment. Due to the importance of reclamation in this region, all the oil sands mining operations in Alberta have been regulated by Alberta Environment and the Alberta Energy Resources Conservation Board. According to Environmental Protection and Enhancement Act, all oil sands companies must restore the oil sands mining lands to their pre-disturbance conditions. In fact, the disturbed land must be turned into a self-sustaining ecosystem that has the same diversity and function as its previous form (National Energy Board, 2000).

Typically, after oil sands mining, soil is not capable of supporting plant growth because substrates such as TS and OB materials contain low OM and nutrients, high sodium content; and a lack of microbial activities (Fung and Macyk, 2000). As a result, in order to make the soil a proper substrate for plant growth, reclamation materials are required (Li and Fung, 1998). In the mining area, once the oil sands deposits are removed, soil cover materials are used to increase the physical, chemical and biological properties of substrate or soil below the soil cover materials for reclamation. These soil cover materials which may

include mineral soil, peat and LFH (a thin organic layer containing viable seeds and propagules) from stripped areas or undisturbed sites are mixed and are then applied to cover mine residues and wastes TS, OB and CT before revegatation (CEMA, 2009, OSVRC, 1998). The most common coversoil materials which are used in the AOSR are either PMM or LFH placed as coversoil over suitable OB and/or TS (Alberta Environment and Water, 2012).

Generally, various types of materials such as PMM, subsoil over TS and OB and Clearwater shale or lean oil sands are applied in reclaimed areas (Rowland et al. 2009). Among all of the various reclamation amendments which have been investigated, PMM over OB was suggested as the most effective soil amendment in oil sands reclamation (Rowland et al. 2009) However, depending on conditions, the type of materials applied and depth of application is different. In Alberta's oil sands areas, the most common reclamation process is topsoiling. During topsoiling, salvaged soil is reapplied on spoiled soil (DePuit, 1984). Salvaged soil contains native seeds and propagules (MacKenzie and Naeth, 2007) and contributes to the establishment of mycorrizal associations (Palmer, 1992). Topsoiling, or the addition of topsoil by increasing the amount of OM and nutrient availability, helps the microorganisms to develop (Sydnor and Redente, 2002).

Currently in the AOSR, two major types of soil amendments are used: (1) peat salvaged (1-2 m) from lowland pre-mining areas, and (2) LFH salvaged (several centimeters deep) from the adjacent upland forest floor. Both types may be used directly or may be mixed with mineral soil (Fung and Macyk, 2000). Generally, the organic: mineral soil mix ratio in these two amendments ranges approximately between 40% and 70% (Lanoue, 2003). PMM, a mixture of underlying mineral soil and organic peat (Singh, 2007) which contains a high amount of OM (Fung and Macyk, 2000), has been the most common material used in topsoiling as an amendment in this region. As noted above, a study conducted by Naeth and Wilkinson (2004) indicated that the appropriate soil material for improving and establishing plant was a combination of peat over TS

or over OB materials. The reason was that this combination improved soil structure and nutrient availability in TS and OB (Takyi et al. 1977). Typically, after spreading PMM, a cereal cover crop called *Hordeum vulgare* L. (Barley) is planted on reclamation soils which can serve as a soil stabilization cover (Renault et al. 2003). Another common material used in topsoiling in the AOSR is upland surface soil, a coversoil material which is a mixture of plant seeds and LFH (MacKenzie and Naeth, 2007). It has been shown that due to providing a better source of native propagules, LFH mineral soil contributes better to a native revegetation response than peat does in reclamation practices (Mackenzie and Naeth, 2006). Furthermore, it has been found that LFH contributes to greater plant cover and increases species richness and density. However, the effect of either LFH or PMM on microbial communities, and whether they can promote microbial communities more similar to pre-disturbed land, is yet unknown.

1.1.9 Evaluation of reclamation success

According to Alberta's Conservation and Reclamation Regulation, after reclamation disturbed post mining lands must be similar to equivalent undisturbed lands (EPEA, 2009). Thus, the Alberta Conservation and Reclamation Regulation (AR 115/93) has defined land capability as ''the ability of land to support a given land use, based on evaluation of the physical, chemical and biological characteristics of the land, including topography, drainage, hydrology, soils and vegetation'' (EPEA, 2009). Based on this definition, in order to evaluate whether a disturbed site has the ability to be reclaimed over time, a model or reference ecosystem and a target ecosystem are both usually considered. A reference ecosystem generally refers to the land before disturbance, and a target ecosystem addresses the condition that is expected after the land undergoes the reclamation process. Thus, the target land can be used as a model both to plan and conduct an appropriate reclamation process and to evaluate reclamation success. To achieve this goal, a target ecosystem must be identified accurately and in detail regarding its structure and composition. For instance, its significant characteristics, such as soil properties and types of vegetation cover, must be determined.

Determining the effectiveness of reclamation reveals how closely the restored site functions in comparison to the target site. To assess reclamation success, several parameters must be evaluated, since a single parameter does not give enough information about the state of the reclaimed soil. Thus, basic knowledge about reclaimed soil, such as ecological processes and all soil factors and properties, is required. For instance, both the physical and chemical characteristics of reconstructed soil must be accurately analyzed, since this soil with all its biotic and abiotic factors and characteristics, is the basis of the future ecosystem (Chambers and Wade, 1990). The importance of considering both physical and chemical properties of the reclaimed soil, which are affected by organic amendments, topsoiling and fertilizers, is that these properties have a direct impact on the establishment of primary species on reclaimed soil. The Society for Ecological Restoration International Science and Policy Working Group in 2004 published a list of nine criteria of an ecosystem which must be considered for assessing and evaluating reclamation success. If the criteria are within similar ranges in both the reclaimed and natural sites, the reclaimed site is regarded as being equivalent to the natural undisturbed site, and the reclamation is considered successful. The criteria include: "similar diversity and community structure in comparison to reference sites; presence of indigenous species; functional groups necessary for long term stability; capacity of the physical environment to sustain viable populations; regular functioning; integration with the landscape; elimination of potential threats; resilience to natural disturbances; and self-sustainability" (Hendrychová, 2008).

In practice, however, just one to three measures of major ecosystem criteria —species diversity, ecological process and vegetation structure — have been considered. Generally, studies of diversity as a measure or criterion to assess reclamation success have measured plant species richness. Studies of vegetation structure for evaluation of reclamation success have mostly analyzed plant cover, vegetation density, and plant biomass, and studies of ecological processes have concentrated on interspecific interactions between microorganisms and plants.

Finally, nutrient pool and soil OM have been investigated (Ruiz-Jean and Aide, 2005).

In most reclamation assessments, therefore, ecological functions have been neglected. The most important factors that can be investigated to evaluate the functionality of the soil are re-aggregation of soil particles and soil microbial processes (Filip, 2002; Šourková et al. 2005; Heras, 2009). According to a study conducted by Edgerton et al. (1995), there is a direct relationship between re-aggregation of soil particles and MBC, hence the investigation of soil microbial biomass and microbial productivity provide some information about soil structure and also a proper way to assess reclamation success. Furthermore, in order to investigate the biological processes, evaluation of reclaimed soil reconstruction can consider microbial diversity and functions due to the significant roles of microorganisms in decomposition and nutrient cycling. Thus, studying microbial structure and microbial metabolic activities is an appropriate way to assess reclamation success (Mummey et al. 2002a; Izquierdo et al. 2005).

Normally, in the first years after reclamation, because of the loss of tree cover and water limitation, microbial activity is much lower in reclaimed soil than in undisturbed soils (McMillan, 2005). The diversity and structure of microbial communities essentially depends on the disturbance and the type of amendments which have been used through the reclamation processes. Macyk et al. (2004) compared the microbial diversity in the topsoil of a reclaimed soil with that of an undisturbed soil, and their results indicated that the microbial diversity of the both soils was very similar. In one study, McMillan et al. (2007) compared the MBC and respiration in undisturbed and reclaimed soils. Both parameters were significantly higher in undisturbed soil than in reclaimed soil. They also investigated and compared MBC and MBN, as well as respiration rates, in reclaimed soils with LFH and PMM treatments. They indicated that all three factors were higher in LFH than in PMM treatments. These results showed that LFH had good potential to stimulate microbial activity. Macyk et al. (2004) also showed that the lowest amount of microbial biomass was found in PMM covers

and the highest was in stockpiled peat, but in LFH covers, microbial biomass was similar to that of undisturbed soils.

1.1.9.1 Biological indicators to assess soil health and ecosystem reestablishment after reclamation

Soil microbial community indicators such as metabolic activities and the nutrient cycles driven by these communities, have great potential for use in assessing the soil health, and stability of reclaimed ecosystems (Bentham et al. 1992). The reason is that during mining activities, these biological indicators as early indicators of ecosystem disturbance are severely affected and changed. Microbial activity measurements have been suggested as indicators for minesoil genesis (Schafer et al. 1979); however, there are relatively few studies that have investigated the microbial community characteristics of minesoils (McMillan et al. 2007; Hahn and Quideau 2013; Jamro et al. 2014).

Among these biological indicators, soil microbial biomass as an important indicator of nutrient pool and agent of OM cycling reflects soil fertility and is one of the most important microbial properties. Soil respiration essentially resulting from microbial respiration and the product of aerobic catabolic processes in nutrient cycles, indicates total soil microbial activity. Soil microbial enzyme activities integrate information on microbial status and soil physicochemical conditions. Measurements of microbial-catalyzed processes using enzymes can provide information on successional development of microbial communities with time (Tscherko et al. 2004). Nutrient cycles, especially N and C cycles, which are closely related to soil microbial activity, are disrupted during mining activities and disturbances (Sheoran et al. 2010). Shifts in N cycling rates due to the disturbance and environmental changes can alter the N availability, which is an important plant nutrient. Thus, N as the most limiting nutrient in soil is considered as an essential element in maintaining a sustainable ecosystem (Cheng et al. 2011). Functional gene assessment is an emerging tool to evaluate the development of microbial communities in response to changes in the

environment. These functional genes can give valuable information on density and abundance of microbial communities involved in N cycle (Wallenstein and Vilgalys, 2005). Among nitrogen transformation processes, nitrogen fixation, nitrification, and denitrification are of great importance. Quantification of nitrogen functional genes (NFGs) such as, *nifH* (Rösch et al. 2002), *amoA* (Rotthauwe et al. 1997; Francis et al. 2007) and *nirS* (Zumft, 1997) involved in nitrogen fixation, nitrification, and denitrification respectively, may provide some insights and information on density of functional microbial groups to estimate the development of N cycling in ecosystem (Hai et al. 2009).

For these reasons, besides microbial biomass, respiration rate and enzyme activities, N cycling transformation abilities of soil microorganisms were also used in this thesis as the main indicators to evaluate the microbial community development and reclamation success with time.

1.1.10 Statement of objectives

The focus of this research was to investigate the development of microbial communities at an oil sands reclaimed site. In other words, this study aimed to assess the development of soil microbial communities using microbial indicators and the reestablishment of N cycle in a post mining soil with time.

The specific objectives of this thesis project were to:

1. Investigate microbial indicators and metabolic activities to estimate the development of microbial communities with time.
2. Evaluate N transformation potential of key players of N-cycling microbial communities by measuring functional gene abundances involved in nitrification, denitrification, and N_2 fixation due to their sensitivity to N- concentration, pH, and other soil factors.

Typically, soil microbial characteristics have the potential to be indicators for soil quality due to their important role in C and N cycles (Nannipieri et al. 1990). Thus, to achieve these objectives, some functions such as enzyme

activities, microbial respiration and microbial dynamics such as MBC and MBN, and also the abundance of some specific genes such as archaeal and bacterial *amoA*, *nirS*, and *nifH* involved in N cycle were used to assess the development and shifts in functional microbial communities in a reclaimed oil sands soil.

This research is part of the Mega Project "Helmholtz Alberta Initiative" under " Theme 6: Land Reclamation and Landscape Development" aimed to identify the amendments for speeding up the natural processes to achieve the sustainable ecosystem in oil sands and coal mined areas of Canada and Germany.

1.1.11 References

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Chapter 2. Assessment of microbial community dynamics, activity and function in oil sands reclamation

2.1 Introduction

Microbial communities mediate most of the soil biogeochemical processes involved in biogeochemical cycling of C (OM), N and other nutrients (Bandick and Dick, 1999). They play major role in the functioning of soil ecosystem through providing energy and making nutrients available for other biotic processes in the ecosystem (Schoenholtz et al. 2000). Microorganisms also respond quickly to disturbances and environmental changes and can also be used for the assessment of soil quality and health (Scow et al. 1998; Harris, 2003). Soil disturbance causes many groups of microorganisms to be lost from the ecosystem (Degens et al. 2000). It alters the normal soil functioning, ecosystem productivity and stability (Mummey et al. 2002), in terms of total biomass, species composition and activity (Harris et al. 1993; Insam and Domsch, 1988). Surface mining activity in oil sands creates one of the largest anthropogenic disturbances in the world (Alberta Government, 2010). During open-pit mining activity, existing vegetation, surface soil, and OB materials are removed to remove oil sands ores, and the resulting disturbed land requires reclamation (Fung and Macyk, 2000). According to recent estimation, approximately 767 km^2 of land has been disturbed by open-pit mining activity. Oil sands companies are bound to reclaim the disturbed area and restore it to an equivalent land capability as prior to the disturbance (Powter et al. 2012).

Sustainability of oil sands reclamation requires the development of microbial communities, as they are linked to ecosystem productivity (McMillan et al. 2007). The key soil components for understanding the ecosystem sustainability are the microbial communities which mediate the key biogeochemical processes involved in nutrient availabilities (Paul and Clark, 1996). It has been shown that,

the development of ecosystem requires examining the changes in microbial community composition and activity over time (Moynahan et al. 2002). Insam and Domsch (1988) suggested soil microbial biomass as a feasible parameter for reclamation assessment and they found that there was a close relationship between the age of reclaimed soil and microbial biomass. Soil enzyme activities are another tool to assess the reclamation success which can provide a clear picture on how reclamation practices affect soil potential to perform the processes (Bandick and Dick, 1999). Key enzyme involved in organic matter decomposition and nutrient cycling (C and N) are β-glucosidase (BGLU) and N-acetyl-β-D-glucosaminidase, respectively. BGLU activity is important during last step in cellulose degradation (C cycle) and NAGase activity is key in chitin degradation (C and N cycle). Both enzyme activities are considered to be affected in reclamation practices and soil management (Ekenler and Tabatabai, 2002; Jamro et al. 2014). Typically, in order to assess how reclamation affects soil quality, various microbial indicators which are correlated to OM such as MBC and MBN, respiration rate and enzyme activity, were reported to be critical (Gajda et al. 2000; Gregorich et al. 2006; McMillan et al. 2007; Jamro et al. 2014). Thus, measuring soil capacity to catalyze reactions involved in C and N cycles through enzyme assays along with microbial respiration as an index can provide information on soil microbial communities' development (Dick et al. 1997).

Oil sands reclamation practices involves a building-up of functioning ecosystem through genesis of soil profile using two OM sources such as PMM (peat mineral soil mix) and LFH (LFH mineral soil mix) (Mackenzie and Naeth, 2007). Typically, PMM which is highly available at mining foot print is used as common organic capping layer of 20-50 cm thick to cover the reconstructed soils (Fung and Macyk, 2000). The LFH contains fine roots, tree stumps and Ae horizons from Luvisolic soils (Soil Classification Working Group, 1998), stripped from pre-mining areas. In general, PMM and LFH have contrasting soil biological properties such as OM content and nutrient availability to soil microbial communities. The LFH has more decomposed OM than PMM (Jamro et al. 2014). The PMM has low microbial activity due to anaerobic origin and low N

availability (Hemstock et al. 2010) resulting from wider carbon to nitrogen (C:N) ratio than LFH (Jamro et al. 2014).

C:N ratio plays a key role in the OM decomposition (Mohanty et al. 2013). With decrease in C:N ratio in soil, the decomposition of OM increases (Devito et al. 1999). The lower C:N ratio provides the labile C and nutrients (MacKenzie and Naeth, 2010). In general, high OM content increases microbial biomass and respiration (Santos et al. 2012) as in response to the increasing concentration of labile C substrate. Moreover, OM content may also have a role as a precursor of enzyme synthesis (Tabatabai, 1994). Vegetation is another factor which may have key role in the development of microbial communities and their functions (Mummey et al. 2002) such as microbial biomass (MacKenzie and Quideau, 2010), respiration (Norris et al. 2013), and microbial community composition (Hahn and Quideu, 2013) in reclaimed soils. Vegetation via litterfall inputs can alter the quality and quantity of substrate required for microbial activity (Xu et al. 2010). It has been indicated that vegetated plots had greater microbial biomass than non-vegetated plots (Ohtonen et al. 1999; MacKenzie and Quideau, 2010); and respiration rate was associated with stand type and distinct microbial community structure (Norris et al. 2013). The NAGase and BGLU activities were linked to litterfall material (Baldrian et al. 2008; Jamro et al. 2014) and the presence of vegetation (Zhang et al. 2010), respectively. Apart from C:N ratio, LFH may serve as a source of native plant species seed propagules (Mackenzie and Naeth, 2007) and increases vegetation cover and species diversity more than PMM (Brown and Naeth 2014; Forsch, 2014). The greater vegetation cover and species diversity in LFH than PMM may help to add more labile substrate to soil and alter the microbial communities, affecting the ecosystem development (Hahn and Quideau, 2013).

Application of CWD has a potential to accelerate the oil sands reclamation process (Brown and Naeth, 2014). The CWD may increase the development of microsites (Brown and Naeth, 2014) that provide habitat for microbial communities and also increase nutrients availability and OM content (Stevens,

1997; Hicks et al. 2003); consequently it influences the plant productivity and growth (Harmon et al. 1986). All these benefits may enhance the oil sands reclamation process.

Most studies in oil sands reclamation have been focused on the contrasting effects of LFH and PMM on microbial communities composition (Hahn and Quideau, 2013), nutrient availabilities (MacKenzie and Quideau, 2010), microbial biomass and respiration (McMillan et al. 2007; Norris et al. 2013), and soil enzyme activities (Quideau et al. 2013; Jamro et al. 2014). Despite the previous studies, more research is required to understand the effects of capping materials (LFH and PMM) on the development of microbial communities in reclaimed oil sands soils. In addition, effects of CWD on the development of microbial communities have not yet been explored and need to be investigated.

In this chapter, we investigated the effects of capping materials (LFH, PMM) and CWD amendment on the development of microbial community dynamics such as MBC, MBN, respiration, BGLU and NAGase activities in order to assess the success of reclamation practices. Due to the contrasting properties of LFH and PMM and CWD application, it is hypothesized that the reclamation practices would change the microbial community dynamics and their development in the reclaimed oil sands site. The overall objective of the study described in this chapter was to investigate microbial indicators and metabolic activities to evaluate the progress of microbial community development during initial ecosystem development after reclamation of disturbed oil sands site.

2.2 Materials and methods

2.2.1 Research site description

The research site was located at Suncor Energy Inc., Lease 86/17 approximately 24 km north of Fort McMurray, Alberta, Canada within the central mixed-wood subregion of the boreal forest natural region (Natural Regions

Committee, 2006) in AOSR. Generally, in this area summers are short and warm while winters are cold and long. The mean annual temperature in this region was 1.0 ^{o}C from 1981 to 2010. Typically the highest temperatures occur in July (average of 23.7 ^{o}C) and the lowest temperatures occur in January (average of -22.5 ^{o}C). Mean annual precipitation is approximately 418.6 mm from which the majority is as rainfall (316.3 mm) and 134 cm is as snow (Environment Canada, 2014). Central mixed-wood natural subregion is mainly characterized by massive areas of upland forests, wetlands and rolling plains. The upland area is dominated by *Populus tremuloides* L (trembling aspen) and *Picea glauca* L (white spruce) mixed wood stands. Some other tree species such as *Populus balsamifera* L. (balsam poplar) and *Pinus banksiana* Lamb. (jack pine) also can occur in this area. Soils in the upland area mainly consist of Gray Luvisols with Dystric and Eutric Brunisols while lowland area contains mostly organic soils and peaty Gleysolic soil (Natural Regions Committee, 2006). Typically tree species in wetlands are *Picea mariana*, *Ledum glandulosum* Nutt. (Labrador tea), *Salix* sp (willow) and sedge species (Natural Regions Committee, 2006).

2.2.2 Plot establishment, experimental design and soil sampling

Research plots were established on the Southeast Dump (56^{o} 58´N 111^{o} 22´W) at Suncor Energy Inc (Brown and Naeth, 2014). In order to establish the research site, in 1999 the site was cleared and served as a dump for OB waste materials. In 2004, OB materials were placed in the dump. In 2007, two rows of plots (a total of 36 plots; 18 in each row) were overlaid horizontally along the slope of the study site in such a way that each plot was separated from the adjacent plot by a 5 m buffer and the two rows were separated by a 10 m buffer. Each plot was 10 m wide and 30 m long. Eighteen plots in each row contained 3 replicates of each treatment. In November 2007, half of the plots received LFH and half received PMM. The LFH (at a depth of 20 cm) was applied over 30 cm of subsoil and 100 cm of suitable OB. The PMM (at a depth of 30 cm) was applied over 100 cm of suitable OB. In February 2008, 24 of 36 plots were covered with woody debris; in each row, 6 plots were covered with *Picea*

mariana, 6 plots with *Populus tremuloides*, and 6 plots with no woody debris added (Figure 1.1). In June 2008 and August 2009, all plots were fertilized aerially with a N: P: K fertilizer (Brown and Naeth, 2014).

This study was involved with two capping materials (LFH vs. PMM) and three CWD treatments (near and away from CWD, and control without CWD) with three replications in a randomized factorial design. Four 1m × 1m subplots of each near (within 5 cm from CWD), away CWD (more than 1 m away from CWD) and control treatments were set up in each plot of organic capping covered by either LFH or PMM. Sampling was performed bi-annually in June and September during three years from 2011 to 2013. Soils were collected from a depth of 0-10 cm from each subplot. Randomly four soil samples were collected from four subplots in each plot and were then mixed to make a composite sample. A total 84 soil samples were collected during the study period; 12 soil samples in June 2011 and 36 soil samples in each 2012 (18 in June, 18 in September) and 2013 (18 in June, 18 in September). For sampling, soil auger was cleaned with 70% ethanol between treatments to decrease cross contamination. Soil samples were put into individual sterile Whirlpack^{TM} bags and were placed in a cooler containing ice pack and kept cold until their arrival at the laboratory. Fresh soil samples were split into two parts and stored immediately at 4^{o}C (for biochemical analyses) and -20^{o}C (for molecular analyses) until use. Samples were passed through a 2-mm sieve prior to analysis. Fresh samples at 4^{o}C were used for biochemical analyses such MBC, MBN, respiration rate and enzyme activities within 5 days. Samples stored at -20^{o}C were used for gene analyses later. It should be noted that samples for September 2011 were only used for molecular analyses not for biochemical analysis.

2.2.3 Soil samples processing and analysis

In laboratory, the soil samples collected at June and September during 2011 to 2013 were used for biochemical analyses within 5 days after sampling, except for June 2011. Samples collected in June 2011 were stored at 4°C until

May 2012. These samples were pre-incubated and were used for analyses given in the following sections.

2.2.4 Reagents and chemicals

Liquid nitrogen was used to perform the fumigation for the measurement of MBC and MBN. Ethanol-free Chloroform and Potassium Sulphate (K_2SO_4) were purchased from Fischer Scientific (Edmonton, Canada) and were used for the extraction in fumigation. Methylumbelliferyl-β-D-glucopyranoside, methylumbelliferyl-N-acetyl-β-glucosaminide substrates and 4-methylumbelliferone were purchased from Sigma Aldrich (Ontario, Canada), and glacial acetic acid (Acros) and Sodium hydroxide (NaOH) were purchased from Fischer Scientific (Edmonton, Canada). These reagents were used for soil enzyme activity assays. To determine the soil respiration, Hydrochloric acid (HCl) and Barium Chloride ($BaCl_2$) were purchased from Fischer Scientific (Edmonton, Canada) and Phenolphthalein was purchased from Sigma Aldrich (Ontario, Canada).

2.2.5 Biochemical analyses

2.2.5.1 Soil water content measurement

To determine moisture content, a 7-gram soil sample was removed from each stored sample at 4^{o}C one day after sampling and was oven dried at 105^{o}C and was weighed until soil weight became stable (Kalra and Maynard, 1991).

2.2.5.2 Soil microbial biomass measurement

MBC and MBN were determined using chloroform fumigation-extraction method (CFE) as described by Brookes et al. (1985). Briefly, two 5g fresh soil samples were used; one of them was weighed into a Nalgene bottle and another one in a 40 ml glass beaker. The first one was immediately extracted (unfumigated sample) using 50 ml of 0.5 M K_2SO_4 (1:2 soil to solution ratio), and the second one (sample in beaker) was fumigated with chloroform for 24 hours.

After fumigation, the sample was extracted using 50 ml of 0.5 M K_2SO_4, shaken for 30 minutes and vacuum filtered using Whatman 42 filter paper. In the extracted solution, the concentrations of dissolved organic carbon (DOC) and dissolved organic nitrogen (DON) were determined using Shimadzu TOC-VTN instrument (Mandel Scientific Company Inc., ON, Canada). In order to determine MBC and MBN, the difference in concentrations of DOC and DON between fumigated and unfumigated soil samples was calculated (Swallow et al. 2009).

2.2.5.3 Soil microbial respiration

Soil microbial respiration as an indicator of microbial activity, was measured using an alkali absorption method described by Zibilske (1994). Respired CO_2 was trapped by 0.5 M NaOH and the reaction yielded formation of sodium carbonate (Na_2CO_3). In this method, 20 g fresh soil was placed in a 1 L glass incubation jar and then an open cap glass scintillation vial containing NaOH (0.5 M × 20 ml) was placed inside the incubation jar. An additional blank glass scintillation vial also was place in a 1 L glass incubation jar without soil sample. The jar was sealed and left at incubator at 25^{o}C for 7 days. When incubation was completed, the glass scintillation vial was taken out and capped instantly. The CO_2 concentration was determined by titration (with 0.5 M HCl) in the presence of $BaCl_2$ and phenolphthalein indicator (Zibilske, 1994; Hopkins, 2007). Respiration rate was calculated using the total amount of CO_2 trapped over the incubation period (Yiqi and Zhou, 2010).

2.2.5.4 Soil enzyme activity assays

Microbial extracellular soil enzyme activities involved in C and N cycling processes was measured as indices of microbial activity. We followed microplate assays as adapted method from Sinsabaugh et al. (2003). Enzyme activity assay was performed on fresh soil samples within 5 days of collection. The activities of β-glucosidase (BGLUCO; E.C.3.2.1.21) and N-acetyl-β-D-glucosaminidase, a proxy for chitinase (NAG; E.C.3.2.1.30) were measured using 4-methylumbelliferyl-β-D-glucopyranoside and 4-methylumbelliferyl-N-acetyl-β-

glucosaminide, as fluorimetric substrates, respectively. To prepare soil suspension, approximately 1 g soil sample was homogenized in 125 ml of 50 mM sodium acetate buffer having pH 5, and shaken for 30 minutes at room temperature. After shaking, 200 µl of soil suspension and 50 µl of 200 µM substrate solution (either methylumbelliferyl-β-D-glucopyranoside or methylumbelliferyl-N-acetyl-β-glucosaminide) were pipetted into a 96 well plate; for each soil sample 16 replicates were used. In addition to 16 wells, 8 wells as negative control containing 250 µl sodium acetate buffer, 8 wells containing 50 µl substrate solution plus 200 µl sodium acetate buffer, and 8 wells as blank containing 200 µl soil sample suspension plus 50 µl sodium acetate buffer, were used. Also, for each soil sample, 8 wells as reference standards contacting 50 µl 4-methylumbelliferone and 200 µl sodium acetate buffer and 8 quench control wells containing 200 µl soil suspension and 50 µl 4-methylumbelliferone were used. The enzyme assay plates were incubated for 3 hours at 20^{o}C in the dark. After the incubation time, 20 µl of 0.5 M NaOH was added to each well to measure enzyme activity. To read and quantify the fluorescence (F) at 360 nm excitation and 460 nm emission, a spectrophotometer microplate reader was used. Enzyme activity rate were expressed in µmol hr^{-1} g^{-1} of soil.

2.2.6 Statistical analysis

Multivariate ANOVA was used to test the significant effects of reclamation practices on the development of MBC, MBN, respiration rate, BGLU, and NAGase activities. For pair-wise comparison of the significant parameters, LSD was used. All statistical analyses were performed using SAS 9.2, and $P < 0.05$ was considered to be statistically significant.

2.3 Results

2.3.1 Soil microbial biomass C and N

The effect of capping materials (LFH and PMM) on MBC and MBN is shown in Figure 2.2.A and Figure 2.4.A, respectively. Capping materials showed

significant ($P< 0.05$) effect on MBC, which was greater in LFH (0.445 ± 0.040 mg g^{-1}) than in PMM (0.280 ± 0.026 mg g^{-1}) (Figure 2.2.A and Table 2.1). The significant difference in MBC between LFH and PMM was found in last sampling time in September 2013 (Figure 2.2.A). The effect of capping materials on MBC with time from June 2011 to September 2013 was also evaluated. The effect of LFH on MBC with time showed that MBC did not significantly change during June 2011 and 2012; however, a significant increase of MBC occurred in September 2012 which remained unchanged in June 2013 and then significantly increased in last sampling time, September 2013 (Figure 2.2.A). The highest (0.760 ± 0.088 mg g^{-1}) and lowest (0.217 ± 0.048 mg g^{-1}) MBC was found in September 2013 and June 2011. A similar trend in MBC was observed over time in PMM amended plots with exception of 2013 when MBC decreased non significantly from June 2013 (0.327 ± 0.056 mg g^{-1}) to September 2013 (0.191 ± 0.035 mg g^{-1}) (Figure 2.2.A).

CWD (control, away CWD, near CWD) did not show any significant effect ($P> 0.05$) on MBC (Figure 2.2.B and Figure 2.2.C). In other words, there were no significant differences among near CWD, away CWD and control over time. The mean values of MBC were 0.419 ± 0.047 near CWD, 0.346 ± 0.042 away CWD, and 0.322 ± 0.043 mg g^{-1} in control (without CWD).

The effect of seasonality on the MBC showed that there was significantly ($P< 0.05$) greater MBC in September (0.496 ± 0.049 mg g^{-1}) compared to June (0.316 ± 0.023 mg g^{-1}) (Table 2.3). The effect of seasons on MBC was more pronounced in 2012 where September exhibited significantly higher MBC than June. The average values of MBC in June and September were 0.268 ± 0.025 and 0.515 ± 0.055 mg g^{-1}, respectively (Figure 2.3.A).

The change in MBC over three years of sampling from 2011 to 2013 is shown in Figure 2.3.B. A significant increasing trend ($P< 0.05$) of MBC was observed from 2011 to 2013. However, the trend was not significantly different

from 2012 to 2013 (Figure 2.3.B). Mean values of MBC were 0.189 ± 0.028, 0.392 ± 0.037, and 0.420 ± 0.045 mg g^{-1} in 2011, 2012, and 2013, respectively.

Overall, the results showed that MBC was significantly greater in LFH capping material than in PMM. MBC was not significantly affected by CWD in LFH and PMM plots. Overall, September showed greater MBC than June, and a significant increasing trend of MBC was observed especially from 2011 to 2012.

Capping materials (LFH and PMM) exhibited a significant effect ($P<$ 0.05) on MBN over time where MBN was greater in LFH (0.036 ± 0.003 mg g^{-1}) than in PMM (0.023 ± 0.001 mg g^{-1}) (Figure 2.4.A and Table 2.1). In MBN, significant differences between LFH and PMM were observed during 2012 (June and September) (Figure 2.4.A). The effect of capping materials on MBN with time from June 2011 to September 2013 showed that in LFH amended plots, MBN displayed a significant increase initially from June 2011 to June 2012; however, after that it remained unchanged during the rest of sampling times (Figure 2.4.A). In PMM amended plots, MBN did not change significantly over time from June 2011 (0.017 ± 0.002 mg g^{-1}) to September 2013 (0.023 ± 0.004 mg g^{-1}) (Figure 2.4.A).

No significant effect ($P>$ 0.05) of CWD (control, away CWD, near CWD) was found on MBN over time (Figure 2.4.B and 2.4.C). The mean values of MBN were 0.033 ± 0.003 near CWD, 0.025 ± 0.002 away CWD, and 0.028 ± 0.003 mg g^{-1} in control (without CWD) plots.

The effect of seasonality on MBN is shown in Figure 2.5.A. Overall, seasonality did not display any significant effect ($P>$ 0.05) on MBN over time and MBN in September (0.033 ± 0.003 mg g^{-1}) was not significantly different from June (0.031 ± 0.002 mg g^{-1}) (Table 2.3). No effect of seasons was also found in each year during study period from 2011 to 2013 (Figure 2.5.A).

A significant change ($P<$ 0.05) in MBN was found over three years from 2011 to 2013. MBN was significantly lower in 2011 compared to both 2012 and

2013 and it did not show any significant change from 2012 to 2013 (Figure 2.5.B). Mean values of MBN were 0.021 ± 0.003, 0.033 ± 0.003, and 0.030 ± 0.003 mg g^{-1} in 2011, 2012, and 2013, respectively.

Altogether, the results of MBN showed that MBN had significantly greater values in LFH than in PMM capping materials. CWD in both LFH and PMM plots did not show any significant effect on MBN over time. Overall, no significant difference in MBN was found in September and June. However, a significant increasing trend of MBN was observed from 2011 to 2012.

2.3.2 Respiration rate

The effect of capping materials (LFH and PMM) on respiration rate is shown in Figure 2.6.A. Capping materials did not show any significant effect ($P>$ 0.05) on respiration rate. Mean values of respiration rate were 49.16 ± 3.73 in LFH and 54.59 ± 5.59 nmol CO_2 g^{-1} hr^{-1} in PMM (Figure 2.6.A and Table 2.1). No significant difference was also found between LFH and PMM capping materials over time (Figure 2.6.A). The effect of capping materials on respiration rate with time from June 2011 to September 2013 displayed that in both LFH and PMM plots, a significant increase of respiration rate was occurred from June 2011 to June 2012 and it remained unchanged till June 2013 and after that it significantly increased in September 2013 (Figure 2.6.A). The greatest (76.50 ± 8.55 in LFH and 86.13 ± 19.34 in PMM) and lowest (14.94 ± 1.10 in LFH and 12.14 ± 0.52 nmol CO_2 g^{-1} hr^{-1} in PMM) respiration rates were found in September 2013 and June 2011, respectively.

Application of CWD (control, away CWD, near CWD) showed significant effect ($P<$ 0.05) on respiration rate (Figure 2.6.B and Figure 2.6.C). The mean values of respiration rate were 66.44 ± 7.28 near CWD, 50.19 ± 5.07 away CWD, and 38.99 ± 3.40 nmol CO_2 g^{-1} hr^{-1} in control (without CWD) plots. In plots amended with LFH, the significant effect of CWD on respiration rate was observed during 2013 when near CWD showed significantly higher respiration rate than away CWD and control in September 2013 and it showed also

significantly higher respiration rate than control in June 2013 (Figure 2.6.B and Table 2.2). In PMM amended plots, near CWD only showed significantly higher respiration rate as compared to control in September 2013 (Figure 2.6.C and Table 2.2).

Seasonality exhibited a significant effect ($P< 0.05$) on respiration rate over time when greater respiration rate was found in September (71.92 ± 5.77) than in June (51.01 ± 2.37 nmol CO_2 g^{-1} hr^{-1}) (Table 2.3). Among years, the effect of seasons on respiration rate was only observed in 2013 when average values of respiration rate were 48.74 ± 3.393 and 81.31 ± 10.32 nmol CO_2 g^{-1} hr^{-1} in June and September, respectively (Figure 2.7.A).

An increasing trend ($P< 0.05$) in respiration rate was observed over time. However, the significant increase was observed only form 2011 to 2012 (Figure 2.7.B). The average values of respiration rate were 13.54 ± 0.68, 57.89 ± 2.86, and 65.03 ± 6.02 nmol CO_2 g^{-1} hr^{-1} in 2011, 2012, and 2013, respectively.

Overall, the results of respiration rate showed that the effect of LFH on respiration rate was not significantly different from PMM. CWD showed significant effect on respiration rates in both LFH and PMM plots. A significant change in respiration rate was observed in September and June due to the effect of seasonality when greater respiration rate was observed in September compared to June and a significant increasing trend of respiration rate was also observed particularly from 2011 to 2012.

2.3.3 BGLU and NAGase activities

The results of the effect of capping materials (LFH and PMM) on BGLU and NAGase activities are shown in Figure 2.8.A and Figure 2.10.A, respectively. The significant effect ($P< 0.05$) of capping materials on BGLU activity was observed where the enzyme activity was greater in LFH (0.118 ± 0.008 μmol $hr^{-1}g^{-1}$) than in PMM (0.068 ± 0.005 μmol $hr^{-1}g^{-1}$) (Figure 2.8.A and Table 2.1). The significant differences in the enzyme activity between LFH and PMM were found

during 2012 (June and September) and June 2013 (Figure 2.8.A). The effect of LFH on BGLU activity with time from June 2011 to September 2013 showed that BGLU activity significantly increased during 2011 and 2012; however it decreased and remained unchanged during 2013 (Figure 2.8.A). In LFH plots, the highest BGLU activity (0.193 ± 0.011 μmol $hr^{-1}g^{-1}$) was found in September 2012 and the lowest enzyme activity (0.062 ± 0.013 μmol $hr^{-1}g^{-1}$) was observed in June 2011. In PMM plots, BGLU activity did not change during June 2011 and June 2012; however, it increased significantly in September 2012 (0.089 ± 0.008 μmol $hr^{-1}g^{-1}$) and remained unchanged during 2013 (Figure 2.8.A).

CWD (control, away CWD, near CWD) had a significant effect ($P< 0.05$) on BGLU activity over time during study period (Figure 2.8.B and Figure 2.8.C). Mean values of BGLU activity for near CWD, away CWD and control (without CWD) were 0.115 ± 0.009, 0.090 ± 0.009, and 0.072 ± 0.008 μmol $hr^{-1}g^{-1}$, respectively. In LFH plots, CWD showed the significant effects on the enzyme activity in June 2011, June 2012 and during 2013 (June and September). Near CWD exhibited higher enzyme activity compared to away CWD and control in June 2011; however, it showed higher enzyme activity than only control in June 2012 and during 2013 (Figure 2.8.B and Table 2.2). In PMM plots, the only significant effect of CWD was observed during the last sampling time (September 2013) when near CWD displayed higher enzyme activity than away CWD and control (Figure 2.8.C and Table 2.2).

The results of the effect of seasons on BGLU activity revealed that there was a significant effect ($P< 0.05$) of seasonality on the enzyme activity with greater enzyme activity in September (0.122 ± 0.010 μmol $hr^{-1}g^{-1}$) than in June (0.085 ± 0.007 μmol $hr^{-1}g^{-1}$) (Table 2.3). The effect of seasonality on BGLU activity was more noticeable during 2012 when significantly greater enzyme activity was observed in September (0.141 ± 0.014) than in June (0.076 ± 0.009 μmol $hr^{-1}g^{-1}$) (Figure 2.9.A).

BGLU activity showed a significant change ($P< 0.05$) over time during study period when the enzyme activity increased significantly from 2011 to 2012 and did not change from 2012 to 2013 (Figure 2.9.B). Mean values of BGLU activity were 0.050 ± 0.007, 0.109 ± 0.010, and 0.098 ± 0.007 μmol $hr^{-1}g^{-1}$ in 2011, 2012, and 2013, respectively.

Generally, the results showed that LFH capping material significantly increased BGLU activity compared to PMM. CWD exhibited a significant effect on BGLU activity and the enzyme activity was also significantly affected by seasonality and was greater in in September than June. Furthermore, a significant increasing trend of BGLU activity was observed principally from 2011 to 2012.

LFH and PMM as capping materials showed significant effect ($P< 0.05$) on NAGase activity during study period from 2011 to 2013. LFH (0.078 ± 0.005 μmol $hr^{-1}g^{-1}$) showed higher NAGase activity as compared to PMM (0.055 ± 0.004 μmol $hr^{-1}g^{-1}$) (Figure 2.10.A and Table 2.1). The effect of LFH and PMM on the enzyme activity was compared and showed that the significant differences in the enzyme activity between LFH and PMM occurred during 2012 (June and September) and September 2013 (Figure 2.10.A). The effect of LFH and PMM capping materials on NAGase activity was investigated from June 2011 to September 2013. In plots amended with LFH, the enzyme activity did not show any significant change from 2011 (June) to 2012 (June); however it significantly increased in September 2012 and then decreased in June 2013 and after that significantly increased in September 2013 (Figure 2.10.A). In plots amended with PMM, no significant change in enzyme activity occurred from June 2011 to June 2012; however it increased significantly in September 2012 and remained unchanged during 2013 (Figure 2.10.A).

CWD amendment (control, away CWD, near CWD) exhibited a significant effect ($P< 0.05$) on NAGase activity over time (Figure 2.10.B and Figure 2.10.C). The average values of NAGase activity for near CWD, away

CWD and control were 0.077 ± 0.007, 0.063 ± 0.005, and 0.059 ± 0.005 μmol $hr^{-1}g^{-1}$, respectively.

In both LFH and PMM plots, the significant effect of CWD on the enzyme activity was observed during 2011 and 2012. In LFH plots, near CWD exhibited significantly higher enzyme activity compared to away CWD and control in June 2011 (Figure 2.10.B and Table 2.2). In PMM plots, near CWD showed significantly higher enzyme activity than control in June 2012 (Figure 2.10.C and Table 2.2).

Seasonality showed a significant effect ($P< 0.05$) on NAGase activity over time. Greater NAGase activity was observed in September (0.086 ± 0.006 μmol $hr^{-1}g^{-1}$) than in June (0.064 ± 0.005 μmol $hr^{-1}g^{-1}$) (Table 2.3). The effect of seasonality on the enzyme activity was especially observed in 2012 when the enzyme activity in September (0.084 ± 0.006 μmol $hr^{-1}g^{-1}$) was significantly higher than June (0.046 ± 0.005 μmol $hr^{-1}g^{-1}$) (Figure 2.11.A).

The change of NAGase activity is shown in Figure 2.11.B. A significant increasing trend ($P< 0.05$) was observed in the enzyme activity over time from 2011 to 2013. The mean values of NAGase activity in 2011, 2012, and 2013 were 0.035 ± 0.004, 0.066 ± 0.005, and 0.084 ± 0.005 μmol $hr^{-1}g^{-1}$, respectively.

Overall, the results of NAGase activity showed that LFH significantly enhanced the enzyme activity compared to PMM. CWD application showed a significant effect on NAGase activity and greater enzyme activity was observed in September than June. A significant change of NAGase activity was also observed over time from 2011 to 2013.

2.4 Discussion

The reestablishment of the soil microbial communities, which plays a key role in many ecosystem processes such as decomposition, nutrient cycling and enzyme production, is critical in the successful reclamation of disturbed areas

(McMillan et al. 2007; Norris et al. 2013; Jamro et al. 2014). Our results suggest that various aspects of the soil microbial communities recover at different rates and are strongly mediated by the choice of capping material and CWD amendments used for the reclamation practices.

We quantified the development of the soil microbial communities using a suite of indices indicative of fully functioning ecosystems. From 2011 to 2013, the change of NAGase activity was very strong (Figure 2.11.B). The changes of MBC, MBN, respiration rate and BGLU activity were more subtle, but they all developed steadily as well (Figure 2.3.B, 2.5.B, 2.7.B, and 2.9.B, respectively) The differences we observed in the developmental changes of the soil microbial indices were likely linked to: (1) differences in the soil OM content; (2) vegetation community differences; (3) seasonal variation; and (4) differences in supplemental amendment material, such as CWD, applied to the reclamation site (Brown and Naeth, 2014; Jamro et al. 2014). How each point affected the developing soil microbial communities will be discussed in turn.

High OM content tends to be an indicator of high soil microbial activity (Hahn and Quideau, 2013). The OM content and C:N ratio of the capping materials used in this study (i.e. LFH and PMM) are quite different (Jamro et al. 2014) and likely played an important role in the development of soil enzyme activities. The LFH capping material had more decomposed OM (Jamro et al. 2014) and thus yielded greater MBC and MBN than PMM capping material (Figure 2.2.A and 2.4.A). Contrary to other results of microbial indicators mentioned above, respiration rate was not changed between capping materials. However, numerically, it was found greater in PMM than in LFH (Figure 2.6.A). The activities of the BGLU and NAGase enzymes were higher in the LFH (Figure 2.8.A and 2.10.A) and may be due to changes in the MBC or MBN (Andersson et al. 2004; Brockett et al. 2012). The NAGase and BGLU activities have also been linked with the soil fungal biomass (Lucas and Casper, 2008) and may be dependent on mycorrhizal biomass specifically. A previous study using the same sites demonstrated that LFH had greater mycorrhizal biomass than did the PMM

(Brown and Naeth, 2014). Mycorrhizal fungi produce many extracellular enzymes, including NAGase and BGLU, and are closely involved in C and N mineralization activities (Smith and Read, 2010). It has also been suggested that the activities of NAGase and BGLU are greater where fungal dominance is greater (Fichtner et al. 2014). Given the potential importance of mycorrhizal fungi in reestablishing functioning of C and N cycles, it is important to understand their role and their potential for accelerating oil sand reclamation activities by selecting capping materials hospitable to their development or by applying mycorrhizal inoculant materials. It is also possible that fungi are more important than addition of other nutrients to encourage the reclamation process especially by affecting N-cycle processes and extracellular enzyme activities. Further work is needed on this potentially important topic in oil sands reclamation.

The soil microbial communities are highly dependent on the vegetation community (Mummey et al. 2002; Hahn and Quideau, 2013). Through a process known as priming (Kuzyakov et al. 2000), plants influence the development of the soil microbial communities by altering the quality and quantity of litterfall and root exudates, the necessary substrates for microbial function and activities (Xu et al. 2010). In our study, the increase in MBC, MBN, NAGase, BGLU and respiration rate we observed over a time may be associated with differences in vegetation cover. LFH plots were dominated by woody species that covered approximately 65% of the experimental plot land area whereas PMM plots tended to be dominated by bryophytes that covered approximately 33% of the land area (Forsch, 2014). NAGase activity was strongly associated with litterfall material (Baldrian et al. 2008) and BGLU activity was linked with presence of vegetation (Zhang et al. 2010). The lower enzyme activity we observed in the PMM may be associated with higher soil pH in PMM than in LFH (Jamro et al. 2014). The higher pH of PMM is linked to the fact that the salvaged mineral soil which was mixed with peat is alkaline in nature (Fung and Macyk, 2000). Soil pH affects microbial community structure (Will et al. 2010) and nutrient cycling (Kemmit et al. 2006). Generally, fungi grow better in acidic environment than bacteria which prefer neutral or alkaline soil pH (Will et al. 2010). Hence, soil pH may alter soil

microbial composition and may affect the soil enzyme activities since they require pH optima (Baldrian et al. 2008). Seasonal fluctuations have an important role in the reestablishment of soil microbial communities and their associated functions. Typically in the oil sands region of northern Alberta where this study is concentrated, the soil temperature, water content, soil pH and substrate availability vacillate between cold and warm periods (Baldrian et al. 2008). During the summer, soil enzyme activities and other microbial dynamics and functions are regulated by changes in temperature and soil water content (Baldrian et al. 2008, Criquet et al. 2002). The addition of fresh litter inputs, rich in available nutrients and labile C substrate to soil, via litter and root exudates inputs at the end of growing season, provide a fresh energy source to soil heterotrophic microorganisms. We observed the greatest MBC, respiration rate, BGLU and NAGase activities in September (Figure 2.3.A, 2.7.A, 2.9.A, and 2.11.A) throughout our study and this was likely associated with fresh litter inputs and labile C substrate during the autumn. This observation is consistent with Jamro et al. (2014) who observed similar trend during a litter fall period in oil sands reclamation. In support of our observations, the MBC and MBN (Baldrian et al. 2008), CO_2 release (Goupil and Nkongolo, 2014), BGLU (Snajdr et al. 2011) and NAGase (Jamro et al. 2014) activities can be altered by fresh litter inputs. Baldrian et al. (2008) observed the increase in soil enzyme activities and MBC during October in brown coal reclaimed soil. The decomposition of fresh litter likely released labile organic and inorganic compounds in a soil system and consequently increased microbial activities (Fontaine et al. 2004; Burke et al. 2011). Variation in precipitation was also observed during our study (Appendix-I). Precipitation may serve as a trigger of change in water and substrate availability (Austin et al. 2004) and affect the decomposition of OM (Collins et al. 2008). As a result, it may have effect on microbial indicators over the three years of this study. These results are also in accordance with previous findings on the development of microbial community composition in oil sands reclamation (Hahn and Quideau, 2013) which showed that soil microbial composition in oil sands reclamation was greatly responsive to changes in moisture than in natural forest

sites of adjacent area. The application of CWD has been recognized as a means to accelerate the development of soil microbial communities and reclamation processes in the oil sands regions (Brown and Naeth, 2014). Our results showed that there is an increasing trend in respiration rate, BGLU and NAGase activities following CWD application, suggesting the application of CWD results in microsite development and the creation of "carbon islands" which provide a source of C (Spears and Lajtha, 2004; Gonzalez-Polo et al. 2013) and enhance C and N turnover rates. The increase in respiration rate and enzyme activities by CWD could be explained by several mechanisms including buffering of environmental changes and increasing DOC content (Gonzalez-Polo et al. 2013). The application of CWD creates microsites for microbial development, provides an energy source for microbes and increases nutrient availability (Gonzalez-Polo et al. 2013). As a result, it increases plant growth and productivity (Pyle and Brown, 1999) and subsequently alters the soil microenvironment. In a pioneer study focused on oil sands reclamation, Brown and Naeth et al. (2014) showed that CWD increased the recovery of flora as well as the recovery of nutrient cycling and water holding capacity. In addition, Rowland et al. (2009) suggested that greater incidence of CWD on the surface of reclaimed oil sands soils may contribute to ecosystem development through the growth of fungal mycelia, proliferation of chitin degrading bacteria as well as the growth of actinomycetes. The growth of these microbial communities may accelerate the decomposition of OM (Durall et al. 2005). CWD application appeared to enhance litter decomposition and enzyme activities within the forest soil matrix by the leaching of DOC (Spears and Lajtha, 2004). Thus, in this study, we expect labile C availability to increase more in near CWD than in away CWD treatment. As a result, it increased respiration rate and BGLU activity (Figure 2.6.C and 2.8.B). The non-significant changes in MBC and MBN by CWD application likely associated with the legacy effect of CWD where it may take longer time to alter the MBC and MBN. In addition, leaching of DOC and DON from CWD may affect C and N cycling (Bantle et al. 2014) by increasing the C:N ratio and consequently increasing the gross N immobilization (Kwak et al. unpublished).

Moreover, the greater precipitation may enhance the leaching and runoff of DOC and DON from CWD in soil and can affect the soil processes (Hafner et al. 2005). It was observed in concurrent study by Forsch (2014) who showed positive relationship between plant species richness and slope of the block. It was indicated that plant species richness was greater in lower block amended with CWD than in upper block. This was likely due to the leaching and runoff of DOC and DON from CWD to soil from upper block to lower block. Results of this study are in agreement with the leaching of DOC and DON from CWD where no significant differences were observed between near and away CWD treatments in respiration rate and BGLU activity (Figure 2.6.C and 2.8.B). Our results suggest that CWD further enhanced the capacity of the LFH to favor the microbial activities than PMM.

2.5 Conclusion

In conclusion, results of this study indicated that the soil microbial communities and some of their associated functions were successfully reestablished over time. The success of the reestablishment was regulated by the decomposition potential of the capping material, vegetation composition, seasonal variations and the presence of CWD. However, the pace of development is an inherently slow process. Thus, it is recommended that multiple seasonal cycles be allowed for the soil microbial communities to develop in order to re-establish a self-sustaining ecosystem. The LFH capping material has been shown to be a better organic capping material than the PMM in terms of developing functioning soil microbial communities. But the availability of LFH is limited and we do not have a good understanding of the long term benefits of the LFH capping materials. Thus, future work needs to be conducted on the long term benefits of using LFH in oil sands reclamation. Additional application of CWD over capping material can result in the development of microsites and help to accelerate the development of the soil microbial communities in oil sands reclamation. However, it may require longer time periods to explore the real role of CWD in the development of microbial communities and their processes in oil sands

reclamation. Finally, more research is needed to better understand the relationships between vegetation composition, environmental factors and the resulting soil microbial community development. Such knowledge is necessary in order to estimate the development of self-sustaining ecosystem in oil sands reclamation and to further improve current reclamation practices.

Table 2. 1 Effect of capping materials on microbial parameters after oil sands reclamation

Soil microbial parameters[a]	Capping type	
	LFH	PMM
MBC (mg g^{-1})	0.445 (0.040)	0.280 (0.026)
MBN (mg g^{-1})	0.036 (0.003)	0.023 (0.001)
Respiration (nmol CO_2 g^{-1} hr^{-1})	49.16 (3.73)	54.59 (5.59)
BGLU (μmol hr^{-1} g^{-1})	0.118 (0.008)	0.068 (0.005)
NAGase (μmol hr^{-1} g^{-1})	0.078 (0.005)	0.055 (0.004)

[a] Soil microbial parameters: MBC = Microbial Biomass C, MBN = Microbial Biomass N, Respiration = respiration rate, BGLU = 1, 4-β-glucosidase, NAGase = 1, 4-β-N-acetylglucosaminidase.

Table 2. 2 Effect of CWD on microbial parameters after oil sands reclamation

Soil microbial Parameters[+]	Cap. type	CWD	2011 June	2012 June	2012 September	2013 June	2013 September
MBC ($mg\ g^{-1}$)	LFH	Control	0.200 a*	0.244 a	0.401 a	0.314 a	0.890 b
		Away	0.200 a	0.301 ab	0.631 b	0.380 ab	0.598 b
		Near	0.252 a	0.345 ab	0.623 bc	0.510 ac	0.793 c
	PMM	Control	0.139 a	0.199 a	0.371 a	0.300 a	0.167 a
		Away	0.139 a	0.247 ab	0.465 b	0.329 ab	0.173 a
		Near	0.201 a	0.272 a	0.604 b	0.354 ab	0.237 a
MBN ($mg\ g^{-1}$)	LFH	Control	0.021 a	0.037 a	0.042 a	0.046 a	0.036 a
		Away	0.021 a	0.031 a	0.040 a	0.028 a	0.030 a
		Near	0.031 a	0.046 a	0.049 a	0.035 a	0.042 a
	PMM	Control	0.016 a	0.025 a	0.025 a	0.026 a	0.013 a
		Away	0.016 a	0.019 a	0.021 a	0.024 a	0.024 a
		Near	0.020 a	0.021 a	0.035 a	0.027 a	0.032 a
Respiration ($nmol\ CO_2\ g^{-1}\ hr^{-1}$)	LFH	Control	15.24 a	45.56 bc	48.74 bc	33.78 ab A	64.98 c A**
		Away	15.24 a	48.49 bc	49.45 bc	44.39 b AB	67.16 c A
		Near	14.34 a	60.70 b	70.45 b	61.58 b B	97.38 c B
	PMM	Control	11.82 a	40.08 a	45.26 a	37.83 a	46.64 a A
		Away	11.82 a	51.57 ac	72.53 bc	47.32 ab	93.96 c B
		Near	12.79 a	73.20 b	88.69 bc	67.59 b	117.78 c B
BGLU ($\mu mol\ hr^{-1}\ g^{-1}$)	LFH	Control	0.041 a A	0.080 a A	0.178 b	0.082 a A	0.062 a A
		Away	0.041 a A	0.102 b AB	0.199 c	0.105 b AB	0.138 b B
		Near	0.106 a B	0.140 a B	0.204 b	0.141 a B	0.145 a B
	PMM	Control	0.037 a	0.034 a	0.080 b	0.070 ab	0.058 ab A
		Away	0.037 a	0.047 ab	0.087 c	0.074 bc	0.072 ac A
		Near	0.041 a	0.054 ac	0.101 b	0.086 bc	0.140 d B
NAGase ($\mu mol\ hr^{-1}\ g^{-1}$)	LFH	Control	0.030 a A	0.054 ab	0.092 b	0.068 ab	0.099 b
		Away	0.030 a A	0.057 ab	0.108 c	0.081 bc	0.094 bc
		Near	0.057 a B	0.073 a	0.119 b	0.094 ab	0.123 b
	PMM	Control	0.029 a	0.026 a A	0.054 ab	0.070 ab	0.072 ab
		Away	0.029 a	0.029 a AB	0.059 ab	0.083 b	0.067 ab
		Near	0.033 a	0.043 ab B	0.074 bc	0.088 c	0.069 ac

[+] Soil microbial parameters: MBC = Microbial Biomass C, MBN = Microbial Biomass N, Respiration = respiration rate, BGLU = 1, 4-β-glucosidase, NAGase = 1, 4-β-N-acetylglucosaminidase.

* Means with different lowercase letters indicate significant difference between month and year in each row.

** Means with capital letters indicate significant difference among CWD treatments (control, away and near) in each column. Numbers without capital letters indicate no significant difference among CWD treatments.

Table 2. 3 Effect of season (month) on microbial parameters after oil sands reclamation

Soil microbial parameters[a]	Month	
	June	September
MBC (mg g^{-1})	0.316 (0.023)	0.496 (0.049)
MBN (mg g^{-1})	0.031 (0.002)	0.033 (0.003)
Respiration (nmol CO_2 g^{-1} hr^{-1})	51.01 (2.37)	71.92 (5.77)
BGLU (μmol hr^{-1} g^{-1})	0.085 (0.007)	0.122 (0.010)
NAGase (μmol hr^{-1} g^{-1})	0.064 (0.005)	0.086 (0.006)

[a] Soil microbial parameters: MBC = Microbial Biomass C, MBN = Microbial Biomass N, Respiration = respiration rate, BGLU = 1, 4-β-glucosidase, NAGase = 1, 4-β-N-acetylglucosaminidase.

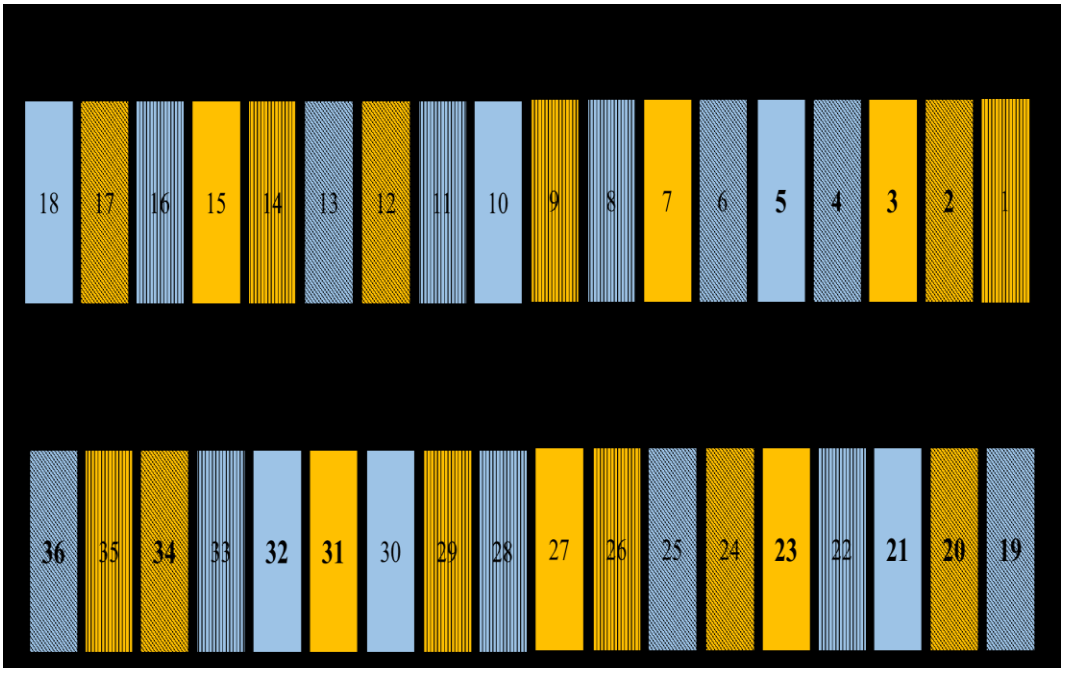

Figure 2. 1 Layout of experimental plots on the reclaimed oil sands site located at southeast dump at Suncor Energy Inc. Fort McMurray Alberta, Canada. Blue: plots covered with LFH (identifiable litter, fragmented and fermented litter and humus); Yellow: plots covered with PMM (peat mineral soil mix). Plots without texture are control (without coarse woody debris). Diagonal texture: plots have *Populus tremuloides* coarse woody debris; Vertical texture: plots have *Picea mariana* coarse woody debris; bold numbered plots (2, 3, 4, 5, 19, 20, 21, 23, 31, 32, 34, and 36) were used for sampling of this study (Brown 2010).

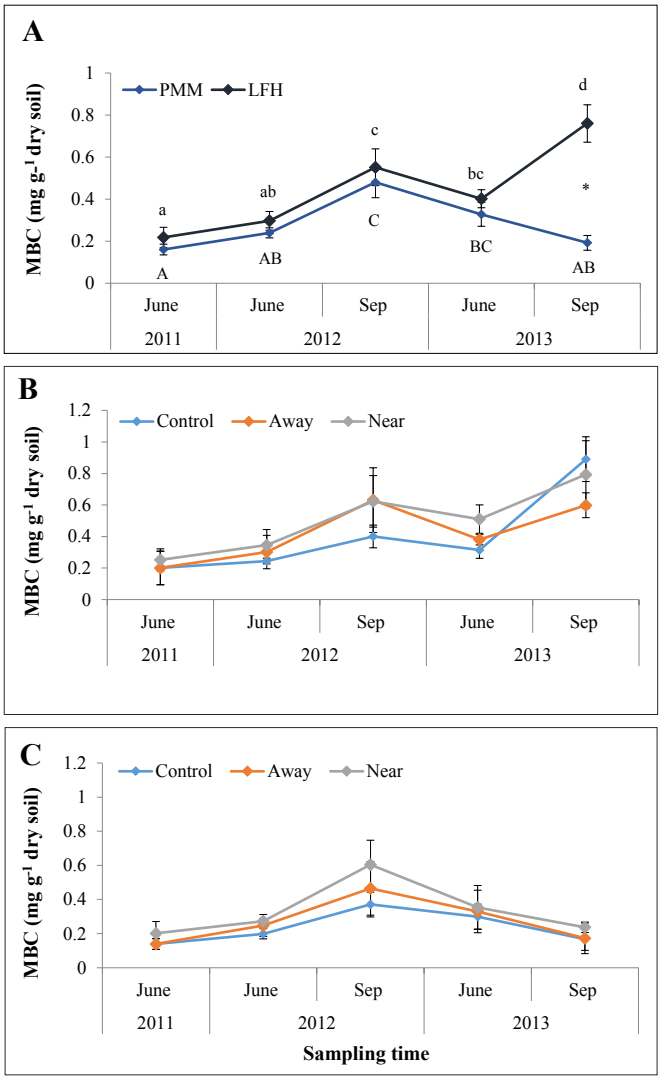

Figure 2. 2. Effect of (A) capping materials [identifiable litter, fragmented and fermented litter and humus (LFH) and peat mineral soil mix (PMM)], (B) coarse woody debris (CWD) over LFH, and (C) CWD over PMM on microbial biomass C (MBC) after reclamation. Soil samples for CWD treatments were collected from control (non-CWD), away from CWD, and near CWD. Error bars represent standard error of the means in treatments. Lowercase letters show differences among means of LFH capping material over time. Capital letters indicate differences among means of PMM capping material over time. * indicates significant differences between LFH and PMM treatments (α= 0.05).

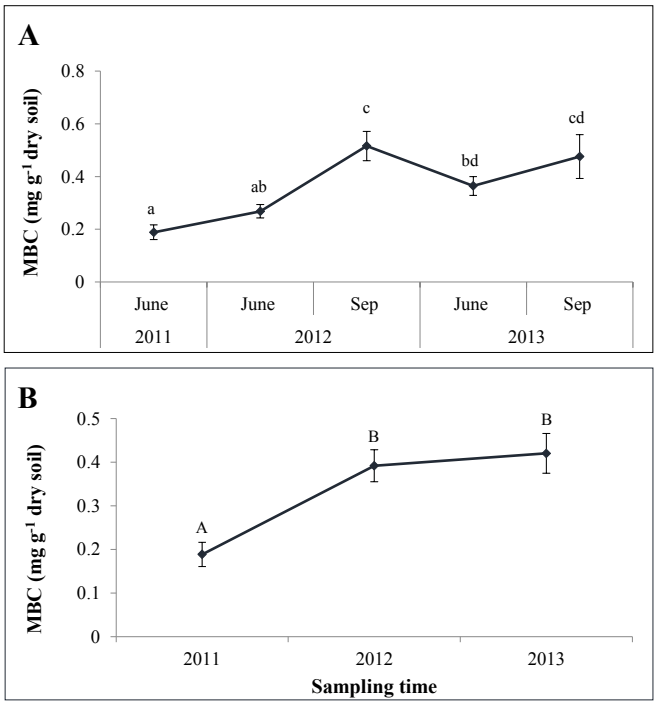

Figure 2. 3. Effect of (A) season (month) and (B) time (year) on microbial biomass C (MBC) after reclamation. Error bars represent standard error of the means calculated for different months and years. Different letters above bars indicate that the values over time are significantly different.

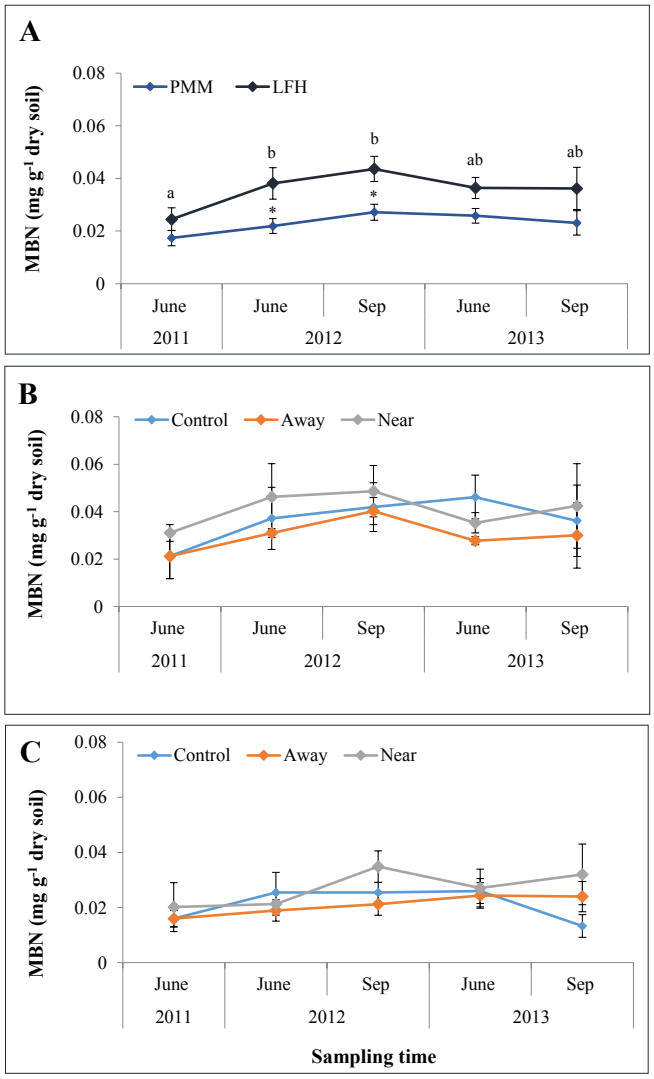

Figure 2. 4. Effect of (A) capping materials [identifiable litter, fragmented and fermented litter and humus (LFH) and peat mineral soil mix (PMM)], (B) coarse woody debris (CWD) over LFH, and (C) CWD over PMM on microbial biomass N (MBN) after reclamation. Soil samples for CWD treatments were collected from control (non-CWD), away from CWD, and near CWD. Error bars represent standard error of the means in treatments. Lowercase letters show differences among means of LFH capping material over time. Capital letters indicate differences among means of PMM capping material over time. * indicates significant differences between LFH and PMM treatments (α= 0.05).

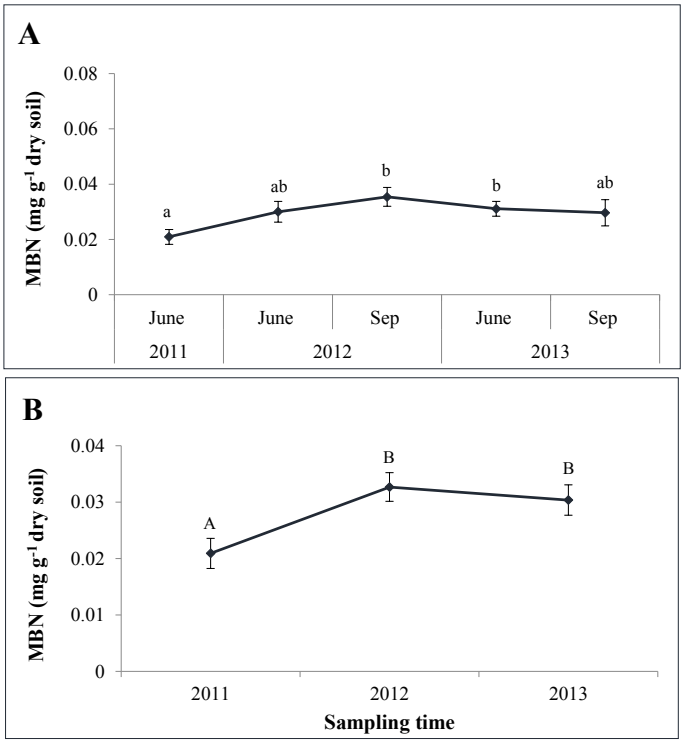

Figure 2. 5 Effect of (A) season (month) and (B) time (year) on microbial biomass N (MBN) after reclamation. Error bars represent standard error of the means calculated for different months and years. Different letters above bars indicate that the values over time are significantly different.

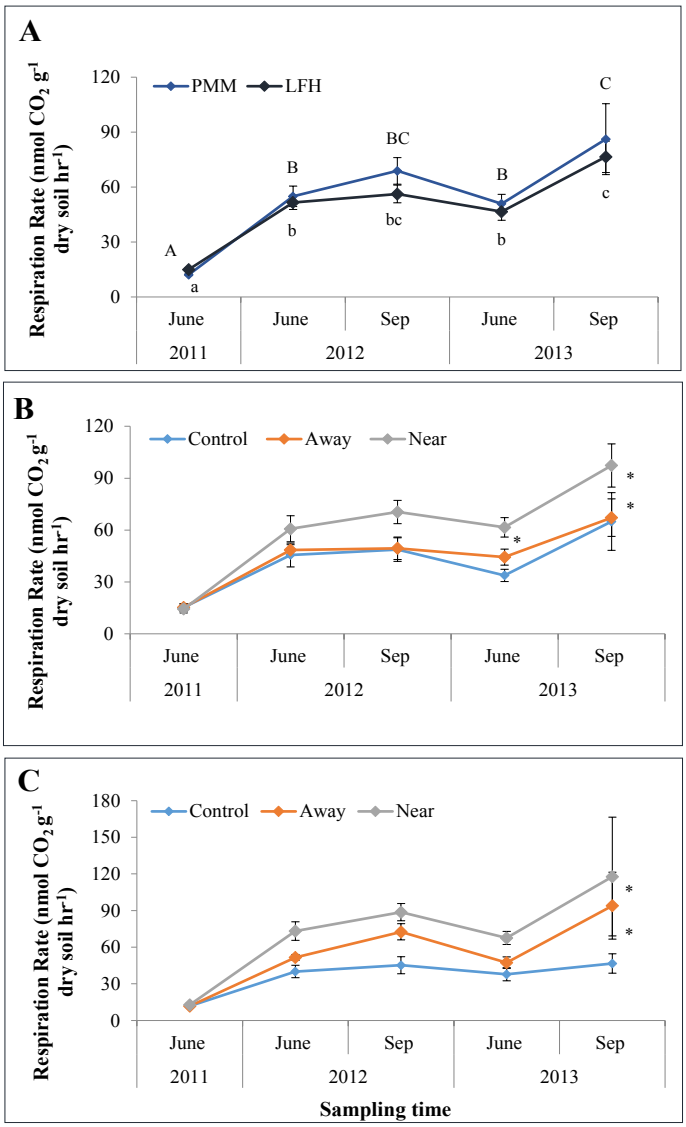

Figure 2. 6. Effect of (A) capping materials [identifiable litter, fragmented and fermented litter and humus (LFH) and peat mineral soil mix (PMM)], (B) coarse woody debris (CWD) over LFH, and (C) CWD over PMM on respiration rate after reclamation. Soil samples for CWD treatments were collected from control (non-CWD), away from CWD, and near CWD. Error bars represent standard error of the means in treatments. Lowercase letters show differences among means of LFH capping material over time. Capital letters indicate differences among means of PMM capping material over time. * indicates significant differences between LFH and PMM treatments (α= 0.05).

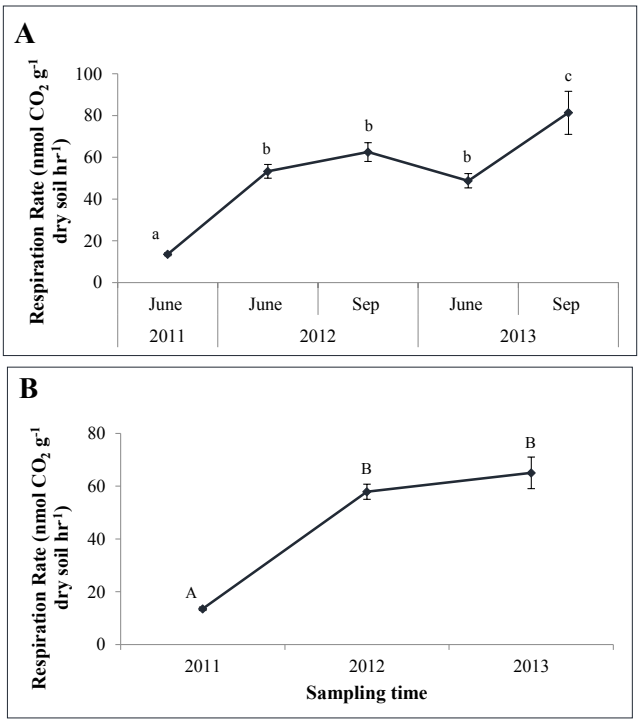

Figure 2. 7 Effect of (A) season (month) and (B) time (year) on respiration rate after reclamation. Error bars represent standard error of the means calculated for different months and years. Different letters above bars indicate that the values over time are significantly different.

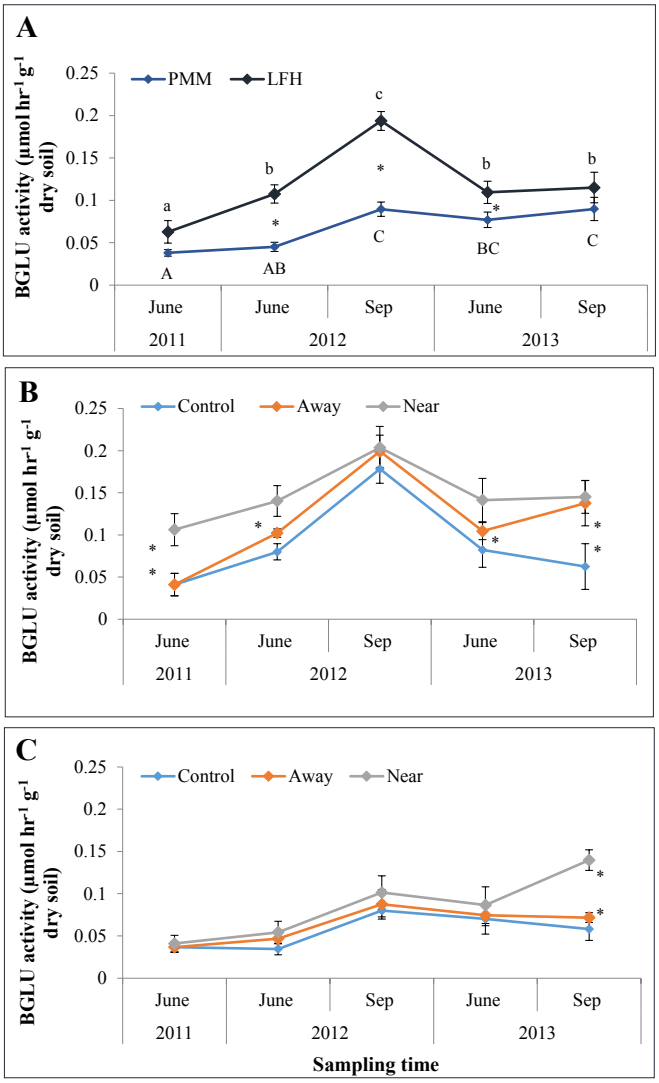

Figure 2. 8 Effect of (A) capping materials [identifiable litter, fragmented and fermented litter and humus (LFH) and peat mineral soil mix (PMM)], (B) coarse woody debris (CWD) over LFH, and (C) CWD over PMM on 1, 4-β-glucosidase (BGLU) activity after reclamation. Soil samples for CWD treatments were collected from control (non-CWD), away from CWD, and near CWD. Error bars represent standard error of the means in treatments. Lowercase letters show differences among means of LFH capping material over time. Capital letters indicate differences among means of PMM capping material over time. * indicates significant differences between LFH and PMM treatments (α= 0.05).

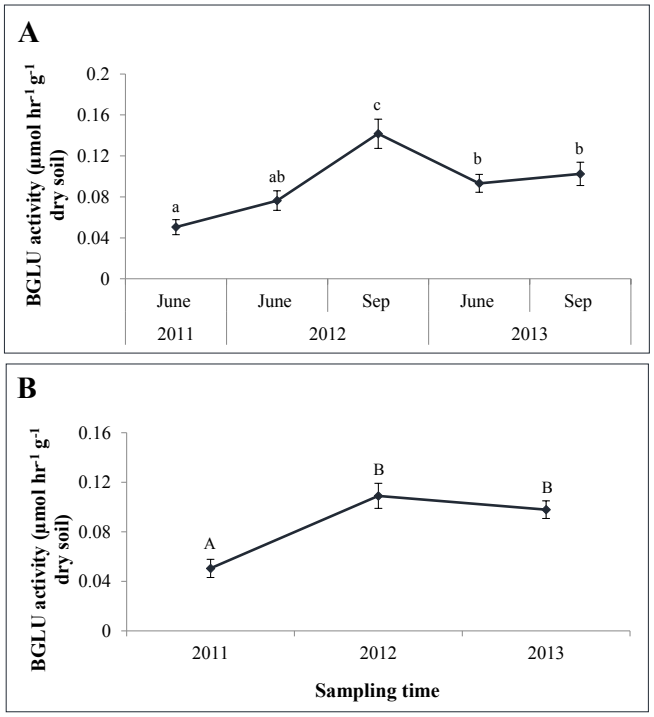

Figure 2. 9 Effect of (A) season (month) and (B) time (year) on 1, 4-β-glucosidase (BGLU) activity after reclamation. Error bars represent standard error of the means calculated for different months and years. Different letters above bars indicate that the values over time are significantly different.

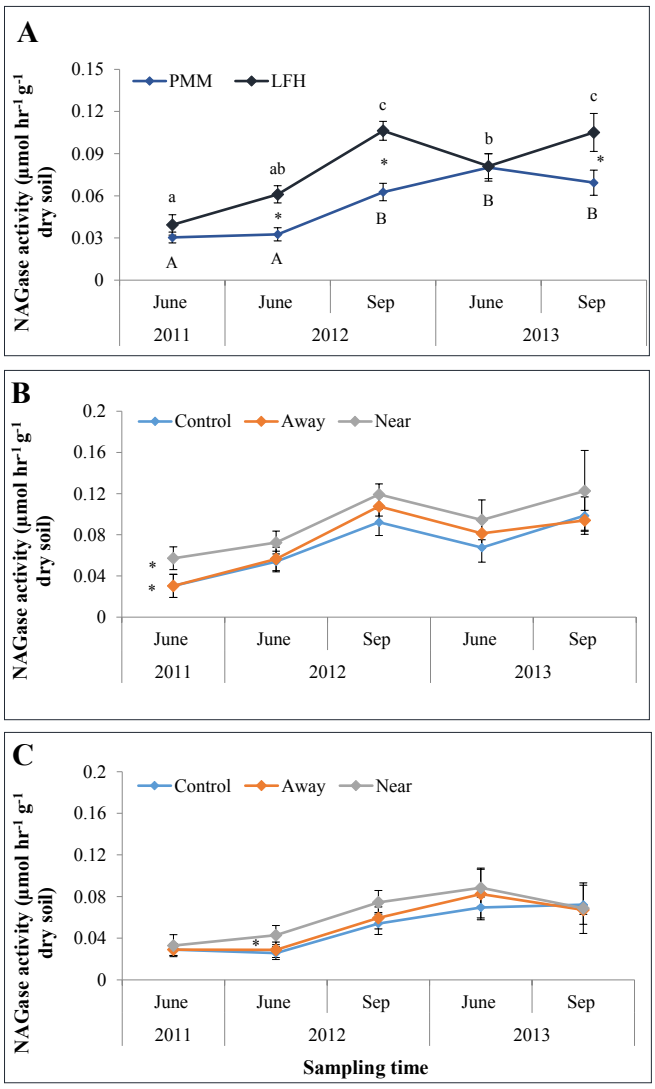

Figure 2. 10 Effect of (A) capping materials [identifiable litter, fragmented and fermented litter and humus (LFH) and peat mineral soil mix (PMM)], (B) coarse woody debris (CWD) over LFH, and (C) CWD over PMM on 1, 4-β-N-acetylglucosaminidase (NAGase) activity after reclamation. Soil samples for CWD treatments were collected from control (non-CWD), away from CWD, and near CWD. Error bars represent standard error of the means in treatments. Lowercase letters show differences among means of LFH capping material over time. Capital letters indicate differences among means of PMM capping material over time. * indicates significant differences between LFH and PMM treatments (α= 0.05).

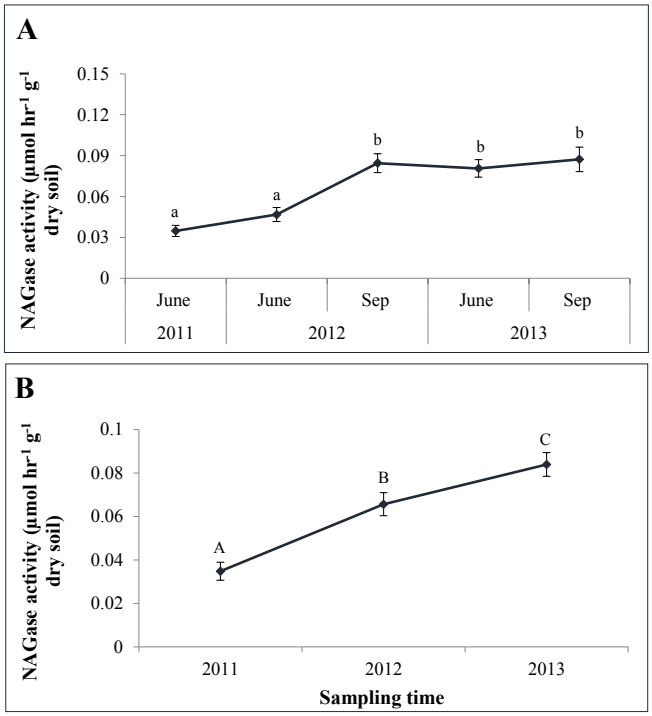

Figure 2. 11 Effect of (A) season (month) and (B) time (year) on 1, 4-β-N-acetylglucosaminidase (NAGase) activity after reclamation. Error bars represent standard error of the means calculated for different months and years. Different letters above bars indicate that the values over time are significantly different.

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Chapter 3. Quantification of functional genes associated with different nitrogen transformation processes in reclaimed oil sands site

3.1 Introduction

Nitrogen (N) cycle is one of the most important ecosystem functions driven by microorganisms (He et al. 2010). For many years, the N cycle was considered as a combination of simple pathways in which free living or symbiotic N_2 fixing microorganisms imparted ammonium (NH_4^+) for assimilation (Beijerinck, 1888), and then excess NH_4^+ was oxidized via nitrite (NO_2^-) to nitrate (NO_3^-) by nitrifying microorganisms (Winogradsky, 1890) and at last, the oxidized nitrogen compounds were converted back to N_2 by denitrifying microorganisms to complete the N cycle (Gayon and Dupetit, 1886). However, advances in molecular methods and new sequence technologies have proved that our understanding of microbial N cycle is not complete yet and more research and studies are required to uncover enormous functional diversity of microbial communities involved in this cycle. Generally, N cycle in soil and terrestrial ecosystems is composed of two main cycles (organic and inorganic) which are interlinked by NH_4^+. Organic N cycle is characterized by the conversion of NH_4^+ to organic N and reconversion of biomass into NH_4^+. However, microbial communities and molecular processes of this cycle are not completely understood (Hai, 2010). Instead, inorganic N cycle and its associated molecular processes and communities involved in, have been better understood and studied. In this study, among all inorganic nitrogen transformation processes, the key processes such as biological nitrogen fixation, nitrification and denitrification and genes involved in these processes, were focused which are mainly impacted by soil management; however other processes are described briefly (Figure 3.1).

3.1.1 Nitrogen fixation

N_2 fixation is considered as the dominant and important natural process in many ecosystems (Houlton et al. 2008). It is the only natural way by which atmospheric N_2 is introduced into the ecosystem through its reduction to NH_4^+ (Turk et al. 2011). N_2 fixation is performed by symbiotic, associative and free living microorganisms generally called diazotrophs. It restores the overall soil available N content which is lost via denitrification and anaerobic NH_4^+ oxidation. Thus, this process is of great importance for ecosystem productivity (Vitousek et al. 2002). Typically, N_2 fixation is performed by phylogenetically diverse microbial groups within the domains Bacteria and Archaea (Young, 1992). Major groups of diazotrophs include Cyanobacteria, Chlorobi (green sulfur bacteria), Archaea, Firmicutes, as well as proteobacterial groups *Azotobacteracea*e, *Rhizobia*, and the Actinobacteria (*Frankia*). In Archaea, N_2 fixation is mainly restricted to methanogens, and was first described in *Methanosarcina barkeri* (Murray and Zinder, 1984), and *Methanococcus thermolithotrophicus* (Belay et al. 1984). All organisms capable of fixing N_2 require the presence and activity of nitrogenase enzyme complex (Raymond et al. 2004). There are four distinct nitrogenases which differ genetically: Iron-based (Fe-), molybdenum-based (Mo-), vanadium-based (V-) nitrogenase (Newton 2006), and the fourth one, MoFeS-nitrogenase has been found in *Streptomyces thermoautotrophicus*, and has a totally different structure from those three types (Ribbe et al. 1997). Among these nitrogenases, Mo-nitrogenase has been most studied and all N_2 fixing bacteria except for the ones which carry the fourth nitrogenase have this enzyme (Newton, 2006). This enzyme has two protein parts: one part "MoFe- protein" encoded by *nifD* and *nifK* genes, is called dinitrogenase and another part "Fe-protein" encoded by *nifH* gene, is known as dinitrogenase reductase (Burris, 1991). The presence of both parts is required for activity of the enzyme. *nifH*, known as N_2 fixation gene, highly conserved among diverse microorganisms, is one of the widely used biomarkers to identify N_2 fixing microorganisms and monitor their potential for N_2 fixation (Rosado et al. 1998; Rösch et al. 2002). Typically, N_2

fixing communities are susceptible to external changes and disturbances, and have been classified as potential key indicators for assessing soil health (Doran et al. 1997). In several studies, *nifH* has been used as a proxy to monitor the abundance and diversity of N_2 fixing communities (Diallo et al. 2004; Patra et al. 2006; Poly et al. 2001; Taketani et al. 2009).

3.1.2 Nitrification

Nitrification is a two-step process; the first and rate limiting step "ammonia-oxidation", is the conversion of NH_4^+ to NO_2^-, and the second step "NO_2^- oxidation", is subsequent conversion of NO_2^- to NO_3^- (Bothe et al. 2000; Prosser and Embley, 2002). The NH_4^+ is first oxidized to hydroxylamine (NH_2OH) by ammonia monooxygenase enzyme (AMO) before its conversion to NO_2^-. This enzyme is a membrane-bound enzyme and consists of three subunits (α, β, and γ), which are encoded by genes *amoA*, *amoB*, and *amoC*, respectively. The first gene, *amoA* has a well-established database than *amoB* and *amoC* (Francis et al. 2005), and is functionally significant and highly conserved. It is mostly used as a functional molecular marker for detecting the abundance of ammonia oxidizers (Rotthauwe et al. 1997).

All ammonia-oxidizers possess the *amoA* gene which encodes the "α-subunit of AMO and is considered as the key gene regulating the ammonia oxidation process (Hyman and Wood, 1985). Ammonia oxidation is performed by microbial groups which belong to both Bacteria and Archaea (Schleper et al. 2005; Francis et al. 2007). However, until recently it was believed that this process was restricted to chemolithoautotrophic ammonia-oxidizing bacteria (AOB) belonging to two monophyletic lineages (Head et al. 1993; Purkhold et al. 2000): the first lineage belongs to betaproteobacteria and contains *Nitrosomonas* spp. (including *Nitrosococcus mobilis*) and *Nitrosospira* spp. (including *Nitrosolobus* and *Nitrosovibrio*); the second lineage belongs to gammaproteobacteria and comprises *Nitrosococcus* spp. In the past few years, metagenomic studies from seawater and soil (Venter et al. 2004; Treusch et al.

2005) showed that ammonia-oxidizing archaea (AOA), a more taxonomically diverse group within the phylum Thaumarchaeota, also possess the metabolic capacity of ammonia-oxidation; and homologs of *amoA* gene was found in the members of this phylum (Treusch et al. 2005). Cultivation of an ammonia-oxidizing archaeon called *Nitrosopumilus martimus* SCM1 confirmed the findings of the metagenomic analyses (Könneke et al. 2005). These findings resulted in the discovery of AOA in marine environments (Francis et al. 2005). Studies have shown that AOA are found in various environments and ecosystems (Leininger et al. 2006; He et al. 2007; Nicol et al. 2008).

In some studies, it has been proved that AOA are even more dominant than AOB especially in some soil types (Leininger et al. 2006; Nicol et al. 2008; Zhang et al. 2012). However, knowledge of the relative importance of AOB and AOA in ammonia-oxidation process is not well studied. In other words, it is not clear if nitrification process widely is linked to archaea or bacteria (Jia and Conrad, 2009). It is believed that while occupying different environmental niches, AOB and AOA can respond in a different way to environmental changes and conditions. Di et al. (2010) found that AOB prefer surface soils and environments rich in nutrients while AOA favor deeper soil and environments low in nutrients. Some studies have shown that environmental changes can result in shifts in AOB and AOA composition and abundance (Avrahami et al. 2003; Horz et al. 2004). Thus, for better understanding of environmental changes and their consequences which affect the relationship between nitrification and microbial communities, both AOB and AOA for ecosystem functioning can be examined and evaluated (Kowalchuk and Stephen, 2001).

In the next step of nitrification, NH_2OH is oxidized to NO_2^-; this reaction is catalyzed by hydroxylamine dehydrogenase (HAO) (Wood, 1986). This enzyme is encoded by the *hao* gene (Bergmann et al. 2005). To date, some studies have been performed to investigate this biochemical pathway and its associated gene, *hao*, in some strains such as *Nitrosomonas europaea* (Arciero and Hooper, 1993; Sayavedra-Soto et al. 1994), *Nitrosococcus oceani*, and *Nitrosospira*

multiformis (Bergmann et al. 2005). However, the most ecological studies have focused on the first step of nitrification (Hai, 2010) and *amoA* gene.

Oxidation of NO_2^- to NO_3^- is the third step in nitrification process. This step is catalyzed by nitrite oxidoredoctase enzyme (NXR) and performed by a group of bacteria called nitrite-oxidizing bacteria (NOB). Among this phylum, *Nitrospira* spp. are the most diverse and abundant species. However, NXR enzyme has been well studied in *Nitrobacter* and consists of two subunits, NxrA and NxrB encoded by *nxrA* and *nxrB* genes, respectively (Sundermeyer-Klinger et al. 1984). However, studies on biochemical properties of NOB are limited and data on the enzyme and gene encoding the enzyme may not be generalized for all of NOB (Hai, 2010).

3.1.3 Anaerobic ammonia oxidation (Anammox)

Anammox can be considered as one of the significant sinks of inorganic N. In order to oxidize NH_4^+ to dinitrogen (N_2) gas, NO_2^- (instead of oxygen) is used as the electron acceptor in this process (Strous et al. 1999; Kartal et al. 2008). For a long time, enrichment and detection of microorganisms on NH_4^+ under anoxic conditions was unsuccessful. For this reason, it was believed that NH_4^+ could not activate under anaerobic conditions. However, this belief was changed (Strous and Jetten, 2004). For the first time, Mulder et al. (1995) found this process in wastewater treatment systems. Presently, different aspects of anammox process are investigating by many researchers and some strains of anammox bacteria have been detected and identified in various aquatic ecosystems (Kuypers et al. 2005; Penton et al. 2006; Kuenen, 2008). Generally, anammox bacteria are grouped as chemolithoautotrophic or mixotrophic bacteria (Kartal et al. 2008). Based on 16S rRNA gene sequence analyses, they form a monophyletic clade within the phylum *Planctomycetes* which belong to genera Brocadia, Scalindua and Kuenenia (Schmid et al. 2005). Significant population of this community exists in wastewater treatment plants and aquatic ecosystems.

However, they have not been isolated in pure culture, and their diversity, distribution and activity still are relatively unknown (Jetten, 2008).

3.1.4 Denitrification

Denitrification pathway, the major processes in the N cycle, is considered as the main sources of nitric oxide (NO), and nitrous oxide (N_2O) emissions to the atmosphere. This pathway is the stepwise reduction of NO_3^- and NO_2^- to gaseous forms of N such as NO, N_2O and N_2, by a respiratory process when oxygen (O_2) is limiting (Zumft, 1997). In this process, under O_2 limiting conditions, oxidized form of N is used as an alternative electron acceptor for energy production (Heylen et al. 2006). Typically, due to the loss of N from soil, denitrification is of great importance to soil N cycle. This process can be performed by a wide range of microorganisms known as denitrifiers, which are taxonomically very diverse (Zumft, 1997), and are within α and β sub-classes of proteobacteria. However, the genus *Pseudomonas* of γ-proteobacteria can also contribute with many of denitrifying bacteria (Zumft, 1997). Since there are various phylogenetic groups of bacteria capable of dentrification, 16S rRNA genes are not appropriate to be targeted for assessing denitrifiers (Wallenstein et al. 2006). Therefore, most investigation on this process in natural environments has focused on evaluation of functional genes which encode structural subunits of denitrification enzymes (Pastorelli et al. 2011). Four enzymatic reactions are involved in the process, and are catalyzed by four metalloproteins. The first step "the reduction of NO_3^- to NO_2^-" is catalyzed by nitrate reductase. This enzyme has been found in two major types of molybdo-enzymes known as NAR and NAP (Zumft, 1997). NAR is a membrane-bound enzyme involving in anaerobic nitrate respiration and denitrification, while NAP is a preplasmic enzyme involved in redox balancing. It has been suggested that nitrate reductase NAP may participate in denitrification under aerobic and anaerobic conditions. It has been proved that denitrifiers can carry either one or both of these enzymes. Genes *narG* and *napA* encode the active subunits of NAR and NAP, respectively (Philippot, 2002).

The second step, the reduction of NO_2^- to NO, is considered as a key step of denitrification, because of its role in producing gaseous forms of N and the removal of N from the pool of fixed N (Fang et al. 2010). Two structural different but functionally and physiologically equivalent types of nitrite reductase (Nir) have been found, which are involved in catalyzing nitrite reduction (Glockner et al. 1993): nitrite reductase contains either copper (NirK) (Fenderson et al. 1991) or cytochrome cd1 (NirS) (Zumft, 1997), which are encoded by *nirK* and *nirS* genes, respectively (Tavares et al. 2006; Wallenstein et al. 2006). It is assumed that cd1-type nitrite reductase is dominant in denitrifying bacteria, Cu-type nitrite reductase having more variations in immunological reactions and molecular weight, is found in more diverse taxa (Coyne et al. 1989). However, with only one exception, in each bacterial strain only one of these two types of nitrite reductase has been found, and within the same bacterial genus and even within the same bacterial species, the type of nitrite reductase (Nir type) may be different (Coyne et al. 1989; Heylen et al. 2006).

In the next step of denitrification, NO is catalyzed to N_2O by three types of metallo-enzymes, known as nitric oxide reductase (NOR) (Zumft, 1993). The difference of these enzymes is based on the type of electron donors which they use. For instance, cNOR (cytochrome nitric oxide reductase) enzyme, uses soluble c-type cytochromes or pseudoazurin; qNOR uses hydroquinones; and qCU_ANOR (quinol nitric oxide reductase) uses two kinds of electron donors called MQH_2 and cyctochrome c551 (Zumft, 1993). It is believed that even non-denitrifying organisms may also have NOR to use it in detoxification of toxic NO. Although, in a study conducted by Kwiatkowski et al (1997), it was stated that in *Rhodobacter sphaeroides*, this enzyme uses environmental NO for gaining energy instead of using it for detoxifying process.

The last step of denitrification, the conversion of N_2O to N_2 is catalyzed by nitrous oxide reductase enzyme (NOS). This enzyme is a periplasmic protein which is encoded by *nosZ* gene. It has been found that some of denitrifiers lack *nosZ* and some of them which possess the gene, do not use the enzyme, and N_2O

is the end product of denitrification in those denitrifiers (Stouthamer, 1988). Generally, a very diverse taxonomic group of bacteria, such as members of Proteobacteria, Actinobacteria, Aquificae, Deinococcus Thermus, Firmicutes, and Bacteroides are capable of denitrification (Zumft, 1997). Moreover, some fungi such as *Fusarium oxysporum* and *Fusarium solani* (Bollage and Tung, 1972; Shoun et al. 1992) and archaea (Zumft, 1997) have been found to have the ability to denitrify. However, it is believed that bacteria are the dominant microorganisms which are responsible of performing denitrification in most environments (Wallenstein et al. 2006). Very few studies have examined the contribution of archaea to denitrification in natural ecosystems especially in soil ecosystems (Wallenstein et al. 2006); however, it is believed that they also perform the pathway similar to bacteria, but with differences in structure and regulation of the denitrifying genes and enzymes (Philippot, 2002). Some extremophile archaea, such as hyperthermophile *Pyrobaculum aerophilum* and halophile *Haloferax denitrificans* have the ability of denitrification (Cabello et al. 2004). However, the extent of denitrification in non-extremophile archaea is still unknown (Wallenstein et al. 2006). Since denitrifiers belong to very diverse phylogenetic groups which do not have close phylogenetic relationship, 16S rRNA gene study is not a proper approach for detection of them in the environment. Thus, many studies have focused on functional genes as the main targets coding for structural subunits of the reductases (Zumft, 1997) such as *napA*, *narG*, *nirS*, *nirK*, *cnorB*, *qnorB*, and *nosZ* in denitrification process (Barker et al. 1998; Barker and Tiedje, 2003; Demanèche et al. 2009; Enwall et al. 2010; Philippot, 2002).

3.1.5 Aerobic denitrification

Normally, denitrification occurs under anaerobic conditions. Under oxic conditions, this process is repressed by inhibition of NO_3^- transport through cytoplasmic membrane (Moir and Wood, 2001). However, it has been revealed that this process can also occur when O_2 is present; for instance, in the presence of O_2, several isolated bacterial species were capable of reducing NO_2^- or NO_3^- to

gaseous N compounds (N_2O and N_2) (Bell et al. 1990; Kuenen, 2008). Among them, *Paracocus denitrificans* as a representative aerobic denitrifying bacterium is well known which can express a periplasmic nitrate reductase in the presence of O_2, this enzyme is not dependent on NO_3^- transport through the cytoplasmic membrane and is able to take over the first step of denitrification (Hayatsu et al. 2008). Typically, aerobic denitrifying bacteria are adapted to fluctuating oxic-anoxic conditions and have been isolated from different ecosystems (Patureau et al. 2000). *Mesorhizobium* sp. and *burkholderia cepacia* are two aerobic denitrifiers isolated from soils (Okada et al. 2005; Matsuzaka et al. 2003). These results prove that various bacteria are able to perform aerobic denitrification especially in arable soil (Hayatsu et al. 2008). However, in many species, the last enzyme in the denitrification process (NOS) is oxygen sensitive. For this reason, in most cases, aerobic denitrification is not complete and whenever the conditions switch from anaerobic to aerobic, an increase in amount of N_2O formation is detected (Frette et al. 1997).

3.1.6 Dissimilatory nitrate reduction to ammonia (DNRA)

This pathway is another process of NO_3^- reduction which results in the formation of reduced nitrogen compounds (NH_4^+) (Chen et al. 1995). Like denitrification, this process occurs under low O_2 concentration whenever NO_3^- in comparison to organic C is limiting (Kraft et al. 2011). The process is performed in two steps; the first step is the reduction of NO_3^- to NO_2^- which is followed by the second step of the reduction of NO_2^- to NH_4^+ (Mohan and Cole, 2007). It is believed that DNRA may be a way to conserve N in the ecosystem because NH_4^+ is less mobile than NO_3^- (Buresh and Patrick, 1978). In contrast to denitrification in which NO_3^- is utilized respiratorily as terminal electron acceptor, NO_3^- is used fermentatively as electron sink (Cole, 1990) in DNRA. In soil, under low nitrate concentrations and severely reducing conditions, DNRA occurs where there is an electron acceptor-limiting condition. In other words, when the concentration of NO_3^- as an electron acceptor is low, DNRA takes place under high C:NO_3^- ratios. In environments with high amounts of available organic C and low NO_3^-

concentration, DNRA is favored (Tiedje et al. 1983). The reduction of NO_3^- to NO_2^- in DNRA is supposed to being catalyzed by enzymes nitrite reductase NapAB, and NrfA nitrite reductase catalyzing the reduction of nitrite to ammonium. The functional gene *nrfA*, encoding NrfA nitrite reductase occurs in different groups of bacteria such as Gamma-, Delta- and Epsilon-proteobacteria (Smith et al. 2007) and some members of Bacteroides (Mohan et al. 2004). However, in most cases, the structural and functional characterization of this gene remains the task of more research (Kraft et al. 2011). DNRA also has been found in some denitrifying fungi. The fungi carry out this process under conditions which are more anoxic than conditions of denitrification (Zhou et al. 2001). Also, it has been shown that some denitrifying archaea are able to carry out DNRA similar to bacteria. In addition to denitrifiers, nitrate reductase also has been found in dissimilatory nitrate reductases with the ability of performing DNRA (Stolz and Basu, 2002). In order to investigate both denitrifiers and dissimilatory nitrate reductases, it has been shown that *narG* gene can be used as a proper molecular marker, while a gene such as *nosZ* is specific to denitrifiers and cannot be used as a marker to assess dissimilatory nitrate reductases (Horn et al. 2006; Philippot, 2002).

AOSR in northeastern Alberta, Canada is one of the single largest recoverable oil reserves of the world (Alberta Environment, 2006). Approximately 20% of oil reserves of AOSR are surface minable (Fung and Macyk, 2000). Open-pit mining technique of oil sands extraction leaves severe impacts on ecosystem sustainability and integrity. The disturbed land needs to be reconstructed completely to a level prior to the disturbance and oil sands companies are legally obligated to reclaime the disturbed land to pre disturbance conditions which have equivalent land capability (Alberta Environment, 2006) and can support self-sustaining ecosystem. To achieve the level of normal ecosystem functioning, it is utmost important to evaluate the success of reclamation practices by understanding the development of N cycle and to insure the ecosystem sustainability of re-constructed soils (McMillan et al. 2007). In general, N cycling processes are severely altered by anthropogenic disturbances

(Galloway et al. 2008) such as oil sands disturbance. Functional genes are known to be one of the best predictors of development of N-cycling processes, because their abundance and quantification can give information about the changes incurred by the environment (Colloff et al. 2008) and reclamation practices (Li et al. 2012; Li et al. 2014).

Reclamation practices include applying PMM and LFH which are common source of OM in oil sands reclamation (Mackenzie and Naeth, 2007). PMM is traditional OM source and salvaged from lowlands at mining site and LFH is salvaged from upland boreal forest of same eco-region (MacKanzie and Quideau, 2012). These both organic material may differ in organic matter content, soil pH (Jamro et al. 2014) and microbial community composition (Hahn and Quideau, 2013).These properties can affect N cycle transformation processes, their associated microbial communities and gene abundances in oil sands reclamation. LFH has greater decomposed OM content, nitrate supply, lower soil pH (Jamro et al. 2014), more diverse microorganisms (Hahn and Quideau, 2013) and vegetation cover, and species diversity and richness (Brown and Naeth et al. 2014) than PMM, suggesting that LFH has large capacity of OM mineralization (Van Cleve et al. 1993), and may have greater impact on the establishment of microbial community and eventually on the abundance of N cycling functional genes. Generally, the distribution and abundance of functional genes involved in N cycle (*amoA*, *nirS* and *nifH*) are associated to the rate of biogeochemical nutrient cycling (Burgmann et al. 2003; Wallenstein and Vilgalys, 2005) and can provide information about ecosystem functions and ecological interactions (Colloff et al. 2008).

Nitrification as one the most important processes in N cycle is more dominant in reclaimed oil sands soils than in natural forest soils (McMillan et al. 2007), suggesting that the reclaimed soils may favor nitrifiers and the associated gene abundance, *amoA* gene, and this is due to higher soil pH in reclaimed than in natural forest soils (McMillan et al. 2007). The activity of ammonia oxidizers is reduced at pH below 5.5 (Hunik et al. 1992). Moreover, in reclaimed soils,

nitrification is affected by C:N ratio. Lower C:N ratio may increase the N supply and increase the ammonia oxidizers (Verhamme et al. 2011). Furthermore, nitrification which is the upstream process of denitrification may also provide nitrate substrate for denitrification in oil sands reclamation since nitrate supply is regulator of denitrification (Hartmann et al. 2013) and this can also affect the *nirS* gene abundance. Denitrification rate can also be high in the presence of high OM and anaerobic environment (Wray and Bayley, 2007). The N_2 fixation process in oil sands reclamation can be evaluated by *nifH* gene abundance. The *nifH* gene abundance itself is also regulated by many factors including C:N ratio, changes in plant community composition (Tan et al. 2003), environmental variables such as soil pH (Nelson and Mele, 2006), soil water content, O_2 concentration (Hsu and Buckley, 2009), and soil N status (Mergel et al. 2001). Vegetation cover and species diversity are also strong regulators of N_2 fixation and *nifH* gene abundance (Menge and Hedin, 2009) in oil sands reclamation.

Recently, CWD as a reclamation material has been introduced in oil sands reclamation (Brown and Naeth, 2014) to expedite the establishment of ecosystem functions. The CWD increases microsites development (Brown and Naeth, 2014), alters soil environment (Goldin and Hutchinson, 2014) and affects nitrifiers (Ayrahami and Bohannan, 2007), denitrifiers (Silvester et al. 1982) and N_2 fixers (Brunner and Kimmins, 2003). In addition, the leaching of DOC and DON from CWD may also affect the soil N cycling processes (Hafner et al. 2005)

Numerous studies exist on comparing effects of LFH and PMM on N mineralization (McMillan et al. 2007; MacKenzie and Quideau, 2012), N availability (Jamro et al. 2014), or peat types on N mineralization (Hemstock et al. 2010) in oil sands reclamation. Recently, the importance of functional genes as appropriate biomarkers has been explored to evaluate the biological potential of N transformation (Colloff et al. 2008) and ecosystem functioning (Bastida et al. 2009). However, there is a lack of research pertaining to quantification of functional gene abundance involved in N cycling in reclaimed oil sands sites. We tested how *amoA*, *nirS* and *nifH* genes would respond to LFH, PMM and CWD in

oil sands reclamation in order to evaluate the changes caused by reclamation practices. We hypothesized that due to different characteristics of reclamation materials, functional gene abundances and their development would also be affected and altered. The main objective of this chapter was to investigate the changes of N cycling microbial communities focusing on key functional gene abundances at a newly reclaimed oil sands site. The results will help to understand the effects of reclamation practices on the abundances of functional genes and their relationships to the properties of reclamation materials in order to develop guidelines for N cycle re-establishment in oil sands reclamation.

3.2 Materials and methods

Soil samples stored at -20^{o}C were used for carrying out molecular analyses. Soil samples were sieved with a 2-mm sieve prior to further analysis. Between soil samples, sieves were sterilized by rinsing them with 70% ethanol and then dried in an oven before use in order to avoid cross contamination.

3.2.1 Molecular analyses

3.2.1.1 Soil DNA extraction

DNA was extracted from the soil samples (0.25 g) using the MoBio PwerLyzer^{TM} PowerSoil DNA Isolation Kit (MOBiO Laboratories, Inc. Carlsbad, CA, USA) according to the manufacturer's instructions. To determine the quality and size of DNA, extracts were run in 1% (w/v) agarose gels. At last, DNA yield was quantified by means of a Nanodrop® ND-1000 spectrophotometer (NanoDrop Technologies). All extracted DNAs were frozen and stored at -20^{o}C for further analysis.

3.2.1.2 Bacterial cultures and genomic DNA extraction

In order to generate the standard curves for real–time quantitative PCR, the following bacterial reference strains were used: the denitrifying bacterium *Pseudomonas stutzeri* ATTC 14405, and the N_2 fixing bacterium *Ensifer meliloti*

ATCC 9930 were purchased from the American Type Culture Collection. DNA of the ammonia oxidizer *Nitrosomonas europaea* was kindly provided by Dr. Lisa Stein's lab, Department of Biological Sciences, University of Alberta (Alberta, Canada). To prepare stock cultures, the freeze-dried original reference strains from vials, *Pseudomonas stutzeri* (Lehmann and Neumann) Sijderius (ATCC®1445^{TM}) and *Ensifer meliloti* (Dangered) Young (ATCC® 9930^{TM}) were grown in fresh media. *Pseudomonas stutzeri* was cultured on ATCC Medium Marine Broth/Agar 2216; Marine Broth 2216; 37.4 g L^{-1} and Marine Agar 2216(BD 212185; 55.1 g L^{-1}. *Ensifer meliloti* was cultured on ATCC Medium 111 Rhizobium X Medium; Yeast Extract, 1.0 g L^{-1}; Manitol, 10.0 g L^{-1}; Agar, 15.0 g L^{-1}; Soil Extract, 200.0 g $Liter^{-1}$ (African violet soil, 77.0 g L^{-1}; Na_2CO_3, 0.2 g L^{-1}). Subcultures were prepared from stock cultures as working cultures. Genomic DNA was extracted according to (William S. and Helene Feil, A. Copeland, Bacterial genomic DNA isolation using CTAB). Gel quantification (1% w/v) was used to determine the quality and size of the extracted genomic DNAs. The concentration of DNA was measured by using a Nanodrop® ND-1000 spectrophotometer (NanoDrop Technologies). DNA was stored at -20^{o}C prior to PCR.

3.2.1.3 Preparation of standards for generating standard curves for real-time PCR

Bacterial purified DNA extracts were used as templates for amplification bacterial *amoA*, *nirS*, and *nifH* genes by PCR on S1000^{TM} Thermal Cycler (Bio-Rad Laboratories, Mississauga, Ontario, Canada) using following primer sets respectively: amoA-1F/amoA-2R (Rotthauwe et al. 1997); nirScd3AF/nirSR3cd (Throbäck et al. 2004); and nifH-F/nifH-R (Rösch et al. 2002). The primers and thermal profiles used for PCR amplification of functional genes are summarized in Table 3.1. PCR amplification of functional genes was performed to prepare real-time PCR standards. PCR amplification of all genes was conducted at a total volume of 20 µL reaction mixture containing 10 µL of Master Mix (Go Taq® Hot Start Green Master Mix, 2X, Promega, USA), 1 µL of 10µM of each forward and

reverse primers, 5 µL of Nuclease-Free water (Integrated DNA Technologies), 2 µL of template DNA (10 ng μL^{-1}), and 1 µL of BSA (Fisher Bioreagents™ bovine serum albumin; 800ng μL^{-1}). PCR runs for bacterial *amoA*, *nirS* and *nifH* started with an initial enzyme activation step performed at 95°C for 15 min. The subsequent thermal profile was different for each gene and is shown in Table 3.1, following by a final extension of 10 min at 72°C. Each PCR product was run on 1% agarose gel electrophoresis to determine the size. The single bands of the target DNA fragments (491 bp, 425 bp, and 458 bp, for bacterial *amoA*, *nirS* and *nifH*, respectively) were cut using a sterilized scalpel and the excised gel bands were purified with Qiagen Gel Extraction Kit (Qiagen, Canada) according to manufactur's innstructions. The purified products were amplified again and purified using Qiagen PCR Purification Kit (Qiagen, Canada) to ensure specificity. At last, the PCR purified products were quantified and used as standards. The copy number for each gene was calculated from the concentration of the PCR purified product and the length of the fragment. Serial dilutions of the standards from 10^8 to 10^1 copies were prepared and used for qPCR. Reproducibility of the standards was verified at least three times before running them with actual samples.

For archaeal *amoA*, the sample-derived standards were used instead of standards prepared from PCR product of genomic DNA from a single bacterial strain. In other words, using the primers Arch-amoAF/ Arch-amoAR (Francis et al. 2005), a 635 bp fragment of DNA from soil sample was amplified on S1000™ Thermal Cycler (Bio-Rad Laboratories, Mississauga, Ontario, Canada). PCR run for archaeal *amoA* started with an initial enzyme activation step performed at 95°C for 10 min. The subsequent thermal profile is shown in Table 3.1, following by a final extension of 10 min at 72°C. The PCR product was visualized on 1% agarose gel, and amplification product was cut out and gel purified using the Qiagen Gel Extraction Kit (Qiagen, Canada). The gel purified product was amplified and purified using Qiagen PCR Purification Kit (Qiagen, Canada). Purified PCR product was ligated into vector pGEM-T Easy (Promega, USA) and ligation products were transformed into competent *Escherichia coli* JM109 cells

(Promega, USA) following the manufacturer's instructions. Transformants were selected on Luria-Bertani agar plates (Bacto®- tryptone, 10 g L^{-1}; Bacto®- yeast extract, 5, 5 g L^{-1}; NaCl 5 g L^{-1}) containing ampicillin (100 µg mL^{-1}), 20 µL of X-gal (50 mg mL^{-1}), and 100 µL of IPTG (24 mg mL^{-1}). Single white colony which contained the recombinant plasmids was inoculated into 400µL fresh LB broth medium containing ampicillin (100µg mL^{-1}) and incubated overnight at 37^{o}C. Subsequently, the plasmid DNA was extracted and purified using a Minipreps DNA Purification System (Promega, USA) according to manufacturer's instructions. In order to verify the presence of appropriate insert in recombinant plasmids, PCR was performed using the corresponding primers. In addition, recombinant plasmids were sequenced with the vector-specific primers T7 promoter primer and SP6. The sequences were analyzed on ABI Hitachi 3730 capillary DNA Analyzer (Applied Biosystems, Canada) using BigDye Terminator V3.1 and then sequences were checked and compared with sequences in the database NCBI BLAST, Software. The PCR products (inserts) were most closely related to archaeal *amoA* sequences. Therefore, the plasmid containing archaeal *amoA* gene was used as standard. The concentration of plasmid DNA was determined by using a Nanodrop® ND-1000 spectrophotometer (NanoDrop Technologies). The archaeal *amoA* copy number was calculated from the plasmid concentration and the length of vector with a 635 bp fragment as the insert. Serial dilutions of the standards were prepared from 10^8 to 10^1 copies and used for qPCR. Reproducibility and consistency of the standards was verified at least three times before running them with actual samples.

3.2.1.4 Real time quantitative PCR of functional genes

In order to quantify potential AOB and AOA, denitrifiers and N_2 fixing microbial populations, genes encoding the catalytic enzymes responsible for ammonia oxidation (*amoA* encoding the ammonia monooxygenase), denitrification (*nirS* encoding cytochrome cd1 nitrite reductase) and N_2 fixation (*nifH* encoding nitrogenase reductase) were used as functional molecular markers. Quantification of all investigated genes was performed in triplicate on a

StepOnePlus™ Real-Time PCR system (Applied Biosystems, Foster City, CA, USA) using QuantiTect SYBR Green PCR Master Mix real-time PCR kit. Real-time PCR was carried out in a 10 µL assay mixture consisted of 5 µL of the SYBR Green PCR Master Mix, 0.5 µL of 10 µM of each forward and reverse primers, 2.5 µL of Nuclease-Free water (Integrated DNA Technologies), 1 µL of template DNA (1 ng μL^{-1}) and 1 µL of BSA (Fisher Bioreagents™ bovine serum albumin; 800 ng μL^{-1}). The primers and qPCR conditions used to assess microbial communities involved in N cycle were the same as those of the regular PCR described in Table 3.1 with the addtional melting curve program at the end which consisted of 95°C for 15 s, 60°C for 1 min and a subsequent temperature increase until 95°C for 15 s with a rate of ramping of 0.03°C s^{-1}. Fluorescence data were collected during the extension phase at 72°C and end point of melting curve stage during the ramping from 60°C to 95°C. Standard curves were generated using serial dilutions containing the investigated genes and were used as references for estimating the abundances of functional genes of environmental DNA samples. The specificity and purity of amplified products were checked using melt curve analysis and also were confirmed by the presence of a unique band of the expected size on a 1% agarose gel.

3.2.2 Statistical analysis

Mean values of gene abundances, archaeal and bacterial *amoA*, *nirS*, and *nifH*, were compared using MANOVA, and pair-wise comparisons of the mean values were performed using LSD. All statistical analyses were performed using SAS 9.2, and $P < 0.05$ was considered to be statistically significant. To determine the relationship between nitrogen cycle functional gene abundances such as *amoA*, *nirS*, *nifH* and microbial indicators such as MBC, MBN, respiration rate, BGLU and NAGase activities, correlation analysis was conducted using Pearson's correlation analyses.

3.3 Results

In this study, microbial community development was evaluated by quantification of the N-cycling functional genes such as AOA and AOB *amoA*, *nirS*, and *nifH*. The effects of LFH, PMM, CWD application and seasonality on the development of the microbial community were also investigated. For this study, soil samples were collected from either LFH or PMM plots amended by away CWD, near CWD and control (without CWD). A total 96 soil samples were collected during the study period; 24 soil samples in 2011 (12 in June, 12 in September) and 36 soil samples in each 2012 (18 in June, 18 in September) and 2013 (18 in June, 18 September).

3.3.1 Archaeal and bacterial *amoA* gene abundances

The effect of capping materials (LFH and PMM) on AOA and AOB *amoA* gene abundances is shown in Figure 3.2.A and Figure 3.4.A, respectively. Capping materials showed significant ($P< 0.05$) effect on AOA *amoA* gene copy numbers which were greater in LFH ($5.8352 \times 10^4 \pm 7.469 \times 10^3$ copies ng^{-1} soil DNA) than in PMM ($2.3771 \times 10^4 \pm 2.907 \times 10^3$ copies ng^{-1} soil DNA) (Figure 3.2.A and Table 3.2). The significant differences in AOA *amoA* gene abundances between LFH and PMM were found during the last three sampling times (September 2012, June 2013, and September 2013) (Figure 3.2.A). The effect of capping materials on AOA *amoA* gene abundance with time (from June 2011 to September 2013) was also determined. The effect of LFH on AOA *amoA* gene abundance with time showed that *amoA* gene abundance did not significantly change initially during 2011; however a significant increase in *amoA* gene abundance was observed in 2012 which continued, with the exception of June 2013, until September 2013 when last soil sampling was performed (Figure 3.2.A). Therefore, the greatest ($1.26469 \times 10^5 \pm 2.4482 \times 10^4$ copies ng^{-1} soil DNA) and lowest gene abundance ($6.355 \times 10^3 \pm 1.282 \times 10^3$ copies ng^{-1} soil DNA) in LFH was found in September 2013 and June 2011, respectively. A similar trend in AOA *amoA* gene abundance was observed in experimental plots

amended with PMM. AOA *amoA* gene abundance observed in June 2011 (5.070 x $10^3 \pm 1.115 \times 10^3$ copies ng^{-1} soil DNA did not change significantly during 2011, however, it increased significantly in June 2012 ($2.9592 \times 10^4 \pm 3.645 \times 10^3$ copies ng^{-1} soil DNA) and remained unchanged till June 2013. After that, a significant increase in gene abundance ($5.4275 \times 10^4 \pm 6.986 \times 10^3$ copies ng^{-1} soil DNA) was observed in September 2013 (Figure 3.2.A).

CWD (control, away CWD, near CWD) showed significant effect ($P< 0.05$) on *amoA* gene abundance. The average values of *amoA* gene abundance were $5.8508 \times 10^4 \pm 1.0267 \times 10^4$ near CWD, $3.1901 \times 10^4 \pm 5.219 \times 10^3$ away CWD, and $3.1293 \times 10^4 \pm 4.400 \times 10^3$ copies ng^{-1} soil DNA in control plots (without CWD). In LFH plots, soil near to CWD showed significantly higher *amoA* gene abundance as compared to control and soil away from CWD particularly during June 2012 and September 2013; however, in September 2012, near CWD was significantly higher than only control and in June 2013, near CWD was significantly higher than only away CWD (Figure 3.2.B and Table 3.3). In PMM plots, the effect of CWD on *amoA* gene abundance was significant in June 2012 and September 2013 when soil near CWD showed higher *amoA* gene abundance than soil away CWD in June 2012 and it also showed higher gene abundance than control in September 2013 (Figure 3.2.C and Table 3.3).

The effect of seasonality on *amoA* gene abundance is shown in Figure 3.3.A. The results showed that there was significantly ($P< 0.05$) greater *amoA* gene abundance in September ($5.4487 \times 10^4 \pm 7.532 \times 10^3$ copies ng^{-1} soil DNA) than in June ($2.7421 \times 10^4 \pm 3.442 \times 10^3$ copies ng^{-1} soil DNA) (Table 3.4). The effect of seasons on gene copy numbers was more conspicuous in 2013 where mean values of *amoA* gene abundance in June and September were $3.5609 \times 10^4 \pm 5.804 \times 10^3$ and $9.0372 \times 10^4 \pm 1.5138 \times 10^4$ copies ng^{-1} soil DNA, respectively.

The change of AOA *amoA* gene numbers over three years from 2011 to 2013 is shown in Figure 3.3.B. A general trend of increasing AOA *amoA* genes was observed over time. Though gene copy numbers increased during the study

period, the significant ($P< 0.05$) increase in AOA *amoA* genes was only observed from 2011 to 2012. Mean values of the gene copy numbers of *amoA* in 2011, 2012, and 2013 were $5.851 \times 10^3 \pm 790$, $4.9969 \times 10^4 \pm 4.770 \times 10^3$, and $6.2990 \times 10^4 \pm 9.233 \times 10^3$ copies ng^{-1} soil DNA, respectively.

Overall, the results showed that *amoA* gene abundance was significantly greater in LFH than in PMM. AOA *amoA* gene abundance was significantly changed by CWD in both LFH and PMM. Overall, September showed greater *amoA* gene abundance than June especially in 2013 and a significant increasing trend of *amoA* gene abundance occurred from 2011 to 2012.

The results of the effect of capping materials (LFH and PMM) exhibited a significant ($P< 0.05$) effect on AOB *amoA* gene abundances where LFH showed greater AOB *amoA* gene abundances ($1.0697 \times 10^4 \pm 1.123 \times 10^3$ copies ng^{-1} soil DNA) than PMM ($6.558 \times 10^3 \pm 504$ copies ng^{-1} soil DNA) (Figure 3.4.A and Table 3.2). The significant differences of AOB *amoA* gene abundances between capping materials, LFH and PMM, were observed during the last two sampling times (June and September 2013) (Figure 3.4.A). The effect of capping materials on AOB *amoA* gene abundance with time from June 2011 to September 2013 showed that in LFH, AOB *amoA* gene abundance did not significantly change during 2011 and June 2012 ($6.436 \times 10^3 \pm 542$ copies ng^{-1} soil DNA); however, it showed a significant increase in September 2012 ($1.2618 \times 10^4 \pm 1.410 \times 10^3$ copies ng^{-1} soil DNA) and remained unchanged till June 2013 and then significantly increased in September 2013($2.1629 \times 10^4 \pm 4.084 \times 10^3$ copies ng^{-1} soil DNA) (Figure 3.4.A). Thus, in LFH, the greatest ($2.1629 \times 10^4 \pm 4.084 \times 10^3$ copies ng^{-1} soil DNA) and lowest ($4.079 \times 10^3 \pm 380$ copies ng^{-1} soil DNA) *amoA* gene abundance were observed in September 2013 and June 2011, respectively. In plots amended with PMM, *amoA* gene abundance did not significantly change during 2011, 2012 and June 2013 ($6.492 \times 10^3 \pm 668$ copies ng^{-1} soil DNA); however, it significantly increased in September 2013 ($1.0768 \times 10^4 \pm 1.235 \times 10^3$ copies ng^{-1} soil DNA) (Figure 3.4.A).

The application of CWD (control, away CWD, near CWD) showed a significant ($P< 0.05$) increase in AOB *amoA* gene abundance over time. The mean values of AOB *amoA* gene abundances were $1.2094 \times 10^4 \pm 1.501 \times 10^3$ near CWD, $7.454 \times 10^3 \pm 683$ away CWD, and $6.157 \times 10^3 \pm 630$ copies ng^{-1} soil DNA in control plots (without CWD). In plots amended with LFH, near CWD exhibited significantly greater gene abundance than away CWD and control especially during September 2011, 2012, and 2013 (Figure 3.4.B and Table 3.3). In PMM plots, the significant effect of CWD on gene abundance was found from September 2011 to September 2013 where near CWD were significantly higher than both away CWD and control in September 2011 and 2013; and it was also higher than control during 2012 and June 2013 (Figure 3.4.C and Table 3.3).

Seasonality showed a significant ($P< 0.05$) effect on the AOB *amoA* gene abundance. The results showed that there was a significant difference between the AOB *amoA* gene abundance in September ($1.1133 \times 10^4 \pm 1.115 \times 10^3$ copies ng^{-1} soil DNA) compared to June ($6.146 \times 10^3 \pm 446$ copies ng^{-1} soil DNA) (Table 3.4). The effect of seasonality especially was observed in 2012 and 2013 where the average values of the gene abundance in June and September were $6.154 \times 10^3 \pm 535$ and $1.0572 \times 10^4 \pm 1.052 \times 10^3$ in 2012, and $8.899 \times 10^3 \pm 774$ and $1.6199 \times 10^4 \pm 2.453 \times 10^3$ copies ng^{-1} soil DNA in 2013, respectively (Figure 3.5.A).

A significant increase ($P< 0.05$) of AOB *amoA* gene abundance was observed over time from 2011 to 2013 (Figure 3.5.B). The average values of the gene abundance in 2011, 2012, and 2013 were $4.450 \times 10^3 \pm 382$, $8.363 \times 10^3 \pm 691$, and $1.2549 \times 10^4 \pm 1.410 \times 10^3$ copies ng^{-1} soil DNA, respectively.

Altogether, the results revealed that AOB *amoA* gene abundances were significantly greater in LFH than in PMM and CWD displayed significant effect on AOB *amoA* gene abundance. Seasonality also showed a significant effect on the gene numbers where greater gene abundance was observed in September than in June especially in 2012 and 2013. Significant change of AOB *amoA* gene abundances was also found from 2011 to 2013.

3.3.2 *nirS* gene abundance

Capping materials (LFH and PMM) showed a significant effect ($P< 0.05$) on *nirS* gene abundance where LFH ($2.0015 \times 10^4 \pm 1.630 \times 10^3$ copies ng^{-1} soil DNA) exhibited significantly greater gene abundance than PMM ($8.724 \times 10^3 \pm$ 680 copies ng^{-1} soil DNA) (Figure 3.6.A and Table 3.2). The significant differences ($P< 0.05$) in *nirS* gene abundance between capping materials (LFH and PMM) were observed over time from June 2011 to September 2013 (Figure 3.6.A). The effect of capping materials on *nirS* gene abundance with time (from June 2011 to September 2013) showed that in plots amended with LFH, *nirS* gene abundance did not significantly change during 2011 and also in June 2012 ($1.3542 \times 10^4 \pm 881$ copies ng^{-1} soil DNA); however, the gene abundance increased significantly in September 2012 ($2.0578 \times 10^4 \pm 2.497 \times 10^3$ copies ng^{-1} soil DNA) and remained unchanged in June 2013; after that, it increased significantly in September 2013 ($3.8031 \times 10^4 \pm 4.662 \times 10^3$ copies ng^{-1} soil DNA) (Figure 3.6.A). In plots amended with PMM, the gene abundance did not change during 2011, 2012, and June 2013; however, it significantly increased in last sampling time in September 2013 (Figure 3.6.A). The lowest ($4.023 \times 10^3 \pm 1.073 \times 10^3$ copies ng^{-1} soil DNA) and highest ($1.7172 \times 10^4 \pm 1.030 \times 10^3$ copies ng^{-1} soil DNA) gene copy numbers were found in June 2011 and September 2013, respectively.

CWD (control, away CWD, near CWD) significantly ($P< 0.05$) had effect on relative abundance of *nirS* gene. The mean values of *nirS* gene abundance were $1.8609 \times 10^4 \pm 2.079 \times 10^3$ near CWD, $1.2745 \times 10^4 \pm 1.832 \times 10^3$ away CWD, and $1.1270 \times 10^4 \pm 1.025 \times 10^3$ copies ng^{-1} soil DNA in control (without CWD). In LFH plots, near CWD exhibited significantly greater gene abundance than away CWD and control during September 2011, 2012, and June 2013; however in September 2013, near CWD showed significantly higher gene abundance than only control (Figure 3.6.B and Table 3.3). In plots amended with PMM, the significant effect of CWD on *nirS* gene abundance was observed during 2011 and September 2013 where near CWD was significantly higher than

away CWD and control in 2011, and it was significantly higher than control in September 2013 (Figure 3.6.C and Table 3.3).

The significant effect ($P< 0.05$) of seasons on the abundance of *nirS* gene was observed over time where greater gene abundance were observed in September ($1.7538 \times 10^4 \pm 1.812 \times 10^3$ copies ng^{-1} soil DNA) than in June ($1.1063 \times 10^4 \pm 830$ copies ng^{-1} soil DNA) (Table 3.4). The effect of seasonality on the gene abundance was more noticeable in 2013, where the difference of mean values of the gene abundance in June and September were $1.4341 \times 10^4 \pm 1.788 \times 10^3$ and $2.7601 \times 10^4 \pm 3.430 \times 10^3$ copies ng^{-1} soil DNA, respectively (Figure 3.7.A).

The significant increasing trend ($P< 0.05$) of realative abundance in *nirS* gene was found over time which is shown in Figure 3.7.B. Although the increasing trend was detected over time, the significant change of the gene abundance was observed only from 2012 to 2013 (Figure 3.7.B). The mean values of *nirS* gene abundance were $8.506 \times 10^3 \pm 960$, $1.2672 \times 10^4 \pm 1.092 \times 10^3$, and $2.0971 \times 10^4 \pm 2.211 \times 10^3$ copies ng^{-1} soil DNA in 2011, 2012, and 2013, respectively.

Overall, the results of *nirS* gene abundance showed that capping materials had significant effect on the gene abundance where LFH had greater gene abundance than PMM. CWD also showed significant effect on the gene abundance in both LFH and PMM capping materials and a significant difference of *nirS* gene abundance was observed in June and September due to the effect of seasonality and *nirS* gene abundance exhibited a significant change particularly from 2012 to 2013.

3.3.3 *nifH* gene abundance

The significant effect ($P< 0.05$) of capping materials (LFH and PMM) was observed on *nifH* gene abundance over time (Figure 3.8.A). LFH exhibited greater *nifH* gene abundance as compared to PMM where the average values of the gene

abundance were $1.1024 \times 10^4 \pm 1.156 \times 10^3$ and $6.389 \times 10^3 \pm 562$ copies ng^{-1} soil DNA in LFH and PMM, respectively (Figure 3.8.A and Table 3.2). The significant differences in *nifH* gene abundance between LFH and PMM capping materials were found during September 2011 and the last three sampling times (September 2012, June and September 2013) (Figure 3.8.A). The effect of capping materials on the gene abundance with time from June 2011 to September 2013 was evaluated and showed that in LFH plots, the gene abundance initially increased in 2011 and remained unchanged in June 2012; however it increased in September 2012 and did not change in June 2013 and then increased significantly in September 2013 (Figure 3.8.A). In PMM plots, no significant change of the gene abundance was observed during 2011 and June 2012 ($4.863 \times 10^3 \pm 685$ copies ng^{-1} soil DNA), the significant increase of the gene abundance occurred in September 2012 ($8.590 \times 10^3 \pm 545$ copies ng^{-1} soil DNA) and September 2013 ($1.2909 \times 10^4 \pm 763$ copies ng^{-1} soil DNA) (Figure 3.8.A).

CWD amendment (control, away CWD, near CWD) showed a significant ($P< 0.05$) effect on *nifH* gene abundance over time. The mean values of *nifH* gene abundance were $1.0570 \times 10^4 \pm 1.451 \times 10^3$ near CWD, $7.248 \times 10^3 \pm 829$ away CWD, and $8.104 \times 10^3 \pm 1.062 \times 10^3$ copies ng^{-1} soil DNA in control (without CWD). In LFH amended plots, near CWD exhibited significantly higher gene abundance than away CWD and control in September 2011 and 2013. However, in June 2013 near CWD showed significantly higher gene abundance than only control (Figure 3.8.B and Table 3.3). In PMM amended plots, the significant effect of CWD was observed only in June 2012 and 2013 where near CWD was significantly higher than away CWD in June 2012 and it was significantly higher than both away CWD and control in June 2013 (Figure 3.8.C and Table 3.3).

A significant effect ($P< 0.05$) of seasonality was observed on *nifH* gene abundance with greater gene abundance in September ($1.1868 \times 10^4 \pm 1.121 \times 10^3$ copies ng^{-1} soil DNA) than in June ($5.593 \times 10^3 \pm 472$ copies ng^{-1} soil DNA) (Table 3.4). The significant effect of seasonality on *nifH* gene abundance was pronounced in all three years from 2011 to 2013, where the mean values of the

gene abundance in June and September were 2.183 x 10^3 ± 208 and 5.147 x 10^3 ± 689 copies ng^{-1} soil DNA in 2011, 5.956 x 10^3 ± 451 and 1.0057 x 10^4 ± 558 copies ng^{-1} soil DNA in 2012, and 8.639 x 10^3 ± 774 and 1.9279 x 10^4 ± 2.048 x 10^3 copies ng^{-1} soil DNA in 2013, respectively (Figure 3.9.A).

nifH gene abundance showed a significant (P< 0.05) increasing trend from 2011 to 2013 (Figure 3.9.B). Average values of the gene copy numbers were 3.530 x 10^3 ± 418, 8.007 x 10^3 ± 495, and 1.3959 x 10^4 ± 1.405 x 10^3 copies ng^{-1} soil DNA in 2011, 2012, and 2013, respectively.

Altogether, the results showed the significant effect of capping materials on *nifH* gene abundance with LFH having greater *nifH* gene abundances than PMM. CWD and seasonality also showed significant effects on the gene abundance and a significant change of the relative gene abundance was observed over time from 2011 to 2013.

3.3.4 Relationships between functional genes and microbial indicators

There were significant correlations between AOA and AOB *amo* genes, *nirS*, *nifH* and MBC, respiration rate, BGLU and NAGase activities (Table 3.5). However, *amo* AOA gene and MBN were not correlated (P<0.05).

3.4 Discussion

3.4.1 Abundances of bacterial and archaeal *amoA* gene

Nitrification, a dominant process of the N cycle in reclaimed soils (McMillan et al. 2007), is partially mediated by ammonia-oxidizing microbial populations. Following three years of data collection, our results revealed that there was an increasing trend in the AOA and AOB *amoA* gene abundances in reclaimed soils. The greater changes in AOB *amoA* gene than AOA *amoA* gene over time (Figure 3.5.B and 3.3.B) may be explained by their different responses to environmental fluctuations suggesting their adaptation to different environmental factors within the soil due to differences in niche preference

between AOA and AOB (Erguder et al. 2009). Substrate kinetic differences such as ammonium concentration or pH play key role in niche specialization and differentiation between soil AOA and AOB (Prosser and Nicol 2012). Thus, the greater changes in AOB than AOA in this study indicating that AOB are more likely to have higher sensitivity to environmental factors, such as ammonium availability, pH and vegetation diversity by reclamation practices (Forsch 2014; Jamro et al. 2014; Kwak et al. unpublished).

The increasing trend we observed in the abundance of AOA and AOB *amoA* is likely associated with the type of capping materials (Jamro et al. 2014). The greater abundance of these genes in LFH than in PMM likely is attributed to the properties of LFH capping material such as lower C:N ratio, greater N mineralization and lower soil pH than in PMM which support higher AOA *amoA* than AOB *amoA* in LFH than PMM (McMillan et al. 2007; Jamro et al. 2014). The C:N ratio has been shown to be the single best predictor of relative abundance of AOA and AOB *amoA* (Bates et al. 2011; Adair and Schwartz, 2008) and in general, the abundance of AOA and AOB *amoA* tends to be oppositely correlated with C:N ratio. The lower C:N ratio may increase the N mineralization; consequently, it may increase the NH_4^+ availability. The LFH had lower C:N ratio and greater NH_4^+ supply than PMM (Jamro et al. 2014). Hence, greater abundance of AOB and AOA *amoA* in LFH than in PMM was attributed to lower C:N ratio and greater NH_4^+ supply. Vermhamme et al. (2011) observed a greater abundance of AOB *amoA* at high NH_4^+ supply than AOA *amoA*. Whereas, AOA *amoA* gene abundance increased in low NH_4^+ supply (Valentine, 2007; Zhang et al. 2010), suggesting the differences in NH_4^+ uptake and accessibility by AOA versus AOB. This is due to the higher-affinity of NH_4^+ transporters in AOA compared to AOB even in low NH_4^+ concentarations (Offre et al. 2014). In reclaimed oil sands soils, NH_4^+ supply was lower than NO_3^- supply (Jamro et al. 2014) and this could cause less abundance of AOB relative to AOA *amoA* gene abundance. Since AOA *amoA* gene abundance is positively correlated to nitrification activity (Tourna et al. 2008), this would suggest that AOA *amoA* is likely to be predominant in soils with an higher nitrification rate such as

reclaimed oil sands soils than in natural boreal forest (McMillan et al. 2007). Under low NH_4^+ supply, AOA which prefer low NH_4^+ concentration (Gubry-Rangin et al. 2010) are able to thrive and have a dominant role in driving nitrification process than AOB in oil sands reclamation.

Soil pH differences between capping materials are potentially a major drivers of genesis, adaptation, and diversity of ammonia oxidizers in oil sands reclamation, which regulate the nitrification process (Gubry-Rangin et al. 2010). Soil pH alters the chemical form, concentration and availability of substrates (Nicol et al. 2008). Generally, lower pH is usually a detriment to AOB due to soil acidity pressure, and low ammonia substrate availability (De Boer and Kowalchuk, 2001). Furthermore, lower pH is also known as a boon to AOA owing to the fact that ammonia released from mineralization of organic matter could be more available to archaeal cells than inorganic ammonia from NH_3/NH_4 (Levicnik-Höfferle et al. 2012). Previous studies indicated that AOB could not survive below pH 7 (Jiang and Bakken, 1999), whereas, AOA showed nitrification activity in acidic pH soil (Nicol et al. 2008). Similarly, in oil sands reclamation, soil pH is also major determinant factor for microbial processes development especially for N availability. It was further showed that LFH had significantly lower pH than PMM (Jamro et al. 2014). Therefore, the greater abundance of AOA than AOB in LFH was likely due to pH. As such, higher abundance of AOB is expected in PMM due to higher pH than in LFH. However, it was not the case, since PMM is associated to anaerobic environment (Andersen et al. 2013) and it has low rates of net N mineralization and nitrification (Hemstock et al. 2010) which causes lower abundance of AOB in PMM than in LFH. The mechanism by which pH can affect the ammonia oxidizers is that pH may reduce the NH_3 availability through its ionization to NH_4^+ (Frijlink et al. 1992). Under this condition, NH_3 diffusion decreases and the required energy for NH_4^+ transformation increases. The changes in NH_3 diffusion may affect the AOB and AOA abundances due to their differences in NH_4^+ affinity (Offre et al. 2014). The vegetation cover of the capping material has a large influence on the microbial community structure and abundance in reclaimed soils (Hogberg and

Read, 2006; Mackenzie and Quideau, 2010). Vegetation communities can affect the soil microbial communities by regulating the availability and quantity of the substrate, altering the soil pH, temperature, and soil moisture (Wallenius et al. 2011; Levy-Booth et al. 2014). Through a process known as belowground priming, the aboveground vegetation can have a profound impact on the soil microbial community by adding fresh labile C and organic N (e.g. amino acids) as root exudates or litterfall materials to soil to become substrate for the soil microbial communities (Kuzyakov et al. 2000). Labile OM substrates tend to have a greater priming effect than less degradable substrates due to their ease of accessibility by microorganisms (Kuzyakov et al. 2000). The increase in AOA and AOB abundance we observed during September was likely associated with the priming effect. In addition, a greater priming effect in LFH was likely associated with the greater vegetation cover and species diversity and richness (Naeth et al. 2013). Diversity of plant species may add diverse substrates in soil (Wardle and Zackrisson, 2005) and may influence microbial composition and decomposition of OM (Orwin et al. 2006) which affects N availability (Kuzyakov et al. 2000; Austin et al. 2004). Jamro et al. (2014) demonstrated that N availability was associated with the priming effect in oil sands reclamation, and showed that greater N availability was due to greater priming effect in LFH than in PMM. Therefore, the increase in AOA and AOB to priming effects may be associated with the differences in utilization of organic C substrates during fall season. However, it is difficult to quantify the magnitude of the priming effect on AOA and AOB but this information would be useful to ensure the success of future oil sand reclamation practices.

Vegetation type also modulates the correlations between AOA and environmental influences such as precipitation (Bates et al. 2011). Precipitation affects the plant community composition and structure (Zak et al. 2003; Kardol et al. 2010) and governs diverse substrate inputs into the soil system (Schlesinger et al. 1996). Differences in precipitation are one of the main contributors to seasonal variations in soil processes and functions in oil sand reclamation (Mackenzie and Quideau, 2010). Thus, the seasonal changes we observed in AOA and AOB gene

abundances (Figure 3.3.A and 3.5.A) were likely associated with variation in precipitation (Appendix- I). The variation in precipitation may increase the water and substrate availabilities for microbial activities and affect the N mineralization (Collins et al. 2008) and it likely increased the abundance of the genes where temperature was not varied from 2011 to 2013.

Another factor which can accelerate the abundance of functional genes in oil sands reclamation is the presence of CWD. CWD may help to develop microsites with differing substrate availabilities which may ultimately influence the existence of AOB and AOA communities. The application of CWD to reclamation sites increased soil water content (Kwak et al. unpublished), altered soil temperature (Goldin and Hutchinson, 2014), and has been shown to be important for ammonia oxidizers which increased with increasing soil water content (30-60%) (Ayrahami and Bohannan, 2007). Differences in the abundance of AOA and AOB are associated with "niche complementarity theory" (Hector et al. 1999), where spatial differences in AOB and AOA would be due to differential utilization of the substrate. Our results are in consistent with this body of literature suggesting that AOA and AOB abundances are different in their association with CWD regardless of the capping materials. Likely associated with greater microsite development, altering the soil microenvironment, and consequently observed greater nitrification rate by Kwak et al. "collaborators on the same study research site" (unpublished), we also found AOA and AOB *amoA* abundance was greater in LFH than in PMM in near CWD than away CWD and control treatments. Our results are in agreement with Hafner et al. (2005) who found that there was 15 times higher DOC concentration in soils near woody logs than in litter layer (without logs) in lowland forest. On the other hand, significant differences were not found between away CWD and control in this study except for some cases where away CWD and control were significantly different. These differences might be due to leaching and runoff from CWD to soil through precipitation. The leaching of DOC or DON from CWD may alter the C and N cycling (Hafner et al. 2005) through increasing the labile substrates for AOA and AOB activities. Availability of labile substrates might have caused the increase in

AOA and AOB gene abundances in this study (Figure 3.2.C and 3.4.C). The probability of leaching and runoff of DOC and DON from CWD in soil is supported by Forsch (2014) who observed the significant relationship between plant species richness and slope of block on similar site, with greater plant species richness in lower block than upper block.

3.4.2 Abundance of *nirS* gene

The *nirS* gene encodes for the nitrite reductase enzyme. This enzyme controls the key process of denitrification which results in gaseous losses of N from terrestrial ecosystems (Braker and Tiedje, 2003). Denitrifiers, components of the soil microbial communities that possess the *nirS* gene, are particularly sensitive to environmental conditions such as alterations in O_2 concentration, soil moisture content, pH, as well as C and N substrate availability which all play roles in the expression of the gene (Hai et al. 2009). This can be seen in our study. The increased precipitation received in 2013 relative to 2011 and 2012 likely resulted in the creation of more anaerobic microenvironments altering vegetation cover and labile substrate input (Kardol et al. 2010) which in turn enhanced O_2 depletion in soil pores (Sikora and Yakovchenko, 1996; Bastida et al. 2009) and could explain why we observed greater *nirS* gene abundance in 2013 than in 2012 and 2011 (Figure 3.7.B).

The activity of denitrifiers is enhanced in the presence of labile C (Pérez et al. 2010). In oil sands reclamation, the emission of NO and N_2O is likely associated with differences in labile C availability, decomposed OM content in capping material types (Jamro et al. 2014) and high nitrification rate (McMillan et al. 2007). However, decomposed OM content could be the dominant factor in regulating the *nirS* gene abundance in oil sands reclamation since various facultative denitrifiers have *nirS* gene.The greater *nirS* gene abundance we observed in LFH than in PMM might be related to differences in C substrate availability in the capping materials (Jamro et al. 2014). The C:N ratio of the LFH was lower because these soils contained more labile C than did the PMM (Brown

and Naeth, 2014; Jamro et al. 2014). The abudance of *nirS* gene has been attributed to greater OM (Wray and Bayley, 2007) and addition of an organic amendment (Bastida et al. 2009). The organic amendment may reduce O_2 availability (consumption of O_2 during organic substrate's oxidation) and establish new niches where O_2 diffusion could be restricted (He´nault et al. 2001) and may therefore create the conditions conducive to reduce NO_3^- present in soil into NO and N_2O. Therefore, it is assumed that LFH might have created new niches which favors denitrification and may increase the *nirS* gene abundances.

The high abundance of the *nirS* gene in the LFH may also be linked to higher NO_3^- availability (Wallenstein et al. 2006). In general, NO_3^- is the predominant form of available N in reclaimed oil sand soils (Jamro et al. 2014). The greater nitrification rates observed in oil sand reclamation compared to nearby undisturbed forest soil (McMillan et al. 2007) can provide the NO_3^- necessary for high denitrification in these soils since nitrification is a strong regulator of denitrification (Hartmann et al. 2013). The differences in the nitrification rates between reclaimed and undisturbed sites could be due to the differences in vegetation cover. Greater plant species richness in the LFH treatment than PMM treatment (Forsch, 2014) may have caused greater *nirS* gene abundances. This is likely associated with the increase in C substrate availability through plant root system (Chang et al. 2014). Addition of labile C substrate in the form of root exudates may in turn enhances O_2 utilization in soil pores, thereby establishing an anaerobic microenvironment (Bastida et al. 2009). Consequently, *nirS* gene abundance could also increase.

CWD can retain the moisture (Baier et al. 2005) and may create the anaerobic microsite (Parkin, 1987) which could also affect the *nirS* gene abundance. Therefore, the greater *nirS* gene abundance in near CWD than away CWD treatment (Figure 3.6.B and 3.6.C) is likely associated with microsites development (Brown and Naeth, 2014). Microsites have ten times greater denitrifying activity as indicated in Scot pine and Douglas fir needle litter layer than fragmented materials (Laverman et al. 2000). In addition, CWD may also

increase the availability of DOC or DON which may lead to increase in C substrate for denitrification process (Henrich and Haselwandter, 1997). The greater abundance of *nirS* in away CWD than control treatments (e.g. LFH) (Figure 3.6.B) is likely resulting from DOC or DON leaching from CWD to soil in some cases in this study.

3.4.3 *nifH* gene abundance

The *nifH* gene is associated with N_2 fixation. A number of factors can affect the abundance of *nifH* gene in the soils of reclaimed areas including the C:N ratio of the capping material, changes in plant community composition (Tan et al. 2003), environmental variables such as soil pH (Nelson and Mele, 2006), soil water content, O_2 concentration (Hsu and Buckley, 2009), and soil N status (Mergel et al. 2001). In the boreal forest, free diazotrophs are known to be dominant N_2 fixers (Levy-Booth, 2014) and they are considered to be most active when N demand by microorganisms is high (Hedin et al. 2009). In the present study, we found that there was greater *nifH* gene abundance in LFH than in PMM. The LFH had a lower C:N ratio than the PMM treatment (Jamro et al. 2014), and lower C:N ratio suggests that LFH has greater labile C substrate which may affect the activity of free living bacteria and enhance the *nifH* gene abundance. N_2 fixation of free living bacteria can be induced by increasing labile C substrate availability (Bürgmann et al. 2003).

Given that N_2 fixation is known to be an energy-intensive process that requires adequate C substrate availability (Hayden et al. 2010), it is not surprising that *nifH* gene abundance has been correlated with organic C content in soil (Huhe et al. 2014) as well as plant species and abundance (Hayden et al. 2010; Schaaf et al. 2011; Reed et al. 2011). Accordingly, *nifH* gene abundance in reclaimed oil sands soils may be strongly related to vegetation cover and diversity, which affect associated microbial community composition involved in N_2 fixation (Menge and Hedin, 2009). Following the reclamation, the LFH treatment has greater plant diversity in terms of grasses, forbs, shrubs, and tree

species than the PMM treatment. The PMM treatment has more bryophytes than the LFH treatment. The greater shrub and mixed forest content in LFH treatment than in PMM in this study site (Forsch, 2014) likely resulted in increased *nifH* gene abundance as it is well known that mixed conifer forests host a variety of N_2 fixing species such as *Frankia* sp., *Paenibacillus* sp., and *Clostridium pasteurianum* (Yeager et al. 2005). Previous studies indicated that shrubs and mixed forest plantations (Li et al. 2011), forbs (Orwin et al. 2014), diazotrophic bacteria embedded in ectomycorhizal tissue (Frey-Klett et al. 2007), and mixed conifer soil (Yeager et al. 2005) contribute to N_2 fixation in forest ecosystems (Bürgmann et al. 2003). Some of the forb species [i.e. *Medicago sativa* L. (alfalfa), *Melilotus alba* Medick (sweet clover), and *Trifolium* sp.] observed on this study site (Forsch, 2014) might have a role in symbiotic N_2 fixation. In the PMM treatment, *nifH* gene was likely linked to presence of bryophytes. Moss species in boreal forests can have an important role in N_2 fixation and have been reported to fix 1.5 to 2 kg N ha^{-1} ye^{-1} (DeLuca et al. 2002). The greater abundance of *nifH* gene in September than June we observed (Figure 3.9.A) may be attributed to seasonal changes in substrate availability accessed by free living N_2 fixing bacteria (Hobbie and Vitousek, 2000; Reed et al. 2007). The changes in vegetation cover from June to September may have provided the labile C substrate from rhizodeposition and litterfall needed to support a free-living N_2 fixing bacterial community (Hayden et al. 2010) and thereby lead to an increase in *nifH* gene abundance in September.

CWD in this study did not directly change the vegetation cover or nutrients' availability (Forsch, 2014; Kwak et al. unpublished) but might have had an indirect effect on *nifH* gene abundance through microsite development, affecting species richness, and altering the soil moisture content (Forsch, 2014). The presence of CWD has been observed to affect nitrogenase activity via an alteration in the soil moisture content (Brunner and Kimmins, 2003). Additionally, CWD may also affect O_2 diffusion and alter the nitrogenase activity (Hicks, 2000). The reduction of O_2 availability may favor anaerobic and microaerophilic bacteria which are involved in a symbiotically N_2 fixation

(Silvester et al. 1982). In addition, decaying of CWD may also affect the C substrate availability and can increase the nitrogenase activity (Means et al. 1992).

3.4.4 Linkages among functional gene abundances and microbial indicators

The strong positive correlations between N functional gene abundances and microbial indicators such as MBC, MBN, respiration rate, and BGLU and NAGase activities suggest that N cycle functional gene abundances are linked to overall microbial community biomass and activities. Greater N functional gene abundances in soil may indicate a larger microbial community population and biomass and consequently may reflect the more microbial processes performance potential and activities (Rocca et al. 2014) such as enzyme activities and respiration rate.

3.5 Conclusion

A key finding of this study is that there are changes in N cycle gene abundances during three years of study. In this study, the average values of the AOA *amoA* gene abundances ranged from 10^3- 10^5 and for the rest of genes (AOB *amoA*, *nirS* and *nifH*) ranged from 10^3- 10^4 copies ng^{-1} soil DNA from 2011 to 2013. Previous studies indicated that AOA and AOB *amoA* and *nifH* gene abundances were in order of magnitude of 10^3 copies ng^{-1} soil DNA and the *nirS* gene abundance was found in order of magnitude of 10^4 copies ng^{-1} soil DNA in wetland and paddy soils (Bannert et al. 2011). However, the orders of magnitude of all measured genes in our study are comparably higher than this study. It can be due to the fact that reclaimed oil sands ecosystem is under fluctuations of different environmental variables and substrate inputs by reclamation practices.

Based on the results of the functional gene abundances in this study, it is also concluded that N cycling functional genes were influenced by characteristics of the capping materials used to complete the reclamation process. Furthermore,

differences in vegetation cover, as well as environmental factors such as microenvironment which were in turn influenced by CWD presence. The initial responses of gene abundances suggest that microbial communities associated with *amoA*, *nirS,* and *nifH* genes need some time to become fully re-established. However, the estimation of the exact time for microbial community development and re-establishment requires longer period of assessment. Seasonal dynamics are known to be obstacles in the accurate prediction and assessment of changes in gene abundances. Among the reclamation practices investigated in this study, the LFH treatment demonstrated superior material for the gene abundances over the PMM treatment. When combined with CWD application, improvement in functional gene abundances is even better. Our results suggest that reclamation policies should incorporate CWD in future oil sand reclamation practices to accelerate the development of microbial communities in terms of functional genes which can potentially be used as integrative ecological tools to predict the assessment of reclamation practices required for proper functioning of reclaimed ecosystems. In this regard, it is also suggested that C and N substrates may be applied to the reclamation materials for evaluating the dependency of functional genes on different substrates. Abiotic stresses such effects of soil temperature; water content and soil pH on functional genes may also be detrmined to evaluate their role in reestablishment of N cycle in the ecosystem. Moreover, two other aspects could be useful in the evaluation of development of N cycling functional groups. One is to study relative abundances of N cycling functional genes to the quantity of the total microbial community 16S rRNA gene. Another would be the study of the microbial community composition of N cycling functional groups using high-throughput sequencing techniques to track their changes under different reclamation practices over time.

Table 3. 1 The primers and thermal profiles used for PCR and real-time PCR of nitrogen functional genes

Target gene	Primer	Sequence (5′to3′)	Amplicon length (bp)	Thermal cycling profile	No. of cycles	Reference
amoA AOA	Arch-amoAF	5′-STAATGGTCTGGCTTAGACG-3′	635	95°C, 60 s; 56°C, 60 s; 72°C, 30 s	40	Francis et al. (2005)
	Arch-amoAR	5′-GCGGCCATCCATCTGTATGT-3′				
amoA AOB	amoA-1F	5′-GGGGTTTCTACTGGTGGT-3′	491	95°C, 60 s; 54°C, 60 s; 72°C, 60 s	40	Rotthauwe et al. (1997)
	amoA-2R	5′-CCCCTCKGSAAAGCCTTCTTC-3′				
nifH	nifH-F	5′-AAAGGYGGWATCGGYAARTCCACCAC-3′	458	94°C, 30 s; 53°C, 45 s; 72°C, 30 s	40	Rösch et al. (2002)
	nifH-R	5′-TTGTTSGCSGCRTACATSGCCATCAT-3′				
nirS	nirScd3AF	5′-GTSAACGTSAAGGARACSGG-3′	425	94°C, 60 s; 57°C, 60 s; 72°C, 60 s	40	Throbäck et al. (2004)
	nirSR3cd	5′-GASTTCGGRTGSGTCTTGA-3′				

Table 3. 2 Effect of capping materials on gene abundances after oil sands reclamation

Functional gene[a]	Capping type	
	LFH	PMM
Archaeal *amoA* (genes ng^{-1} DNA)[b]	5.8352×10^4 (7.469×10^3)	2.3771×10^4 (2.907×10^3)
Bacterial *amoA* (genes ng^{-1} DNA)	1.0697×10^4 (1.123×10^3)	6.558×10^3 (504)
nifH (genes ng^{-1} DNA)	1.1024×10^4 (1.156×10^3)	6.389×10^3 (562)
nirS (genes ng^{-1} DNA)	2.0015×10^4 (1.630×10^3)	8.724×10^3 (680)

[a] Functional gene: Archaeal *amoA*: Archaeal ammonia monooxygenase, Bacterial *amoA*: Bacterial ammonia monooxygenase, *nifH*: nitrogenase reductase, *nirS*: cytochrome cd1 nitrite reductase.

[b] Gene abundances are the total gene copy numbers divided by the measured DNA concentration used per reaction to give gene copies/ng of DNA.

Table 3. 3 Effect of CWD on gene abundances after oil sands reclamation

Functional gene[+]	Capping type	CWD	2011 June	2011 September	2012 June	2012 September	2013 June	2013 September
Archaeal *amoA* (genes ng^{-1} DNA)[***]	LFH	Control	3.9×10^3a*	1.1×10^4ab	3.3×10^4bc A	7.1×10^4d A**	5.6×10^4cd AB	6.9×10^4d A
		Away	3.9×10^3a	1.1×10^4ab	3.5×10^4b A	8.0×10^4c AB	3.1 x 10 b A	9.2×10^4c A
		Near	1.1×10^4 a	1.5×10^4a	8.8×10^4bc B	1.0×10^5b B	7.3×10^4c B	2.2×10^5d B
	PMM	Control	2.9×10^3ac	6.0×10^2a	3.6×10^4b A	3.2×10^4b	1.9×10^4bc	3.4×10^4 b A
		Away	2.9×10^3a	6.0×10^2a	1.6×10^4ab B	3.1×10^4b	1.4×10^4ab	5.9 X 10^4c B
		Near	9.4×10^3a	4.8×10^3a	3.7×10^4 b A	3.9×10^4b	2.0×10^4a	6.9×10^4c B
Bacterial *amoA* (genes ng^{-1} DNA)	LFH	Control	3.3×10^3a	5.2×10^3a A	5.9×10^3a	1.1×10^4b A	1.1×10^4b	1.3×10^4b A
		Away	3.3×10^3a	5.2×10^3a A	5.8×10^3a	9.3×10^3b A	1.0×10^4b	1.5×10^4c A
		Near	5.6×10^3a	1.0×10^4bd B	7.7×10^3ab	1.8×10^4c B	1.3×10^4d	3.7×10^4e B
	PMM	Control	2.2×10^3a	3.8×10^3a A	3.1×10^3a A	4.3×10^3ab A	4.9×10^3ab A	7.1×10^3b A
		Away	2.2×10^3a	3.8×10^3ab A	6.4×10^3b B	1.0×10^4 cd B	6.3×10^3b AB	1.1×10^4d B
		Near	3.7×10^3a	7.4×10^3b B	8.1×10^3bc B	1.1×10^4c B	8.3×10^3bc B	1.4×10^4d C
nifH (genes ng^{-1} DNA)	LFH	Control	2.9×10^3a	5.5×10^3ab A	6.9 X 10^3b	1.2×10^4 c	7.2×10^3b A	2.5×10^4 d A
		Away	2.9×10^3a	5.5×10^3ab A	7.1×10^3 bc	9.7×10^3cd	1.1×10^4d B	1.8×10^4e B
		Near	3.2×10^3a	1.1×10^4b B	7.2×10^3c	1.3×10^4b	1.3×10^4b B	3.5×10^4d C
	PMM	Control	1.3×10^3a	3.2×10^3ab	5.8×10^3bc A	8.1×10^3c	5.9×10^3c A	1.3×10^4d
		Away	1.3×10^3a	3.2×10^3ac	2.8×10^3ac B	8.2×10^3b	5.0×10^3c A	1.3×10^4d
		Near	1.6×10^3a	4.6×10^3b	6.0×10^3b A	9.4×10^3c	9.8×10^3c B	1.4×10^4d
nirS (genes ng^{-1} DNA)	LFH	Control	1.1×10^4a	1.2×10^4a A	1.4×10^4a	1.7×10^4ab A	1.5×10^4ab A	2.2×10^4b A
		Away	1.1×10^4a	1.2×10^4a A	1.2×10^4a	1.5×10^4a A	1.3×10^4a A	4.4×10^4b B
		Near	1.4×10^4a	2.1×10^4a B	$1.5x\ 10^4$a	2.9×10^4b B	2.9×10^4b B	4.8×10^4c B
	PMM	Control	2.0×10^3a A	3.2×10^3a A	7.0×10^3b	7.9×10^3b	8.8×10^3b	1.5×10^4c A
		Away	2.0×10^3a A	3.2×10^3a A	7.2×10^3b	8.0×10^3b	8.3×10^3b	1.7×10^4c AB
		Near	8.1×10^3a B	9.3×10^3a B	9.8×10^3a	9.8×10^3a	1.1×10^4a	1.9×10^4b B

[+] Functional gene: Archaeal *amoA*: Archaeal ammonia monooxygenase, Bacterial *amoA*: Bacterial ammonia monooxygenase, *nifH*: nitrogenase reductase, *nirS*: cytochrome cd1 nitrite reductase.

* Means with different lowercase letters indicate significant difference between month and year in each row.

** Means with capital letters indicate significant difference among CWD treatments (control, away and near) in each column. Numbers without capital letters indicate no significant difference among CWD treatments.

*** Gene abundances are the total gene copy numbers divided by the measured DNA concentration used per reaction to give gene copies/ng of DNA.

Table 3. 4 Effect of season (month) on gene abundances after oil sands reclamation

Functional gene[a]	Month	
	June	September
Archaeal *amoA* (genes ng^{-1} DNA)[b]	2.7421×10^4 (3.442×10^3)	5.4487×10^4 (7.532×10^3)
Bacterial *amoA* (genes ng^{-1} DNA)	6.146×10^3 (446)	1.1133×10^4 (1.115×10^3)
nifH (genes ng^{-1} DNA)	5.593×10^3 (472)	1.1868×10^4 (1.121×10^3)
nirS (genes ng^{-1} DNA)	1.1063×10^4 (830)	1.7538×10^4 (1.812×10^3)

[a] Functional gene: Archaeal *amoA*: Archaeal ammonia monooxygenase, Bacterial *amoA*: Bacterial ammonia monooxygenase, *nifH*: nitrogenase reductase, *nirS*: cytochrome cd1 nitrite reductase.

[b] Gene abundances are the total gene copy numbers divided by the measured DNA concentration used per reaction to give gene copies/ng of DNA.

Table 3. 5 Pearson Correlation Coefficients (r- value) and significance among functional genes and microbial indicators after oil sands reclamation (n= 90)

Variables[a]	MBC	MBN	Respiration rate	BGLU	NAGase	Archaeal *amoA*	Bacterial *amoA*	*nifH*	*nirS*
MBC									
MBN	0.2624								
	0.0125								
Respiration	0.3633	0.2736							
	0.0004	**0.0091**							
BGLU	0.4578	0.5089	0.3979						
	<.0001	**<.0001**	**0.0001**						
NAGase	0.6305	0.4506	0.5201	0.6923					
	<.0001	**<.0001**	**<.0001**	**<.0001**					
Archaeal *amoA*	0.2779	0.1670	0.3284	0.2402	0.3654				
	0.0080	0.1158	**0.0016**	**0.0226**	**0.0004**				
Bacterial *amoA*	0.4122	0.2192	0.3830	0.3050	0.5434	0.8771			
	<.0001	**0.0379**	**0.0002**	**0.0035**	**<.0001**	**<.0001**			
nifH	0.5236	0.2092	0.4976	0.3343	0.5720	0.8054	0.8453		
	<.0001	**0.0478**	**<.0001**	**0.0013**	**<.0001**	**<.0001**	**<.0001**		
nirS	0.4375	0.2301	0.3798	0.4027	0.5078	0.7820	0.8162	0.7945	
	<.0001	**0.0291**	**0.0002**	**<.0001**	**<.0001**	**<.0001**	**<.0001**	**<.0001**	

[a] Variables: MBC: microbial biomass C, MBN: microbial biomass N, Respiration = respiration rate, BGLU = 1,4-β-glucosidase, NAGase = 1,4-β-N-acetylglucosaminidase, Archaeal *amoA*: Archaeal ammonia monooxygenase, Bacterial *amoA*: Bacterial ammonia monooxygenase, *nifH*: nitrogenase reductase, *nirS*: cytochrome cd1 nitrite reductase.

Bold indicates significant coorelation (α= 0.05).

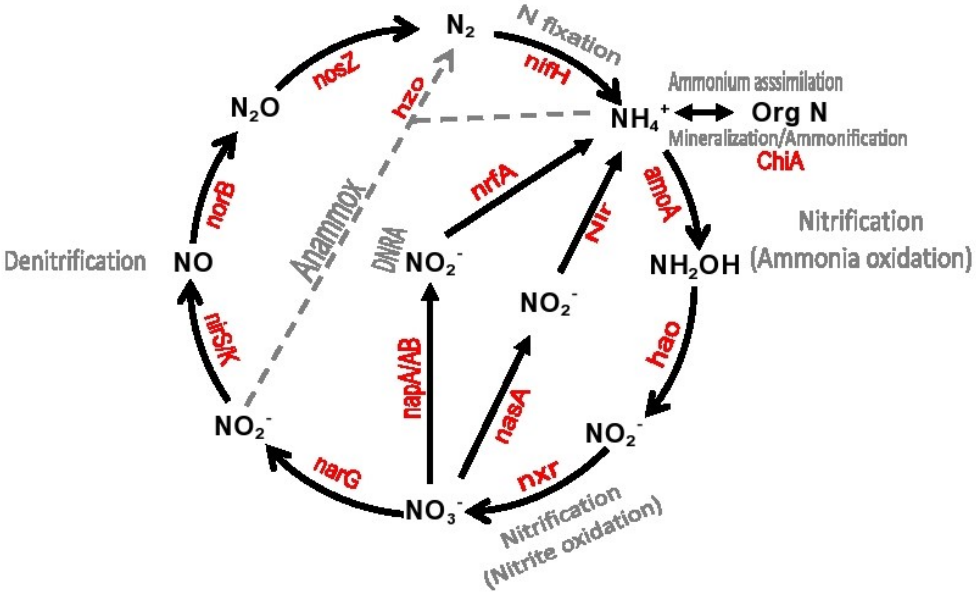

Figure 3. 1 Processes and the genes involved in the nitrogen cycle. *nifH*: encoding nitrogenase; *amoA*: encoding ammonia monooxygenase; *hao*: encoding hydroxylamine dehydrogenase; *nxr*: encoding nitrite oxidoredoctase; *narG*: encoding nitrate reductase; *nirS*: encoding cytochrome cd1 nitrite reductase; *nirK*: encoding copper nitrite reductase; *norB*: encoding nitric oxide reducatse; *nosZ*: encoding nitrous oxide reductase; *napA*: encoding periplasmic nitrate reductase; *nrfA*: encoding cytochrome cd1 nitrite reductase; *nasA*: encoding nitrate reductase, *Nir*: encoding nitrite reductase; *hzo*: encoding hydrazine oxidizing enzyme. Modified from Lu et al. (2012).

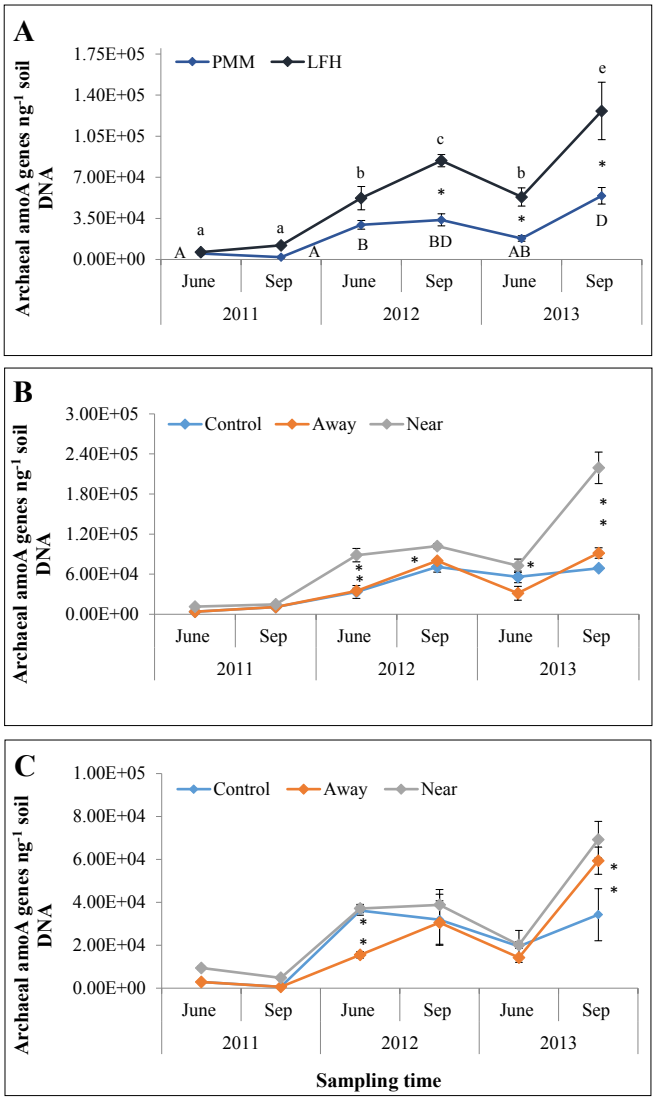

Figure 3. 2 Effect of (A) capping materials [identifiable litter, fragmented and fermented litter and humus (LFH) and peat mineral soil mix (PMM)], (B) coarse woody debris (CWD) over LFH, and (C) CWD over PMM on *amoA* (archaeal ammonia monooxygenase) gene abundance after reclamation. Soil samples for CWD treatments were collected from control (non-CWD), away from CWD, and near CWD. Error bars represent standard error of the means in treatments. Lowercase letters show differences among means of LFH capping material over time. Capital letters indicate differences among means of PMM capping material over time. * indicates significant differences between LFH and PMM treatments (α= 0.05).

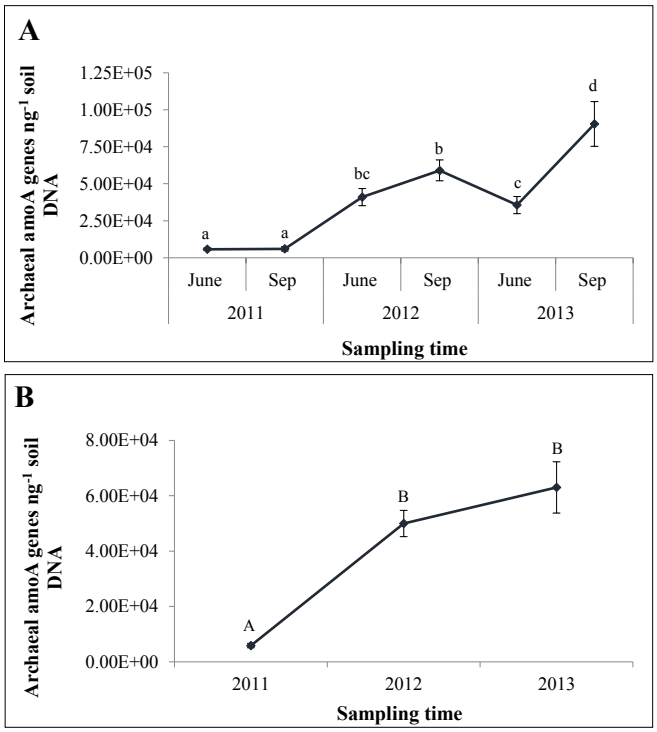

Figure 3. 3 Effect of (A) season (month) and (B) time (year), on *amoA* (archaeal ammonia monooxygenase) gene abundance after reclamation. Error bars represent standard error of the means calculated for different months and years. Different letters above bars indicate that the values over time are significantly different.

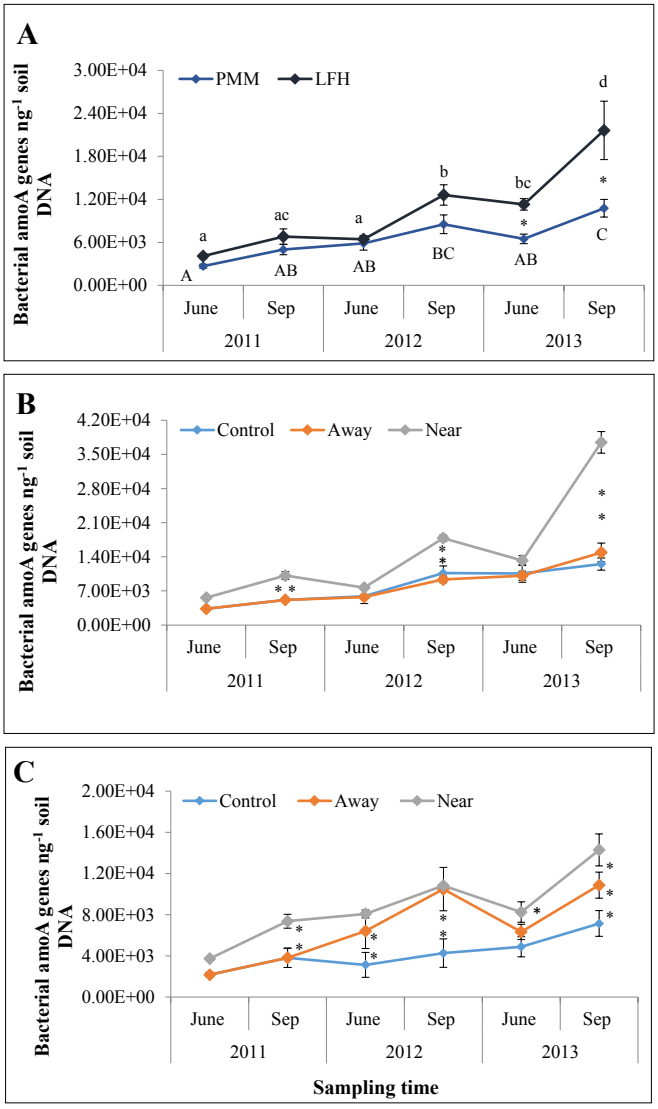

Figure 3. 4 Effect of (A) capping materials [identifiable litter, fragmented and fermented litter and humus (LFH) and peat mineral soil mix (PMM)], (B) coarse woody debris (CWD) over LFH, and (C) CWD over PMM on *amoA* (bacterial ammonia monooxygenase) gene abundance after reclamation. Soil samples for CWD treatments were collected from control (non-CWD), away from CWD, and near CWD. Error bars represent standard error of the means in treatments. Lowercase letters show differences among means of LFH capping material over time. Capital letters indicate differences among means of PMM capping material over time. * indicates significant differences between LFH and PMM treatments (α= 0.05).

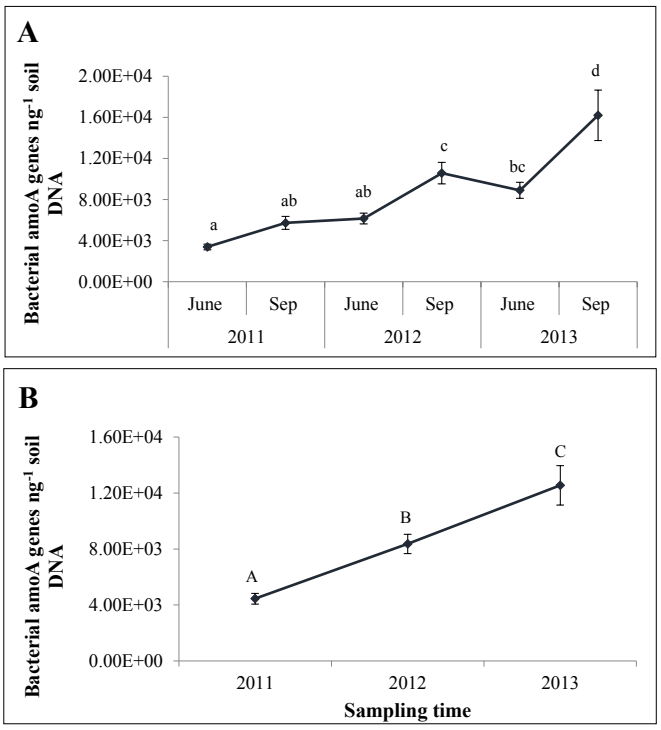

Figure 3. 5 Effect of (A) season (month) and (B) time (year), on *amoA* (bacterial ammonia monooxygenase) gene abundance after reclamation. Error bars represent standard error of the means calculated for different months and years. Different letters above bars indicate that the values over time are significantly different.

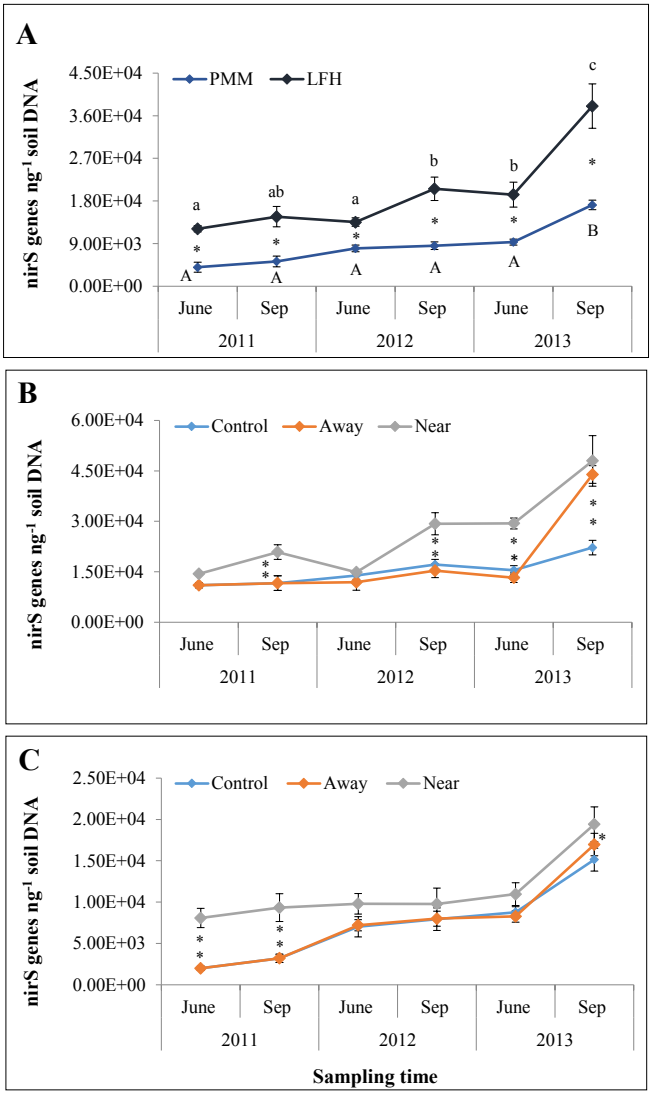

Figure 3. 6 Effect of (A) capping materials [identifiable litter, fragmented and fermented litter and humus (LFH) and peat mineral soil mix (PMM)], (B) coarse woody debris (CWD) over LFH, and (C) CWD over PMM on *nirS* (nitrite reductase) gene abundance after reclamation. Soil samples for CWD treatments were collected from control (non-CWD), away from CWD, and near CWD. Error bars represent standard error of the means in treatments. Lowercase letters show differences among means of LFH capping material over time. Capital letters indicate differences among means of PMM capping material over time. * indicates significant differences between LFH and PMM treatments (α= 0.05).

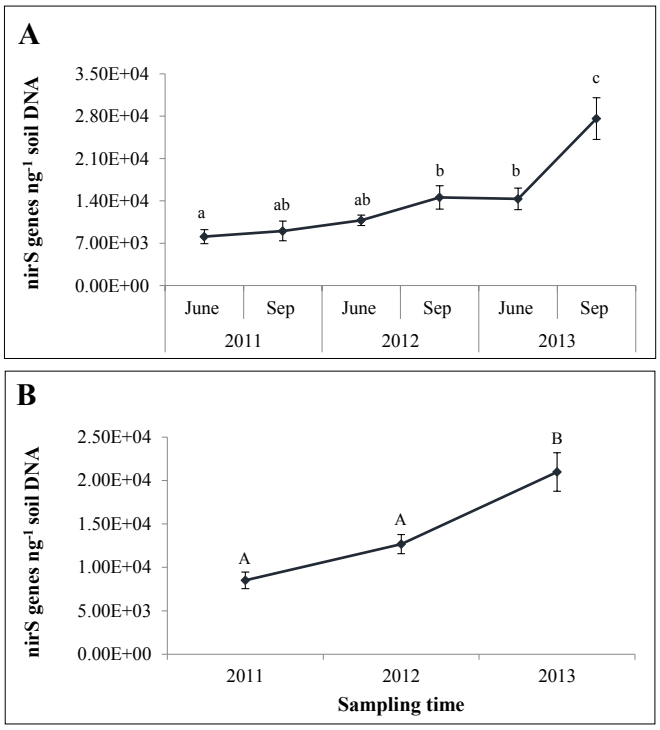

Figure 3. 7 Effect of (A) season (month) and (B) time (year), on *nirS* (nitrite reductase) gene abundance after reclamation. Error bars represent standard error of the means calculated for different months and years. Different letters above bars indicate that the values over time are significantly different.

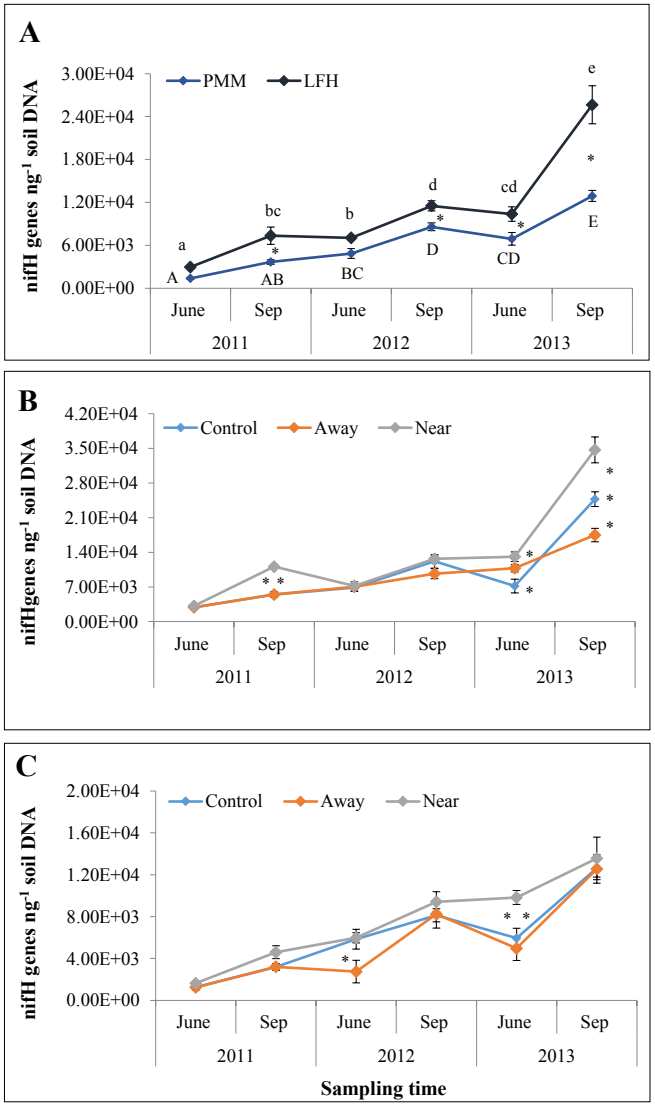

Figure 3. 8 Effect of (A) capping materials [identifiable litter, fragmented and fermented litter and humus (LFH) and peat mineral soil mix (PMM)], (B) coarse woody debris (CWD) over LFH, and (C) CWD over PMM on *nifH* (nitrogenase) gene abundance after reclamation. Soil samples for CWD treatments were collected from control (non-CWD), away from CWD, and near CWD. Error bars represent standard error of the means in treatments. Lowercase letters show differences among means of LFH capping material over time. Capital letters indicate differences among means of PMM capping material over time. * indicates significant differences between LFH and PMM treatments (α= 0.05).

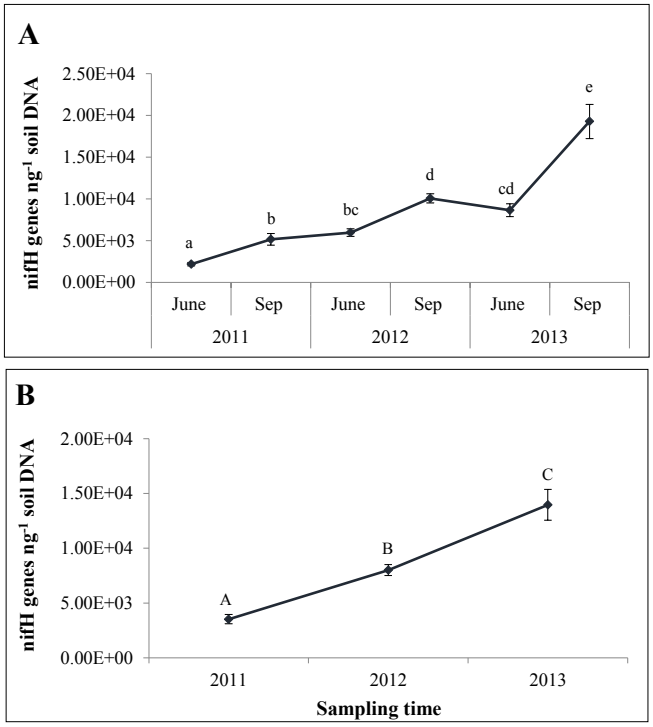

Figure 3. 9 Effect of (A) season (month) and (B) time (year), on *nifH* (nitrogenase) gene abundance after reclamation. Error bars represent standard error of the means calculated for different months and years. Different letters above bars indicate that the values over time are significantly different.

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Chapter 4. Summary and Future Research

4.1 Summary

To assess the re-establishment of the functional ecosystem during reclamation of disturbed land after oil sands operation, it is necessary to evaluate the reclamation processes which affect the reestablishment of the ecosystem. Microbial communities have important role in the functioning of soil ecosystem by providing energy and making nutrients available for other biogeochemical processes in the ecosystem (Schoenholtz et al. 2000). They respond quickly to disturbances and environmental changes and can also be used as indicators of soil quality development (Harris, 2003). Therefore, the assessment of the microbial community dynamics can help to assess the success of reclamation. Key objective of this study was to investigate the development of microbial communities through their dynamics in reclaimed oil sands soils. Currently, in oil sands reclamation, PMM and LFH are used as capping materials. They have contrasting soil properties with LFH having more decomposed OM than PMM. Consequently, they may affect the soil microbial dynamics and functional gene abundances and their development. Recently, the potential of CWD has also been explored to accelerate the oil sands reclamation process by changing the microenvironment (Brown and Naeth, 2014). To assess the role of reclamation practices in the development of microbial community dynamics, soil samples were collected consecutively from a newly reclaimed oil sands site, Fort McMurray, Alberta, Canada in June and September from 2011 to 2013. During this study, effects of capping materials (LFH and PMM), CWD application and seasonal variations on microbial community dynamics were evaluated. This study was comprised of two data chapters, in first data chapter, the development of MBC, MBN, respiration rate, BGLU and NAGase activities were determined. In second data chapter, the abundance of N- cycling functional genes such as AOB and AOA *amoA*, *nirS* and *nifH* were quantified.

The results of this study showed that LFH had greater MBC, MBN, BGLU, NAGase activities and abundances of AOB and AOA *amo*A, *nirS* and *nifH* genes than PMM. These results might be attributed to the narrower C:N ratio in LFH than in PMM (Jamro et al. 2014) which may favor the faster decomposition of OM. This may yield greater MBC and MBN and may provide substrate for BGLU and NAGase activities. In addition, the greater activities of BGLU and NAGase were also likely associated to the greater mycorrhizal biomass in LFH than in PMM (Brown and Naeth, 2014; Jamro et al. 2014). The greater abundances of AOB and AOA *amoA* in LFH than in PMM is associated with greater NH_4^+ supply (Jamro et al. 2014) and higher nitrification rate (McMillan et al. 2007). The greater abundance of *nirS* gene observed in LFH was due to high nitrification rate and more decomposed OM which can provide substrates (C and NO_3^-) for denitrification process. Moreover, LFH had greater plant species diversity such as grasses, forbs, shrubs, and tree species than PMM (Forsch, 2014) which likely supported the presence of symbiotic and non-symbiotic bacteria and consequently the increased *nifH* gene abundances. The application of CWD increased respiration rate, BGLU andNAGase activities, and all measured functional genes abundances. However, it did not affect MBC and MBN during the study period. The increase in microbial dynamics and gene abundances with the application of CWD was likely linked to the changes in microsite development which may alter the soil water content and temperature (Brown and Naeth, 2014). In addition, greater plant species richness in the presence of CWD over capping materials (Forsch, 2014) might have increased the substrate availability for microbial activities and processes.

Seasonal changes are also recognized as key factors interfering with the accurate evaluation of the effects of reclamation practices on development of microbial community dynamics (Dimitriu et al. 2010). The results of this study indicated that seasonality contributed to significant increase in MBC, respiration rate, BGLU, NAGase activities, AOA and AOB *amoA*, *nirS*, and *nifH* gene abundances in September than June, however, it did not affect MBN during the study period. These results showed that the seasonal variations may alter soil

temperature and water content and also increase substrate availability in the form of labile C substrate via fresh litter inputs and root exudates. This consequently can increase microbial metabolism and activity which is reflected by increase in microbial community dynamics in September sampling in this study.

Overall, significant changes were observed in all assessed soil microbial parameters including MBC, MBN, respiration rate, BGLU, NAGase activities and abundances of functional genes including AOB and AOA *amoA*, *nirS* and *nifH*. Such changes were likely appeared to be influenced by the differences in C:N ratio, pH, N supply, vegetation cover, plant diversity and richness in capping materials and microsite development by CWD application.

4.2 Future Research

The results of this study reported in this thesis may provide base knowledge and guidelines for the effects of reclamation practices on the development of microbial community indicators and N-cycling functional gene abundances. However, two major research areas could be included in future work. One could be identification and characterization of microbial community structure via 16S rRNA gene analyses to track the microbial community diversity and composition overtime. The other would be characterization of functional genes involved in N transformations by sequencing of functional genes and their phylogenetic analyses in order to link the microbial functions to their composition which is prerequisite for the assessment of success of reclamation practices and ecosystem development.

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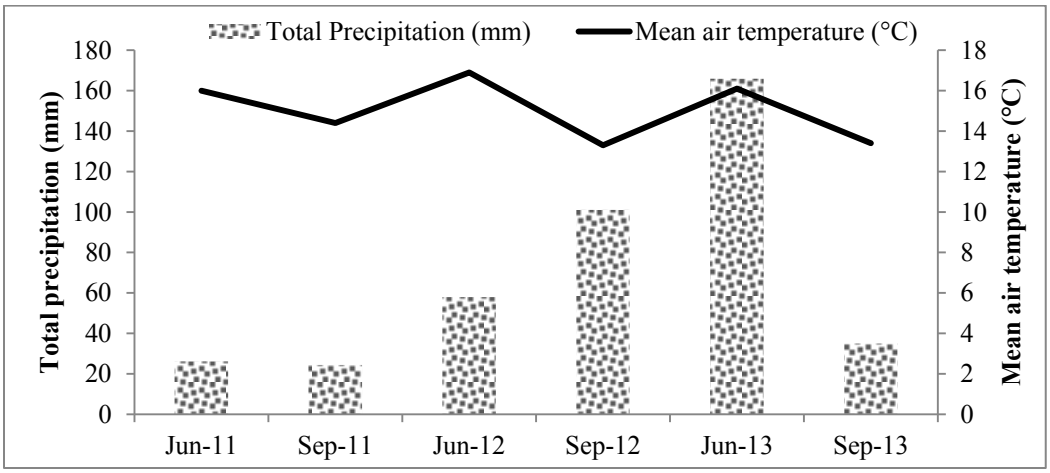
Appendices



Appendix- I. Monthly precipitation (bars) and mean monthly air temperature (lines) during sampling period.