University of Alberta

Microbial communities in organic substrates used for oil sands reclamation and their link to boreal seedling growth.

by

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ABSTRACT

Soil reconstruction in the Athabasca oil sands region utilises forest floor and peat materials as surface organic amendments to help reclaim decommissioned mine sites to upland boreal forests. The objective of this study was to characterize the microbial community in these two organic materials, and to determine the relationships between two boreal tree seedlings, aspen (*Populus Tremuloides* Michx.) and alder (*Alnus crispa* Ait), and their respective rhizosphere microbial communities. The forest floor exhibited a greater basal respiration than the peat, and a distinct microbial community structure as seen with phospholipid fatty acid analysis. Stable isotope probing showed greater carbon flow between trees and their rhizosphere communities when seedlings were grown in forest floor material. However, only the alder seedlings demonstrated correspondingly greater growth in the forest floor material. These results suggest that forest floor material fosters a microbial community which interacts more closely with boreal tree seedlings than peat does.

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LIST OF SYMBOLS AND ABBREVIATIONS

^{13}C	Stable carbon isotope with one additional neutron
$^{13}CO_2$	Carbon dioxide with a ^{13}C isotope
AOSR	Athabasca oil sands region
$CaCl_2$	Calcium chloride
C/N	Carbon to nitrogen ratio
FFM	Forest floor mineral (stockpiled material)
GC	Gas chromatograph
ISA	Indicator species analysis
LFH	Forest floor organic layer (natural)
MRPP	Multi-response permutation procedure
NMR	Nuclear magnetic resonance
NMS	Non-metric multidimensional scaling
PDB	Pee dee belemnite
PLFA	Phospholipid fatty acids
SIP	Stable isotope probing
SMC	Soil microbial community
WHC	Water holding capacity

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I. INTRODUCTION

The Athabasca oil sands deposits of northeastern Alberta contain a proven oil reserve of over 171 billion barrels. This extensive oil sands deposit underlies 142,200 km^2 of the Canadian boreal forest, and as of December 31, 2010, 715 km^2 of the 4,800 km^2 that can be surface mined economically have been disturbed by surface mining (Department of Natural Resources 2012).

At the end of the mine life, materials are required to reclaim the spent mine and return it to a "productive state". Standard industry practice is to salvage the topsoil, store it until the end of mining activities and place it as a cover material for revegetation of the spent mine (Ziemkiewicz et al. 1980). The topsoil is the weathered vestige of the parent geological material, and embodies the chemical and physical characteristics that will provide an immediately reclaimable plant growth medium to support sustained ecosystem productivity. It is comprised of glacial till blankets, eolian sand dunes and postglacial fluvial and lacustrine plains (Crown and Twardy 1970)

The overburden, located below the topsoil and above the bituminous sands is of marine origin (Crown and Twardy 1970) and was deposited during the Cretaceous (Monenco 1980). By itself, overburden represents a poor medium for plant growth, as it either contains coarse textured sandy material having poor water and nutrient retention capacity, or medium to fine textured materials that are saline and sodic with a pH near 8 (Monenco 1980, Alberta Environment 2007). Whereas overburden may not represent a favorable medium for plant growth, early oil sands reclamation research investigated the

incorporation of organic matter to ameliorate reclamation materials and improve soil tilth, available water and nutrient retention (Kong et al. 1980, Land Resources Network 1993).

Danielson (1991) conducted an early oil sands experiment investigating the affect of sewage sludge, chemical fertilizer and peat on the growth of jack pine (*Pinus banksiana* Lamb.) and white spruce (*Picea glauca* (Moench) Voss), and the consequent litter development and soil mycorrhizal growth. The results were mixed. Peat showed greater mycorrhizal growth but sewage sludge produced substantially greater plant growth and litter development. Although sewage sludge showed as good or better results, the remoteness of the source from the mine site and the refusal of the labour force to participate in the application of the material due to its potential to harbor pathogens has eliminated sewage sludge as a reclamation amendment in the oil sands (Visser et al. 1984).

Although soil reclamation is often restricted by the availability of organic matter sources (Wullschleger et al. 2004), this is not the case for the Athabasca oil sands, which occurs within the Wabasca lowlands ecoregion, a poorly drained discharge area dominantly covered by peatlands (Ecological stratification working group 1995). Therefore, peat is in abundant supply in the area and was initially described as "the only organic material naturally available for rebuilding soil fertility" (Kong et al. 1980).

HBT AGRA Ltd. (1992) conducted a five-year soil reconstruction field trial in the oil sands that was concurrent to the work of Danielson (1991), focusing on peat as an organic amendment. The experimental design included three applications rates of peat

and non-saline/sodic overburden at two different application depths overtop tailings sand. Chemical analyses of the constructed materials showed that peat increased the total quantities of nitrogen and sulphur, while overburden increased total phosphorus and potassium. Soil physical analyses demonstrated that while peat increased available water, this was not the case for the dominantly clay overburden. While the materials were rotovated, the clay overburden material and the peat were not mixed homogeneously; instead the overburden remained in "clods" due to its high cohesive nature (HBT AGRA Ltd. 1992).

The continued failure to develop operational scale strategies for homogenizing the reconstructed soils resulted in the over-salvaging of peat layers to include underlying mineral material as a "peat-mineral mix" (HBT AGRA Ltd. 1992) and finally the complete abandonment of overburden incorporation in favor of layering (Marty Yarmuch, Syncrude Canada, *personal communications*, 2012). Current oil sands environment approvals for peat application require 0.5 to 1 m of approved cover material with 0.3 m of peat on the surface (Alberta Environment 2007).

While field trials have produced results showing substantial improvements in soil fertility from the application of peat in reconstructing soils, surface mining of the oil sands has created disturbance on a landscape scale. The mine excavations are measured in square kilometers and commonly exceed 80 m in depth (Department of Natural Resources, Canada 2012). Further, glacial till expands or "bulks" by approximately 20% after excavation. This increase in volume creates the potential for the post mining landscape to

be 20% higher than pre-disturbance. For instance in the case of a 80 m excavation the increase in height would be 16 metres, corresponding to the height of a six story building. Therefore, it will be impossible to return the entirety of the post-disturbance landscape to a lowland setting.

The majority of the post mining landscape will include, by necessity, an upland topography and will have to support a plant community different from that of a peatland. Moreover, peat is the organic residue from a plant community dominated by mosses, and is very different from the target community on the reclaimed landscape, which will be one of upland boreal forest. Research has shown that soil organic matter (SOM) originating from different plant sources can have a different composition (Hannam et al. 2004, Turcotte et al. 2009), and this composition strongly influences both the abiotic and biotic properties of the soil (Quideau et al. 2000). For instance, the field trials conducted by HBT AGRA Ltd. (1992) on soil reconstructed using peat reported mixed results of revegetation efforts with woody boreal species. While a number of species showed good survival and even infilling during the five year course of the experiment, all three actinorhizal species (silverberry, buffaloberry & alder) showed a continued decline in survival throughout the experiment. Further, while not statistically significant, both the crown diameter and total root weight of these species showed declines as the application rate of peat increased.

All three of these woody species (silverberry, buffaloberry & alder) form a tripartite relationship with mycorrhizae and actinomycetes (Visser et al. 1991, Yamanaka et al.

2005). Microorganisms found in their rhizospheres provide them with services such as nitrogen fixing or increased nutrient absorption in exchange for labile carbon compounds supplied by the host plant (Darrah 1991, Neumann & Romheld 2007, Hartmann et al. 2008). These exchanges are beneficial to all partners and result in a competitive advantage, which increases their survival. However, there is a large body of seminal literature pointing to the anti-microbial properties of peat (Banerjee & Sen 1979, Johnson & Damman 1991, Painter 1991, Verhoeven & Toth 1995, Aerts et al. 1999) and research has shown that without its microbial partners, the survival and/or growth of the plant partner is compromised (Visser et al. 1991, Yamanaka et al. 2005).

Oil sands research into the microbial community of reconstructed soils produced results suggesting that mycorrhizae potential (i.e. the percentage of colonization of each mycorrhizal type per root length) is inhibited by peat (Danielson *et al.* 1983). Vesicular-arbuscular mycorrhizae and ectomycorrhizae were found to be missing or had the lowest values in the peat treatment compared to overburden treatments (fine and coarse textured overburden, and fine textured overburden from greater than two m depth). Further, poor fungal survival was observed following inoculation by nine different mycorrhizal species, as the introduced fungi did not survive past three years (Danielson & Visser 1989). The authors concluded that this failure was not due to competitive replacement, as in some cases as much as 50% of the root remained uninfected. The only mycorrhizae that were successful in the reconstructed soils were those that were endemic to the peat material. This suggests that the soil conditions; i.e., the chemical and/or physical characteristics of

the peat, were inhibiting the success of the mycorrhizae otherwise known to be associated with the target upland plant community (Danielson & Visser 1989).

More recently, development of alternative reclamation strategies during the last decade has included research on the utilization of forest floor mineral (FFM) material as a soil amendment. Upland sites within the ASOR are forested by aspen-white spruce mixed forests or jack pine stands established on Gray Luvisolic and Eluviated Dystic Brunisolic soils, respectively (Crown and Twardy 1970). Both soil types form an L, F and H organic layer composed of leaves, twigs and woody material on top of an eluviated Ae mineral horizon (Soil Classification Working Group 1998). FFM is a heterogeneous mixture of the upland forest organic layer (LFH), coarse woody debris and Ae mineral horizon salvaged from upland sites designated for future mining.

This FFM material was originally adopted as an economical source of plant propagules (Lanoue & Qualizza 2001, as cited in MacKenzie 2006). Reclamation trials have reported significantly greater plant diversity and cover in FFM amended sites compared to peat (Mackenzie 2006). However, the success of FFM is tempered by its limited quantity. Since most of the pre-mining landscape is dominated by peatlands, there is not enough FFM to reclaim all the post-mining areas.

Given results broadly reported in seminal literature pointing to the anti-microbial properties of peat (Banerjee & Sen 1979, Johnson & Damman 1991, Painter 1991, Verhoeven & Toth 1995, Aerts et al. 1999) and research showing the greater reclamation success of FFM, a comparison of the microbial community between these two materials

is a natural continuation of the development of reclamation strategies in the oil sands. My first investigation focused on confirming the broad findings of previous researchers and tested for evidence of reduced activity in peat microbial communities compared to upland forest floor and FFM material. Since microbes respire approximately 50% of the carbon that they mineralize, soil respiration rate can be used as a proxy of the amount of organic matter that is mineralized (Margesin & Schinner 2005). Using this experimental approach, I investigated and compared the activity of the microbial communities in FFM and peat by measuring their respiration rates (as a proportion of the total soil organic carbon).

Additionally, as it is unavoidable that peat will be used for reclamation in the oil sands region, for the second part of my thesis, I wanted to investigate how the FFM and peat interact as mixing of the two materials may be a management option to optimize the limited volume of FFM. While characterization of peat for oil sands reclamation described its high nutrient potential, it was acknowledged that the majority of these nutrients, i.e. N & S, was likely in a form unavailable to plants (Land Resources Network 1993). Field trials illustrated peat improvements to soil tilth, available water and nutrient retention (HBT AGRA 1992) and also described the slow decay of the material as a benefit since the amendment would have a long lasting effect (Land Resources Network 1993).

Decomposition is limited by three factors: 1) the quantity and quality of the substrate available, 2) the number of microbes present, and 3) the activity level of the microbes (Vecchioli et al. 1990). Previous research has shown less $CO_{2\ (g)}$ mineralized per gram of

organic carbon in *Sphagnum* peat than in deciduous, coniferous or graminoid litter (Aerts et al. 1999), which would imply less activity in the associated microbial community. However, decomposition rates can potentially be increased by an incorporation of a more labile carbon source, which is called the "priming effect".

Positive soil priming occurs when newly incorporated litter stimulates the microbial community to greater activity. The resulting increase in concentration of catabolic enzymes breaks down more recalcitrant carbon compounds in the existing soil carbon pool (Fontaine et al. 1999). For instance, previous research has found positive soil priming after the incorporation of *Zea mays* L. litter into a brown forest soil, which resulted in an accelerated decomposition of the native soil organic matter (Nottingham et al. 2009). Therefore, it is possible that the addition of forest floor to peat will produce priming and stimulate the microbial community to mineralize a greater portion of the peat. An examination of the available literature investigating peat and FFM in the oil sands illustrates the broad perspective taken in examining the benefits and limitations of each material (Table 1). While FFM contains more available nutrients and more active microbial community, it lacks the water holding capacity of peat. Moisture was identified as the primary limiting factor in oil sands reclamation in the early phases of reclamation research (Monenco 1983). Therefore, a mixture of the two materials could ameliorate the shortcomings of each individual material.

However, given the reports of anti-bacterial properties of peat and the failure of allochthonous bacteria to survive or flourish when introduced to a foreign microbial

community (Walter et al. 1987), a high dilution of FFM material by peat may serve to just stimulate the autochthonous peat microbial community and fail to reproduce an upland forest microbial community. Therefore, a mixture of equal parts of FFM and peat (by organic carbon content) should create an operationally viable increase in volume while the greater activity in forest floor material should still give the mixture the greatest chance of producing a microbial community representative of an upland forest soil.

The next step of my thesis was to investigate the composition of the microbial communities. For this I used phospholipid fatty acid (PLFA) analysis. Briefly, phospholipids present in the cell membranes of the microbes endemic to the two materials can be chemically extracted and analysed (Tunlid et al. 1989). Results provide the molecular concentration of a series of PLFAs, some of which are characteristic of a distinct group of microorganisms, hence can be used as indicators of these groups (White et al. 1979). The combination of these indicators and their molar concentrations creates a profile or fingerprint of the community composition that can be compared to that of another community (Frostegård et al. 1993).

Results from this analysis enabled me to compare the composition of the communities as a whole to see if they differed and at the same time allowed me to analyse individual PLFAs for differences between peat and forest floor materials. However, this does not address the specific impact of the organic material type on the mutualistic segment of the microbial community. While there are indicator PLFAs for mycorrhizae (16:1ω5c and 18:1ω9c), not all mycorrhizae form mutualistic relationships with the plant host and some

can even be parasitic (Newcombe et al. 2010). Further, there is no direct indicator for actinorhizal bacteria. These are gram positive, but several PLFA indicators exist for gram positive bacteria. Thus, changes in the actinorhizal segment of the community could be lost within the larger gram positive population, and are unlikely to be detected with PLFA analysis.

To address these limitations in the PLFA technique I used stable isotope probing to introduce ^{13}C, a stable isotope of carbon, to the rhizosphere to specifically label the active microorganisms in close association with the roots of the host plant. This was accomplished by growing plants in each material (peat, forest floor, layered treatment of the two), enclosing them in a container and introducing $^{13}CO_{2\,(g)}$ to the closed container. The actively photosynthesizing plant absorbs the $^{13}CO_{2\,(g)}$ through its stomata and incorporates it into glucose.

Glucose is then either transformed or transported through the plant, some of which to the roots (Martin & Kemp 1986), where a mixture of simple and complex saccharides, amino acids and organic acids (Griffiths et al. 1999) is exuded into the rhizosphere. The composition and quantity of the exudates are dependent upon the host species (Biondini et al. 1988, Griffiths et al. 1999) and are mediated by the host in response to environmental stimuli such as nutrient availability (Nagahashi et al. 1996, Wall 2000) and the size of the rhizosphere microbial community (Biondini et al. 1988). In actinorhizal woody species, exudates could represent as much as 40% of the total carbon fixed by the host (VanVeen et al. 1991). As mycorrhizae and actinorhizae are both

located within the rhizosphere, carbon from the root exudates will be available to them for uptake and some of it will be utilized to build cell membranes which should include phospholipids.

The resulting PLFAs can be extracted and analysed via compound specific isotope ratio mass spectrometry to quantify their ^{13}C enrichment. Isotopes of the element carbon differ in the number of neutrons contained in the nucleus (Fry 2006). Of the stable carbon isotopes, carbon 12 (^{12}C) contains 12 neutrons and makes up almost 99% of the naturally occurring carbon whereas carbon 13 (^{13}C) contains 13 neutrons and is less common (~1%).

Carbon-13 enrichment of PLFAs above the background level can be indicative of the quantity of ^{13}C being transferred through the plants from the ^{13}C-enriched CO_2. Given that an equal volume and concentration of $^{13}CO_{2\,(g)}$ was introduced to each seedling and that it constituted the only vector of ^{13}C to the rhizosphere, the resulting PLFA ^{13}C enrichments are representative of the interaction occurring between the plant and the rhizosphere community. PLFAs known to be indicative of mutualistic segments of the microbial community were separated and compared across organic treatments to determine how they differed between peat and FFM. Finally, the growth of the plants was measured and compared to the enrichment of the PLFAs to determine if the nutrient exchange occurring was indeed mutualistic and aiding the plant growth.

The main objectives of this research were to determine if organic amendments of different botanical origins influenced the composition and activity of the soil microbial

community in reconstructed top soils of the Athabasca oil sands and, if so, to determine if the differences were related to the growth of boreal plant seedlings. Specifically, the objectives of the research were investigated by:

1) Incubation of two different sources of peat as well as forest floor material to assess the differences in the composition and activity of their microbial communities.
2) Incubation of mixtures of peat and forest floor to assess the impact of mixing on the composition and activity of the soil microbial community.
3) Stable isotope probing of the rhizosphere of alder (*Alnus crispa* Ait.) and aspen (*Populus tremuloides* Michx.) with ^{13}C to trace the carbon flow from the plant to the microbial community and assess the differences between plants grown in peat material or material from the forest floor layer.

This thesis is divided into four chapters. Following this introductory chapter, chapter two contains details of an incubation experiment which used basal respiration and phospholipid fatty analysis to investigate the activity and composition of the soil microbial communities in pure and mixed organic materials from natural sites and stockpiles common to the Athabasca oil sands. Chapter three investigated how the differences seen in the composition of the microbial community were related to the growth of boreal tree species using stable isotope probing. I hypothesized that the peat materials would foster a microbial community that will be less active and would have a soil microbial community with a different composition compared to that of the forest

floor material. As such, I also expected that mixing peat and the forest floor would create greater variability in the composition of the mixed treatment mixture compared to the pure materials. Finally, I hypothesized that the tree seedlings grown in peat would display reduced growth compared to those grown in the material from the forest organic layer, and would correspond to less carbon flow from the plant to the soil microbial community. Chapter four is a summary of my findings and also contains recommendations for future research and the application of these findings for practical implementation in soil reclamation.

CHAPTER I. TABLES AND FIGURES

Table 1.1. Summary of physical and chemical properties of peat and forest floor mineral (FFM) material from previous research.

Property	Peat	Forest Floor Mineral
Water Holding Capacity (%)	550-1200 (Kong et al. 1980)	~44 (McMillan et al. 2007)
Carbon/ Nitrogen Ratio	20-80 (Kong et al. 1980)	20-25 (MacKenzie 2006)
Available Phosphorus (mg kg^{-1})	~2 (MacKenzie 2006)	4-5 (MacKenzie 2006)
Exchangeable Potassium (mg kg^{-1})	3-5 (MacKenzie 2006)	8-15 (MacKenzie 2006)
Nitrogen Mineralization Rate (mg_N $kg^{-1}mo^{-1}$)	0-5 (McMillan et al. 2007)	5-15 (McMillan et al. 2007)
Microbial Biomass C (mg_C kg^{-1})	~300 (McMillan et al. 2007)	~400 (McMillan et al. 2007)
pH	3.7-6.6 (Kong et al. 1980)	5.5-6.1 (MacKenzie 2006)

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II. Mixing Organic Amendments For Oil Sands Reclamation Affects Soil Microbial Communities.

1. Introduction

Surface mining in the Athabasca oil sands region (AOSR) of north-eastern Alberta, Canada, creates landscape scale disturbance which removes the topsoil, the parent geologic material, and the overburden, to a depth that often exceeds 80 m. Before this scale of excavation can begin, the mine site is drained, resulting in a lowering of the water table both on the mine site and adjacent property (Millennium 2005a). A new landscape is further created throughout the operation and reclamation of the mine site. Excavation of the overburden results in "bulking", an increase in volume per unit of soil that can reach 20%. This increase in volume produces topographic and surface expression changes that constitute a significant departure from the lowland peatlands characteristic of the pre-disturbance landscape. The most recent mine approval estimates that the area re-vegetated to upland forests will be double that of the original area (Millennium 2005b).

The very different plant communities found in peatlands and upland boreal forests produce distinct litter compositions. Characterization of the molecular carbon structure of these litters using nuclear magnetic resonance (NMR) yields unique spectra that indicate quantifiable differences among them (Turcotte et al. 2009). Plant litter is the primary source of soil organic matter (Six et al. 2001), and is also the primary driver of many biological processes occurring in the soil (Quideau et al. 2000). Hence, forest floor native to the target ecosystems would be the ideal source of organic amendment to cap the

reconstructed soils. However, soil reclamation practices in the AOSR have primarily used peat to build soil fertility, due to the shortage of forest floor in the landscape undergoing mining. Widespread occurrence of organic soils on the landscape makes peat abundantly available and economical to salvage during the excavation of the mine site. Following a period of stockpiling, peat is applied in a variety of ways depending upon the regulatory approval for each mining company, which includes different thicknesses and mixtures of the material.

Recent studies in the AOSR have used phospholipid fatty acid (PLFA) analysis, a chemical characterization of cell membrane phospholipids that can be specific to a type or genus of microorganisms, to investigate the composition of the soil microbial communities (SMC) in reconstructed soils (Dimitriu et al. 2010; Sorenson 2011; MacKenzie and Quideau 2012; Hahn and Quideau 2012). Results of such studies demonstrate that SMC from peat amended reclaimed sites differs from natural boreal forests, and that time since reclamation is overridden by the influence of the organic reclamation material (Dimitriu et al. 2010). This raises the concern that the use of peat as an organic amendment may not support a soil microbial community consistent with forest development.

Since 1997, the Cumulative Environmental Management Association (CEMA), an association of stakeholders in the AOSR, has explored the use of natural forest floor material (LFH) as an organic soil amendment for oil sands reclamation (Oil Sands Revegetation Committee 1998). Although this material is more management intensive to

salvage than peat, and not as common in the landscape, it was explored as a cost effective source for plant propagules consistent with an upland plant community (Lanoue & Qualizza 2001, as cited in MacKenzie 2006). It has proven more successful than peat in establishing woody plants in field trials (Mackenzie & Naeth 2007). The LFH material is salvaged by scraping the top layer of forest floor to a 10 to 30 cm depth and contains a heterogeneous mixture of forest litter, coarse woody debris and underlying mineral substrate. Results from a recent study comparing paired peat and forest floor mineral (FFM) mix amended plots suggest that FFM puts the SMC and plant community of the reclaimed sites on a faster trajectory toward a natural forest than peat does (Hahn et al. 2012). With the necessity to reclaim large areas, the best use of the limited forest floor material may be to combine it with the more abundant peat. However, so far, limited investigations have looked at how the two materials interact when mixed. MacKenzie and Quideau (2012) reported that the microbial community from a mixture of the two materials (FFM and peat) showed no significant difference compared to FFM, suggesting that the mixing of the two materials may be a viable option for reclamation.

The first objective of this study was to examine and compare the microbial communities from forest floor and peat materials, either sampled from undisturbed ecosystems, or following salvage and stockpiling. The second objective was to investigate how mixing of the two materials would influence their biodegradation and microbial communities. Thirdly, as moisture is an important environmental factor influencing SMC, additional treatments were included to understand the specific response of these organic amendments to different moisture levels.

2. Materials and Methods

2.1. Site description and sample collection

The AOSR is located within the Wabasca Lowlands, an ecoregion of north-central Alberta which is characterized as a poorly drained, undulating lowland that is dominated by organic soils associated with fens and bogs (Ecological stratification working group 1995). Vegetation common to these peatlands includes an overstory of black spruce (*Picea mariana* (Mill.) Britton), and tamarack (*Larix laricina* (Du Roi) K.Koch), an understory of Labrador tea *(Ledum groenlandicum* Nutt.), and a groundcover of *Pleurozium* and *Sphagnum* mosses. The subregion is characterized by short, warm summers with 105 to 115 frost free days and a mean air temperature of 15.9°C for the warmest month of the year (Natural Regions Committee 2006). Winters are long and cold with a mean air temperature of -18.7°C for the coldest month. The mean annual precipitation is 478 mm with an average of 336 mm falling during the growing season.

Four different materials were collected for this study, all within a 20 km radius on or around the Mildred Lake Plant Site (Syncrude Canada Ltd.) located 50 km north of the town of Fort McMurray (56°43′35″N 111°22′49″W). These included materials sampled from two undisturbed natural sites (LFH and *Sphagnum*), as well as two stockpiled materials (FFM and Peat). The *Sphagnum* material (*Sphagnum*) was collected in the spring of 2003 from a very-poorly drained, lowland depression where a thick (> 1 m) Histosol (FAO 2006) had developed on top of a clay loam. Vegetation included black spruce, the dominant tree species, with the presence of some larch, and an understory dominated by low bush cranberry, willow (*Salix spp.*), bog birch (*Betula glandulosa*

Michx.), and moss primarily composed of *Sphagnum spp*. A large, composite sample was collected from the central area that was representative of the site and placed in a clean, dry container for transport to the laboratory.

The undisturbed forest floor layer (LFH) was collected in May 2010 from an aspen (*Populus tremuloides* Michx.) dominated site (latitude 56.95°N, longitude -111.71°E) with few (~10% canopy cover) white spruce (*Picea glauca* (Moench) Voss) trees. The soil at the site was classified as a Luvisol (FAO 2006), with a texture of clay loam over clay, and was moderately well drained. Understory species included bearberry (*Arctostaphylos uva ursi* L.), common blueberry, low bush cranberry and buffalo berry (*Shepherdia canadensis* (L.) Nutt.). The groundcover was dominated by Schreber's moss (*Pleurozium spp*.). Forest floor material was collected 40 m from the forest stand edge to avoid edge effect. Live plants and fresh litter were removed before the organic layer was collected in a clean, dry container for transport back to the laboratory.

The stockpiled forest floor mineral material (FFM) and stockpiled peat (Peat) had been salvaged the previous winter from an aspen-dominated site and wooded fen, respectively, in areas scheduled to be mined near the Mildred Lake settling basin (MLSB) located on Syncrude (Marty Yarmuch, Syncrude Canada, *personal communication*, 2012). These materials were selected as salvaged organic materials common to the area, which had undergone stockpiling, a common practice for oil sands operators. The FFM and Peat stockpiles were systematically sampled in May 2010 by taking many (> 20) samples from

the surface of both stockpiles and compositing them to yield a large representative volume of each.

2.2. Laboratory analyses

All samples were dried at room temperature for a period of 15 days, sieved to 4 mm, and carefully mixed to yield buckets of homogenized materials. Water holding capacity was determined on subsamples of each material (n=5) using Puustjarvi's (1973) "soak and drain" method. Carbon content on three subsamples for each material was determined by dry combustion using a Costech elemental combustion system (Model 4010; Valencia, CA). All materials are of organic origin and preliminary analysis established that carbonate content was minimal; hence total carbon is equivalent to organic carbon for these samples. A 0.01 M $CaCl_2$, solution was used to measure pH using a 2:1 solution:sample ratio (Kalra and Maynard 1991).

Three subsamples from the LFH, *Sphagnum*, FFM and Peat materials were analysed as described in Thiffault et al. (2008) via ramped-amplitude cross-polarization carbon-13 nuclear magnetic resonance (RAMP CP ^{13}C NMR) spectroscopy to describe their macromolecular composition. Five carbon types were identified based on their chemical shifts, including aliphatics (Alkyls) from 0 to 44 ppm, carbohydrates (O-Alkyls) from 45 to 111 pm, aromatics from 112 to 139 ppm, phenolics from 140 to 165 ppm, and carbonyls from 165 to 192 ppm.

2.3. Incubation experiment

This experiment was separated into two separate incubations including the following materials: 1. the natural materials collected from the two undisturbed natural sites (LFH and *Sphagnum*), and 2. the stockpiled materials (FFM and Peat). Within the first incubation, three treatments were created: LFH, LFH + *Sphagnum*, and *Sphagnum*. Similarly, the second incubation consisted of the following three treatments: FFM, FFM + Peat, and Peat. Replicate samples (n=5) of each treatment were further incubated at three different water contents, corresponding to 30, 40 and 50% of field capacity (for a total of 90 samples).

The amount of organic material (g) needed for each replicate sample was calculated to be equal with 4 g of organic carbon and mixed in a 2:1 ratio of sand to organic matter (Campbell et al. 1993). The mixed treatments consisted of equal parts of organic carbon from the LFH and *Sphagnum* (LFH + *Sphagnum*), and FFM and Peat (FFM + Peat) materials. Samples were incubated in 1 L glass mason jars for two weeks at room temperature to allow the microbial community to reactivate and come to equilibrium (Campbell et al. 1993). Flasks were watered daily by weight to maintain a constant water content.

Basal respiration was determined using a modified closed chamber and the CO_2 accumulation method (Hopkins 2008). Each sample was measured weekly for 5 weeks by closing off the flask for 48 hours with an air-tight lid fitted with a septum. At the end of the 48-hour period, 1 ml of the headspace was removed using a Gastight™ syringe and

injected into a Hewlett Packard 5890 Series II thermal conductivity detector (TCD) gas chromatograph (GC). The $CO_{2\ (g)}$ concentration (ppm) was then determined from the integration results using a calibration curve generated from a series of external standards. Results were converted to $\mu g_{CO2}/g_{OC}/h$ by taking into account the volume of the container (1 L), the respiration period (48 hours), and the amount of carbon in each sample (4 g).

2.4. Phospholipid fatty acids (PLFA) analysis

At the end of the 5-week incubation experiment, all replicates were collected and freeze-dried to prepare them for chemical extraction. Briefly, 1 g subsamples of material were extracted with a modified Bligh and Dyer extractant (Frostegård and Bååth 1996). The resulting lipid mixture was fractionated with solid phase silica extraction columns (Agilent Technologies, Wilmington, DE, USA) to separate the phospholipids from the neutral lipids and glycolipids (Tunlid et al. 1989). The PLFAs were depolarized in a mildly alkaline solution to replace the phosphate group with methyl group and form fatty acid methyl esters (White et al. 1979). Separation and identification of the FAMEs were performed using the MIDI 1200-A prokaryotic standard on an Agilent 6890 Series gas chromatograph. Results were reported in % mole relative to the integrated value measured for all phospholipids. PLFAs were named using the x:ywz convention, with 'x' representing the number of carbons in the molecule, 'y' representing the number of double bonds and 'z' describing the location(s) of the double bond(s) from the aliphatic end (ω) of the molecule.

2.5. Statistical analyses

Basal respiration data gathered over the 5-week incubation experiment were analysed with a repeated measures ANOVA using the fixed model of the mixed procedure of the Statistical Analysis Software (SAS) version 9.2. Comparisons were limited to within treatment groups, i.e. natural and stockpiled, and were adjusted using a Bonferroni correction to account for multiple comparisons ($\alpha=0.05/18=0.0028$).

The % mole values from the PLFA analysis were analysed using non-metric multidimensional scaling (NMS) ordinations, indicator species analysis (ISA), and multi-response permutation procedure (MRPP) using the PC-ORD software, version 5.33. NMS is a non-parametric analytical technique that produces 2 or 3-dimensional positioning of data points with a Bray-Curtis distance measurement based on the similarity of ranking scores between samples (Peck 2010). The main matrix contained the % mole of each PLFA, while the secondary matrix included organic material type, water content, and the combination of material type by water content to determine what groupings, if any, separated within the ordination space. Differences among groupings were further tested using MRPP, also a non-parametric analytical technique adapted to multivariate data which produces three values: 1) a P-value, specifying the overall significance of the comparison, 2) a T-value, which indicates the separation between groups, and 3) an A-value, which shows the homogeneity within groups (Peck 2010). Indicator species analysis (ISA) combined the mean abundance and relative frequency of each PLFA among the different incubation treatments to produce an indicator value for each PLFA within each material type (Dufrene et al. 1997). The PLFAs (% mole) found

to be significant using ISA (i.e., having a P-value ≤ 0.05 and an indicator value>25) were then compared across treatments using a Kruskal-Wallis ranked ANOVA using the Statistical Analysis Software (SAS) version 9.2.

3. Results

3.1. Physical and chemical properties

Water holding capacity (WHC) differed widely between the two natural materials, and the *Sphagnum* peat (1591%) possessed a WHC more than seven times greater than that of the forest floor (Table 2.1). The stockpiled materials were found to have lower water holding capacities than their respective natural materials, with 77% and 251% for the forest floor mineral material (FFM) mix and the Peat, respectively. A similar trend was reflected in the carbon contents of the materials, where both stockpiled materials showed considerably less carbon than the natural materials. The *Sphagnum* and peat materials also showed higher carbon contents than their forest floor counterparts (LFH and FFM).

Other measured properties, including pH, C/N ratio, and carbon structure as seen with solid state NMR, outlined the differences between the *Sphagnum* peat and the three other materials (Table 2.1). The LFH, FFM and stockpiled Peat had comparable C/N ratios ranging from 18 to 24, while *Sphagnum* displayed a remarkably higher C/N ratio (i.e.; 78); pH was also comparable for LFH, FFM, and Peat (5.1-5.9) but markedly lower for *Sphagnum* (3.6). Finally, LFH, FFM, and Peat showed similarity in their macromolecular carbon structure across all five carbon types, while *Sphagnum* contained significantly more O-alkyl carbon (Table 2.1). This was further illustrated on the solid-state CPMAS

^{13}C NMR spectra displayed on Figure 2.1. Major signals in the O-alkyl carbon area were found around 73 ppm, and are characteristic of the C-2, C-3, and C-5 carbons of cellulose and hemicelluloses. The peak at 73 ppm clearly dominated the *Sphagnum* spectrum, and none of the other carbon types exceeded 12% of the total spectral area for this material (Table 2.1). Also prominent on the *Sphagnum* spectrum, the peak at 105 ppm was likely derived from the anomeric carbon of cellulose and hemicelluloses (Figure 2.1).

The second most intense signals on the Peat, FFM, and LFH spectra were found in the alkyl carbon region (Figure 2.1). The main peak occurred around 30 ppm, suggesting that alkyl carbons present in these materials were mainly of the polymethylene type. In the aromatic carbon region, the peak at 130 ppm probably originated from C-substituted aromatic carbons, such as the C-1 carbon of guaiacyl and syringyl units, or the C-1, C-2, and C-6 carbons of p-hydroxyphenyl lignin moieties. When present, the peak at 57 ppm can be assigned to the methoxyl carbon signal of lignins. Finally, the peak at 174 ppm, indicative of carbonyl carbon in acetyl and ester moieties was particularly intense on the Peat spectrum.

3.2. Basal respiration

Basal respiration ($\mu g_{CO2}/g_{OC}/h$) of the fresh LFH was significantly greater than that of the stockpiled FFM for all water content treatments (Figures 2.2 & 2.3). The natural materials showed significant differences among treatments (Figures 2.2a, b & c). However, significant differences by water content were only observed within the pure LFH treatment (Figure 2.2c). Specifically, the LFH treatments with 30% and 40% water

content showed significantly lower basal respiration than the 50% treatment, indicating that water availability was limiting microbial activity at these lower water contents.

The calculated additive respiration ($\mu g_{CO2}/g_{OC}/h$) for the LFH and *Sphagnum* treatments were compared to measured results for the mixture (Figure 2.3). The comparison showed that the actual respiration rates were greater than the calculated sum in both the 30 and 40% water contents (Figure 2.3a & b). This was not the case, however, for the 50% water content (Figure 2.3c). Given that the water content of the mixed treatment is an average of the LFH and *Sphagnum* materials, the higher water holding capacity of the *Sphagnum* material created a larger total water content in the mixed treatment than in the pure LFH material. Even with a fractional difference in matric potentials between LFH and *Sphagnum,* it is likely that some water diffused toward the LFH material and increased its available water, at least in the lower moisture treatments (30% and 40%).

Similarly to the basal respiration results of the natural materials, the stockpiled materials also showed significant differences across materials (Figures 2.4a, b & c), with differences by water content only appearing in the FFM treatments (Figure 2.4c). Conversely to the results of the natural materials, mixing of the stockpiled materials failed to produce a greater than additive effect at any water content.

3.3. Phospholipid fatty acid analysis

Non-metric multivariate scaling (NMS) analysis of the phospholipid fatty acid (PLFA) profiles produced a 2-D ordination with a final stress of 8.2 and an instability of $<10^{-5}$ after 75 iterations (Figure 2.5). Results clearly illustrated that both natural materials

separated from the stockpiled materials within the ordination space. However, there was no separation linked to water content (data not shown).

As the mixed treatments are balanced with equal amounts of organic carbon from each parent material, each treatment group (either natural and stockpiled) represents a gradient of material. In the case of the natural treatment group, the LFH and *Sphagnum* groups represent polar differences in community composition, which was reflected in their opposite positioning within the ordination space (Figure 2.5). Although the mixed treatments constituted equal proportions of their parent materials, they did not occupy a medium position between the opposing materials. Rather, they appeared to cluster in closer proximity to the aspen derived materials (LFH and FFM) than the moss derived materials (*Sphagnum* and Peat). Given the statistical technique underlying the illustration of the ordination, the Bray-Curtis distance measurement, this positioning suggests that the composition of the microbial community in the mixed treatments was more similar to that of the aspen derived materials. This observation was supported by results of a multi-response permutation procedure (MRPP) analysis of the PLFA data (Table 2.2). In particular, the T-value, a indication of the separation between groups demonstrated that the mixed treatment for the natural materials was closer to LFH (T= -16.46) than to *Sphagnum* (T= -26.48). For the stockpiled materials, a smaller T value was also found between the mixed treatment and FFM when compared to the Peat, although the difference was not as marked.

Indicator species analysis (ISA) for the natural materials identified ten PLFAs showing a progressive increase or decrease in indicator values across organic matter materials that paralleled the gradient seen in the NMS ordination (Figure 2.5). Results of the Kruskal-Wallis ranked ANOVAs confirmed that seven of these PLFAs were statistically different among materials (Figure 2.6). Two of these PLFAs (15:0 and 18:1ω9c) increased in abundance with increasing peat content, while the other five showed the opposite trend. Indicator species analysis (ISA) of the stockpiled materials similarly identified three lipids, 18:1ω9c, 10Me18:0 and 20:1ω9c, which showed a strong trend mirroring the gradient among natural materials. However, none of these PLFAs were statistically significant for the stockpiled materials (data not shown).

4. Discussion

The lower basal respiration in the FFM material compared to that of the LFH is consistent with a decline in material quality during stockpiling, which could be due to nutrient losses linked to increased mineralization (Reicosky 1997). However, the FFM material did not display significant differences in its macromolecular carbon structure when compared to LFH; the O-alkyl/alkyl carbon ratio, which is commonly used as an index of organic matter decomposition (Baldock et al. 1997) was similar for both materials (Table 1). This is likely due to the relatively short period since soil salvaging (several months), although even this short period of stockpiling was enough to cause a measurable difference in the composition of the microbial community (Figure 2.5).

The NMS analysis of the PLFA data did not show treatment separation by water content (Figure 2.5). This is consistent with analogous studies which examined microbial community composition using either PLFA and DNA based analyses and demonstrated that community composition does not change markedly until the saturation point is reached (Bachar et al. 2010; Carson et al. 2010). As the soil becomes saturated, oxygen is no longer available as a terminal electron acceptor and aerobic microorganisms decline as conditions begin to favour anaerobic microorganisms. Until this point is reached, the composition may not fluctuate significantly. The water contents used in this study never exceeded 50% of field capacity, hence stayed far from anaerobic conditions. These results also support those of Brockett et al. (2012), who found that ordination of the PLFA profiles of the microbial community in the humified forest floor 'H' layers of seven forest types in western Canada separated by forest type but did not correlate strongly with any of the measured environmental factors, including moisture content.

In contrast to community composition, basal respiration responded to changes in moisture and increased with increasing water content in both the LFH and FFM treatments (Figures 2 and 4). As basal respiration can be used as an indicator of the catabolic activity of the microbial community (Margesin & Schinner 2005), these results suggest that while the community is more active in the LFH and FFM treatments, its composition as seen by PLFA profiling is remaining stable. Again, these results are consistent with those of Brockett et al. (2012), who reported that enzymatic activities in all seven forest types separated into groups which were most correlated with moisture content.

The activity of the microbial community is one of the most influential features controlling the rate of organic matter decomposition (Vecchioli et al. 1990), and both aspen derived materials demonstrated greater activity than their counterparts during the basal respiration portion of the experiment. A combination of amendments has often been found to be of greater benefit than each material applied separately (Larney and Angers 2012). For instance, field trials by de Varennes et al. (2010) showed that a combination of compost, polyacrylate polymers and chemical fertilizer produced greater microbial enzymatic activity and total accumulated biomass than the separate amendments alone. In our study, comparison of the basal respiration results for the natural materials showed a greater than additive respiration rate in the LFH + *Sphagnum* treatment (Figure 2.3); this was likely linked to the greater total available water supplied by the *Sphagnum* material in this mixed treatment.

Mixing of the organic substrates was also found to have a definite effect upon the composition of the soil microbial communities (Figure 2.5). The distribution of the natural materials aligned along a gradient between the pure LFH group and the *Sphagnum* treatment. Similarly, the stockpiled materials showed a gradient between the FFM and the Peat. The orientation of the groups in ordination space conforms to results reported by Drenovsky et al. (2004), who stated that adding organic carbon "substantially alters" community composition. This follows results of Griffiths et al. (1999), who showed that the composition of a rhizosphere community progressively shifted along a "coherent gradient" as the concentration of an organic solution was increased. This is illustrated in the NMS ordination of the PLFA profiles of both the natural and stockpiled materials

(Figure 2.5), which shows distinct separations by organic matter types, although the mixed treatments occupied a position clearly closer to the aspen-derived forest floors than to the peat materials.

Seven PLFAs were found to be statistically different among the natural materials (Figure 2.6). Two PLFAs showed an increase in abundance with increasing *Sphagnum*, one of which was 18:1ω9c, primarily an indicator of saprophytic fungi (Hill et al. 2000; Leckie 2005; Högberg 2006, Joergensen and Wichern 2008). Although this PLFA has also been found in plant tissues (Zelles 1997), studies have shown its occurrence to be negligible (Kaiser et al. 2010). Of the five PLFAs shown to increase in relative abundance as LFH loading increased, two were indicators of actinomycetes (16:0 10 methyl & TBSA 18:0 10 methyl). This group of gram positive bacteria inhabit numerous soil types and have also been shown to be sensitive to low pH (Waksman 1959). The natural materials proved to be widely divergent in this regard with an average pH of 5.9 in the LFH and 3.6 in the *Sphagnum* material (Table 2.1). Thus, it is likely that pH was driving the observed shifts in the microbial communities for the natural materials. On the other hand, the average pHs for the stockpiled materials were both 5.1, which is likely why no such PLFA indicators were found to differ between these treatments.

The PLFA 16:1ω5c, an indicator for arbuscular mycorrhizal fungi (AMF) (Haack et al. 1994; Olsson 1999), also showed an increase with the addition of LFH (Figure 2.6). Arbuscular mycorrhizal fungi are ubiquitous in the soil environment and colonize the roots and rhizosphere of a wide variety of vascular plants (Singh et al. 2005). Their

filamentous networks extend into the surrounding soil to adsorb water and minerals that are supplied to host plants in exchange for sugars, amino acids and vitamins (Harley and Smith 1983). Previous research in oil sands reclamation has reported that peat may negatively affect the mycorrhizal colonization of roots. For instance, Danielson et al. (1983) found that vesicular-arbuscular mycorrhizae had the lowest counts in the peat treatment compared to several overburden treatments, including a range of texture and depth of salvage. Therefore, it is possible that the anti-microbial properties of *Sphagnum* cited by previous researchers (Banerjee and Sen 1979; Johnson and Damman 1991; Painter 1991; Verhoeven & Toth 1995; Aerts et al. 1999) were reducing the growth of arbuscular mycorrhizae in this material.

Conversely, the stockpiled peat material had been salvaged from a wooded fen, with an assemblage of moss species dominated by Schreber's moss (*Pleurosium* spp.) While the 16:1ω5c PLFA showed a statistically different concentration in the FFM and Peat materials, as well as in the mixed treatment and Peat ($p<0.0001$), the FFM and mixed treatment were similar ($p=0.56$). As there were no obvious differences in pH or carbon structural composition between the stockpiled materials, this suggests that other (unmeasured) characteristics of the botanical origin of these organic substrates also influenced the composition of the microbial community.

5. Conclusions

Results from the basal respiration experiment and the PLFA analysis illustrated that the microbial community in the forest floor materials was more active and was distinct

structurally from that of peat. These characteristics were more prominent in the fresh, natural materials and appeared to a lesser extent in the stockpiled materials.

Basal respiration increased with LFH addition and only responded to water content within the pure LFH treatment. Mixed treatments of the fresh materials produced a greater than additive respiration. Combining forest floor material and peat further created a mixture with a soil microbial community more similar to that of forest floor material than to peat. Given operation considerations and the shortage of FFM material available for soil reclamation, a FFM:Peat mixture has the potential to increase the volume of material available while increasing water holding capacity and producing a microbial community more analogous to an upland forest community than peat alone.

CHAPTER II. TABLES AND FIGURES

Table 2.1. Physical and chemical properties of the organic materials, including two natural (forest organic layer (LFH) and *Sphagnum*) and two stockpiled materials (Forest Floor Mineral (FFM) and Peat). Numbers in parentheses represent one standard error from the mean (n=3).

	WHC (%)	C (g kg^{-1})	N (g kg^{-1})	C/N	pH	Carbon type (% of total NMR spectral area)				
						Alkyl	O-alkyl	Aromatic	Phenolic	Carbonyl
LFH	216 (29)	243 (1.2)	9.9 (0.09)	24 (0.3)	5.9 (0.1)	21 (0.4)	56 (0.2)	12 (0.3)	5 (0.1)	4 (0.1)
Sphagnum	1591 (70)	428 (5.5)	5.5 (0.04)	78 (0.7)	3.6 (0.2)	12 (0.5)	75 (0.3)	8 (0.4)	3 (0.2)	2 (0.1)
FFM	77 (11)	101 (1.6)	4.5 (0.03)	23 (0.2)	5.1 (0.1)	20 (0.9)	56 (0.3)	14 (0.3)	5 (0.3)	4 (0.2)
Peat	251 (23)	164 (2.8)	9.3 (0.04)	18 (0.3)	5.1 (0.1)	27 (0.8)	50 (0.2)	12 (0.4)	5 (0.1)	5 (0.2)

Table 2.2. Multi-response permutation procedure (MRPP) results comparing the microbial community composition between the two natural materials (forest organic layer (LFH) and *Sphagnum*), the two stockpiled materials (Forest Floor Mineral (FFM) and Peat), and their respective mixed treatments (LFH + *Sphagnum*, FFM + Peat). The P-value specifies the overall significance of the comparison, the T-value indicates the separation between groups (more negative values equal larger separation), and the A-value shows the homogeneity within groups.

Pair-wise Comparison	**T**	**A**	**P**
Natural Materials			
LFH *vs.* LFH + *Sphagnum*	-16.46	0.14	$< 10^{-8}$
Sphagnum vs.* LFH + *Sphagnum	-26.48	0.45	$< 10^{-8}$
Stockpiled Materials			
FFM *vs.* FFM + Peat	-11.11	0.14	6.3×10^{-7}
Peat *vs.* FFM + Peat	-13.73	0.21	0.9×10^{-7}

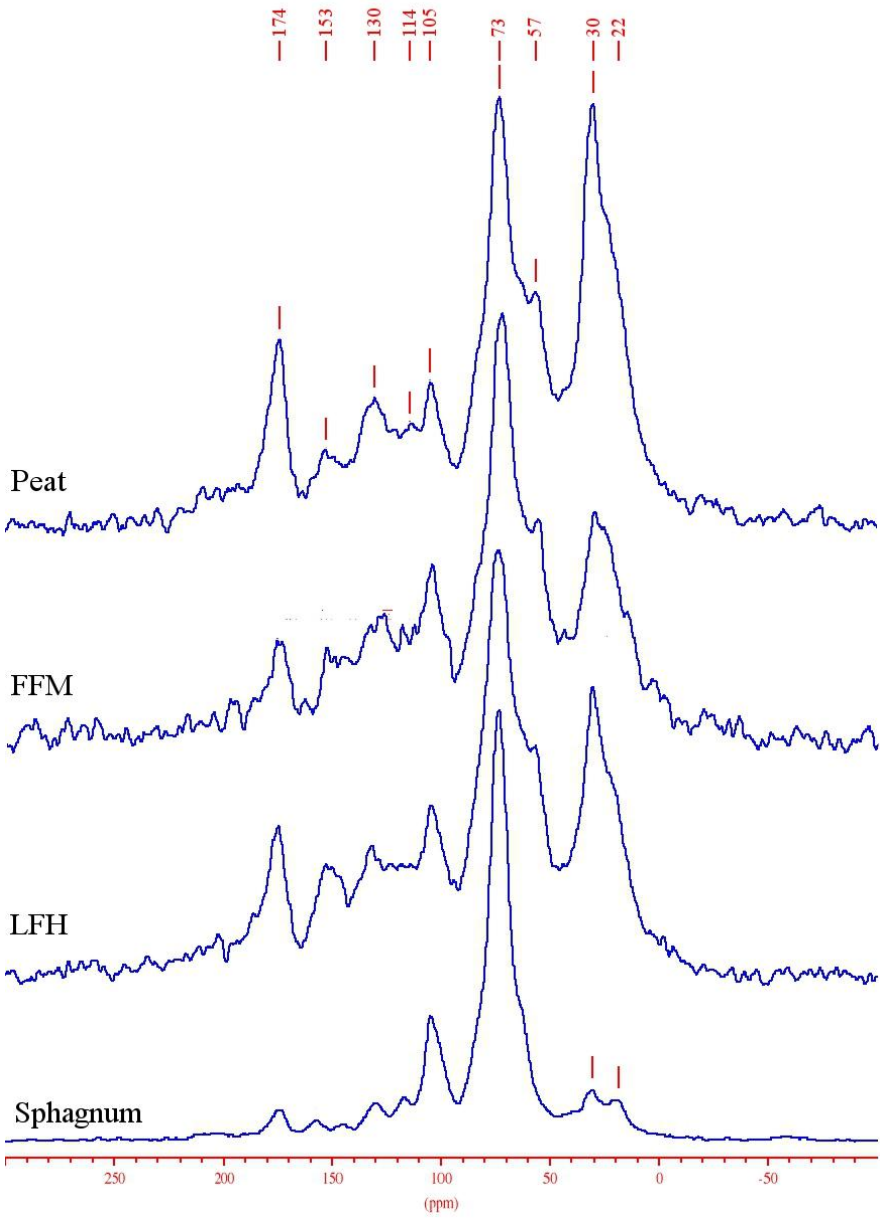


Figure 2.1. Examples of ^{13}C solid state nuclear magnetic spectra for stockpiled peat (Peat), stockpiled forest floor mineral material (FFM), undisturbed forest floor layer (LFH), and *Sphagnum* material (*Sphagnum*).

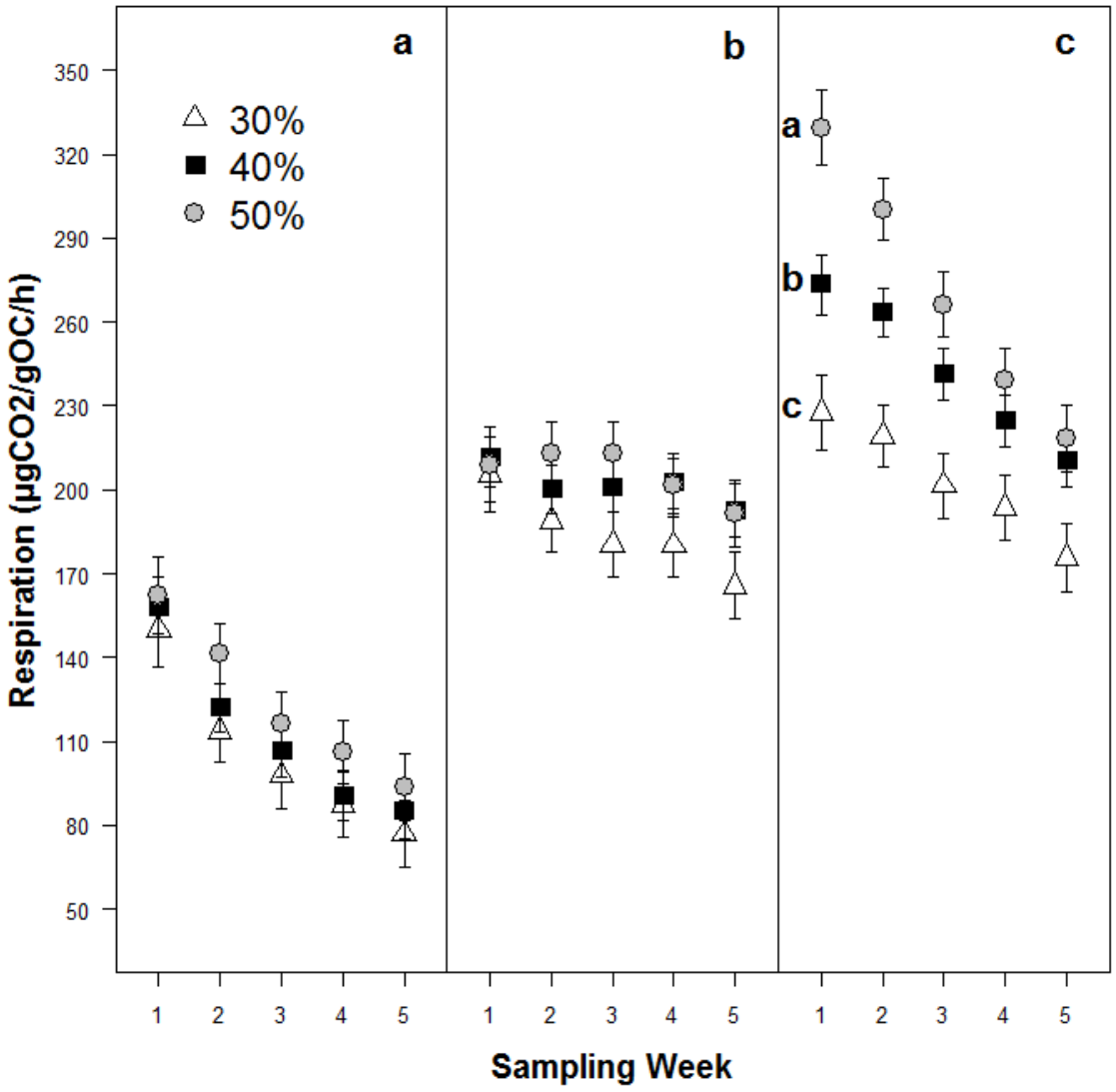


Figure 2.2. Basal respiration of three organic materials, *Sphagnum* peat (panel a), forest floor organic layer (LFH) + *Sphagnum* (panel b) and LFH (panel c), at three different water contents, 30%, 40% and 50% of field capacity, over a 5 week period. Error bars indicate one standard error from the mean (n=5). Different letters on the graph indicate statistical differences ($p \leq 0.05$) using repeated measures ANOVA.

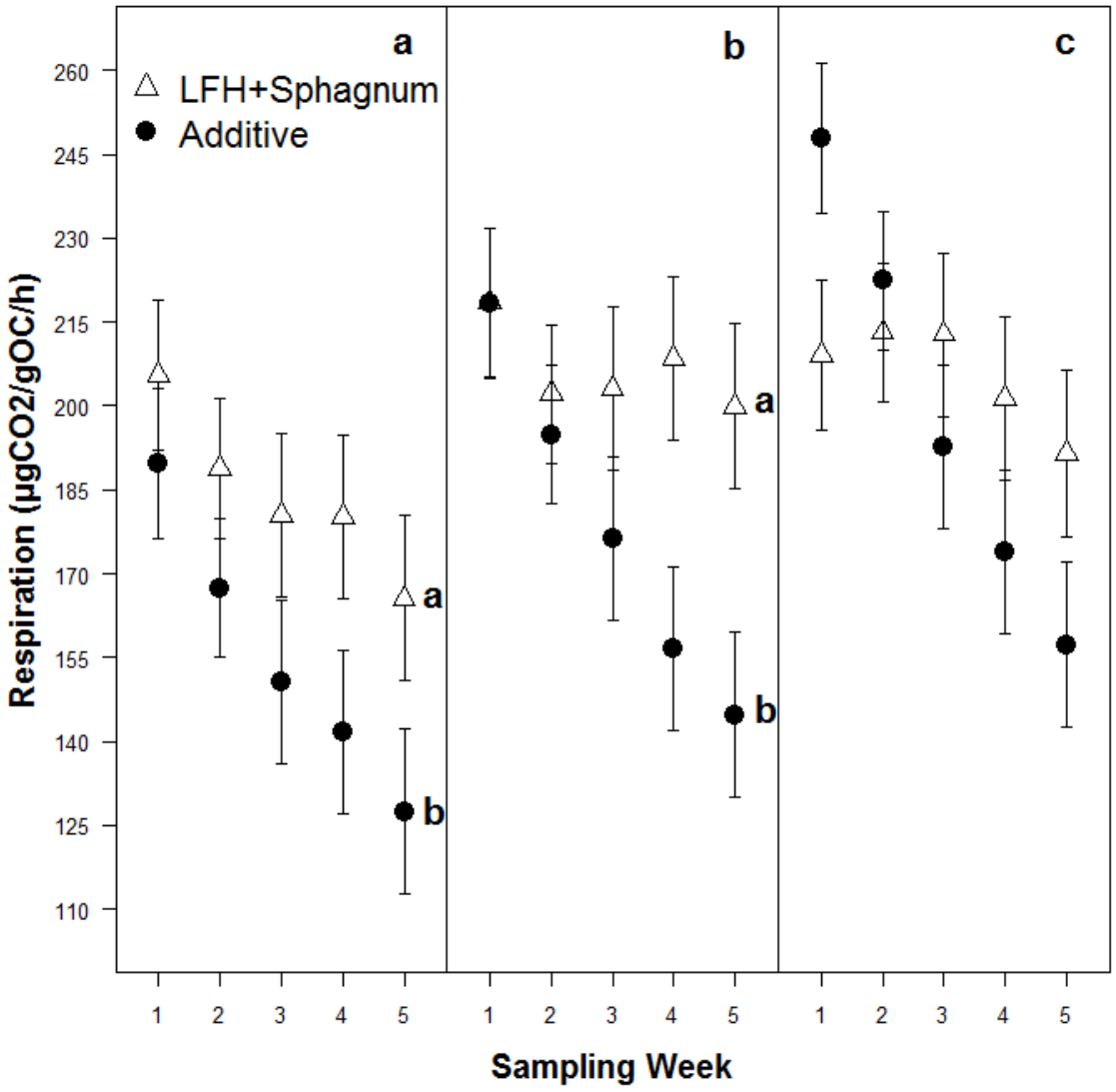


Figure 2.3. Measured basal respiration of the forest floor organic layer (LFH) + *Sphagnum* treatment and the calculated additive respiration of the two separate organic materials at 30%, 40% and 50 % of field capacity (panel a, b & c, respectively). Error bars indicate one standard error from the mean (n=5). Different letters on the graph indicate statistical differences ($p \leq 0.05$) using a repeated measures ANOVA.

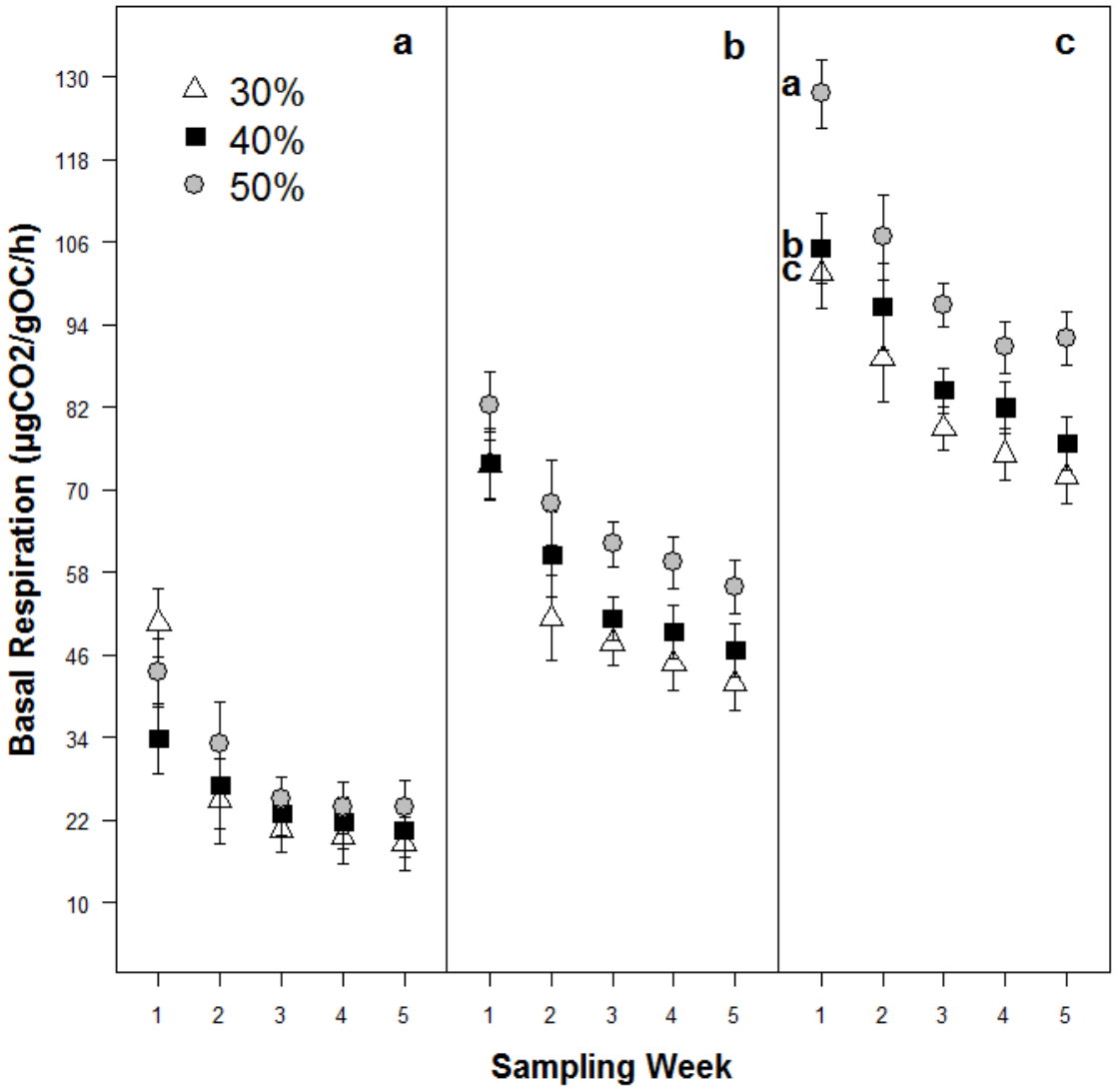


Figure 2.4. Basal respiration of three organic materials, stockpiled Peat (panel a), forest floor mineral material (FFM) + Peat (panel b), and FFM (panel c) at 30%, 40%, and 50% of field capacity over a 5 week period. Error bars indicate one standard error from the mean(n=5). Different letters on the graph indicate statistical differences ($p \leq 0.05$) using a repeated measures ANOVA.

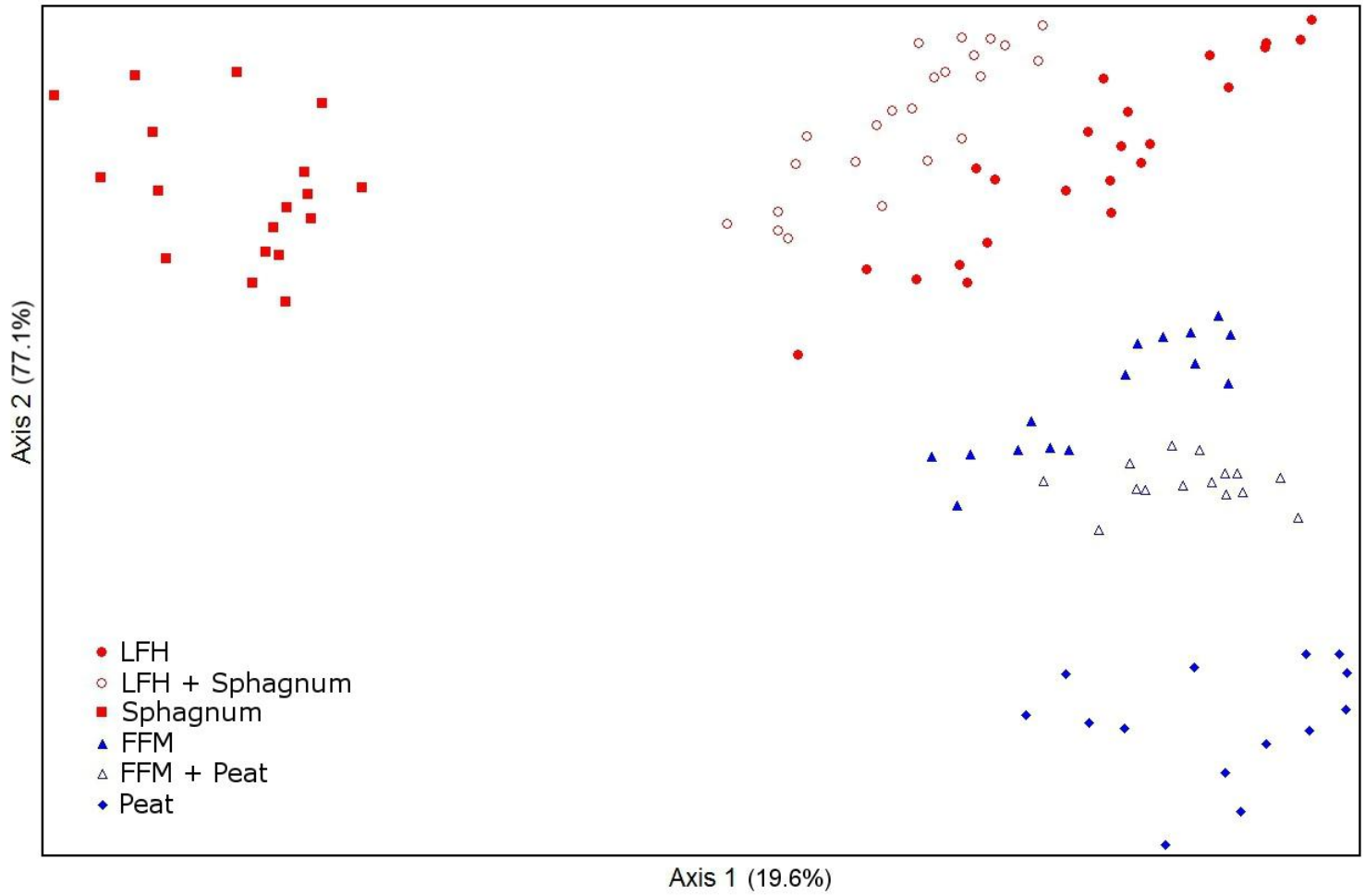
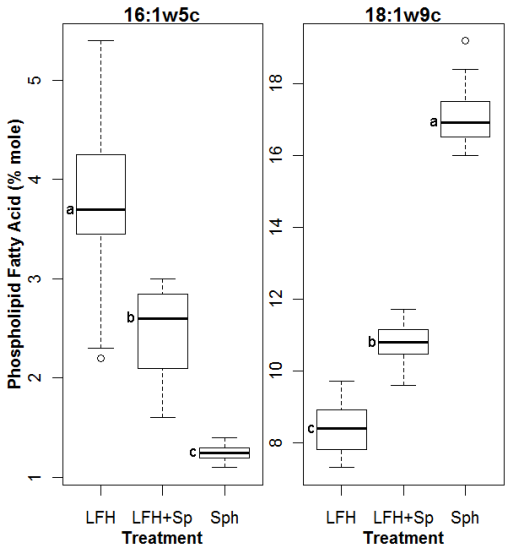


Figure 2.5. Non-metric multivariate scaling ordination (NMS) for the phospholipid fatty acids extracted from the natural and stockpiled materials and their respective mixed treatments.



PLFAs increasing with LFH addition	PLFAs increasing with *Sphagnum* addition
16:1ω5c	15:0
16:0 10 methyl	18:1ω9c
17:0i	
17:0a	
10Me18:0	

Figure 2.6. Boxplot of PLFA biomarkers (% mole) extracted from the forest floor layer (LFH), the *Sphagnum* material (Sph), and the 1:1 mixture (LFH + Sph). Median values are indicated by the horizontal bar within the box. The first and third quartiles are denoted by the lower and upper box limits respectively, and the 5th and 95th percentiles by the bars at the end of the vertical bars projecting from the box. Outliers are identified by open circles. Different letters on the graph indicate statistical differences ($p \leq 0.05$) using a Kruskal-Wallis pair-wise comparison.

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III. Stable isotope probing of the rhizosphere microbial communities in *Populus tremuloides* Michx. and *Alnus crispa* Ait.

1. Introduction

Resource extraction in the western Canadian boreal forest has created a patchwork of disturbances on the landscape and nowhere is this disturbance more evident than in the Athabasca oil sands region (AOSR) of northern Alberta. The AOSR is situated in the Wabasca Lowlands ecoregion of the Boreal Plains and is characterized as a poorly drained lowland plain comprised of 50% peatlands (Ecological Stratification Working Group 1995). The cumulative impact of open-pit mining projects in this relatively small area has led to the draining of extensive areas of peatlands (CNRL 2003) and has transformed the topography of the area at a landscape scale (Grant et al. 2008). However, the legacy of the previous plant community, i.e.; its organic residues, is still present and even actively placed on the post mining landscape to cap the reconstructed soils. Peat, the organic residue most common to the Wabasca lowlands, constitutes a store of nutrients which can improve soil fertility and increase the water holding capacity of the reconstructed soils (Kong et al. 1980). However, peat is for the most part comprised of moss residues, hence its chemical composition is different from the litters of upland forest communities (Turcotte et al. 2009) that will occupy a large proportion of the reclaimed landscape (Millennium 2005).

Two common groups of mutualistic soil microorganisms associated with boreal forest plants are mycorrhizal fungi and actinorhizal bacteria. Both associate with the roots of

their host plant and exchange water and mineral nutrients for photosynthates (Darrah 1991; Neumann & Romheld 2007; Hartmann et al. 2008). This exchange of resources has been shown to increase growth and pathogen resistance in the host plant (Visser et al. 1991; Yamanaka et al. 2005; Newcombe et al. 2010). As an early to late successional species native to the Wabasca lowlands, aspen (*Populus tremuloides* Michx.), is commonly found on disturbed sites (Newcombe et al. 2010). Alder (*Alnus crispa* Ait.) is also an early successional species which is commonly associated with aspen in mesic boreal ecosites (Beckingham and Archibald 1996). In contrast to aspen that has a facultative mycorrhizal association, alder shows a closer, symbiotic relation with several microorganisms; it is known to form root nodules with the nitrogen-fixing actinomycete *Frankia* and to exchange nutrients with arbuscular mycorrhizae that act to increase the plant nutrient absorption capacity (Yamanaka et al. 2005). As a species with a stronger association with the mutualistic segment of the soil microbial community than aspen, alder may be expected to demonstrate a larger growth response to changes in this community composition.

Analysis of phospholipid fatty acids (PLFAs) found in microbial cell membranes has become a routine, rapid method to fingerprint the structural composition of soil microbial communities (Frostegård et al., 2011). As PLFAs are unstable in soils and break down very quickly, the majority of extracted PLFAs ($\leq$ 20 carbons) represents active microorganisms within the microbial community (Zelles 1999). More recently, pulse labeling of plants with $^{13}CO_{2(g)}$, followed by compound specific-isotopic ratio mass spectrometry of individual PLFAs, has been used to precisely monitor the carbon flow

from plants to the rhizosphere microbial community (Ladygina and Hedlund 2010). Individual PLFAs are often representative of discrete groups of microbes within the community (Zelles 1997) and give researchers the ability to broadly describe what segment of the microbial community is metabolically active. Within the categories of PLFAs, 16:1ω5c is used as an indicator of arbuscular mycorrhizae (Olsson et al. 1995; Olsson 1999), 18:1ω9c as an indicator of saprophytic fungi and the genus of mycorrhizae *Gigaspora* (Bell et al. 2009; Allison and Miller 2005) and several iso & anteiso branched phospholipids have been used as broad indicators of gram positive bacteria (Degrood et al. 2005), including mutualistic gram positive actinomycetes *Frankia spp.*

The overall objective of this study was to examine how the differences in soil material used to grow aspen and alder seedlings may affect the composition of the soil microbial community composition, and more specifically the mutualistic segment of the rhizosphere community. The first step was to determine and compare the overall microbial community composition, or PLFA profile, of the bulk and rhizosphere soils to determine the potential influence exerted by each tree species and how it may differ by soil type. Next, stable isotope probing of individual PLFAs was utilized to quantify the carbon flow from the tree seedlings to the rhizosphere microbial community, in order to identify the metabolically active microorganisms, and how these potentially differed between tree species and soil type. Finally, we determined if the growth of the two tree species was related to the carbon flow from the plant, i.e., the overall ^{13}C enrichment of the rhizosphere microbial community.

2. Materials & Methods

2.1 Sample collection and preparation

The Athabasca oil sands region of northern Alberta occurs within the Wabasca lowlands. The regional climate is characterized by short, warm summers and long, cold winters. The mean air temperature for the warmest month of the year is 15.9°C with the growing season including 105 to 115 frost free days. The coldest month of the year experiences a mean air temperature of -18.7°C. The mean annual precipitation is 478 mm with an average of 336 mm falling during the growing season (Natural Regions Committee 2006). Soils in the oil sands region are dominated by organic soils associated with lowland areas, while Luvisolic soils occur on finer-textured lacustrine deposits and till on upland areas (Turchenek and Lindsay 1982).

The materials used in this experiment were selected as organic materials common to the area, which had undergone stockpiling, a common practice for oil sands operators. They were sampled from two stockpiles on Syncrude Canada's Mildred Lake mine site (57°01'36.24"N, 111°47'9.50"W), and included forest floor mineral (FFM) material and peat (Peat) that had been salvaged during the winter of 2009/2010 from areas scheduled for mining (Marty Yarmuch, Syncrude Canada, personal communication, 2012). The FFM was salvaged from an aspen dominated forest stand with an understory of prickly rose (*Rosa acicularis* Lindl.), low-bush cranberry (*Viburnum edule* (Michx.) Raf.) and Canada buffalo-berry (*Shepherdia canadensis* (L.) Nutt.). Salvaging consisted of stripping the top 10 to 30 cm layer at the soil surface and resulted in a heterogeneous mixture of decomposing forest litter, coarse woody debris and underlying mineral soil.

Soil at the salvage site was a medium textured, Luvisolic soil (FAO 2006). The Peat material was salvaged from a treed, nutrient-poor fen co-dominated by black spruce (*Picea mariana* (Mill.) B.S.P.) and tamarack (*Larix laricina* (Du Roi) K.Koch) with an understory of Labrador tea (*Ledum groenlandicum* Oeder), bog cranberry (*Vaccinium vitis-idaea* L.) and willow (*Salix spp.*). Salvaging commenced three years following the draining of the fen and consisted of the residual peat material with minimal mixing of the underlying mineral horizon. The FFM and Peat stockpiles were systematically sampled in May 2010 by taking many (> 20) samples from the surface of both stockpiles and compositing them to yield a large representative volume of each.

All materials were air dried at room temperature for 15 days and sieved to homogenize samples and remove any particle greater than 4 mm in diameter. Water holding capacity (i.e.; water content at field capacity) was determined on subsamples of each material (n=5) using Puustjarvi's (1973) soak and drain method. Total carbon and nitrogen contents were determined by dry combustion of finely ground subsamples (n=3) of each material using a Costech elemental combustion system (Model 4010; Valencia, CA). Preliminary analysis established that carbonate content was negligible in these samples, hence total carbon is equivalent to organic carbon.

Aspen (*Populus tremuloides* Michx.) seeds were collected from Flatbush, AB in the spring of 2009 and nutlets for alder (*Alnus crispa* Ait.) were collected from the North Saskatchewan River valley near Edmonton, AB in the spring of 2010. Seeds from both species were air dried overnight and refrigerated at 4˚C until planting.

2.2 Greenhouse experiment

Seeds were germinated in trays filled with sterilized sand in a model E8VH Conviron growth chamber at diurnal temperatures of 21˚C (and 18˚C at night) during an 18 hour photoperiod per day at approximately 400 microeinsteins of light. The trays were watered daily to maintain near saturated conditions with a NPK 15:30:15 soluble fertilizer mixed at a ratio of 1/1000 parts fertilizer to water for the first week. Alder seedlings were inoculated with *Frankia spp*. during the second week using root nodules collected from the roots of the parent trees. After four weeks, seedlings were planted in 35 cm sections of PVC piping (diameter 10 cm) filled with three soil types, including: 1. 30 cm of FFM, 2. 30 cm of Peat or 3. 15 cm of FFM over 15 cm of Peat (n=7 for each tree species and each soil material resulting in a total of $7 * 2$ tree species $*$ 3 soil types = 42 seedlings). Aspen seedlings were watered daily to maintain a water content of 50% of field capacity by weight. Alder seedlings were maintained at near field capacity by creating drainage holes 2.5 cm from the bottom of the pipe and daily watering to excess.

At the end of 20 weeks, each seedling was sealed within a 25 cm length of clear plastic tubing and pulse-labelled with 20 ml of 99% $^{13}CO_{2\,(g)}$ (Cambridge Isotope Laboratories, Inc. Andover, MA, USA) every 30 minutes for 3 hours followed by a 3 hour chase period according to Ladygina and Hedlund (2010). At the end of the chase period, the above ground plant biomass was cut off at the soil surface to sever the gradient of water potential within the plant and arrest the flow of carbon compounds to the root (Marschner et al. 1996). The rhizosphere was collected using a method adapted from Zak et al. (1996) by gently separating the bulk soil from the root ball. Samples were further split by depth,

above and below 15 cm. The rhizosphere soil was not separated from the fine roots; together, they were placed in clean, dry plastic bags and preserved on dry ice to fix the microbial community and prevent the further transfer of ^{13}C through trophic levels. Samples were then transferred to a super-freezer and stored at -85°C until phospholipid fatty acid (PLFA) extraction.

Four growth parameters for the seedlings were measured, including above ground dry biomass (following oven drying), basal stem area, plant height, and leaf area. Leaf area for each seedling was measured from a scan of the leaves taken with a Xerox Workcentre 5665 scanner, using Adobe Photoshop CS3 software (Adobe Systems, San Jose, CA), which counted the number of green hued pixels within the pdf scan of the leaves and compared the pixel count to that of a known area (Bradshaw et al. 2007). The basal stem area of each seedling was calculated from the measured basal stem diameter (Haase and Haase 1995) and the entire above ground biomass was oven dried at 65°C for 48 hours and weighed to determine dry biomass.

2.3 Laboratory analyses

All bulk soil and rhizosphere samples were freeze dried using a Labconco Freezone 6 system before they were extracted for phospholipid fatty acids (PLFAs). Briefly, each sample was agitated on a Labquake shaker in a modified Bligh and Dyer extractant (Frostegård and Bååth 1996). The PLFAs were then fractionated from the neutral and glycolipids using solid phase silica extraction columns (Agilent Technologies, Wilmington, DE, USA) and methylated into fatty acid methyl esters (FAMEs) in a mildly

alkaline solution (White et al. 1979; Tunlid et al. 1989). Finally, identification of individual PLFAs was performed by comparison of retention times with that of the MIDI 1200-A prokaryotic standard on an Agilent 6890 gas chromatograph equipped with a flame ionized detector (GC-FID). PLFAs were identified using the convention *x:y*ω*z*, in which '*x*' refers to the length of the carbon chain, '*y*' refers the number of double bonds present and 'z' describes the location of the bond in relation to the aliphatic end (ω) of the molecule. There are a number of suffixes also associated with this convention describing branching and other characteristics that are listed and described in Table A1 of Appendix A. Results were reported in % mole based on the integrated peak area of each PLFA in comparison to the total response measured from each sample.

The δ^{13}C values of individual PLFAs extracted from the rhizosphere and bulk samples were determined by gas chromatography combustion isotope ratio mass spectrometry (GC/C/IRMS) using an Agilent 6890 gas chromatograph coupled to a Finnigan Deltaplus Advantage IRMS (ThermoFinnigan, Bremen, Germany). Retention times for individual PLFAs were determined using the fatty acid methyl ester (F.A.M.E.) mixes GLC-30 and C4-C24 Unsaturates (Sigma Aldrich). Retention times of the peaks from the IRMS were further manually compared to those of the GC-FID to ensure their correct identification and assignment to individual PLFAs. IRMS integration values (peak areas) of less than 300 units were discarded due to low signal to noise ratios. The δ^{13}C levels were relativized against the Pee Dee Belemnite standard to calculate δ^{13}C (‰) using the Peterson & Fry (1987) equation. Resulting δ^{13}C values were corrected for the addition of

a carbon during the methylation step of the PLFA extraction with the Kramer & Gleixner (2008) equation.

2.4 Statistical Analyses

Phospholipid fatty acids (PLFAs) less than 20 carbons in length are largely considered to be of microbial origin (Tunlid et al. 1989), with the exception of 16:0 and 18:0, which are "general membrane lipids" (Dungait et al. 2011) and are common to both prokaryotes and eukaryotes (Zelles et al. 1997). As a portion of these lipids could have been extracted from the seedling roots instead of the rhizosphere soil, they were removed before statistical analysis of the PLFA data using ordination. Conversely, while the PLFA 18:1ω9c has been identified in plant roots (Zelles 1997), further research has shown its occurrence to be negligible (Kaiser 2010), therefore it was included in the analysis. The % mole values from the PLFA analysis were analysed using non-metric multidimensional scaling (NMS) ordinations and multi-response permutation procedure (MRPP) with PC-Ord, version 5.33. The NMS ordination is a non-parametric analytical technique that produces two and three-dimensional plots of data points using Bray-Curtis distance measurements of similarity among samples (Peck 2010). The main matrix contained the % mole measurements of the individual PLFAs profiles in each sample. The following categorical variables were identified: soil type (Peat, FFM); sample type (rhizosphere, bulk soil); and depth of sampling (above and below 15 cm) to identify groupings within the ordination space.

The PLFA data were further analysed for statistical differences among groups using MRPP, a non-parametric technique that generates three values: 1) the P-value, 2) a T-value which describes the difference between groups and 3) an A-value which describes the homogeneity within groups (Peck 2010). Lastly, a Kurskal-Wallis ranked ANOVA analysis of the $\delta^{13}C$ PDB values of all individual PLFAs was performed with the Statistical Analysis Software (SAS) version 9.2 to determine statistical differences using a pairwise comparison of each material against its control and between organic soils.

3. Results

3.1. Soil material and seedling growth characteristics

The Peat material demonstrated a much greater water holding capacity, a greater carbon content, and a slightly lower carbon to nitrogen ratio than the FFM material (Table 3.1). Both materials had an average pH of 5.9. Measurements of plant height, basal stem diameter, leaf area and above ground dry biomass (dry biomass) of the alder seedlings showed all parameters to be consistently less in the Peat treatment than the Layered and FFM treatments (Figure 3.1). Specifically, the leaf area of the alder seedlings demonstrated the greatest difference between treatments, and ranged between 230 cm^2 and 530 cm^2 within the Layered and FFM treatments, while it averaged 130 cm^2 for the Peat. Similarly, the alder above-ground dry biomass ranged between 1.2 g and 3.7 g within the Layered and FFM treatments, and was lowest at 1.0 g for the Peat. Height and basal stem area showed similar trends with the average values for the Peat treatment failing to reach the minimum measurements of either the Layered or FFM treatment.

Conversely, the same measurements for the aspen seedlings showed the FFM and Peat treatments to be the lowest across most parameters and the Layered treatment to be the highest overall (Figure 3.2). Specifically, dry biomass ranged from 1.1 g to 2.1 g in the Layered treatment while the FFM and Peat treatments averaged 0.8 g and 1.2 g, respectively. Height measurements demonstrated a similar pattern with the Layered treatment ranging from 23.1 cm to 32.2 cm, and the FFM and Peat averaging 18 cm and 21.7 cm, respectively.

3.2. Soil microbial community composition

The non-metric multivariate scaling (NMS) analysis of the phospholipid fatty acids (PLFAs) extracted from the rhizosphere and bulk soil samples produced an ordination with a final stress of 10 after 98 iterations for the alder seedlings and a final stress of 9 after 137 iterations for the aspen seedlings (Figures 3.3 and 3.4). Ordinations showed clear groupings by soil material (FFM versus Peat) for both tree species. On the other hand, no distinguishable difference was found between the FFM treatment and the FFM layer of the Layered treatment (data not shown), indicating that layering had no measurable effect upon the microbial community composition. Similarly, no difference could be detected between the Peat treatment and the peat layer of the Layered treatment. Consequently, PLFA data from either the pure or Layered treatment were compiled by material type (FFM and Peat) on the NMS ordinations (Figures 3.3 and 3.4).

Within each soil material (FFM and Peat) used to grow alder seedlings, the corresponding NMS ordination illustrated pronounced differences between the rhizosphere and bulk soil

samples (Figure 3.3). While individual data points from the bulk samples grouped tightly for both the FFM and Peat materials, the rhizosphere samples displayed greater within group variability for both materials, and even more so for the Peat rhizosphere samples. In addition, the Peat rhizosphere samples were distributed in closer proximity to the bulk Peat group than what could be seen between the FFM bulk soil and rhizosphere samples. This pattern was confirmed by the MRPP results, which showed that the pair-wise separation between the group average of the FFM rhizosphere and that of the bulk FFM was much greater, i.e. had a more negative T value, than the comparison between Peat rhizosphere and bulk Peat (Table 3.2).

Similarly to what was observed for the alder seedlings (Figure 3.3), both the ordination and MRPP analysis of the aspen microbial communities showed separation between the rhizosphere and bulk soil samples within both the FFM and Peat materials (Figure 3.4 and Table 3.2). However, as opposed to what was seen for the alder seedlings, separation between the rhizosphere and bulk soil was similar for both materials; in addition, when compared to alder, the aspen p-values were higher and the T values were lower for the comparison between rhizosphere and bulk soil samples, demonstrating that aspen had less of an influence on its rhizosphere microbial PLFA profile.

3.3. Stable isotope probing

As with the PLFA results, and for each soil material (FFM and Peat), no significant difference was observed between the pure treatment and the same material from the Layered treatment; hence results from both treatments were analyzed together.

Compound specific isotope-ratio mass spectrometry of the PLFAs revealed differences in ^{13}C enrichment between soil materials for several PLFAs. Enrichment varied across PLFAs. For instance, 17:0 cyclo and 19:0 cyclo failed to show any enrichment in either soil material or tree species and their median $\delta^{13}C$ values never exceeded -27.6‰ (data not shown). On the other hand, the ubiquitous PLFAs, 16:0 and 18:0 (Zelles 1997), showed $\delta^{13}C$ enrichment in both tree species and soil materials (Figure 3.5). In addition, enrichment of the PLFA indicative of arbuscular mycorrhizae, 16:1ω5*c* (Olsson et al. 1995), was the most enriched of all PLFAs and showed a median value as high as 71.7‰ (Figure 3.6).

Enrichment was higher for both 16:0 and 18:0 in the alder rhizosphere samples than in aspen, with the $\delta^{13}C$ median values being more than twice those recorded in the aspen rhizosphere (Figure 3.5). For instance, 16:0 had a median value of 163.1‰ for the alder rhizosphere, and 69.4‰ for aspen. In addition, while rhizosphere samples from the alder seedlings showed a significantly greater $\delta^{13}C$ enrichment of the 16:0 and 18:0 lipids in the FFM treatment compared to the Peat treatment, the $\delta^{13}C$ enrichment was similar in both soil types for the aspen seedlings.

The PLFAs 15:0a, 15:0i, 17:0a and 17:0i, recognized biomarkers for gram positive bacteria (DeGrood et al. 2005), all showed $\delta^{13}C$ enrichment in the rhizosphere samples of the alder seedlings in both the FFM and Peat materials (Figure 3.7). The PLFAs 15:0a, 15:0i and 17:0i (Figure 3.7a, b & c) showed a significantly greater enrichment in the FFM rhizosphere treatment compared to that of the Peat treatment. Enrichment of ^{13}C within

the aspen rhizosphere PLFAs followed a similar overall trend as the alder seedlings in that the gram positive PLFAs (including 15:0i, 15:0a, 16:0i, 17:0i and 17:0a) all displayed a measurable $\delta^{13}C$ enrichment in both soil types (Figure 3.8). However, only the 15:0a, 16:0i and 17:0i PLFAs displayed a significantly greater ^{13}C enrichment in the FFM material compared to that of the Peat. Furthermore, enrichment in the alder FFM rhizosphere treatment was higher than for aspen; for instance alder median $\delta^{13}C$ values were -18.7‰ (15:0a) and 4.7‰ (16:0i) compared to values slightly more negative than -21‰ for the same two PLFAs extracted from the aspen rhizosphere samples.

For the alder seedlings, the PLFA 16:1ω5c was more enriched in the FFM rhizosphere treatment than in the Peat rhizosphere (Figure 3.6a). There was no difference between soil types for the aspen seedlings (Figure 3.6b). In addition, 16:1ω5c enrichment was much higher for the alder FFM rhizosphere and averaged 285.6‰ compared to the aspen rhizosphere samples (-11.6‰). Lastly, for the aspen seedlings, 18:1ω9*c*, an indicator for saprophytic fungi and a limited number of mycorrhizae (Hill et al. 2000; Allison and Miller 2005; Leckie 2005; Hogberg 2006; Joergensen & Wichern 2008), showed greater enrichment in the FFM treatment compared to Peat (Figure 3.6c).

4. Discussion

Compound specific mass spectrometry of the PLFAs did not produce results that were homogenous across PLFAs. There was no indication that the lipids 17:0 cyclo and 19:0 cyclo were enriched in either the alder or aspen treatments. Conversely, gram positive bacteria lipids showed clear ^{13}C enrichment that varied by material type and tree species.

The variable ^{13}C enrichment level across the PLFAs measured is a good indication that the method was successful in delivering ^{13}C enriched compounds to the rhizosphere microbial community long enough for them to be incorporated into their own biomass but not long enough for the enrichment to diffuse significantly through trophic levels and become ubiquitous. Results from previous studies using comparable isotope probing methodology have demonstrated the differential labelling individual rhizosphere PLFAs (e.g.; Butler et al. 2003; Treonis et al. 2004; Ladygina and Hedlund 2010). Similarly to these studies, we found that fungal PLFA biomarkers (16:1ω5*c*, 18:1ω9*c*) became enriched in ^{13}C, in some cases to a greater extent than the ubiquitous 16:0.

Results from the PLFA analysis clearly indicated differences in the structural composition of the rhizosphere and bulk soil microbial communities for both tree species (Figures 3 & 4). The clear differences seen on these ordinations illustrate the influence that rhizodeposition has on the overall composition of the microbial community and support the results reported for instance by Zak et al. (1996), who measured distinct differences in the microbial PLFA profiles of bulk and rhizosphere soils from *Populus grandidentata* Michx. Similarly, previous experiments examining microbial communities in forest soils have illustrated the strong influence that plant roots have on community composition when analysing sites with and without tree roots (Brant et al. 2006). It is a common strategy for higher plants to use rhizodeposition, the release of labile carbon compounds into their rhizosphere, to encourage the growth of mutualistic microbial species to improve their own access to soil nutrients (Visser et al. 1991; Yamanaka et al. 2005; Necombe et al. 2010).

Conversely, for the alder seedlings, there was less distinction between the microbial community composition of the bulk and rhizosphere soils within the Peat than within the FFM material (Figure 3.3). Furthermore, based on the $\delta^{13}C$ PLFA values (Figures 3.6 & 3.7), incorporation of the ^{13}C label was lower in the peat than in the FFM rhizosphere microorganisms. This was seen, in particular, for the gram positive bacteria lipids 15:0i, 15:0a and 17:0i (DeGrood et al. 2005) as well as the arbuscular mycorrhizae PLFA 16:1ω5c (Haack et al. 1994; Olsson 1999). Taken together, these results would suggest that the alder seedlings and their rhizosphere microbial communities are sharing fewer associations in the Peat treatment than in the FFM treatment. Previous field trials in the oil sands region investigating the influence of peat as a growth substrate on mycorrhizae associations found that none of the nine mycorrhizae species infecting the roots of jack pine (*Pinus banksiana* Lamb.) seedlings survived past three years following planting (Danielson et al. 1989). The authors speculated that changes in host physiology caused by soil conditions interrupted carbon flow to the fungi, resulting in their death; even after 10 years, the inoculation potential of mycorrhizae remained low and was composed of a spectrum of mycorrhizal species characterized as of a "non-forested soil" (Danielson 1991).

Host plants supply labile carbon to mutualistic microbes in exchange for increased nutrient uptake in challenging environments and reduce the amounts of exudates when nutrient levels are not limiting (Nagahashi et al. 1996; Wall 2000). Hence, the increase in the plant-microbial community associations that was observed in this study through a higher ^{13}C enrichment of several PLFAs within the FFM material (e.g., Figure 3.7) could

be interpreted as the result of lower nutrient levels in the FFM compared to that of peat. However, peat studies in the region have consistently reported lower available nutrient levels in peat compared to forest floor (McMillan et al. 2007, Rowland et al. 2009). Alternatively, seminal work by Banerjee & Sen (1979) identified 29 species of moss with anti-microbial properties, which displayed lower decay and mineralization rates compared to other vascular and non-vascular plant species (Aerts et al. 1999). Thus, it is possible that results from our study originated from the peat inhibitory influence on rhizosphere microbial communities that may be reducing their association with the alder seedlings. Further, research in plant-microbial mutualism has established that host plants with a high inoculation of mutualist partners are more resistant to biotic and abiotic stress (Newcombe et al. 2010) and accumulate greater biomass (Bois et al. 2005). In our study, the alder correspondingly grew significantly more in the FFM than the Peat material (Figure 3.1).

For the aspen seedlings, instead of finding a clear influence exerted on the rhizosphere community as seen for the alder seedlings (Figure 3.3), the composition of the microbial community seemed to shift more equally from bulk soil to rhizosphere in both materials than seen in the results for the alder treatment (Figure 3.4). This lack of sensitively in the rhizosphere microbial community composition was supported by the results of the growth measurements which actually showed a lower average growth of aspen grown in FFM compared to the Layered treatment and Peat (Figure 3.2). These results are contrary to previously reported results from the oil sands region which have shown greater growth of aspen in FFM (Pinno et al. 2012). However, Pinno et al. (2012) used a less controlled

watering regime, where they watered the pots to saturation. In our study, where water content was maintained at 50% of field capacity, it may be that water became more of a limiting factor in the FFM than the Peat treatment, considering the greater water holding capacity of the peat material (Table 3.1).

Further, results of stable isotope probing showed significantly greater ^{13}C enrichment in FFM than in Peat in a few PLFAs (Figures 3.6 & 3.8), which indicates a flow of photosynthates to the aspen rhizosphere. However, without corresponding greater aspen growth in the FFM treatment, this does not appear to be indicative of mutualistic associations. As previously mentioned, the host species is influential to the microbial community. While aspen is known to exchange nutrients with mycorrhizae (Lodge 2000), researchers have yet to determine the extent of the mutualism occurring (Newcombe et al. 2010).

Although soil moisture levels have been shown to have a larger impact on arbuscular mycorrhizae colonization than host genetics (Piotrowski et al. 2008), the greater water holding capacity of the peat material (Table 3.1) failed to produce greater ^{13}C flow to the PLFA 16:1ω5*c* (Figure 3.6), an arbuscular mycorrhizae indicator, in the rhizosphere community ($p=0.6$). However, for the aspen seedlings, 18:1ω9*c*, a PLFA which broadly represents saprophytic fungi and some mycorrhizae (Hill et al. 2000; Allison and Miller 2005; Leckie 2005; Hogberg 2006; Joergensen & Wichern 2008), was found to be more enriched in the FFM material than the Peat ($p=0.0276$). While mycorrhizae are known to exchange nutrients with aspen and have been correlated with resistance to a select

number of pathogens, there are a broad range of mycorrhizae that fall into this group and make up a sliding scale of relationships ranging from mutualistic to parasitic (Newcombe et al. 2010).

5. Conclusions

The sharing of photosynthates in the rhizospheres of two early successional boreal species, aspen (*Populus tremuloides* Michx.) and alder (*Alnus crispa* Ait.) was investigated using ^{13}C stable isotope probing to determine how soil material (forest floor and peat from a wooded fen) influenced plant-microbial associations. Seedlings were grown in each separate material and a layered treatment of forest floor material on top of peat for 20 weeks, after which they were pulse labelled with $^{13}CO_{2\,(g)}$. Phospholipid fatty acids were extracted from the rhizosphere to analyse the composition of the community and compound-specific isotope-ratio mass spectrometry was used to determine the $\delta^{13}C$ values of individual phospholipids. Non-metric multidimensional scaling of the phospholipids showed differences in composition of the alder rhizosphere community compared to the bulk soil, in particular when seedlings were grown in forest floor. Differences between bulk soil and rhizosphere microbial communities were not as marked for the aspen seedlings. In addition, although there was evidence of greater ^{13}C flow to the rhizosphere of the aspen grown in the forest floor material, no enrichment was found in PLFAs indicators of known mutualistic microorganisms. On the other hand, for alder, phospholipids commonly used as identifiers for arbuscular-mycorrhizae and gram positive bacteria (e.g.; *Frankia spp.*), were more enriched in the alder rhizosphere grown

in forest floor material, with correspondingly greater growth measured in the alder seedlings than seen in the peat treatment.

CHAPTER III. TABLES AND FIGURES

Table 3.1 Physical and chemical properties of the two stockpiled soil materials used for the study, including peat from a woody fen (Peat) and forest floor material (FFM) from an aspen forest stand. Numbers in parentheses represent one standard error from the mean (n=3).

	WHC (%)	**C (g kg^{-1})**	**N (g kg^{-1})**	**C/N**	**pH**
FFM	77 (11)	101 (2)	4.5 (0.1)	23 (1)	5.1 (0.1)
Peat	251 (23)	164 (3)	9.3 (0.1)	18 (1)	5.1 (0.1)

(WHC = Water Holding Capacity)

Table 3.2 Multi-response permutation procedure (MRPP) results comparing the phospholipid fatty acid (PLFA) profiles extracted from the rhizosphere and bulk soil samples of alder (*Alnus crispa* Ait.) and aspen (*Populus tremuloides* Michx.) grown in the two different stockpiled soil materials, peat from a woody fen (Peat) and forest floor material (FFM) from an aspen forest stand. The P-value specifies the overall significance of the comparison, the T-value indicates the separation between groups (more negative values equal larger separation), and the A-value shows the homogeneity within groups.

Pair-wise comparison	T	A	P-value
Alder			
FFM Rhizosphere vs. Bulk Soil	-26.8	0.44	$<10^{-8}$
Peat Rhizosphere vs. Bulk Soil	-14.7	0.17	$<10^{-7}$
Aspen			
FFM Rhizosphere vs. Bulk Soil	-12.9	0.24	$<10^{-6}$
Peat Rhizosphere vs. Bulk Soil	-14.5	0.28	$<10^{-6}$

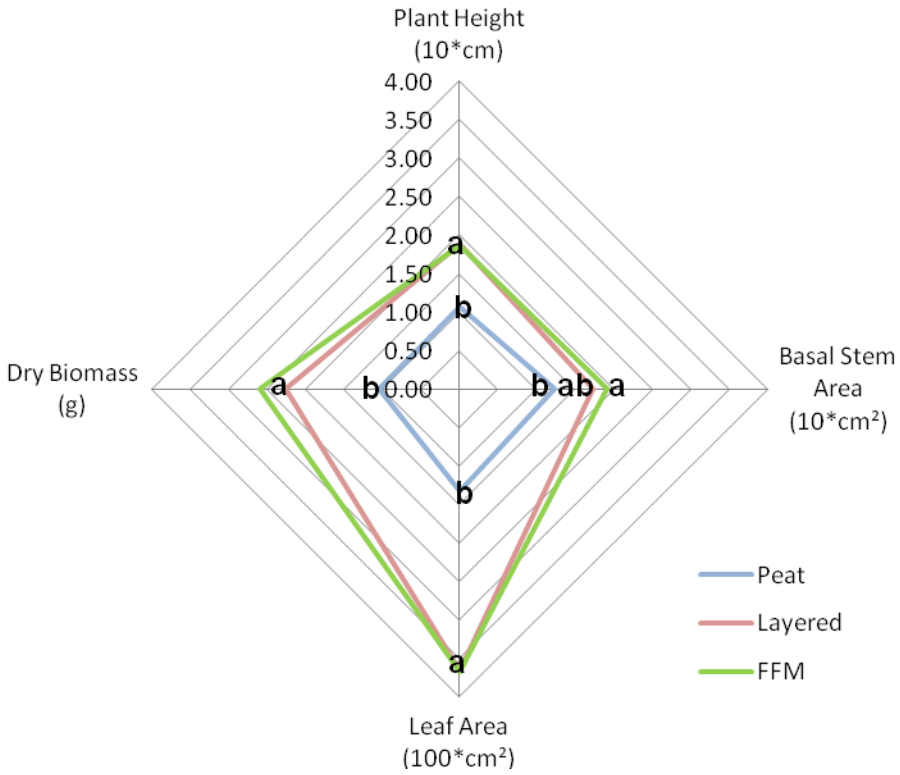

Figure 3.1 Growth measurements of alder (*Alnus crispa* Ait.) seedlings (n = 7) grown for 20 weeks in three organic soil treatments; peat from a wooded fen (Peat), forest floor material (FFM) and a layered treatment of FFM over Peat (Layered).

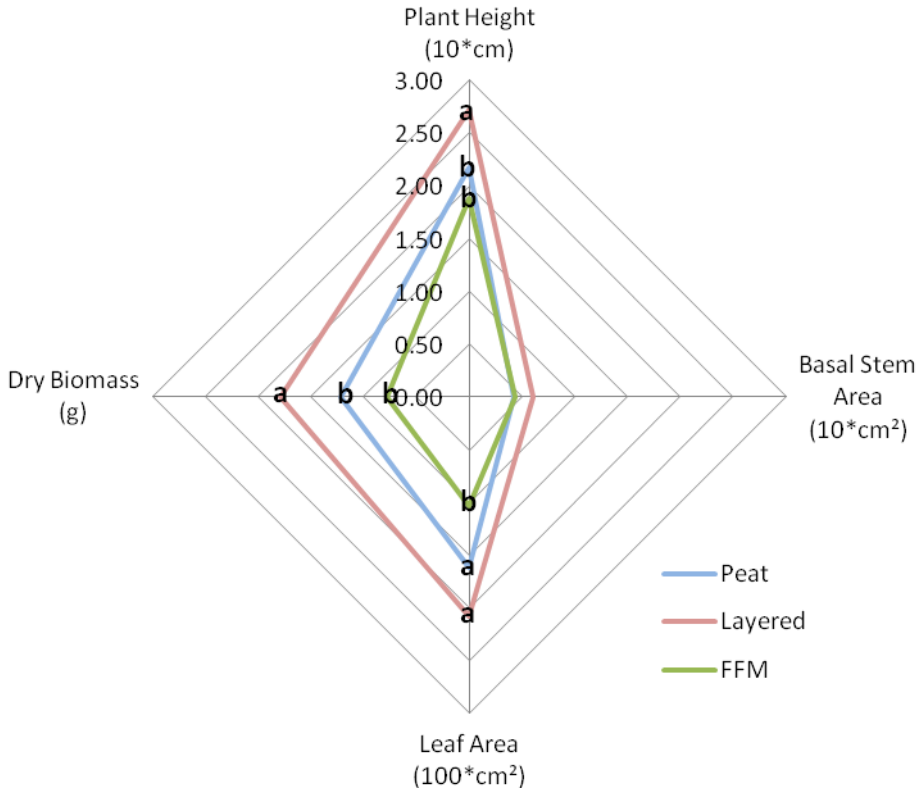

Figure 3.2 Growth measurements of aspen (*Populus tremuloides* Michx.) seedlings (n = 7) grown for 20 weeks in three organic soil treatments; peat from a wooded fen (Peat), forest floor material (FFM) and a layered treatment of FFM over Peat (Layered).

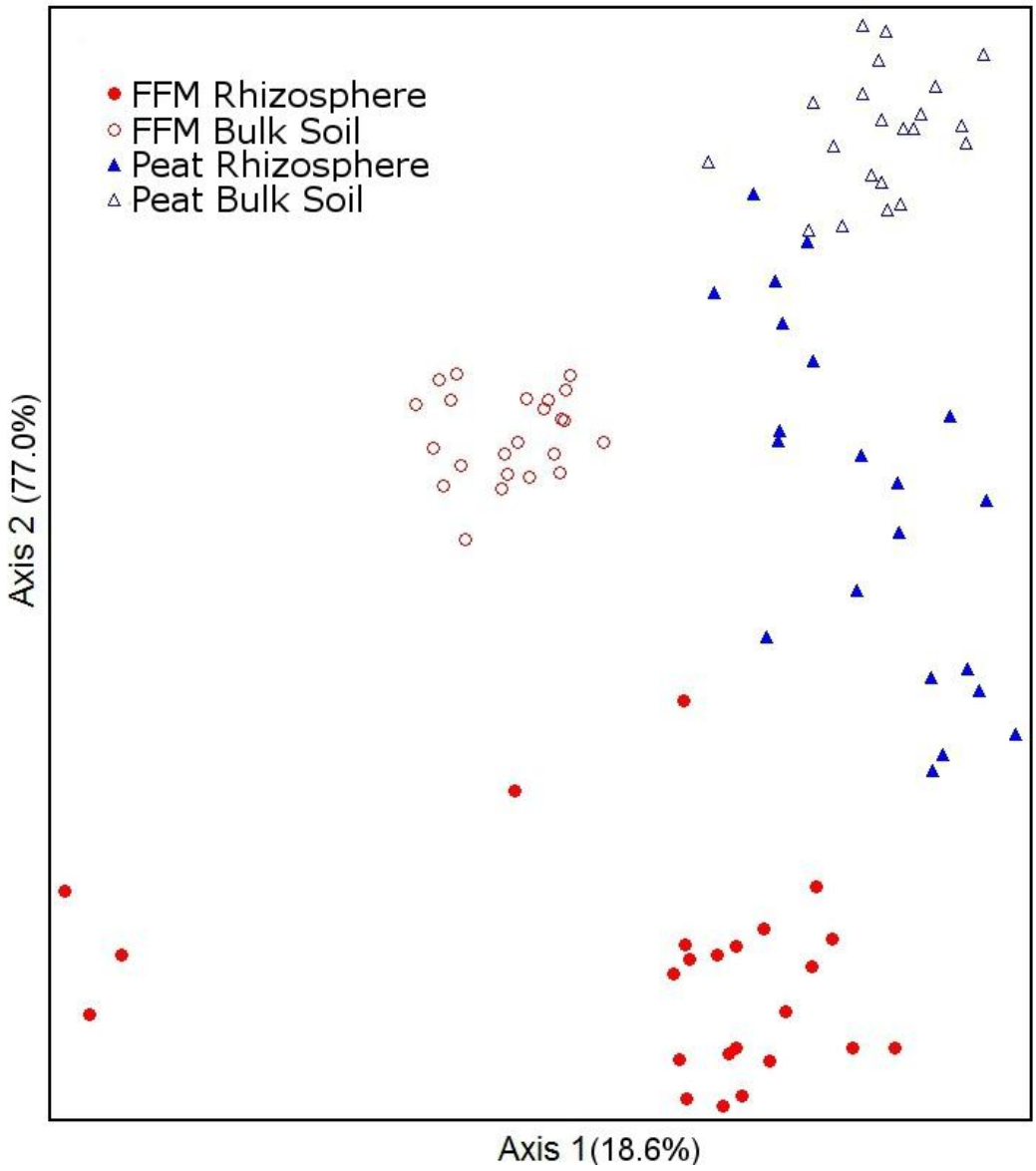

Figure 3.3 Non-metric multidimensional scaling (NMS) ordination of the phospholipid fatty acid (PLFA) profiles of the microbial community in the bulk soil and rhizosphere of alder (*Alnus crispa* Ait.) seedlings grown in forest floor material (FFM) and peat from a wooded fen (Peat).

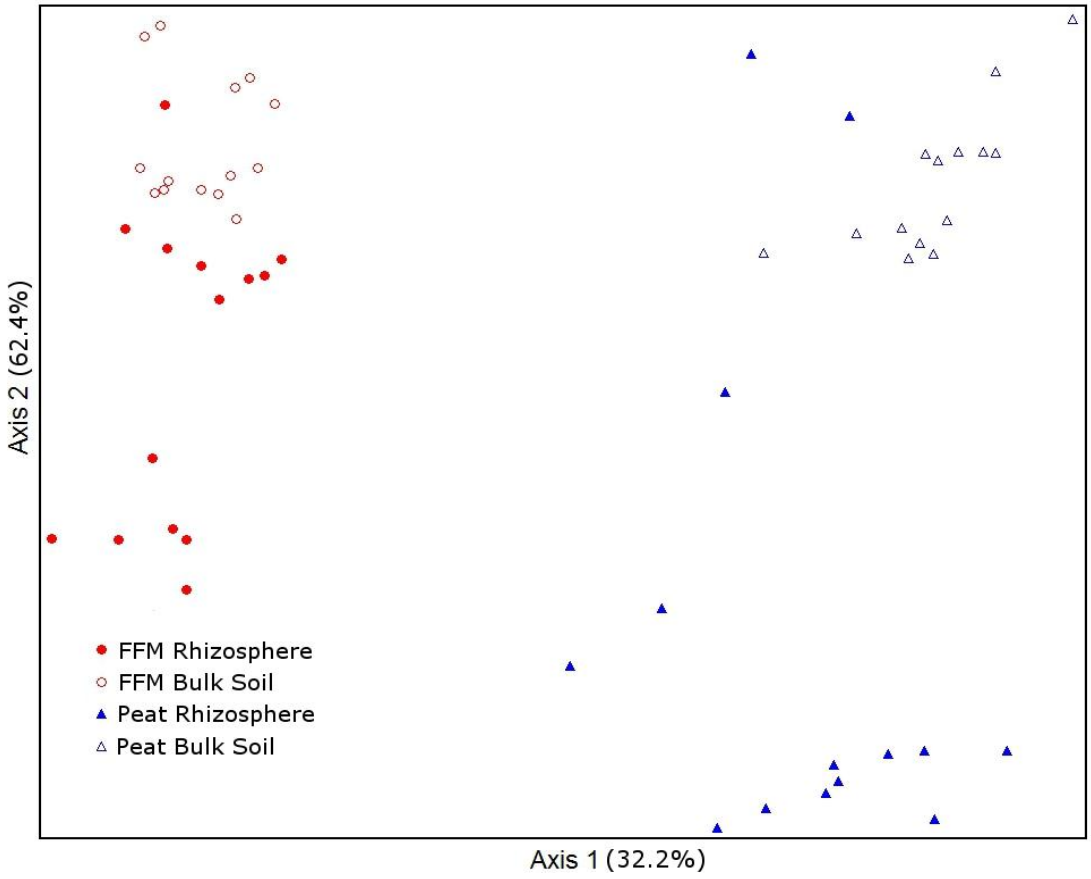

Figure 3.4 Non-metric multidimensional scaling (NMS) ordination of the phospholipid fatty acid (PLFA) profiles in the bulk soil and rhizosphere of aspen (*Populus tremuloides* Michx.*)* seedlings grown in forest floor material (FFM) and peat from a wooded fen (Peat).

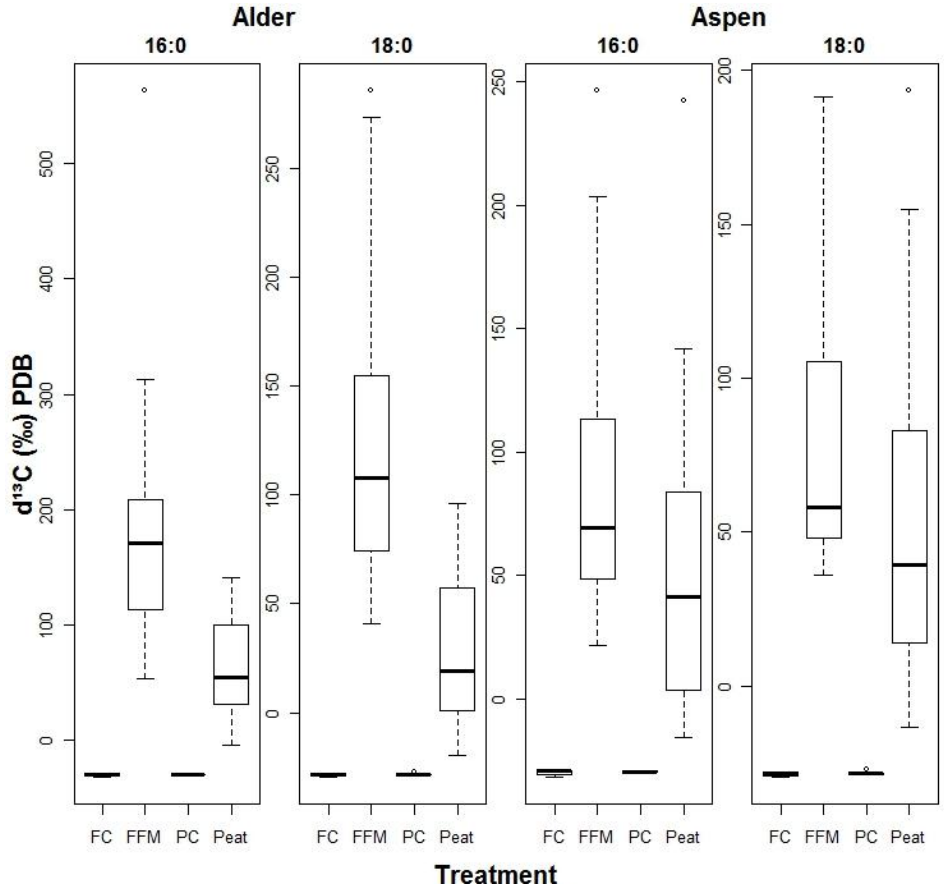

Figure 3.5 Delta ^{13}C values ($\delta^{13}C$ Pee Dee Belemnite (‰)) of the phospholipid fatty acids 16:0 and 18:0 extracted from the bulk soil (FC & PC) and rhizosphere (FFM & Peat) samples (n = 4-14) of alder (*Alnus crispa* Ait.) and aspen (*Populus tremuloides* Michx.) grown in forest floor material (FFM) and peat from a wooded fen (Peat), where FC corresponds to the FFM bulk soil, and PC to the Peat bulk soil. Median values are indicated by the horizontal bar within the box. The first and third quartiles are denoted by the lower and upper box limits respectively, and the 5th and 95th percentiles by the bars at the end of the vertical bars projecting from the box. Outliers are identified by open circles.

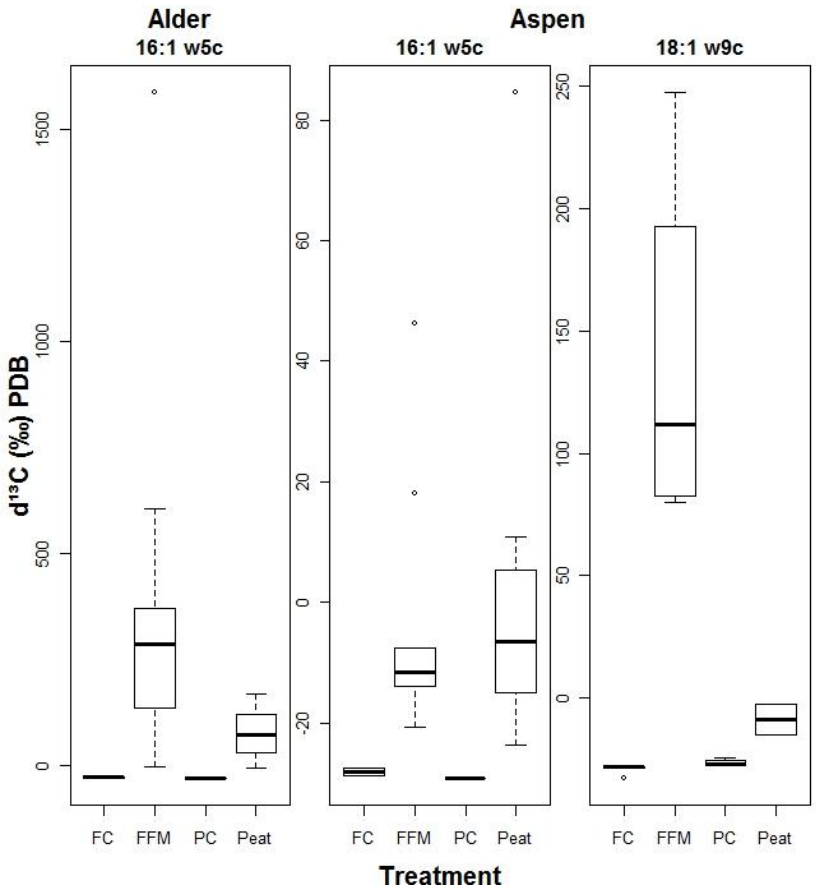

Figure 3.6 Delta ^{13}C values ($\delta^{13}C$ Pee Dee Belemnite (‰)) of the phospholipid fatty acid 16:1ω5*c* extracted from the bulk soil (FC & PC) and rhizosphere (FFM & Peat) samples (n=2-13) of alder (*Alnus crispa* Ait.), and of the phospholipid fatty acids 16:1ω5*c* and 18:1ω9*c* extracted from the bulk soil and rhizosphere samples (n=4-14) of aspen (*Populus tremuloides* Michx.) grown in forest floor material (FFM) and peat from a wooded fen (Peat), where FC corresponds to the FFM bulk soil, and PC to the Peat bulk soil. Median values are indicated by the horizontal bar within the box. The first and third quartiles are denoted by the lower and upper box limits respectively, and the 5th and 95th percentiles by the bars at the end of the vertical bars projecting from the box. Outliers are identified by open circles.

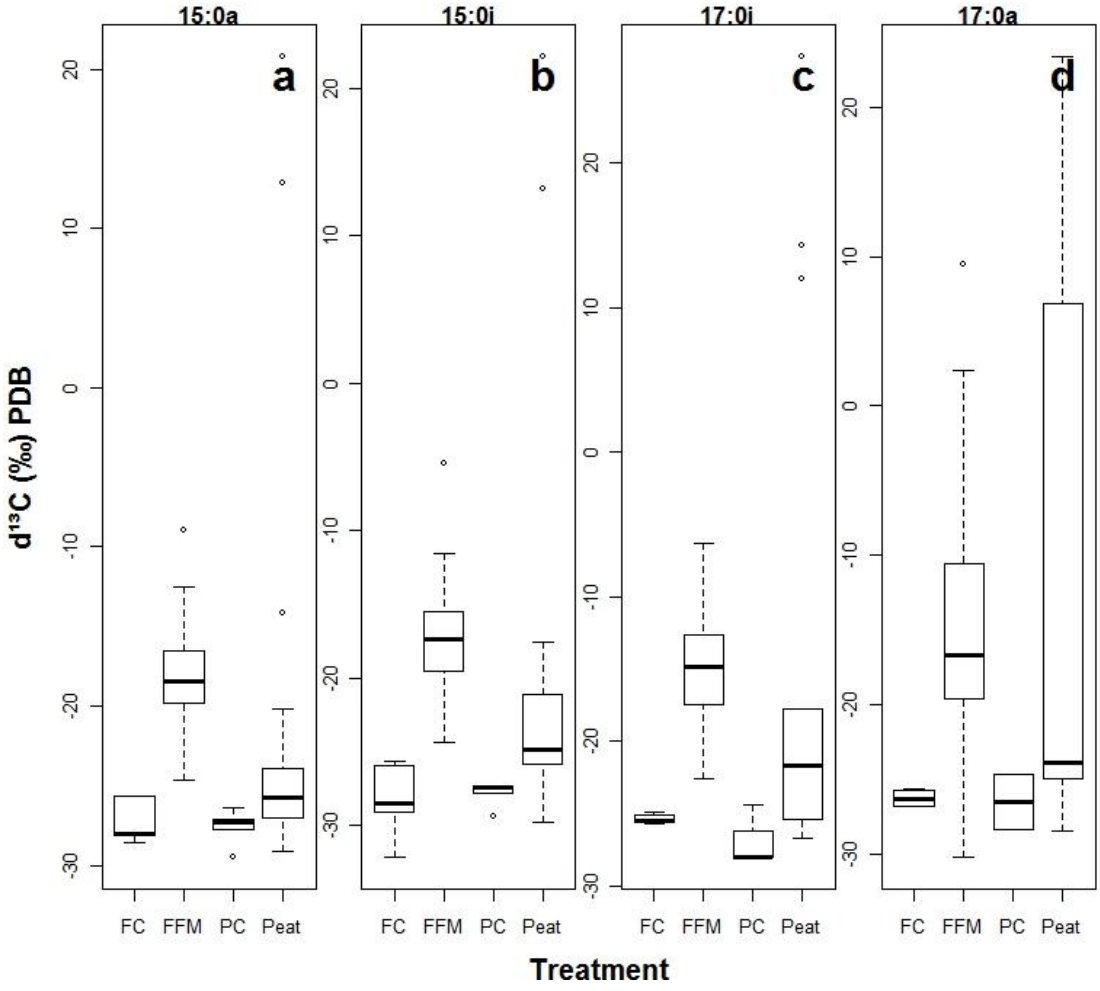

Figure 3.7 Delta ^{13}C values ($\delta^{13}C$ Pee Dee Belemnite (‰)) of the phospholipid fatty acids 15:0*a*, 15:0*i*, 17:0*i* and 17:0*a* extracted from the bulk soil (FC & PC) and rhizosphere (FFM & Peat) samples (n = 4-14) of alder (*Alnus crispa* Ait.) grown in forest floor material (FFM) and peat from a wooded fen (Peat), where FC corresponds to the FFM bulk soil, and PC to the Peat bulk soil. Median values are indicated by the horizontal bar within the box. The first and third quartiles are denoted by the lower and upper box limits respectively, and the 5th and 95th percentiles by the bars at the end of the vertical bars projecting from the box. Outliers are identified by open circles.

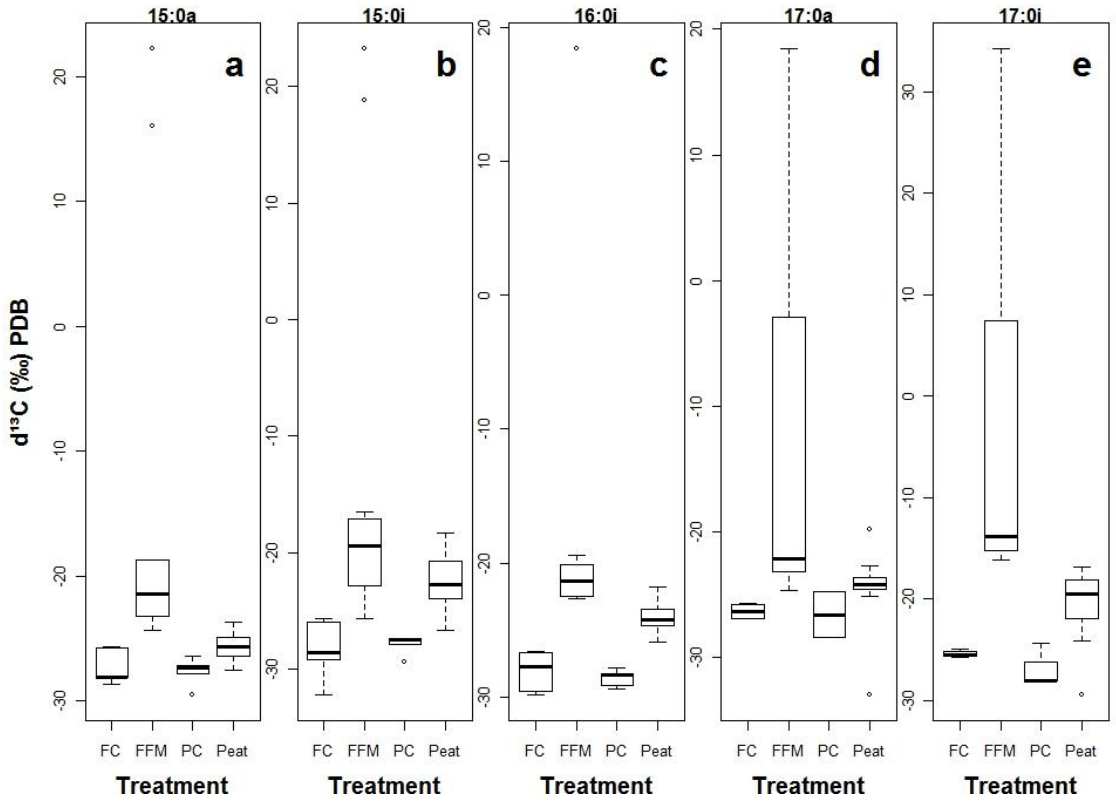

Figure 3.8 Delta ^{13}C values ($\delta^{13}C$ PeeDee Belemnite (‰)) of the phospholipid fatty acids 15:0*a*, 15:0*i*, 16:0*i*, 17:0*a* and 17:0*i* extracted from the bulk soil (FC & PC) and rhizosphere (FFM & Peat) samples (n = 4-14) of aspen (*Populus tremuloides* Michx.) grown in forest floor material (FFM) and peat from a wooded fen (Peat), where FC corresponds to the FFM bulk soil, and PC to the Peat bulk soil. Median values are indicated by the horizontal bar within the box. The first and third quartiles are denoted by the lower and upper box limits respectively, and the 5th and 95th percentiles by the bars at the end of the vertical bars projecting from the box. Outliers are identified by open circles.

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IV. Synthesis

1. Summary

The main objectives of this thesis were to determine if organic amendments of different botanical origins influence the composition and activity of the soil microbial community and to investigate how the differences observed may be related to the growth of boreal plant species.

Incubation of the organic amendments as reported in chapter two showed the activity of the soil microbial community in the peat material to be significantly less than that measured in the forest floor material. Specifically, both the *Sphagnum* peat taken from the natural site and the stockpiled peat had significantly lower respiration rates than the natural forest floor and the stockpiled forest floor material, respectively. Additionally, the materials taken from the stockpiles showed significantly lower respiration rates than the materials taken from the natural sites. Further, ordination of the phospholipid fatty acid profiles of these materials illustrated distinctly different community compositions in organic substrates of differing botanical origins.

Mixing the materials showed what appeared to be a priming effect from the more easily decomposable forest floor material. However, this was more likely a result of a stimulation of the microbial community in the material from the forest floor material by the greater water holding capacity created by the peat material. Concurrent analysis of the soil microbial composition provided supporting evidence and illustrated a microbial community composition more similar to the forest organic layer than the peat in

ordinations of the PLFA data. These results suggest greater growth and activity of the microbial community from the forest floor material in the mixed treatment. An analysis of individual PLFAs showed a coherent gradient across the gradient of materials (forest organic layer > mixed treatment > *Sphagnum* peat), which followed a gradient of pHs across materials. However, the stockpiled materials, which all had a comparable pH, demonstrated the same coherent change in the composition of their microbial communities across treatments (forest floor mineral > mixed treatment > Peat). As such, while pH may be a contributing factor, the observed changes appear to be driven by other factor(s) inherent to the botanical origin of the materials that were not measured in this study.

The greenhouse experiment in chapter three returned stable isotope probing results for the rhizosphere community of alder (*Alnus crispa* Ait.) suggesting that the differences in community composition between peat and forest floor has a meaningful impact on the functioning of such microbial community. There was significantly less ^{13}C enrichment in the microbial community of Peat compared to the forest floor material. This reduced carbon flow in the Peat treatment was most apparent in PLFAs that are indicators of mutualistic microorganisms associated with alder, and corresponded to its lower growth in all measured growth parameters.

In contrast, while characterization of the PLFA profile of the aspen rhizosphere also showed reduced carbon flow in the Peat treatment compared to the forest floor material, there was no evidence of any influence on the growth of the aspen seedlings. These

results suggest that peat has an inhibitory effect upon some segments of the microbial community which may include mutualistic soil microorganisms. This inhibitory effect may reduce the growth of actinorhizal and mycorrhizal plant species but appears to have little effect upon plant species with more facultative associations with the microbial community.

2. Novel findings

This research project has made two main contributions to the body of knowledge that focuses on soil reclamation in the Athabasca oil sands region. The experiment in chapter two was the first experiment within the oil sands which investigated the composition and activity of the microbial community in a mixture of forest floor and peat organic materials. While previous researchers have investigated changes in microbial community composition and soil priming within mineral soils, this investigation within organic materials suggests that, given equal mixing of organic substrates, forest floor material will dominate the composition and activity of the mixture. Similarly, chapter three was also the first time stable isotope probing was used to investigate the soil microbial communities in reconstructed soils of the oil sands region. Stable isotope probing provided new information concerning the associations occurring between the soil microbial community and some boreal plant species used during reclamation.

3. Project limitations and future research

Results support the use of PLFA analysis as an meaningful measure of comparing overall soil community composition and support the hypothesis that the composition of the

microbial community is influenced by the botanical origin of its organic substrate. However, this was a laboratory based research project which focused on the capability of individual organic residues to foster microbial communities. This experiment was conducted under controlled conditions that may be quite different from the variability and competing influences of the environment that are more typical of field conditions.

This M.Sc. program investigated two types of peat, which were derived from different ecological settings and assemblages of plant species, in conjunction with natural and stockpiled materials collected from an aspen stand, one of the dominant forest typed in the region. This allowed for the comparison of each material at its maximum potential, i.e. in fresh material, and its potential following standard operational handling, i.e. in stockpiled material. This yielded valuable information but was limited by both the availability of newly salvaged materials and the scope of a Master's thesis. Thus, a broader range of natural forest floor materials should be included in future research. Specifically, the forest floor materials from forest stands located on coarse textured soils should be investigated, which are not only composed of a different assemblage of tree and understory species, but are also home to mutualistic mycorrhizae which associate with species such as jack pine (Beckingham and Archibald 1996, Singh et al. 2005).

The inclusion of stockpiled material incorporated some of the variability created by the salvaging and handling processes of oil sands operations. However, salvaged materials are often stockpiled for years and the results of this experiment have shown stockpiling to reduce the activity of the microbial community after less than a year of stockpiling. Thus,

it may be valuable to analyze a chronosequence of stockpiled materials to determine the effect of time on the composition and activity of the microbial community. The depth of stockpiling could also be a concern as stockpiles are often several metres or more in depth and can become anoxic in their centers. Thus, it may be valuable to determine if the aerobic microbial community of the stockpiled forest organic layer recovers after placement in soil reclamation to a composition and activity similar to that before stockpiling and how this varies by the depth and duration of stockpiling.

Stable isotope probing of the soil microbial community via carbon flow through the plant illustrated new and meaningful information. However, the higher mass and lower activity of the ^{13}C isotope commonly results in discrimination against the incorporation of the heavier isotope by plants and microorganisms (Fry 2006). This often results in a relative depletion of ^{13}C as it is transferred through trophic levels, i.e. from primary producer to primary consumer, which should be considered during analyses. Additionally, it is likely that root exudates are more depleted in ^{13}C than the plant tissues. Due to mortality of a number of seedlings during the greenhouse experiment, I had to use bulk soil as a control as opposed to the rhizosphere of unlabelled plants that I originally had planned. As such, the control used may be slightly more enriched than the original control from an unlabelled rhizosphere; hence, the calculated differences in enrichment between the labelled and control samples may constitute an underestimation of the actual carbon flow.

Further, while the use of complete PLFA profile has become the most common method of describing the composition of soil microbial communities, there is still some ambiguity

concerning the use of individual PLFAs as specific indicators (Frostegård et al. 2011). Some PLFAs cited as specific indicators of a group or genus of microorganisms rely on a single study, and often an individual PLFA can be associated with more than one segment of the microbial community. The PLFA 16:1ω5c is cited in this thesis as an indicator of arbuscular mycorrhizae as reported in numerous scientific articles (Bååth et al. 1992; Haack et al. 1994; Olsson et al. 1995; Olsson 1999; Allison and Miller 2005). However, it also occurs in benthic bacteria (Nichols et al. 1986), and *Cytophaga hutchinsonii* (Walker 1969), a gram negative soil bacteria which has been characterized as a cellulose degrading specialist, hence may not be relevant to this study. Still, a thorough review of the literature on PLFA indicators always needs to be carefully conducted when using these to characterize microbial communities, and comparison of individual PLFAs should be limited to a qualitative, not quantitative, measure of the microbial community.

Given results of the stable isotope probing of the alder and aspen rhizospheres, which showed a significant reduction in carbon flow to the rhizosphere microbial community of both species when grown in peat but with only a corresponding reduction of growth in alder, I believe that additional research using this technique is warranted, which should encompass a broader range of boreal plant species.

4. Recommendations

Although the stable isotope probing (SIP) results produced valuable data which described the functioning of the microbial community, hindsight suggests that the method used in this experiment could have been improved with the use of a larger soil sample and a

larger label of $^{13}CO_{2\ (g)}$. The results of the PLFA elution described the total number of identifiable PLFAs and reported over 70 individual PLFAs. However, most of these PLFAs were of small magnitude and were discarded during SIP analysis due to low signal to noise ratios. A greater response could conceivably be achieved on these small magnitude PLFAs by extracting a larger soil sample or concentrating the final PLFA extract, thereby increasing the number of PLFAs that can be included in the analyses. The ^{13}C enrichment could also be increased with a larger dose of $^{13}CO_2$ which would increase the $^{13}C/^{12}C$ ratio and improve the IRMS signal.

Due to higher than expected seedling mortality in the greenhouse experiment, the control used for chapter three had to be adjusted from the original plan. The control originally planned was the rhizosphere of seedlings that had undergone the exact same experimental protocol as the enriched samples, except this time with the introduction of $^{12}CO_2$ (g) instead of $^{13}CO_2$ (g). As previously mentioned, discrimination of ^{13}C isotopes commonly occurs between trophic levels and the procedure used could have had an influence upon the physiology of the seedlings. These parameters could have influenced the enrichment levels measured and would not have been represented by the measurements of background ^{13}C enrichment obtained from the bulk soil that was used as a control. Future investigations using stable isotope probing should include controls submitted to the same experimental procedure as the labelled materials, but without the introduction of the tracer isotope.

Similarly, time restrictions and equipment failure experienced during the preliminary investigations of the stable isotope labelling method removed the possibility of calibrating the method to determine the optimum pulse labelling and chase period to create maximum labelling of the microbial community without transfer of the introduced ^{13}C isotopes through trophic levels or lateral transfer within trophic levels (cross-feeding). The experimental design had originally included a suite of treatments using a variety of pulse and chase periods along with different amounts of ^{13}C label. However, unforeseen issues with the IRMS instrument prevented analysis of these samples. Time restrictions required that a choice of the most likely method to produce successful enrichment be made from the available literature. Hence, the pulse and chase periods that were chosen may not represent the optimal solution for the type of materials and plant seedlings that were investigated in this specific study. Future investigations using stable isotope probing should include preliminary testing to calibrate the method as to produce results with optimal ^{13}C signal and minimal amount of cross feeding occurring.

From an oil sands soil reclamation perspective, mixing the FFM material with peat could represent an operational alternative to the pure materials. The mixed treatments displayed a microbial community composition more similar to that of the forest organic layer than the peat material. Mixing the materials most likely needs to be done in equal parts by organic carbon content to maximize the benefits. Given the limited volumes of the forest organic layer available for reclamation this may also provide a means of increasing the volume of reclamation material with the added benefit of increasing its water holding capacity.

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Appendix A. Supplimental Information

Table A1. Suffixes associated with phospholipid fatty acids describing their chemical structure.

Suffix	Meaning
i	• Branching at second carbon from aliphatic end (iso)
a	• Branching at third carbon from aliphatic end (anteiso)
cy	• Cyclopropyl group
***x*OH**	• Hydroxyl group located on the *x* carbon from aliphatic end
10me	• Methyl group located on the 10th carbon from carboxyl end
c	• Double bond in the *cis* configuration
t	• Double bond in the *trans* configuration