# Biodegradation of Hydrocarbons in Bitumen: Exploring Plant-Assisted and Microbial Stimulation Techniques

by

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## Abstract

While bitumen is one of the oldest construction materials in the world and currently provides an important fuel needed to sustain our modern lifestyle, the disturbances caused by extracting and refining this material are considerable, with 895 $km^2$ of land being disturbed in Alberta as of 2018 that needs reclamation. As part of reclaiming the mined landscape, landforms made of lean oil sands (low-grade bitumen containing < 7 % hydrocarbons by volume) are covered with a cap of suitable soil to provide a base for revegetation. However, the effects of hydrocarbons present in lean oil sand are concerning due to their adverse effects on ecological health and plant growth. Revegetating reclaimed areas with native plant species not only supports restoring locally common forests but may also enhance the degradation of hydrocarbons. The goal of my research is twofold: 1) to determine whether trembling aspen (*Populus tremuloides*) and jack pine (*Pinus banksiana*), tree species that are native to the region and commonly used in reclamation, are effective phytoremediation candidates, and 2) to determine the effects of lean oil sands on the soil microbial community and their potential for hydrocarbon degradation. I conducted a growth chamber experiment to assess hydrocarbon degradation over 30 weeks in two grades of LOS, 4.54 % hydrocarbons and 1.95 % hydrocarbons, in the presence and absence of each of the plant species. In addition to hydrocarbon degradation, I also measured biomass of plants in response to the two grades of LOS. Analysis of phospholipid fatty acids (PLFA) was used to determine how the soil microbial community was altered by plant species and LOS grade. While my study determined that the plants studied here did not enhance degradation of hydrocarbons, the addition of nutrients and water to the system may have acted as a biostimulant. This biostimulant effect may have enabled microbes to degrade groups of hydrocarbons generally considered to be recalcitrant. Furthermore, analysis of PLFAs showed that increased concentrations of hydrocarbons

corresponded to increased microbial PLFA concentrations regardless of functional group, potentially indicating that the local microbial community can use hydrocarbons to produce additional biomass. At low concentrations, such as those used in my study, the native microbial community may be stimulated in the presence of hydrocarbons to degrade these compounds and immobilize them via incorporation into new biomass. Taken together, my research indicates that while the plant species used here are not suitable to enhance degradation under the tested conditions, nutrient and moisture amendments may enhance the degradation of hydrocarbons in LOS by stimulating the soil microbial community.

## Preface

No data from this thesis has been published prior to thesis defense and publication.

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## Introduction

### *Bitumen Mining and Mine Site Reclamation*

Bitumen is one of the oldest construction materials used worldwide, though in modern times its use is not limited to construction, instead it forms the energy mix needed to sustain our modern lifestyles (Read, Whiteoak, and Hunter, 2003). Bitumen is defined as "any of various mixtures of hydrocarbons (such as tar) often together with their nonmetallic derivatives that occur naturally or are obtained as residues after heat-refining natural substances (such as petroleum)"(Merriam-Webster, 2020). While useful, the mining and extraction of bitumen is not without consequence, with 895 $km^2$ of disturbed forest present in Alberta, Canada as of 2018 (Government of Alberta, 2017, 2019). This disturbance results in a landscape that must be reclaimed to a state that functions similarly to the surrounding native boreal forest.

In Alberta, reclamation returns degraded systems to an ecologically functional state with equivalent land capability with respect to the original system prior to usage by industry (Government of Alberta, 2019). Reclamation is defined within the *Environmental Protection and Enhancement Act,* section 1 (ddd) as "the removal of equipment or buildings or other structures or other appurtenances, the decontamination of building or other structures or other appurtenances, or land or water; the stabilization, contouring, maintenance, conditioning or reconstruction of the surface of the land; and any other procedure, operation or requirement specified in the regulations." In addition to this, "equivalent land capability" is defined in the *Conservation and Reclamation Regulation,* section 1 (e), as "the ability of the land to support various land uses after conservation and reclamation is similar to the ability that existed prior to an activity being conducted on the land, but that the individual land uses will not necessarily be

identical." In practice, this means in Alberta that the goal of reclamation is to recover degraded landscapes to a point at which they can support a variety of land uses for both humans and the wider environment. While reclamation does not necessarily need to restore land to a point identical to the pre-disturbance state, the ecosystem functions and services provided must be in some form equivalent to those that were lost as a result of disturbance.

As part of reclamation in northern Alberta, lean oil sands (LOS, low-grade bituminous ore containing < 7 % hydrocarbons by volume) is used to reconstruct out-of-pit landforms and provide, in conjunction with a salvaged soil cap, a base for revegetation (MacLennan et al., 2018, and Visser, 2008). Lean oil sands is present as overburden material in the Athabasca Oil Sands Region and is within 75 m of the surface in roughly 20 % of oil sands reserves, namely those that are able to be accessed via open pit mining (Government of Canada, 2016). Lean oil sands is often characterized by the presence of little to no volatile hydrocarbons in the F1 fraction such as benzene, toluene, ethylbenzene and xylene, and usually contains elevated levels of hydrocarbons with a chain length of 16 to 50+ carbon atoms (Visser, 2008). Furthermore, LOS often contains little phosphorus or nitrogen, while sulfates are elevated along with the metals nickel, vanadium, rhenium and molybdenum (Bicalho et al, 2017; Goldschmidt, 1937; Selby and Creaser, 2005). While the placement of LOS is part of reconstructing and recontouring reclamation landforms, elevated levels of F2–F4 hydrocarbons are still present within the mixture (Visser, 2008). It is important to note that while LOS is a natural geologic formation within the Athabasca Oil Sands Region, the concern lies with its removal and placement in newly constructed landscapes, and the potential risk posed by the liberation of naturally present hydrocarbon fractions. These hydrocarbon fractions range from 10–50+ chains of carbons (AMTAG, 2008), with the larger chemical structures being generally more recalcitrant than their lower molecular weight

counterparts. Specifically, these fractions are groups of hydrocarbon compounds with similar amounts of carbon atoms present in the chemical structure, with the groupings defined as follows: F2 (>nC10 to nC16), F3 (>nC16 to nC34) and, F4 (>nC34 to nC50+) (Turle et al, 2007). These fractions of hydrocarbons are so named due to fractional distillation, a method for separating specific groups of compounds from crude oil during the refinement process (Johan, 1942). These low molecular weight compounds may have adverse health effects on many organisms due to a high affinity for organic tissues (Abdel-Shafy and Mansour, 2016, Mehlman, 1990; Blackburn et al., 1984), as well as challenge plant growth on reclamation sites owing to indirect and direct effects of the residual hydrocarbons. In particular, hydrocarbons may form a physical barrier due to soil compaction (Pernitsky et al., 2016), alter the hydrology of soils due to the hydrophobic nature of hydrocarbons (Roy et al., 2003), or directly inhibit plant growth (Chaineau, Morel, and Oudout, 1997; Shiram et al, 2008;, Visser, 2008). Furthermore, previous work has shown that hydrocarbons have growth and metabolism-inhibiting effects on the soil microbial community, even while that community is degrading the compounds in question (Atlas, 1991 and Labud, Garcia, and Hernandez, 2007). Taken together, residual hydrocarbons present in bitumen may impact the long-term success of mine site reclamation if not degraded.

*Phytoremediation in Reclamation*

One possible solution to removing hydrocarbons in LOS is phytoremediation. This technology relies on plants and their associated rhizosphere microbial community to remove, immobilize, or isolate undesirable or harmful compounds from the environment (Salt, Smith, and Raskin, 1998). This technology has been successfully used in several trials to remove organic compounds from soils and shallow aquifers across a wide range of latitudinal, climatic, and chemical site variation (Cook et al., 2010; Macci et al., 2013; Kang, 2014 and Nichols et al.,

2014). Previous work has shown that using native plant species and their associated microbial communities to degrade organic compounds is cost-effective, but concerns remain regarding the expediency of this approach (Azubuike, Chikere, and Okpokwasili, 2016; Kuiper et al., 2004; Vangronsveld et al., 2009). Particularly in northern regions, a short growing season and colder average temperatures can slow metabolic activity in the rhizosphere, hindering phytoremediation efforts (Collins, 2007).

In Alberta, reclamation of mined areas includes planting native species belonging to the regional flora. Some of these plant species may also promote hydrocarbon degradation, a function that could improve long-term reclamation performance when plants begin to interact with LOS material. Native tree species, such as jack pine (*Pinus banksiana*) and trembling aspen (*Populus tremuloides*), are key species in the revegetation phase of oil sands reclamation in northern Alberta (Pinno et al., 2012; Farnden et al., 2013). Little work, however, has been done to assess the phytoremediation potential of these species with respect to hydrocarbons. As both plant species and substrate characteristics influence microbial community structure and therefore degradation (Siciliano et al., 2003), it is important to understand how LOS substrates alter the community composition of soil microorganisms. To this end, phospholipid fatty acid analysis may be used to better understand broad changes occurring within the microbial community in regards to both biomass and composition. Phospholipid fatty acids (PLFAs) are part of microbial cell membranes, and different microorganisms will produce different PLFAs in order to maintain the integrity and function of their cells (Quideau et al, 2016). While they are not able to identify individual species in mixed soil sample, they can be a useful tool for providing a broad overview of the living soil community, as PLFAs are rapidly degraded upon cell death and the risk of capturing dead biomass in a PLFA fingerprint is minimal (Quideau et al, 2016). This technique

has been used extensively to characterize how microbial communities respond to changes in land management such as in reclamation (DeGrood, Claasen, and Scow, 2005; Quideau et al, 2013; Hahn and Quideau, 2013; Margesin, Hämmerle, and Tscherko, 2005).

The rate of phytoremediation is influenced by abiotic factors that affect both the plant and its associated microbial community. These factors include the concentration of hydrocarbons, substrate temperature, nutrients, moisture, and pH, which can alter the composition and metabolic activity of the microbial community as well as the growth of vegetation (Alori, 2016). Therefore, understanding physical and chemical characteristics of LOS can inform how the plant-microbial system degrades hydrocarbons *in situ,* as well as what can be added to the system to achieve the desired degradation of hydrocarbons. Previous work shows that tree species such as jack pine and aspen change their rooting behavior in response to nutrient and water stresses (Tan and Hogan, 1997; Anderegg, 2012), stresses which are also present in unweathered LOS (Visser, 2008). As root surface area correlates with increasing degradation in petroleum-hydrocarbon containing substrates (Merkl, Schultze-Kraft and Infante, 2005), changes in rooting behavior caused by the presence of LOS may influence phytoremediation. For instance, if roots are not interacting with LOS due to a physical or chemical barrier to growth, this will likely result in lower overall degradation rates (Merkl, Schultze-Kraft and Infante, 2005). Therefore, it is important to understand how both biotic and abiotic factors influence the degradation of LOS hydrocarbons.

*Research Objectives*

To address the management of LOS in reclaimed landforms, I aim to answer the following questions: 1) do current tree species used in revegetation degrade hydrocarbons present in LOS. That is, are *Populus tremuloides* and *Pinus banskiana* phytoremediation candidates? Candidates for phytoremediation would be identified as native species capable of either direct degradation through the release of enzymes from the roots (Iimura et al, 2007), or indirectly enhance degradation through the stimulation the soil microbial community by signaling hormones or labile organic compounds (Page, Yergeau and Greer, 2015). These direct and indirect mechanisms in my study can be measured as enhanced degradation of hydrocarbons when a plant species is present compared to its unplanted control. And, 2) how does LOS influence the composition of the microbial community responsible for the bulk of *in situ* hydrocarbon degradation? I hypothesize that the biomass of the microbial community will decrease when subjected to higher hydrocarbon concentrations, as the increased environmental stress placed on the microbial community will result in the die-off of microbial groups that are more sensitive to hydrocarbons. Addressing these questions will enhance our understanding of how plants and their associated microbial community degrade hydrocarbons during phytoremediation, allowing us to more efficiently remove hydrocarbons from a substrate such as LOS. This may allow us to combine remediation and revegetation stages of reclamation into a single step, resulting in a more effective approach to mine site reclamation.

## Methods

### *Field Collection of Growth Substrates*

To measure degradation, I collected 80 L of lean oil sand (LOS) with Syncrude Canada Ltd. from the Aurora North Mine Site (57.3300 º, -111.5222 º) on June 6, 2019, which was then transported back to the University of Alberta within 24 hours of collection. During reclamation, bare overburden (i.e., LOS) is not revegetated, rather a suitable growth substrate is used to cap the landform. Specifically, reclamation of LOS involves placement of an appropriate capping thickness of soil reclamation material that consists of surface soil material that is salvaged within the disturbance area footprint. To model the reclamation practice of a soil reclamation cap over LOS (but not a similar thickness), I placed 2 cm of salvaged peat to the surface of experimental pots containing LOS (see below). The peat was sourced from the Aurora Soil Capping Study reclamation site (57.3300 º, -111.5222 º) also on June 6, 2019, which in turn was sourced earlier from the upper 200 cm of black spruce (*Picea mariana* Miller) lowland, free from mineral material (Scott et al., 2019; Hankin, Karst, and Landhaüsser, 2015) (See Table 1). The vegetation community of this source area was predominantly classified as a shrubby poor fen (j ecosite), which consists of sparse black spruce (*Picea mariana* Miller) and tamarack (*Larix laricina* K.Koch) (Beckingham and Archibald, 1996). Forty L of salvaged peat was collected from within 10 cm of the peat-LOS interface from roughly 30 cm in depth within the soil profile. The interface was chosen to maximize the presence of a LOS-acclimated microbial community, as microbes have been shown to acclimate to hydrocarbons in as little as seven days as shown by enhanced degradation activity (Bauer and Capone, 1988), and this material has been present on-

site since 2012. The collected peat was then transported back to the University of Alberta in coolers within 24 hours and stored at 4 ºC until use on June 19, 2019.

*Experimental Set-up*

To assess plant capacity to promote hydrocarbon degradation, I grew two species, jack pine (*Pinus banksiana* Lambert) and trembling aspen (*Populus tremuloides* Michaux) in LOS capped with peat. I sourced pine seeds from seed lot SYN 26-96-10-4-2008 PJ, zone CM2.1, Smokey Lake Tree Nursery, Alberta, Canada and stratified them within 24 hours of collection. I sterilized the seeds by soaking them in 5% bleach for 15 minutes before rinsing with deionized water, followed by a 24 hour soak in deionized water before cold stratification for 14 days at 5 ºC. I sourced aspen seeds from a population occurring on the northern edge of the University of Alberta, North Campus, Edmonton, Canada and sterilized them in 1% bleach for 15 minutes before being rinsed with deionized water prior to planting. We obtained the aspen seeds from seed zone CP 1.1, (Government of Alberta, 2016) given that we were unable to obtain aspen seeds from the same seed zone as the pine.

Seedlings were grown in LOS of two different grades. The first grade used LOS containing an average total hydrocarbon concentration of 43,533 mg $kg^{-1}$ (4.35 %) ('High LOS'). The second grade was a mix of the LOS with sterilized silica sand (Garden Sand, Kott Holdings Ltd., Edmonton, Alberta, Canada) at 50 % by volume and possessed an average total hydrocarbon concentration of 19,676 mg $kg^{-1}$ (1.96 %) ('Low LOS'). These grades were selected to represent variation the material that is currently used in reclamation to create post-mining landforms, as well as a concentration that is much lower than the current reclamation concentration and commonly used to simulate field hydrocarbon contamination without causing

mortality of plants (Liu et al., 2012; Peng et al., 2008). Silica sand was sourced from Apache Seeds in Edmonton, Alberta, and sterilized twice 24 hours apart via autoclave at 121 ºC for 90 minutes. Sterilized sand was used within seven days of autoclaving.

I used sixty planted and thirty unplanted pots in my experimental design, with treatments consisting of plant identity (pine or aspen) crossed with hydrocarbon concentration ('Low LOS' or 'High LOS'). Each plant species was paired with their own control group, due to the differing nutrient requirements for aspen and pine. Individual seedlings were grown in 656 mL Deepots (Model D60L, Stuewe and Sons., Inc. Tangent, Oregon) that I had sterilized along with mesh bottoms and identification tags in a 10% bleach solution for 10 minutes prior to use. I also set up a unique set of control pots for each species, due to differing nutrient and water requirements. After sterilization, I then filled pots with 450 mL of unpacked LOS (roughly 25 cm) and capped with a 2 cm layer of peat. Twelve seeds were then added to each pot and pots were then positioned in trays within the growth chamber in a randomized design. To minimize the effects of pot position within the growth chamber on the experiment, I shuffled pots randomly to a new location monthly. Aspen seedlings were grown for 120 days, while pine seedlings grew for 212 days. Both groups of plants were grown in the same growth chamber with a 16/8-hour day-night cycle to approximate longer day periods that would be present in northern growing season. The temperature within the growth chamber was kept at 20 ºC during that day and 12 ºC at night on a gradual gradient to best approximate northern growing season conditions within the technical constraints of the growth chamber. Daytime light intensity within the growth chamber was 322 $\mu mol\ s^{-1}\ m^{-2}$ throughout the entire experiment as measured by LI-250A light sensor (model LI-191/R, LI-COR Biosciences, Lincoln, Nebraska). For the first two weeks of growth, seedlings were watered via misting until water began pooling on the pot surface and then covered with

clear plastic wrap to prevent desiccation. After this initial period, aspen seedlings were given 50 mL of water daily while pine were given 50 mL every second day for the next two weeks. Then, both species were given 50 mL of water every third day for the remainder of the experiment. Aspen were given 25 ppm of either 30-10-10 or 10-52-10 fertilizer every two weeks, depending on observable nutrient deficiencies, while pine were given 25 ppm of the same fertilizers every three weeks. Plants were also given 24.3 ppm $MgSO_4*7H_2O$ twice during the experiment to correct observed magnesium deficiencies.

At harvest, shoots and roots were separated, and shoots were stored in paper bags and oven-dried at 65 ºC for 5 days prior to being weighed. Roots were washed clean of potting substrate and stored in 50% ethanol prior to analysis of surface area, length, and biomass. To determine fine root surface area and length for each species, I laid roots in a tray filled with deionized water and imaged them with an Epson Perfection V600 Photo scanner (Epson Canada Limited, Markham, Canada) using 16-bit greyscale and 800 dpi resolution. I then analyzed scanned images using WinRHIZO Pro analysis software (Regent Instruments Inc., Quebec City, Canada) using diameter classes of less than 2 mm for jack pine and 1 mm for aspen to delineate fine roots versus coarse roots. Once imaging was performed, I dried roots at 65 ºC for five days prior to being weighed and recorded.

Peat caps were removed from the control pots using sterilized scoops, re-sterilizing between each pot with 10% bleach to prevent cross-contamination. In planted pots, peat was scooped out as in the controls without damaging the roots, and the remainder of the peat was then extracted with the seedlings. The remaining LOS was then pushed through a 2 mm sieve and partitioned into sample streams for hydrocarbons, soil chemical and physical properties, and phospholipid fatty acid analysis. Sieves and all containers were sterilized prior to use and

between each sample with 10% bleach. Hydrocarbon samples were stored in 118 mL glass jars with Teflon lids (Catalogue Number 120-0060, Thermofisher Scientific, Waltham, MA) at 4 ºC, with samples being submitted for analysis within 48 hours after collection. Phospholipid fatty acid samples were kept within 15 mL centrifuge tubes (Catalogue Number 430766, Corning Science Mexico, Reynosa, Tamaulipas) at -20 ºC until analysis.

*Hydrocarbon Analysis*

Hydrocarbon concentrations were measured before and after the experiment for F2–F4G fractions in accordance with the Reference Method for the Canada-Wide Standard for Petroleum Hydrocarbons in Soil-Tier 1 Method (AMTAG, 2008). Analysis of hydrocarbon concentrations was performed by Bureau Veritas Environmental Services Laboratory in Edmonton, Alberta. Gas chromatography with flame ionization detection was used to determine the concentrations of F2–F4 hydrocarbons in each sample. Samples were prepared by using a Soxhlet apparatus and then recovered. Samples were then transferred to gas chromatography columns and analyzed before drying to determine hydrocarbon concentrations and sample moisture.

*Soil Chemical and Physical Properties*

Baseline soil chemical and physical properties were established prior to the beginning of the experiment, with all but soil texture being reanalyzed after the experiment had concluded. Soil texture was determined via particle size analysis using a hydrometer (ASTM D422-63, 2007; Carter and Gregorich, 2006, and Bouyoucos, 1962). Extractable phosphate was measured via the Modified Kelowna method (Alberta Agriculture, 1995), whereas extractable nitrogen was determined via the 2M KCl method (Maynard, Kalra, and Crumbaugh, 2008; Jones, 2001, and

Kalra and Maynard, 1991). Total nitrogen and total carbon were determined via dry combustion method Using a ThermoScientific Flash 2000 Organic Elemental Analysis instrument (ThermoFisher Scientific, Waltham, MA, USA). Electrical conductivity and pH of soils were determined by preparing a 2:1 soil:water mixture and analyzing with a combination pH/EC meter (Fisher AR20 pH/EC meter, Thermo Fisher Scientific, Inc., Cambridge, UK) as outlined in Method NRAL-009 (Miller and Curtin, 2007; McLean, 1982; Hendershot, Lalande, and Duquette, 2007; Kalra, 1995; Miller and Kissel, 2010; and Rhoades, 1982). Lastly, recoverable metals (Na, K, Ca, Mn, Fe, Cu, Zn, Mg, P) and sulfur were determined by the use of inductively coupled plasma-optical emission spectroscopy analyzed via spectrometer (Thermo iCAP6300 Duo inductively coupled plasma-optical emission spectrometer, Thermo Fisher Inc., Cambridge, UK) (Skoog, Holler, and Crouch, 2007).

*Soil Phospholipid Fatty Acid Analysis*

Samples were lyophilized for 96 hours and stored at -20 ºC prior to analysis. Analysis was performed following the methods of Quideau et al. (2016), using a surrogate standard of 19:0; 1,2-dinonadecanoyl-sn-glycero-3-phosphocholine (Avanti Polar Lipids Inc, Alabaster, USA) that is added prior to the first extraction, and an instrument standard of 10:0 Me; methyl decanoate (Aldrich, St. Louis, USA) that is added before gas chromatography analysis. Analysis of fatty acid methyl esters is conducted via Agilent 6890 Series capillary gas chromatograph (Agilent Technologies, Santa Clara, USA) fitted with a 25 m Ultra 2 column (Crosslinked 5 % Ph-Me-Silicone), and a flame ionization detector. To identify and quantify fatty acid methyl esters, The Sherlock Microbial Identification System Version 6.3 software (MIDI Inc., Newark, USA) was used. The software method MICSOIL3 was used to estimate microbial types using

known marker PLFAs contained within the method that have been shown in previous work to be reasonably specific to the target microbial groups (Frostegård and Bååth, 1996; Ringelberg et al., 1997, and Zelles, 1999) (see Table 2).

These groups of microbes were selected for analysis because all groups could play a potential role in the degradation of organic material, such as hydrocarbons. In the case of eukaryotes, macro-organisms have been shown to increase the rate of hydrocarbon degradation, as well as enhance microbial respiration in hydrocarbon-containing soils (Schaefer and Juliane, 2007). Facultative anaerobes have also been shown to have the capacity to degrade hydrocarbons and may even degrade at a faster rate under aerobic conditions (Grishchenkov et al., 2000). Actinomycetes were also of interest due to the ability of species across several genera such as *Rhodococcus, Gordonia, Nocardia,* and *Dietzia* to facilitate complete hydrocarbon degradation via the β-oxidation enzymatic pathway (Alvarez, 2003). Ectomycorrhizal and saprotrophic fungi were chosen due to their ability to produce extra-cellular enzymes that are capable of degrading complex organic material (Field et al., 1992 and Hammel, 1995), while AM have been shown to enhance plant's tolerance to hydrocarbons and may indirectly improve the rate at which hydrocarbons are dissipated by the plant host (Zhou et al., 2013). Finally, while gram positive and gram negative bacteria are a broad classification that may include members that can degrade hydrocarbons, these classifications were chosen instead to provide a more clear estimate of how the bacterial biomass in the soil was responding to the change in hydrocarbon concentration.

*Data Analysis*

All statistical analyses were conducted in R version 4.0.0 (R Core Team, 2019 and RStudio Team, 2020) using the packages tidyverse (Wickam, 2019), geoR (Ribeiro Jr. et al., 2020), car (Fox and Weisberg, 2019), ggpubr (Kassambara, 2020), vegan (Oksanen, 2019), and ecodist (Goslee and Urban, 2007).

To analyze the hydrocarbon degradation data, I quantified degradation as the difference between the pre- and post-experiment concentrations of hydrocarbons. Box-cox transformation was used for both the pine and aspen datasets to meet the assumptions of normality and homogeneity of variances that a two-way ANOVA requires. After data transformation, data from the aspen treatment group and the pine treatment group were analyzed separately using two-way ANOVA with the factors LOS grade (high or low) and species presence (planted or unplanted). Pairwise comparisons were performed using Tukey's HSD as an *a priori* test. To test if fine root surface area influenced the rate of hydrocarbon degradation, correlations were performed using the box-cox transformed data and Pearson's correlation coefficient. To test if LOS had a significant effect on plant biomass, single factor ANOVAs were used to assess the effects of LOS grade on both shoot and root biomass.

I first analyzed microbial PLFA data for each unplanted control to test if the different LOS grades influenced the soil microbial community. I chose not to run PLFAs on planted treatments as I had observed no significant difference in degradation between the planted and unplanted treatments. These data were transformed using the Hellinger transformation to handle the numerous 0-values that are present within PLFA data. Next, a Bray-Curtis dissimilarity matrix was calculated to prepare the data for Non-metric multidimensional scaling (NMDS)

analysis and to allow me to determine the stress of the NMDS. Finally, to determine if LOS grade had an influence on microbial PLFA composition, a permANOVA using the Bray-Curtis dissimilarity matrix was performed, making use of the "adonis" function in R (Oksanen, 2019).

## Results

### *Soil Characteristics*

Texture analysis of the LOS confirmed that it was primarily composed of sand, with under 5% clay being present in both the high- and low-grade LOS (Table 3). The LOS grades ranged from neutral to slightly alkaline pH, with the high-grade LOS possessing a pH of 7.23, and the low-grade LOS possessing a pH of 8.25. Dry combustion confirmed that the LOS grades were extremely low in terms of nitrogen content, with both grades being under the instrumental limit of detection (Table 6). In addition to low nitrogen, combustion also showed the mean organic carbon content of the LOS grades to be 1.95 % and 4.54 % for low- and high-grade LOS, respectively (Table 4), roughly the same values as those established for the hydrocarbon concentrations. These results parallel those of pre-experimental hydrocarbon analysis, where the low-grade LOS contained a mean hydrocarbon concentration of 19,677 (1.97 %) ± 1,722 mg $kg^{-1}$ and the high-grade LOS contained a mean hydrocarbon concentration of 43,533 (4.35 %) ± 1,234 mg $kg^{-1}$. We found hydrocarbons within the peat as well, with the peat containing a mean concentration of 15,143 (1.51 %) ± 1,976 mg $kg^{-1}$, which was primarily composed of hydrocarbons in the F4G fraction (Table 5). However, it is important to note that the origin of hydrocarbons in the peat remains unclear. Nutrients in the LOS grades were generally low, with

bioavailable nitrogen being (ammonia [$NH_4$-N] and nitrate [$NO_3$-N]) < 5 mg $kg^{-1}$ and bioavailable phosphorus being (phosphate [$PO_4$-P]) < 4 mg $kg^{-1}$ in both grades of LOS (Table 6).

*Hydrocarbon Degradation*

Both planted and unplanted treatments were significantly lower than the baseline hydrocarbon concentrations in both grades of LOS, but there was no significant difference between planted and unplanted treatments. I observed in the aspen treatment groups a mean degradation of 9,153 ± 3,159 mg $kg^{-1}$ in the high-grade LOS, and a mean degradation of 9,916 ± 1,498 mg $kg^{-1}$ in the low-grade compared to the pre-experiment baseline (Fig. 1). Interestingly, degradation occurred in proportionally similar amounts in the F4G fraction, which is generally considered to be highly recalcitrant (Figs. 3 & 4). Although degradation occurred within both grades of LOS, there was no significant difference in the degradation rates of those treatments that contained a live aspen seedling versus the unplanted controls ($F_{1,42}$ = 0.054, p = 0.818) (Table 7). Similar results were observed for pine, with the high-grade LOS degrading by 11,975 ± 4,512 mg $kg^{-1}$ and the low-grade LOS degrading by 9,499 ± 1,882 mg $kg^{-1}$ on average when compared to the pre-experiment baseline (Fig. 2). The presence of pine had no significant effect on degradation compared to the unplanted control ($F_{1,42}$ = 0.384, p = 0.5386) (Table 8). Although there was a marginally significant effect of LOS grade on degradation within the pine treatment, Tukey's HSD failed to show any significance when pairwise comparisons were performed (Table 9). No significant correlation was found between fine root surface area and the observed degradation in either the aspen ($r$ = -0.0043, p = 0.98) or pine ($r$ = 0.13 p = 0.48) treatments.

Biomass measurements show that the high-grade LOS was associated with a significant reduction in both shoot ($F_{1,30}$ = 5.175, p = 0.0302) and root ($F_{1,30}$ = 5.303, p = 0.028) biomass of

pine (Tables 10 & 11). Pine had a mean shoot biomass of 0.47 ± 0.12 g and 0.72 ± 0.17 g and a mean root biomass of 0.30 ± 0.06 and 0.41 ± 0.07 in the high- and low- grade LOS, respectively (Figs. 5 & 6). Aspen biomass measurements yielded similar results, with a mean shoot biomass of 0.43 ± 0.12 g and 1.00 ± 0.08 g and a mean root biomass of 0.38 ± 0.09 and 0.76 ± 0.11 in the high- and low- grade LOS, respectively (Figs. 7 & 8). High-grade LOS reduced both shoot ($F_{1,30}$ = 57.8, $p < 0.001$) and root ($F_{1,30}$ = 27.3, $p < 0.001$) aspen biomass (Tables 12 & 13). Leaves of aspen showed evidence of pathogens such as black and brown spots that expanded with time and curling of leaf edges, regardless of what grade of LOS they were grown in.

*Microbial Phospholipid Fatty Acids*

To address my secondary objective, I found that community composition differed between the two LOS grades in unplanted controls of pine and aspen (Fig. 9 & 10). The permANOVA analysis of the pine control showed that the difference was due to the high-grade LOS containing a higher concentration of microbial PLFAs than the low-grade LOS ($F_{1,12}$ = 21.907, $R^2$ = 0.64609, $p < 0.001$) (Fig. 11, Table 14). For the aspen controls, I obtained similar results. High-grade LOS had a higher concentration of microbial PLFAs compared to the low-grade LOS ($F_{1,14}$ = 25.226, $R^2$ = 0.6431, $p < 0.001$) (Fig. 12, Table 15). However, as a percentage of the total PLFAs present in each LOS grade and control treatment, no group of microbes appeared to respond disproportionately to the LOS grade when compared to all other groups in the same watering and fertilizer treatment (Figs. 13 & 14).

## Discussion

### *Hydrocarbon Degradation*

Focusing on my primary objective, I determined the tree species used in this study are not suitable for phytoremediation under the conditions or timeline used in this study, despite their use in revegetation. I chose these species due to being widely used in northern boreal reclamation (Pinno et al., 2012; Farnden et al., 2013), therefore making their status as potential phytoremediation candidates of interest from a management perspective due to a limited selection of local native species. Furthermore, aspen and other members of the *Populus* genus have been shown to release labile root exudates such as geraniol (Owen et al., 2007) that may act as a primer for microbial degradation of hydrocarbons as well as possess endophytic organisms such as *Burkholderia* spp. that can degrade hydrocarbons (Yrjälä et al., 2010). Despite these attributes, their function in phytoremediation appears limited across the conditions and timeline used in this study.

That the presence of aspen and jack pine did not increase degradation may be the consequence of two core issues. Firstly, due to disease in the case of the aspen, as well as the potential growth-inhibiting effects of LOS on aspen and jack pine, any ability that these plant species may possess to enhance hydrocarbon degradation could have been suppressed. In the case of pine, it is possible that this species may not possess the necessary metabolic processes, such as degradation-promoting compounds exuded from their roots (Toussaint et al., 2012 and Miya and Firestone, 2001) to degrade hydrocarbons. In the case of aspen and other *Populus* species where numerous successful phytoremediation studies have been carried out, most use transgenic aspen (Van Dillewijn et al., 2008; Couselo, Navarro-Avino, and Ballester, 2010;

Ruttens et al., 2011; and Doty, et al., 2007). For example, aspen that have been made to express a gene for fungal manganese peroxidase are known to be effective in facilitating the degradation of specific hydrocarbon groups (Iimura et al, 2007). In addition, it should be noted that the biomass measurements obtained here indicated that both jack pine and aspen are highly sensitive to the presence of LOS, although whether or not the hydrocarbons were solely responsible cannot be determined due to other differences in the substrates such as nutrient status (Table 6). Specifically, increasing hydrocarbon concentrations decreased both shoot and root mass, with a similar magnitude of decrease as the results observed in another study using the same plant species (Visser, 2008). This reduction in biomass may also adversely affect the ability of plants to degrade hydrocarbons, especially in regard to the roots, where previous work has shown that larger root systems tend to result in higher rates of degradation (Merkl, Schultze-Kraft and Infante, 2005). I speculate however, that the lack of degradation observed may also be attributable to the fact that the seedlings did not have time to mature, and that a thicker topsoil cap may create conditions that are more conducive to the establishment of phytoremediation-capable plant species. Specifically, conditions that are more conducive to the establishment and maturation of tree species will likely reduce the effect of stresses present in LOS, and providing the resources necessary to carry out the processes of phytoremediation, be it the release of enzymes to directly degrade hydrocarbons or the stimulation of the local microbial community.

However, it is important to note that substantial degradation was observed to have occurred within the study system, regardless of whether there was a plant present or not. This result is contrary to the results of degradation experiments performed by Visser, (2008), in which very little degradation was found over the course of a 130-day incubation period in a similar study system, particularly within the F4 and F4G fractions. However, the difference in outcomes

can be attributed to three main reasons. Firstly, in Visser, (2008) the starting LOS material in some cases contained very high concentrations of hydrocarbons of greater than 5.3 %, with some samples returning nearly ore-grade concentrations of hydrocarbons. Therefore, it is possible that these treatments contained a hydrocarbon concentration that was too high for the microbes to effectively degrade. Secondly, combining means across treatments despite differences in starting hydrocarbon concentrations likely obscured any degradation that was occurring in individual treatments. Finally, given that the F4 and F4G fractions contain such a wide range of compounds, there may be differences in the chemical composition of the LOS used in both Visser, (2008) and my study that were not entirely captured by describing hydrocarbons by fraction.

The hydrocarbon degradation observed in my study is potentially due to the nutrient and water additions acting as a biostimulant, providing the missing components in the microbial metabolism needed to effectively degrade hydrocarbons. The use of nutrient and moisture amendments in order to efficiently remove unwanted compounds from soils has been established in many cases across a wide variety of site conditions (Wu et al, 2016; Xu and Lu, 2010; Margesin and Schinner, 2001). This mechanism is likely a key driver of the degradation observed here for all hydrocarbon fractions given the low initial nutrient concentrations present in the LOS, especially fractions such as the F4G which are generally considered to be highly recalcitrant (Visser, 2008). However, it may be possible to target and isolate bacterial species that are able to degrade heavy hydrocarbons, such as those in the F4G fraction. Recent work by Ksirsagar et al., (2020) shows that not only are some bacteria able to directly degrade heavy hydrocarbon mixtures in an efficient manner, they can also be isolated via specific hydrocarbon

substrates for future use in degradation, enhancing the removal of compounds that are traditionally considered recalcitrant.

While the pine planting treatment showed marginally significant differences in degradation between the LOS grades, it is possible that over a longer duration, degradation may become more pronounced. Therefore, a longer experimental duration is recommended to further study the possible phytoremediation capabilities of pine in LOS. It is important to note that a slow degradation rate of the hydrocarbons present in LOS may indicate that LOS itself is a relatively stable material that does not readily interact with ecological receptors such as plants and wildlife. Additional work should be carried out exploring a wider potential range of phytoremediation candidates, focusing on native species that possess extensive root systems and appear to be performing well when interacting with both LOS as well as naturally occurring bituminous soils. Furthermore, it would be worthwhile to study mature trees to determine if any phytoremediation capabilities arise as root networks become more robust and interact with soil microbes such as ectomycorrhizal fungi. This would allow us to determine if revegetation species have the potential to enhance degradation over a longer time period than what was assessed in this study. Another avenue of research that requires further study is that of applying transgenic plants that express specific degradation enzymes such as polyphenol oxidase and enzymes possessed by asphaltene-degrading bacteria to achieve greater levels of degradation in the F4 and greater hydrocarbon fractions.

*Microbial Community Composition*

Addressing my secondary objective, I determined that the concentration of hydrocarbons present in the LOS greatly impacted microbial biomass in unplanted control pots, with the high-grade LOS (4.54 % hydrocarbons) having far greater concentrations of microbial PLFAs than the low-grade LOS (1.95 % hydrocarbons). This effect was observed regardless of the nutrient and watering regime, making it likely that higher hydrocarbon concentration in the high-grade LOS was more conducive to the growth and proliferation of the microbial community. While this finding goes against some established literature (Labud, Garcia and Hernandez, 2007; Phelps et al, 1988), these other studies used higher hydrocarbon concentrations than those assessed here, as well as chlorinated substances that may yield toxic byproducts as they degrade, hampering the rate of degradation over time (Travis and Rosenberg, 1997). The gram positive and gram negative classifications for bacteria in the PLFA analysis was quite broad compared to those of fungal groups, with more marker PLFAs for bacteria being utilized in the MICSOIL3 method, and this may be responsible for the dominance of these two groups over the others analyzed via this method. However, the lack of mycorrhizal fungi present in this system is perhaps unsurprising given that the LOS originated from an unreclaimed landform and would have therefore likely been colonized by bacteria to a greater degree than mycorrhizal fungi. Similar fungal to bacterial ratios have been observed in reclaimed peat mineral mix soils sampled from the same region (MacKenzie and Quideau, 2010). Other research has shown that microbes are able to acclimate to, and utilize, petroleum hydrocarbons as a source of carbon and energy provided that the concentrations do not exceed roughly 10 % by weight (Labud, Garcia, and Hernandez, 2007). This is especially the case in the presence of other labile organic material, such as the peat used in my study system, leading to increased microbial PLFAs and biomass in

soils that contain higher concentrations of hydrocarbons (Alrumman, Standing, and Paton, 2015; Franco et al, 2004; Langworthy et al, 2002). While bacterial communities have reduced biomass in response to increasing hydrocarbon concentrations, it has also been demonstrated that the expression of hydrocarbon degrading genes increases in the presence of higher bitumen concentrations (Yergeau et al., 2013). Therefore, given that the LOS used in my study system had been sitting in place for several years and not covered by reclamation soil, it is possible that the *in situ* microbial community had already acclimated to this hydrocarbon rich environment. Therefore, when the hydrocarbon concentrations in the LOS were diluted, microbial composition remained unchanged, but biomass was reduced in response to the lack of usable carbon substrate. It is recommended that future studies with the objective of removing hydrocarbons from LOS focus on additional molecular tools to gain a more in-depth understanding of what microbial components of the soil community comprise the majority of hydrocarbon degraders. This approach may allow us to develop a system to better target those microbial groups' needs, thereby making a biostimulation approach to degrading hydrocarbons in LOS more effective.

*Conclusions and Future Recommendations*

The results of my research, as they relate to my primary objective of determining whether aspen or jack pine were suitable phytoremediation candidates, show that neither tree species was effective in enhancing the degradation of hydrocarbons in LOS in the short term of one growing season. However, given adequate quantities of nutrients and moisture, hydrocarbon degradation can be accomplished in this material due to the presence of microbes with a metabolism capable of effectively degrading hydrocarbons. Referring to my secondary objective, the addition of moisture and nutrients appears especially effective in the case of higher-grade LOS, where the higher concentration of a carbon source results in increased microbial biomass, provided other

growth restrictions are mitigated. This increase in microbial biomass perhaps suggests that suitable soil and vegetation covers with higher nutrient concentrations could be used to provide the resources necessary to stimulate the degradation of hydrocarbons in LOS. If future studies are seeking to further enhance the rates at which hydrocarbons are degrading in this system, I recommend that further native plant candidates be assessed for phytoremediation capabilities by using an annotated plant genome database such as Phytozome v13 (Joint Genome Institute, 2020). Further molecular work should also be done on both bacteria and fungi to determine the identity and method of action of specific hydrocarbon degrading organisms. This will allow us to identify to a much finer resolution species in LOS such as asphaltene degraders that are capable of degrading groups of hydrocarbons such as the F4G fraction, that are normally considered to be highly recalcitrant. Exploring which plant-microbe combinations can degrade hydrocarbons and determining the mechanisms by which this is accomplished may allow us to combine remediation and revegetation into one step during reclamation.

## Figures

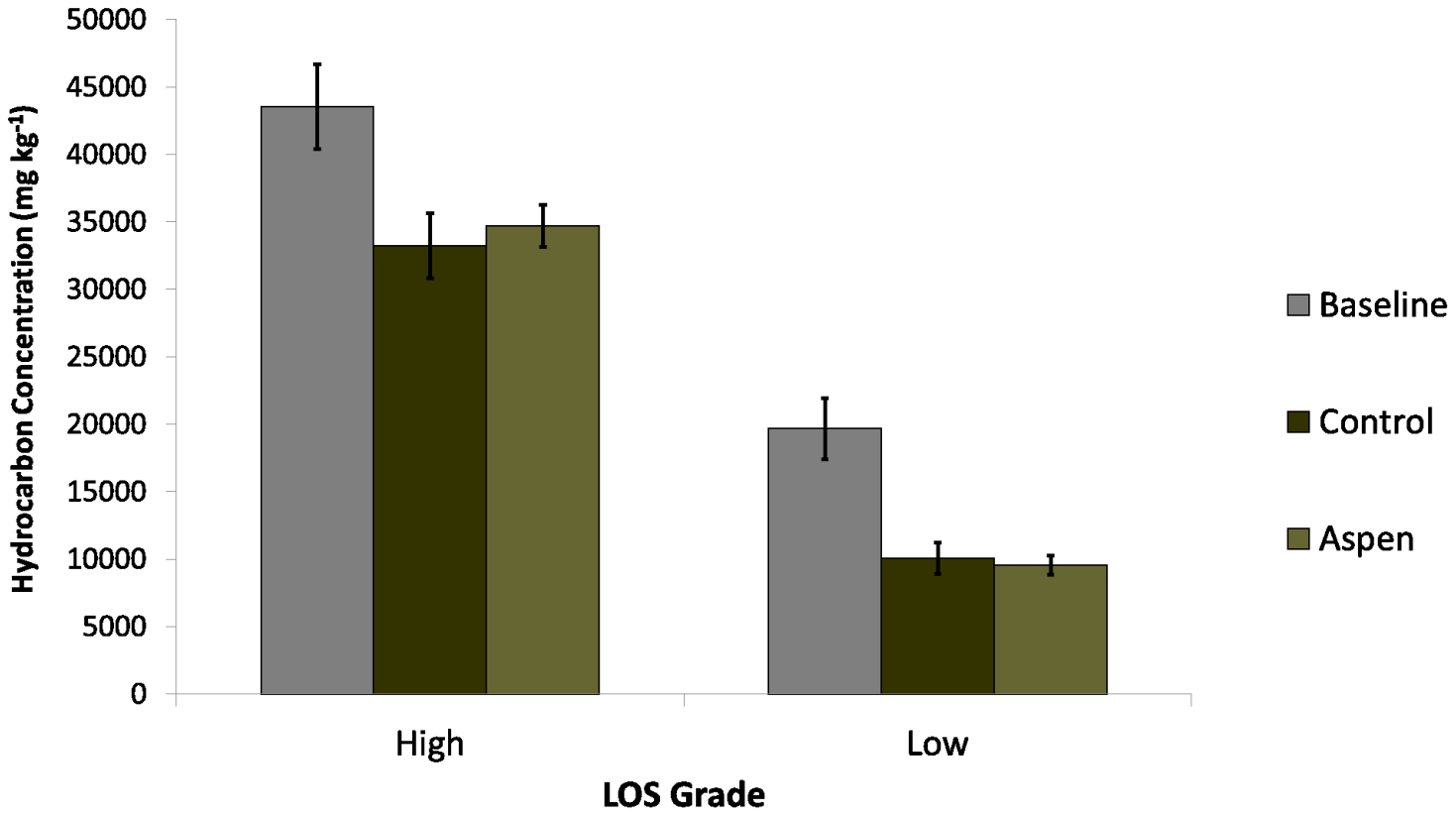


Figure 1. Mean soil concentrations of cumulative hydrocarbon fractions F2–F4G in the aspen (*Populus tremuloides*) treatment. Grey represents baseline concentration (n = 3), while dark green and light green bars represent planted (n = 16) and unplanted treatments, respectively (n = 7). Error bars represent 95 C.I. of the means.

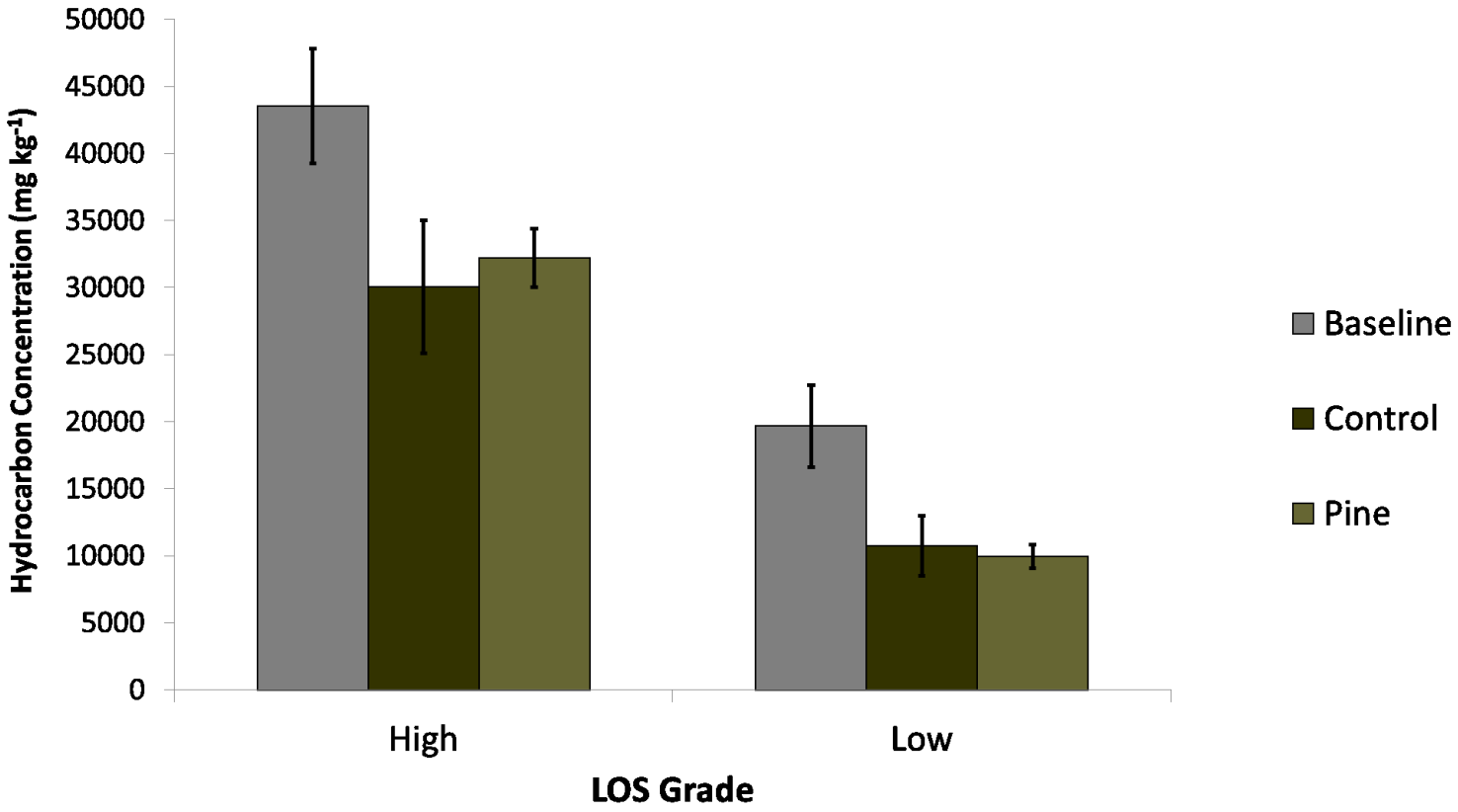


Figure 2. Mean soil concentrations of cumulative hydrocarbon fractions F2–F4G in the pine (*Pinus banksiana*) treatment. Grey represents baseline concentration (n = 3), while dark green and light green bars represent planted (n = 16) and unplanted (n = 7) treatments respectively. Error bars represent 95 C.I. of the means.

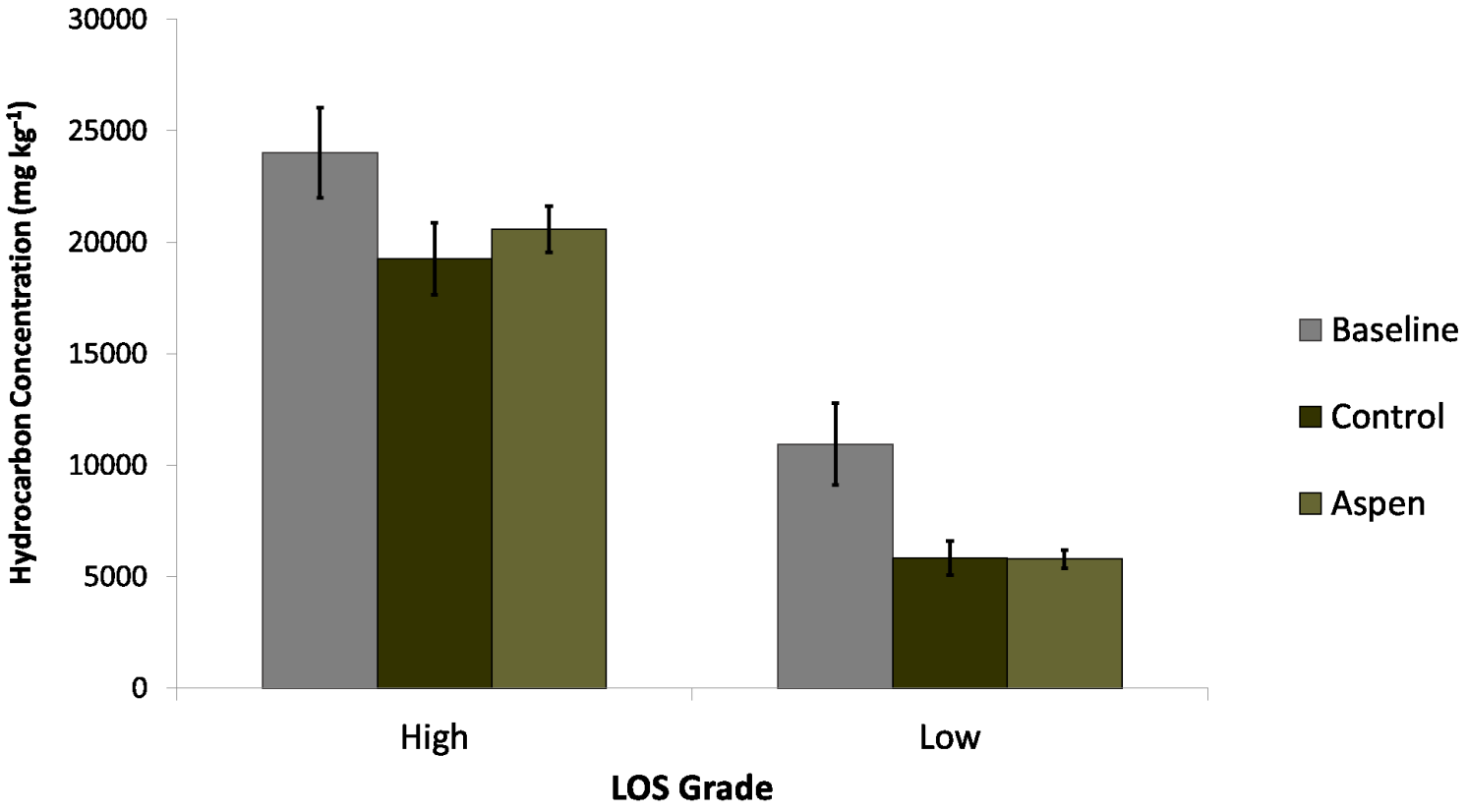


Figure 3. Mean soil concentrations of hydrocarbon fraction F4G in the aspen (*Populus tremuloides*) treatment. Grey represents baseline concentration (n = 3), while dark green and light green bars represent planted (n = 16) and unplanted (n = 7) treatments respectively. Error bars represent 95 C.I. of the means.

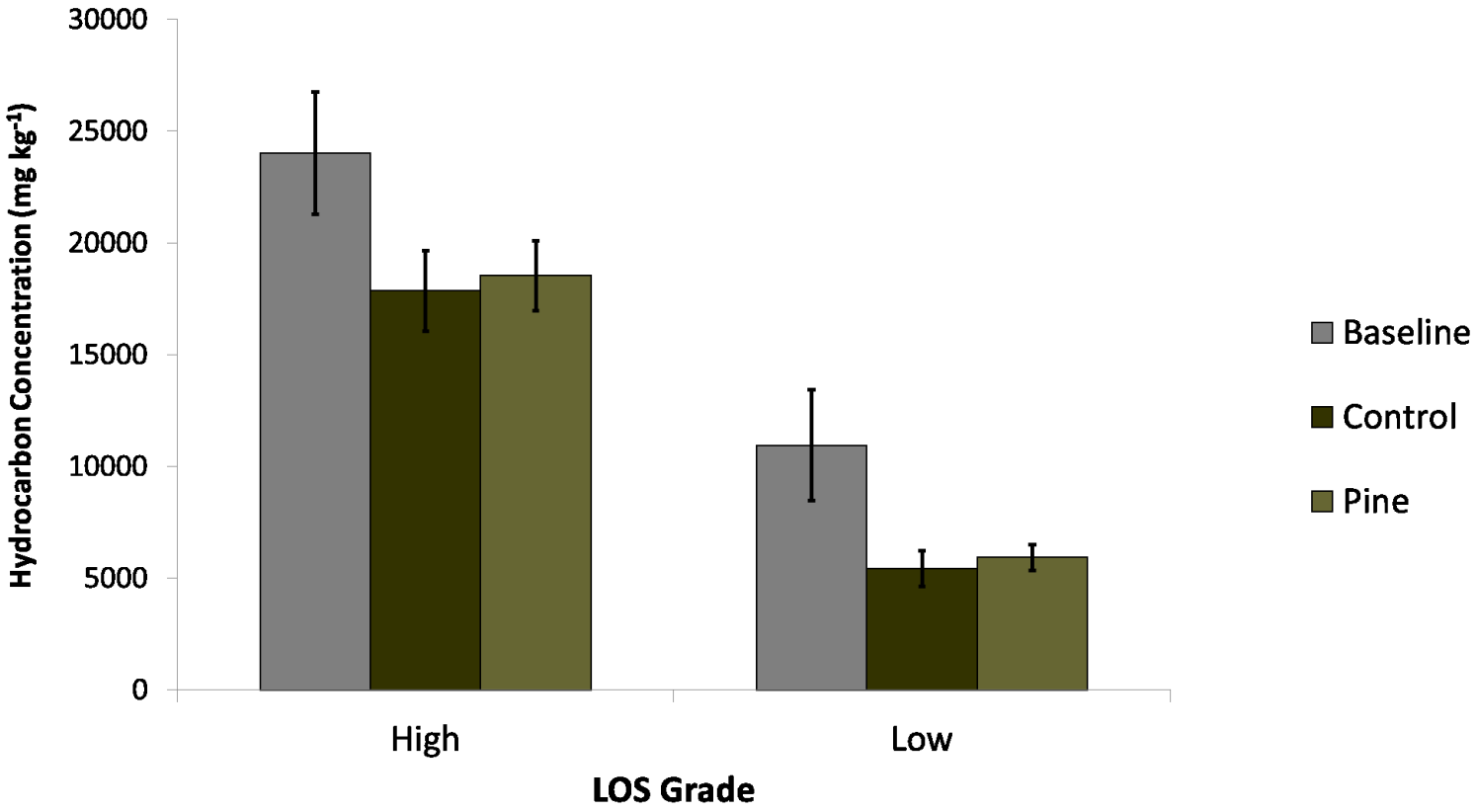


Figure 4. Mean soil concentrations of hydrocarbon fraction F4G in the pine (*Pinus banksiana*) treatment. Grey represents baseline concentration (n = 3), while dark green and light green bars represent planted (n = 16) and unplanted (n = 7) treatments respectively. Error bars are 95 C.I. of the means.

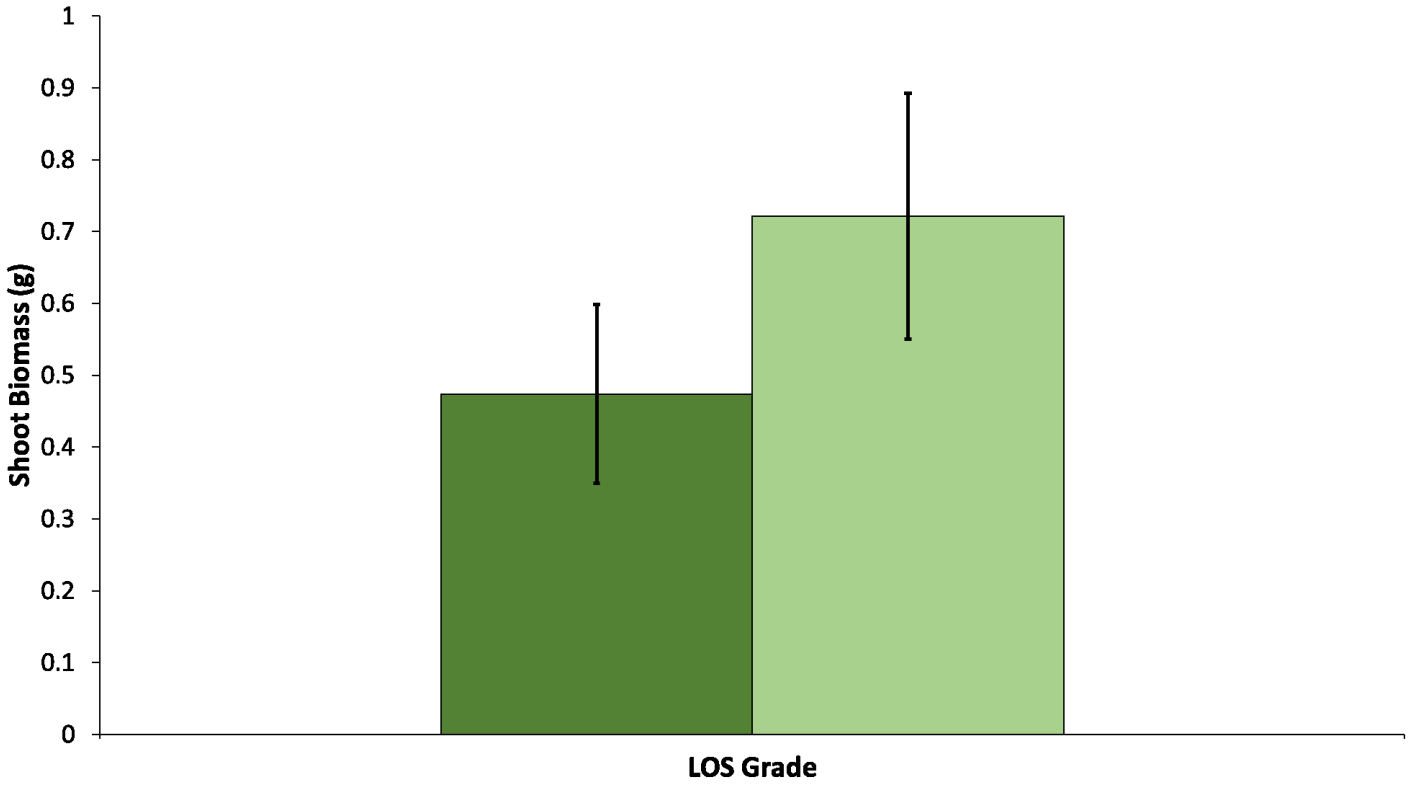


Figure 5. Mean shoot biomass measurements for the pine (*Pinus banksiana*) treatment. Dark green represents the 'High' (4.54 % hydrocarbons) grade lean oil sands, while light green represents the 'Low' (1.95 % hydrocarbons) grade lean oil sands. Error bars are 95 C.I. of the means.

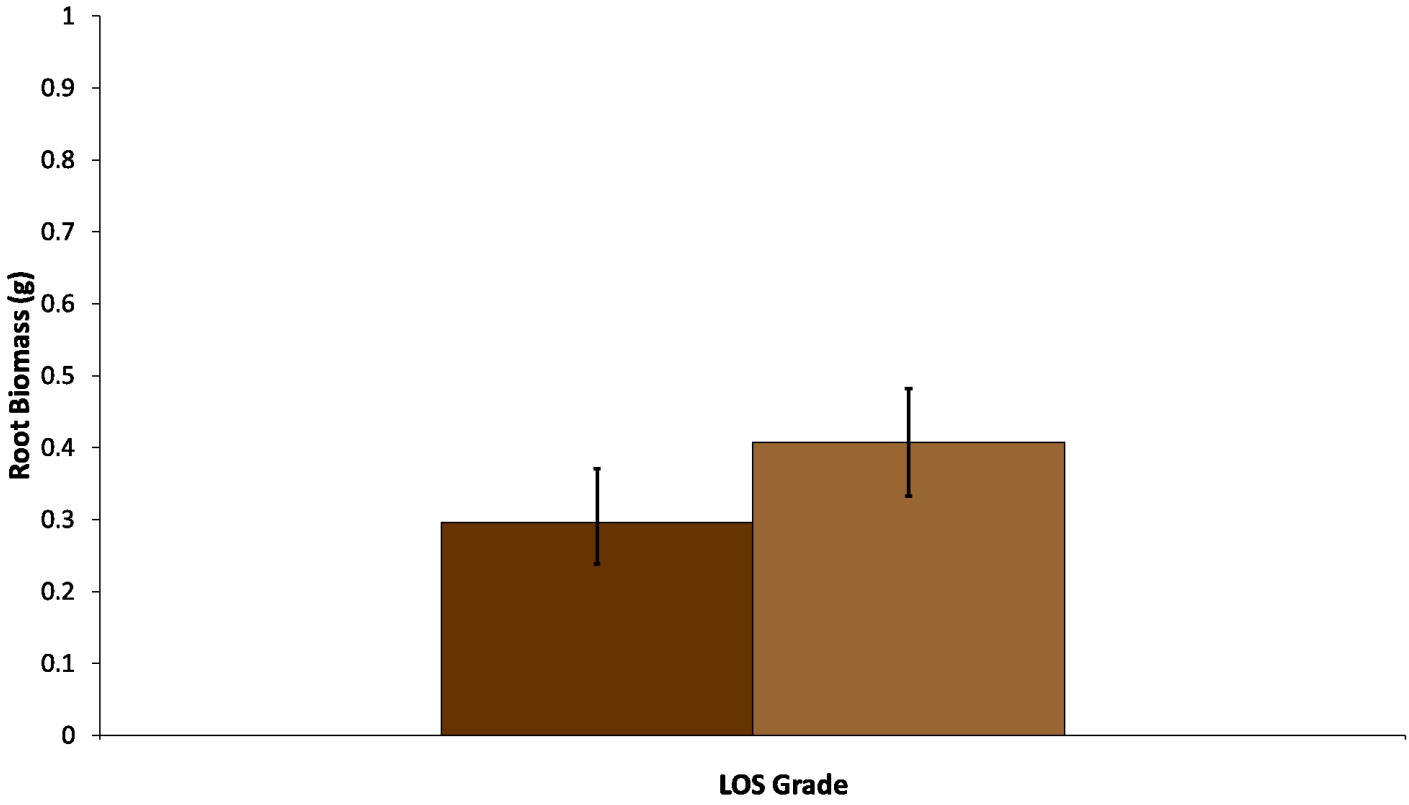


Figure 6. Mean root biomass measurements for the pine (*Pinus banksiana*) treatment. Dark brown represents the 'High' (4.54 % hydrocarbons) grade lean oil sands, while light brown represents the 'Low' (1.95 % hydrocarbons) grade lean oil sands. Error bars are 95 C.I. of the means.

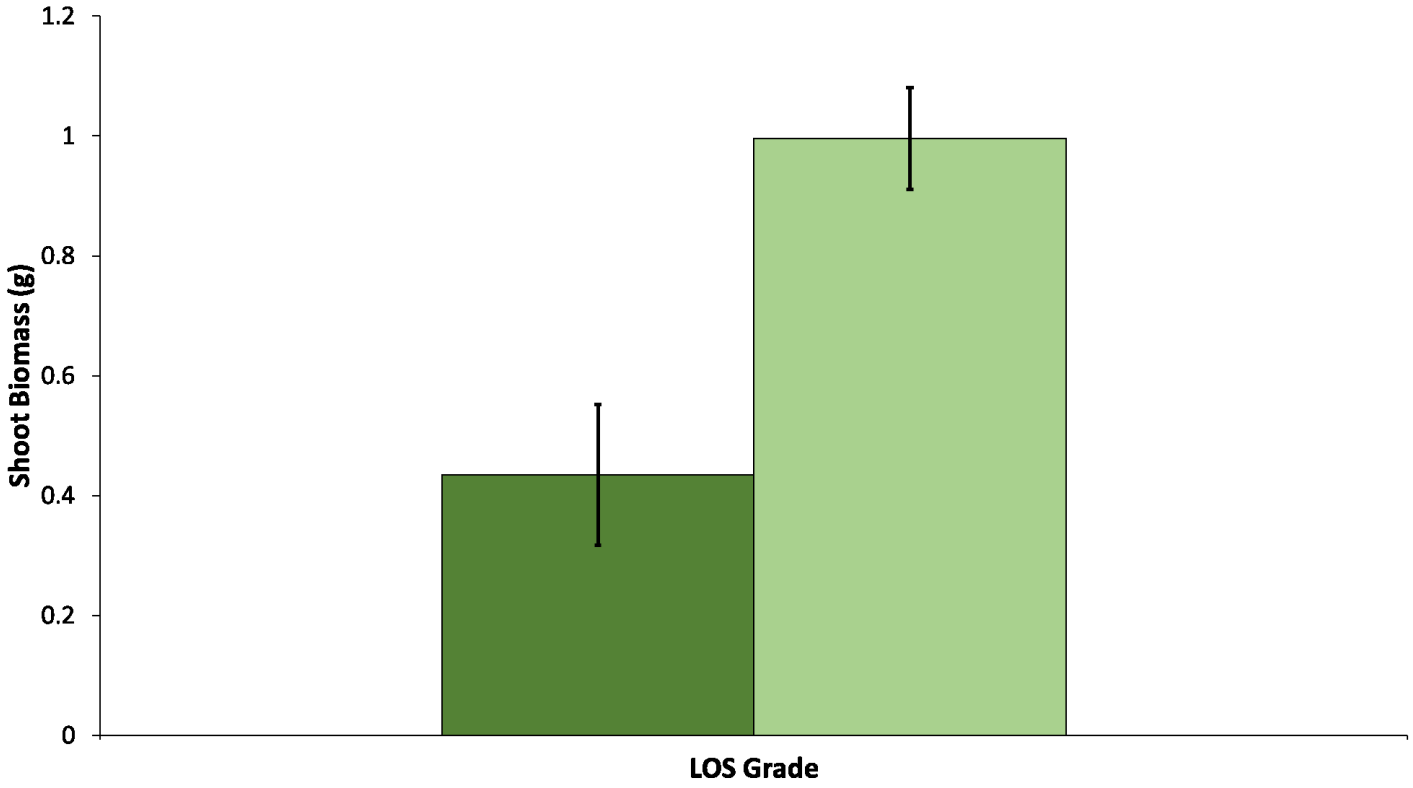


Figure 7. Mean shoot biomass measurements for the aspen (*Populus tremuloides*) treatment. Dark green represents the 'High' (4.54 % hydrocarbons) grade lean oil sands, while light green represents the 'Low' (1.95 % hydrocarbons) grade lean oil sands. Error bars are 95 C.I. of the means.

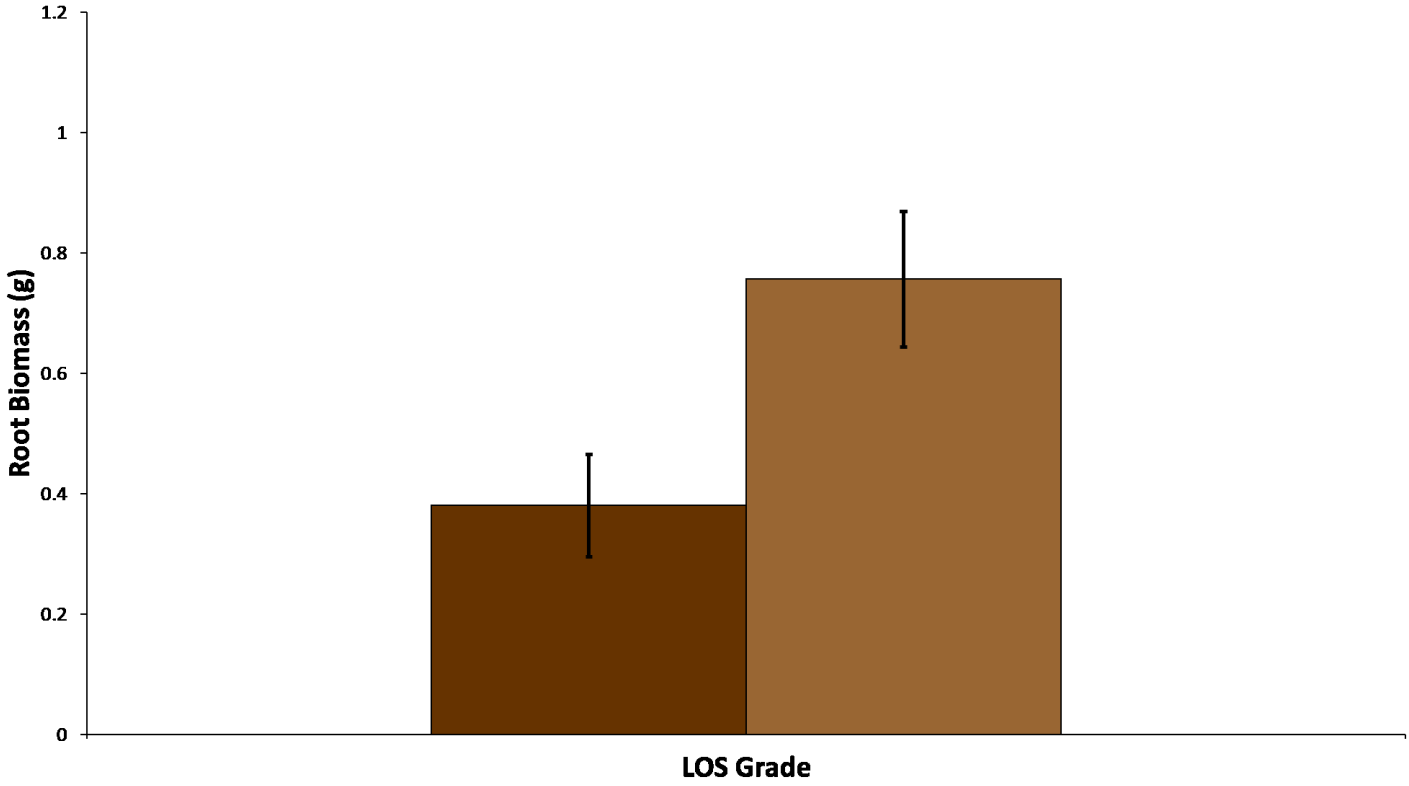


Figure 8. Mean root biomass measurements for the aspen (*Populus tremuloides*) treatment. Dark brown represents the 'High' (4.54 % hydrocarbons) grade lean oil sands, while light brown represents the 'Low' (1.95 % hydrocarbons) grade lean oil sands. Error bars are 95 C.I. of the means.

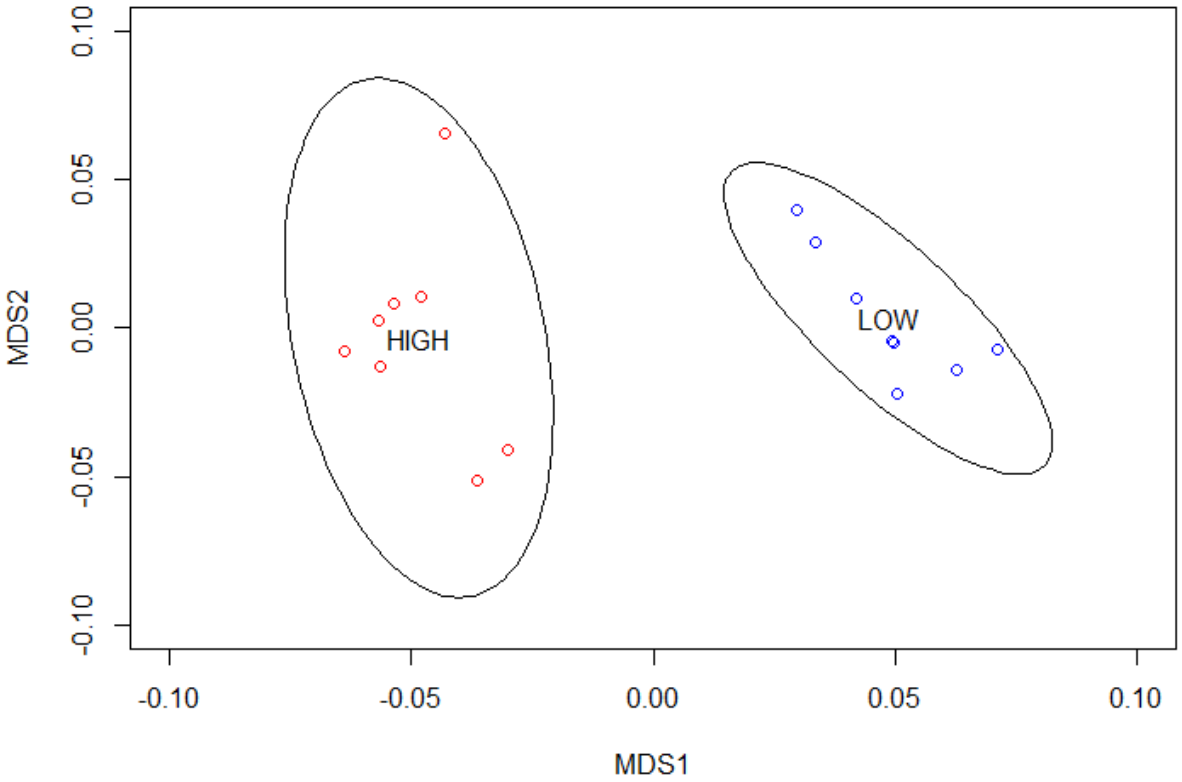


Figure 9. Non-metric multidimensional scaling analysis of phospholipid fatty acids by lean oil sand grade (n = 16) in soils of unplanted controls subjected to the fertilizer and watering regime of the aspen (*Populus tremuloides*) planted trials. Each point represents a sampled pot's microbial phospholipid fatty acid profile, with red points representing those from high-grade (4.54 %) lean oil sands, and blue representing those from low-grade (1.95 %). Bray-Curtis stress was 0.07, and ellipses represent a confidence interval of 0.95.

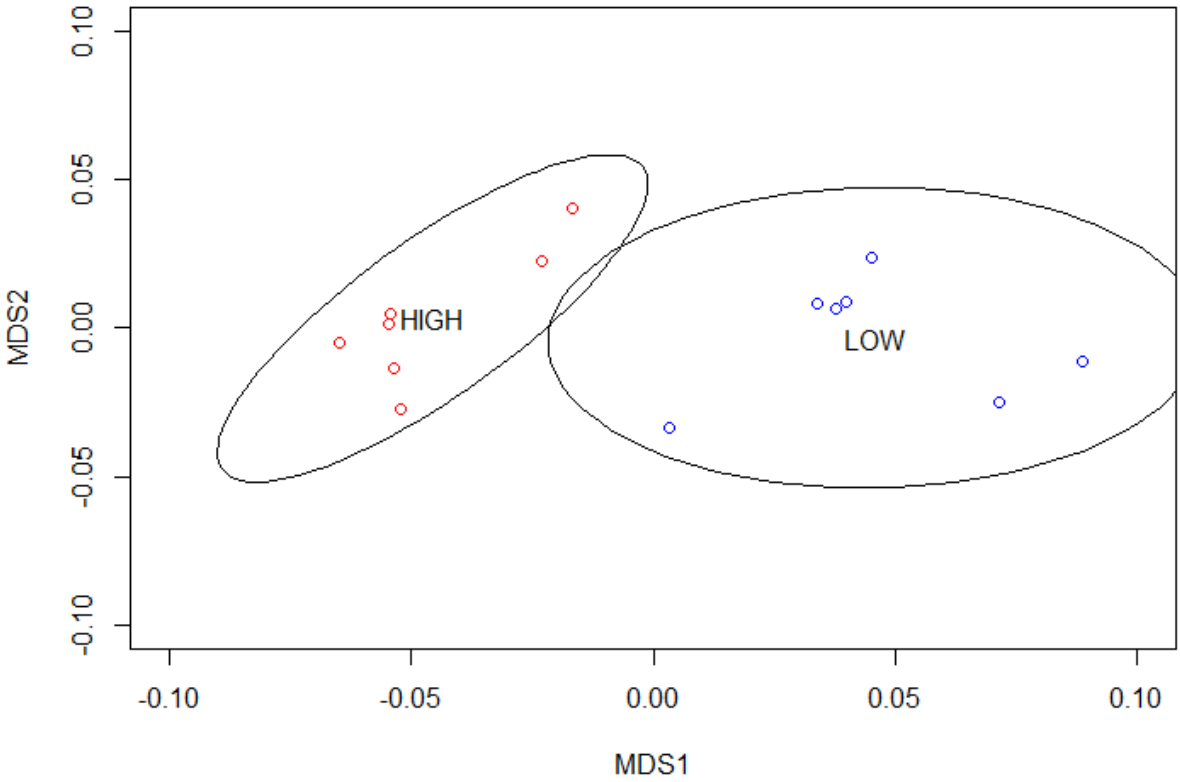


Figure 10. Non-metric multidimensional scaling analysis of phospholipid fatty acids by lean oil sands grade (n = 14) in soils of unplanted controls subjected to the fertilizer and watering regime of the pine (*Pinus banksiana*) planted trials. Each point represents a sampled pot's microbial phospholipid fatty acid profile with red points representing those from high-grade lean oil sands (4.54 %), and blue representing those from low-grade (1.95 %). Bray-Curtis stress was 0.05, and ellipses represent a confidence interval of 0.95.

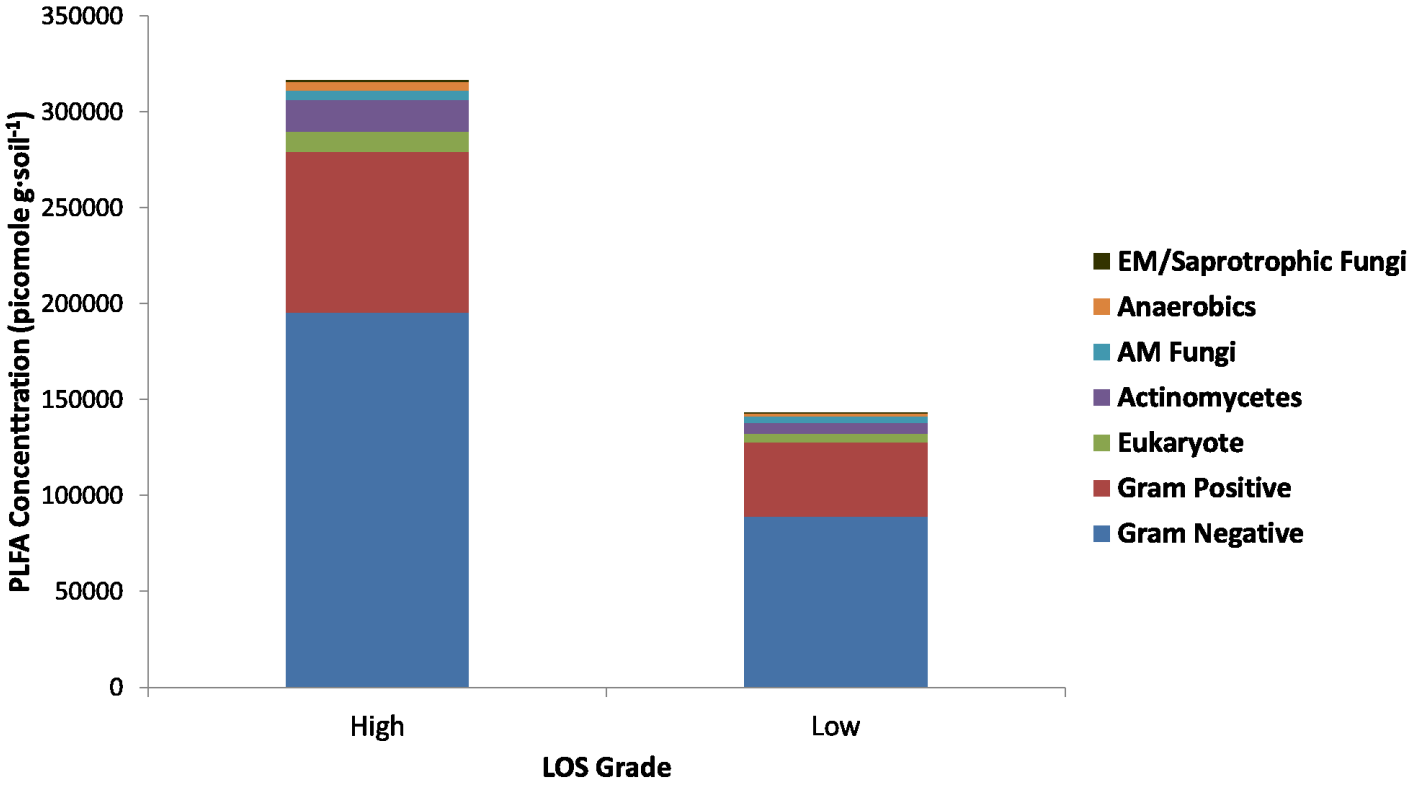


Figure 11. Mean phospholipid fatty acid concentrations corresponding to microbial groups present in soils of unplanted controls subjected to the fertilizer and watering regime of the pine (*Pinus banksiana*) planted trials. 'EM' signifies ectomycorrhizal fungi, whereas 'AM' signifies arbuscular mycorrhizal fungi.

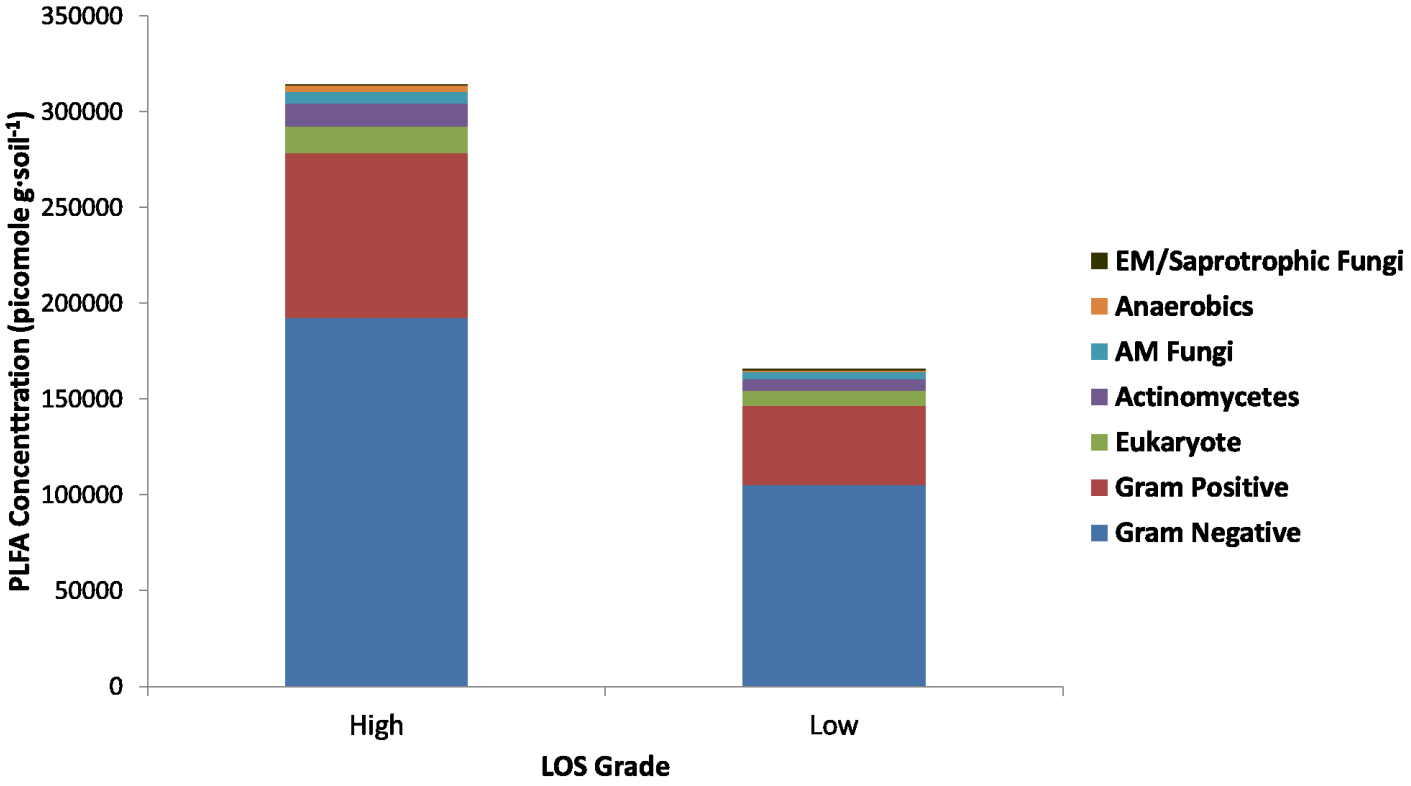


Figure 12. Mean phospholipid fatty acid concentrations corresponding to microbial groups present in soils of unplanted controls subjected to the fertilizer and watering regime of the aspen (*Populus tremuloides*) planted trials. 'EM' signifies ectomycorrhizal fungi, whereas 'AM' signifies arbuscular mycorrhizal fungi.

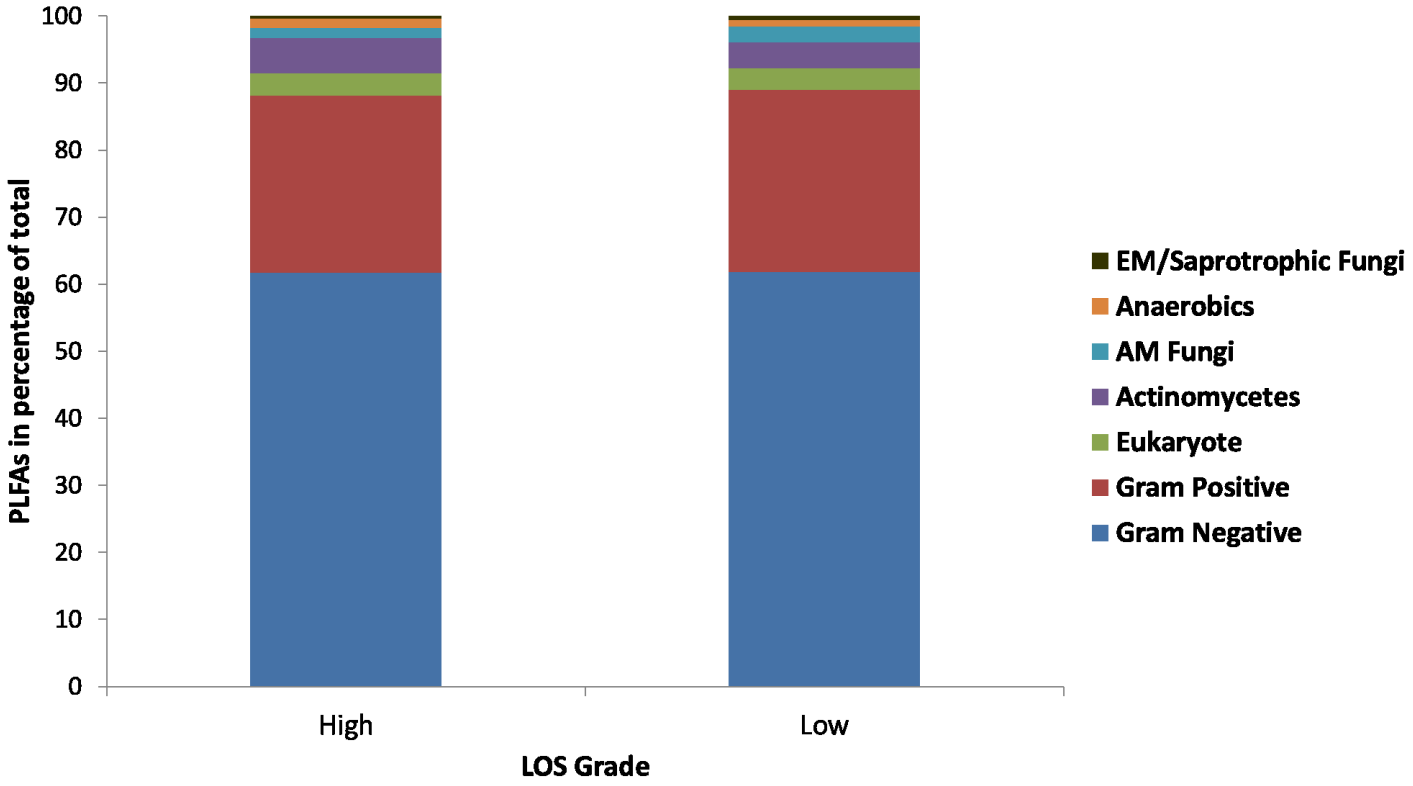


Figure 13. Phospholipid fatty acid concentrations as a percentage of the total corresponding to microbial groups present in soils of unplanted controls subjected to the fertilizer and watering regime of the pine (*Pinus banksiana*) planted trials. 'EM' signifies ectomycorrhizal fungi, whereas 'AM' signifies arbuscular mycorrhizal fungi.

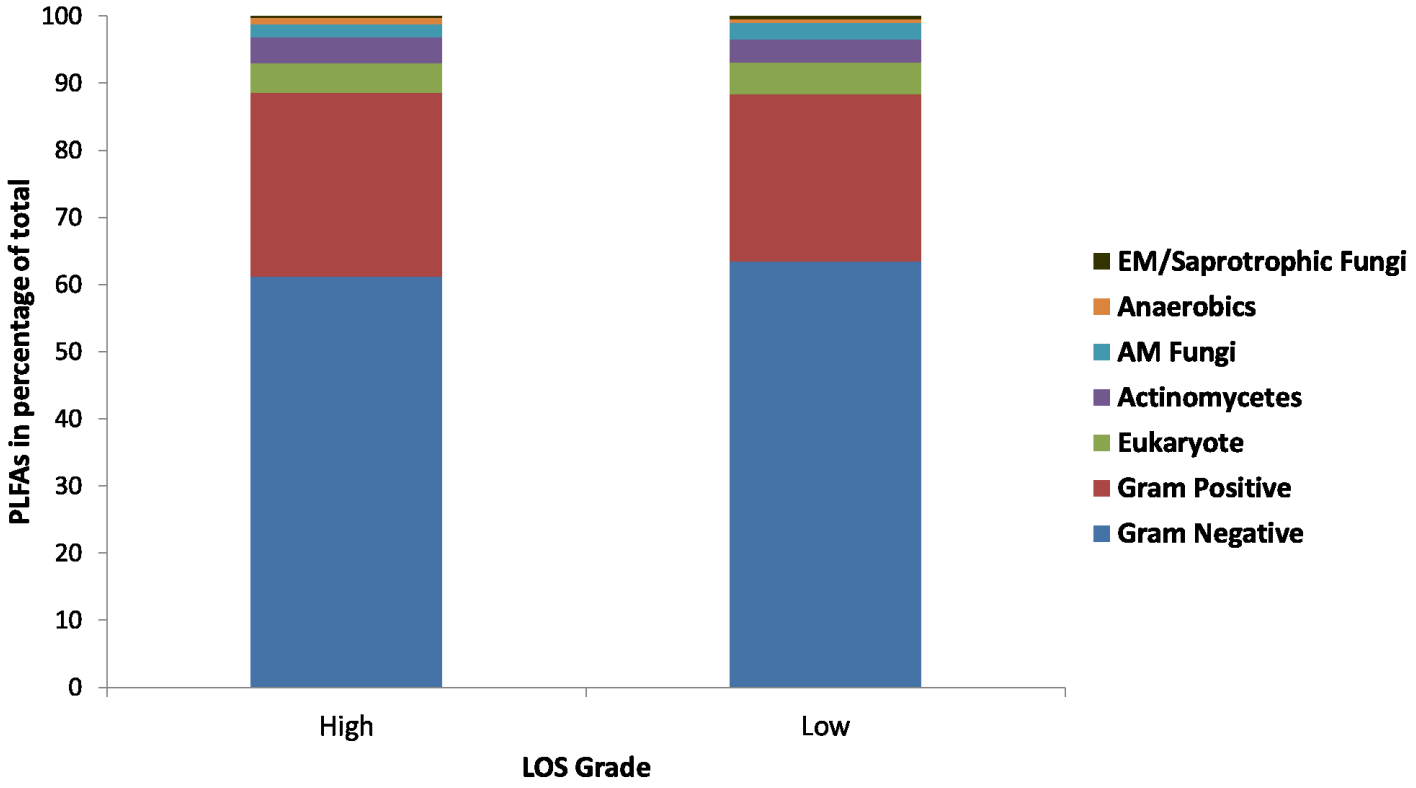


Figure 14. Phospholipid fatty acid concentrations as a percentage of the total corresponding to microbial groups present in soils of unplanted controls subjected to the fertilizer and watering regime of the aspen (*Populus tremuloides*) planted trials. 'EM' signifies ectomycorrhizal fungi, whereas 'AM' signifies arbuscular mycorrhizal fungi.

## Tables

Table 1. Physical and chemical attributes of salvaged peat used in the Aurora Soils Capping Study reclamation site. Adapted from Hankin, Karst, and Landhaüsser, (2015) and Pec et al., (Unpublished).

| **Source** | **Depth (cm)** | **Mean pH** | **Min pH** | **Max pH** | **Mean EC (dS $m^{-1}$)** | **Min EC (dS $m^{-1}$)** | **Max EC (dS $m^{-1}$)** | **Texture** | **Mean Organic Matter (%)** | **Mean Total Organic Carbon (%)** | **Mean Nitrogen (%)** | **Mean C:N ratio** | **Phosphorus (mg $kg^{-1}$)** |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| ***Picea mariana* lowland** | 0–200 | 7.4 | 5.0 | 7.8 | 1.2 | 0.4 | 2.3 | Sand | 34.1 | 17.0 | 0.75 | 25.4 | 5.0 |

Table 2. Marker phospholipid fatty acids and associated microbial types. Obtained by the MICSOIL3 Method within Sherlock Microbial Identification System Version 6.3 software (MIDI, Inc., Newark, USA).

| Category | Peaks | | | |
|---|---|---|---|---|
| **AM Fungi** | 16:1 w5c | | | |
| **Gram Negative** | 10:0 2OH | 10:0 3OH | 12:1 w8c | 12:1 w5c |
| | 13:1 w5c | 13:1 w4c | 13:1 w3c | 12:0 2OH |
| | 14:1 w9c | 14:1 w8c | 14:1 w7c | 14:1 w5c |
| | 15:1 w9c | 15:1 w8c | 15:1 w7c | 15:1 w6c |
| | 15:1 w5c | 14:0 2OH | 16:1 w9c | 16:1 w7c |
| | 16:1 w6c | 16:1 w4c | 16:1 w3c | 17:1 w9c |
| | 17:1 w8c | 17:1 w7c | 17:1 w6c | 17:0 cyclo w7c |
| | 17:1 w5c | 17:1 w4c | 17:1 w3c | 16:0 2OH |
| | 18:0 cyclo w6c | 18:1 w8c | 18:1 w7c | 18:1 w6c |
| | 18:1 w5c | 18:1 w3c | 19:1 w9c | 19:1 w8c |
| | 19:1 w7c | 19:1 w6c | 19:0 cyclo w7c | 19:0 cyclo w6c |
| | 20:1 w9c | 20:1 w8c | 20:1 w6c | |
| | 20:1 w4c | 20:0 cyclo w6c | 21:1 w9c | 21:1 w8c |
| | 21:1 w6c | 21:1 w5c | 21:1 w4c | 21:1 w3c |
| | 22:1 w9c | 22:1 w8c | 22:1 w6c | 22:1 w5c |
| | 22:1 w3c | 22:0 cyclo w6c | 24:1 w9c | 24:1 w7c |
| | 11:0 iso 3OH | 14:0 iso 3OH | 17:0 iso 3OH | |
| **Methanotroph** | 16:1 w8c | | | |
| **Eukaryote** | 15:4 w3c | 15:3 w3c | 16:4 w3c | 16:3 w6c |
| | 18:3 w6c | 19:4 w6c | 19:3 w6c | 19:3 w3c |
| | 20:4 w6c | 20:5 w3c | 20:3 w6c | 20:2 w6c |
| | 21:3 w6c | 21:3 w3c | 22:5 w6c | 22:6 w3c |
| | 22:4 w6c | 22:5 w3c | 22:2 w6c | 23:4 w6c |
| | 23:3 w6c | 23:3 w3c | 23:1 w5c | 23:1 w4c |
| | 24:4 w6c | 24:3 w6c | 24:3 w3c | 24:1 w3c |
| | 18:4 w3c | | | |
| **Fungi** | 18:2 w6c | | | |
| **Gram Positive** | 11:0 iso | 11:0 anteiso | 12:0 iso | 12:0 anteiso |
| | 13:0 iso | 13:0 anteiso | 14:1 iso w7c | 14:0 iso |
| | 14:0 anteiso | 15:1 iso w9c | 15:1 iso w6c | 15:1 anteiso w9c |
| | 15:0 iso | 15:0 anteiso | 16:0 iso | 16:0 anteiso |
| | 17:1 iso w9c | 17:0 iso | 17:0 anteiso | 18:0 iso |
| | 17:1 anteiso w9c | 17:1 iso w10c | 17:1 anteiso w7c | |
| | 18:1 w9c | 19:0 cyclo w9c | | |
| | 19:0 iso | 19:0 anteiso | 20:0 iso | 22:0 iso |
| **Anaerobe** | 12:0 DMA | 13:0 DMA | 14:1 w7c DMA | 14:0 DMA |
| | 15:0 iso DMA | 15:0 DMA | 16:2 DMA | 17:0 DMA |
| | 16:1 w9c DMA | 16:1 w7c DMA | 16:1 w5c DMA | 16:0 DMA |
| | 18:2 DMA | 18:1 w9c DMA | 18:1 w7c DMA | 18:1 w5c DMA |
| | 18:0 DMA | 19:0 cyclo 9,10 DMA | | |
| **Actinomycetes** | 16:0 10-methyl | 17:1 w7c 10-methyl | 17:0 10-methyl | 22:0 10-methyl |
| | 18:1 w7c 10-methyl | 18:0 10-methyl | 19:1 w7c 10-methyl | 20:0 10-methyl |

Table 3. Texture analysis of high- (4.54 % hydrocarbons) and low-grade (1.95 % hydrocarbons) lean oil sands (LOS) treatments (n = 1).

| **LOS grade** | **Clay % (< 2 µm)** | **Silt % (2–50 µm)** | **Sand % (> 50 µm)** |
|---|---|---|---|
| **Low** | 4.5 | 10.6 | 84.9 |
| **High** | 3.6 | 22.6 | 73.9 |

Table 4. Total carbon (TC), and total organic carbon (TOC) contents of peat and lean oil sand (LOS) of high- (4.54 % hydrocarbons) and low-grade (1.95 % hydrocarbons) pre-experiment. (n = 3).

| **Substrate** | **TC (w/w %)** | **TOC (w/w %)** |
|---|---|---|
| **Low LOS** | 2.32 | 1.95 |
| **High LOS** | 4.81 | 4.54 |
| **Peat** | 29.49 | 28.26 |

Table 5. Mean baseline hydrocarbon concentrations for low- (1.95 % hydrocarbons) and high-grade (4.54 % hydrocarbons) lean oil sands (LOS) as well as peat pre-experiment. (n = 3).

| **Substrate** | **F2 (C10-C16) mg kg$^{-1}$** | **F3 (C16-C34) mg kg$^{-1}$** | **F4 (C34-C50) mg kg$^{-1}$** | **F4G (C50+) mg kg$^{-1}$** | **Total mg kg$^{-1}$** |
|---|---|---|---|---|---|
| **Low LOS** | 943 | 5,233 | 2,567 | 10,933 | 19,677 |
| **High LOS** | 2,100 | 11,667 | 5,767 | 24,000 | 43,533 |
| **Peat** | 109 | 3,500 | 2,133 | 9,400 | 15,143 |

Table 6. Peat and lean oil sands (LOS)) (low- (1.95 % hydrocarbons) and high-grade (4.54 % hydrocarbons) nutrients and extractable metals pre-experiment.

| **Sample** | **NH4-N mg kg$^{-1}$** | **NO3-N mg kg$^{-1}$** | **PO4-P mg kg$^{-1}$** | **Na mg kg$^{-1}$** | **K mg kg$^{-1}$** | **Ca mg kg$^{-1}$** | **Mn mg kg$^{-1}$** | **Fe mg kg$^{-1}$** | **Zn mg kg$^{-1}$** | **Mg mg kg$^{-1}$** | **S mg kg$^{-1}$** |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **Low LOS** | 4.42 | 0.38 | 3.76 | 88.82 | 690 | 11,980 | 266.15 | 8,001.62 | 23.58 | 2,780 | 1,150 |
| **High LOS** | 2.94 | 0.11 | 0.65 | 54.97 | 850 | 1150 | 216.39 | 5,460.79 | 17.86 | 600 | 2,830 |
| **Peat** | 14.36 | 39.51 | 1.86 | 149.90 | 480 | 23,880 | 209.64 | 6,463.84 | 24.11 | 1,230 | 7,600 |

Table 7. Two-way ANOVA for planting state (planted or unplanted) and lean oil sands (LOS) grade (low (1.95 % hydrocarbons) or high (4.54 % hydrocarbons) effects on hydrocarbon degradation in pots of the aspen (*Populus tremuloides*) group post-box-cox transformation.

| | **df** | **SS** | **MS** | **F** | **p-value** |
|---|---|---|---|---|---|
| **Planting State** | 1 | 332679 | 332679 | 0.054 | 0.818 |
| **LOS Grade** | 1 | 6685537 | 6685537 | 1.076 | 0.305 |
| **Planting State × LOS Grade** | 1 | 7654118 | 7654118 | 1.232 | 0.273 |
| **Residuals** | 42 | 260865143 | 6211075 | | |

Table 8. Two-way ANOVA for planting state (planted or unplanted) and lean oil sands (LOS) grade (low (1.95 % hydrocarbons) or high (4.54 % hydrocarbons) effects on hydrocarbon degradation in pots of the pine (*Pinus banksiana*) group post-box-cox transformation. Significant P values are marked with an asterix.

| | **df** | **SS** | **MS** | **F** | **p-value** |
|---|---|---|---|---|---|
| **Planting state** | 1 | 4571214 | 4571214 | 0.384 | 0.538 |
| **LOS Grade** | 1 | 70514830 | 70514830 | 5.929 | 0.019* |
| **Planting state × LOS Grade** | 1 | 21737440 | 21737440 | 1.828 | 0.183 |
| **Residuals** | 42 | 499549396 | 11894033 | | |

Table 9. Tukey HSD comparisons for planting state (planted or unplanted) and lean oil sands (LOS) grade (low (1.95 % hydrocarbons) or high (4.54 % hydrocarbons)) effects on hydrocarbon degradation in pots of the pine (*Pinus banksiana*) group post-box-cox transformation of data.

| **Comparison** | **Difference** | **Lower** | **Upper** | **p-value adjusted** |
|---|---|---|---|---|
| **Planted × High - Unplanted × High** | -2179.080 | -6359.658 | 2001.497 | 0.509 |
| **Unplanted × Low - Unplanted × High** | -4554.810 | -9485.950 | 376.331 | 0.079 |
| **Planted × Low - Unplanted × High** | -3745.935 | -7926.512 | 434.643 | 0.093 |
| **Unplanted × Low - Planted × High** | -2375.729 | -6556.307 | 1804.848 | 0.434 |
| **Planted × Low - Planted × High** | -1566.854 | -4828.497 | 1694.788 | 0.577 |
| **Planted × Low - Unplanted × Low** | 808.875 | -3371.703 | 4989.452 | 0.954 |

Table 10. Single factor ANOVA for lean oil sands grade (low (1.95 % hydrocarbons) or high (4.54 % hydrocarbons)) effects on the shoot biomass of pine (*Pinus banksiana*).

| | **df** | **SS** | **MS** | **F** | **p-value** | **F crit** |
|---|---|---|---|---|---|---|
| **Between Groups** | 1 | 0.49005 | 0.49005 | 5.175 | 0.0302* | 4.170 |
| **Within Groups** | 30 | 2.84075 | 0.094692 | | | |
| | | | | | | |
| **Total** | 31 | 3.3308 | | | | |

Table 11. Single factor ANOVA for lean oil sands grade (low (1.95 % hydrocarbons) or high (4.54 % hydrocarbons)) effects on the root biomass of pine (*Pinus banksiana*). Significant P values are marked with an asterix.

| | **df** | **SS** | **MS** | **F** | **p-value** | **F crit** |
|---|---|---|---|---|---|---|
| **Between Groups** | 1 | 0.099013 | 0.099013 | 5.303 | 0.028* | 4.170 |
| **Within Groups** | 30 | 0.560075 | 0.018669 | | | |
| | | | | | | |
| **Total** | 31 | 0.659088 | | | | |

Table 12. Single factor ANOVA for lean oil sands grade (low (1.95 % hydrocarbons) or high (4.54 % hydrocarbons)) effects on the shoot biomass of aspen (*Populus tremuloides*).

| | **df** | **SS** | **MS** | **F** | **p-value** | **F crit** |
|---|---|---|---|---|---|---|
| **Between Groups** | 1 | 2.525628 | 2.525628 | 57.804 | <0.001* | 4.170 |
| **Within Groups** | 30 | 1.310769 | 0.043692 | | | |
| | | | | | | |
| **Total** | 31 | 3.836397 | | | | |

Table 13. Single factor ANOVA for lean oil sands grade (low (1.95 % hydrocarbons) or high (4.54 % hydrocarbons)) effects on the root biomass of aspen (*Populus tremuloides*).

| | **df** | **SS** | **MS** | **F** | **p-value** | **F crit** |
|---|---|---|---|---|---|---|
| **Between Groups** | 1 | 1.132513 | 1.132513 | 27.319 | <0.001* | 4.170 |
| **Within Groups** | 30 | 1.243638 | 0.041455 | | | |
| | | | | | | |
| **Total** | 31 | 2.37615 | | | | |

Table 14. permANOVA for unplanted controls subjected to the fertilizer and watering regime for the pine (*Pinus banksiana*) planted trials, analyzing the effect of low (1.95 % hydrocarbons) and high-grade (4.54 % hydrocarbons) lean oil sands (LOS) on microbial phospholipid fatty acid profiles post-Hellinger transformation.

| | **df** | **SS** | **MS** | **F** | **$R^2$** | **p-value** |
|---|---|---|---|---|---|---|
| **Unplanted x LOS Grade** | 1 | 0.034525 | 0.034525 | 21.907 | 0.646 | <0.001* |
| **Residuals** | 12 | 0.018912 | 0.001576 | | 0.353 | |
| **Total** | 13 | 0.053437 | | | 1.000 | |

Table 15. permANOVA for unplanted controls subjected to the fertilizer and watering regime for the aspen (*Populus tremuloides*) planted trials, analyzing the effect of low (1.95 % hydrocarbons) and high-grade (4.54 % hydrocarbons) lean oil sands (LOS) on microbial phospholipid fatty acid profiles post-Hellinger transformation.

| | **df** | **SS** | **MS** | **F** | **$R^2$** | **p-value** |
|---|---|---|---|---|---|---|
| **Unplanted x LOS Grade** | 1 | 0.034470 | 0.034470 | 25.226 | 0.643 | <0.001* |
| **Residuals** | 14 | 0.019130 | 0.001366 | | 0.356 | |
| **Total** | 15 | 0.053601 | | | 1.000 | |

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