Cutin and suberin in mixed-wood boreal forest plants and their use as markers for origin of soil organic matter

by

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Abstract

Quantifying above and belowground inputs to soil organic matter is important to assess forest soil health and to develop practices that increase the soil organic matter present in forest soils. Cutin and suberin are biopolymers found in leaves and roots, respectively, that protect plants from desiccation. Due to their specific locations within plant tissues, the presence of the polymers cutin and suberin in the soil is used to confirm the contributions of these leaves and roots to organic matter. Specifically, the identity of the monomers comprising these biopolymers is used to infer the contributions of leaves and roots to organic matter. However, previous studies have identified monomers in tissues of leaves or roots that do not concur with published lists of markers, highlighting the importance of using region- and species-specific markers. Cutin and suberin were extracted from leaf, root, and bark samples from the eight most dominant species in a boreal mixed-wood stand in Alberta using hydrolysis. Additionally, I sampled soils from the interface of the organic and mineral soil, treated with the same hydrolysis process to investigate if the monomers present in the plant tissue samples were also detectable in soils. The monomers were identified using GCMS and compared to published lists of cutin and suberin markers. Across the roots and leaves of the eight species, a total of 142 monomers were identified. In soil samples, only 48 monomers were observed, five of which were not present in any of the plant tissues. Due to their presence in other plant tissues aside from leaves and roots, and in microorganisms, several classes of compounds identified in this study cannot determine the origin of soil organic matter. When compared to published lists of cutin and suberin markers, I found that while a select number of markers held true for the samples analysed in this study, others are not appropriate for use in study areas with vegetation similar to the one in this study due to their presence in multiple tissue types. The inconsistencies between the monomers identified in this study and those in published reports highlight the importance of using cutin and suberin markers specific to the dominant species of plants present in the area of interest.

Preface

This thesis is an original work by Ariel Brown. No part of this thesis has been previously published.

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Table of Contents

List of Tables

List of Figures

Introduction

Soil organic matter (SOM) plays several important roles in a forest ecosystem, such as serving as a major source of nutrients, increasing the water holding capacity of soils, as well as influencing the soil temperature regime and nutrient exchange capacity (Kononova, 1966). Any carbon-containing compound in the soil can be considered part of the SOM, with the exception of living roots and inorganic carbonates, such as those derived from minerals (Binkley and Fisher, 2013). Organic matter can enter the soil via plant litter and exudates, microbial and fungal inputs, as well as animal sources. Organic horizons are defined at those that contain more than 17% organic carbon by weight and are defined based on the origin of the organic material that contributes to them (Soil Classification Working Group, 1998). The organic horizon can be split into three sub-horizons: litter (L), fibric (F) and the humus layer (H), wherein with each subsequent layer the origin of the organic material becomes less discernable, as they are broken down into humic substances and other simpler compounds. The exception to this rule for identifying organic horizon layers is roots – which can be deposited as fresh organic material at any depth that roots are present. The organic matter nutrient pool is influenced by factors other than just the input of organic materials into the soil, such as climate, soil type, and the vegetation present in the area (Kuzyakov et al. 2000). Deriving the origin of soil organic matter is important because studies have suggested that roots, and not leaves, may be the main contributor to the stable carbon pool in soils (Crow et al., 2009; Hamer et al., 2012; Spielvogel et al. 2014). One way to investigate proportions of root vs. leaf into the carbon pool of soils is through cutin and suberin analysis.

With respect to the plant origins of SOM, the relationship between aboveground and belowground inputs is not fully understood. It has been suggested that roots contribute more carbon to soil (Rasse, 2005), while aboveground plant tissue predominantly release their carbon back into the environment as CO_2 in the atmosphere (Binkley and Fisher, 2013). A study of a deciduous forest by Crow

et al. (2009), determined that belowground inputs may be the driving force of stabilized soil organic matter, while in an old growth coniferous forest, it was found that aboveground inputs appeared to be the main source of stabilized organic matter. Suberin concentrations in soils have also demonstrated that roots are a major contributor to soil organic carbon (SOC) in grassland soils (Bull et al., 2000; Nierop et al. 2003). To complicate matters, studies have also suggested that root-derived carbon is preferentially preserved in soils due to its chemical structure or location within the soil, where soil minerals may provide physio-chemical protection (Rasse et al., 2005). Differences in the primary source of stable organic matter in different ecosystems highlights the importance of investigating carbon inputs in multiple forest types as a key element in improving our understanding of carbon input and stabilization of forest soils (Binkley and Fisher, 2013).

Many studies investigating the inputs of aboveground and belowground plant tissues use the biopolymers cutin and suberin as markers for the tissue of origin (Reiderer et al., 1993; Rasse et al., 2005; Crow et al., 2009; Mendez-Millan et al., 2010a,b; Hamer et al., 2012; Spielvogel et al., 2014), as they are poorly soluble in organic solvents and are water insoluble in nature and can be detected in soil samples. The recalcitrance of cutin and suberin is not entirely agreed upon (Tegelaar et al., 1989; Kögel-Knabner, 2002), however, it has been noted that they have potential for preservation in soils (Kögel-Knabner et al., 1989). Cutin is found in the cuticle, which is a layer of tissue that covers the epidermis of aboveground plant structures to protect against desiccation (Kögel-Knabner, 2002; Beck, 2010). It is comprised of fatty acids and chain lengths of C_{16} and C_{18} linked by ester bonds (Kolattukudy, 1981). These fatty acids typically belong to the di- and trihydroxy and epoxy groups (Kögel-Knabner, 2002). Suberin is found in the belowground parts of plants, such as the epidermis, endodermis and phellem of roots, as well as the cork of aboveground tissues of woody plants that undergo secondary thickening (reviewed in Bernards, 2002). Unlike cutin, which consists of aliphatic monomers, the suberin polymer has both an aliphatic and aromatic domain (Bernards, 2002; Kögel-Knabner, 2002). Monomers that

make-up suberin commonly have longer chain lengths (C_{20}-C_{30}), and can include phenolic acids (Kögel-Knabner, 2002). Suberin is also present in the tissues of bark, which may hinder the use of suberin as a marker for belowground tissues in areas dominated by woody species (Rasse et al. 2005). Similar to cutin, suberin appears to play a role in water retention and microbial protection (reviewed in Bernards, 2002; Beck, 2010). While many of the monomers that make up cutin and suberin overlap, some appear to be specific to each polymer (Nierop et al., 2003; Otto et al., 2005; Otto and Simpson, 2006; Mendez-Millan et al., 2010b). Additionally, some monomers may even be unique to the cutin and/or suberin of specific species (Hamer et al., 2012). The particular monomer composition of cutin and suberin allow for their use as not only markers of aboveground plant inputs into the soil, and also may be able to act as markers for species-specific inputs. Due to the variation of monomers among species, it is important to verify the markers used for cutin and suberin for the dominant species in a given study area (Hamer et al., 2012).

Soil organic carbon can come from a variety of sources — and to understand its origins, we need reliable markers for different inputs. Studies have been done on monomers found in plant waxes (Bull et al. 2000, Kunst and Samuels, 2003), monomers from microbial or fungal inputs (Otto and Simpson 2005) and monomers present in cutin and suberin (such as: Otto and Simpson 2005; Nierop, 1998; Mendez-Millan et al. 2010a,b). Using these monomers, studies have identified some that are only present in one organism, tissue type, or polymer, and as a result, these have been used as markers for specific sources. However, studies have also shown that there is variation in the monomers depending on the species of plant present in the study area (Hamer et al. 2012). The presence of suberin in bark also presents a challenge, as it limits the conclusions one can draw regarding root input into forest soils when using suberin as a marker in wooded environments.

This study aims to investigate the monomers derived from the hydrolysis of cutin and suberin from the leaf, root and bark (when applicable) of seven understory species and one tree species common to the boreal mixed-wood forests of Alberta, Canada. Specifically, I will first compare monomers produced from these tissues to those identified as markers for cutin or suberin in previous studies, with the objective of identifying markers that work across species. Second, I searched for species-specific monomers; these would allow for a higher resolution of sourcing organic matter from plants. Additionally, the suberin monomers from the bark and roots of a single widespread woody species, *Populus tremuloides* (aspen) were compared to examine differences in the composition of suberin based on its source tissue. Finally, I will evaluate this information towards deriving the source of organic matter in soils from a boreal mixed-wood stand.

Methods

Study location

Study sites are located at the University of Alberta Woodbend Research Forest (53.3946, -113.7528) and within the Cooking Lake Blackfoot recreation area (53.5047, -112.9433) both near Edmonton, Alberta, Canada. The Woodbend Research Forest consists of low sandy ridges interspersed by bogs and marshes. Aspen (*Populus tremuloides*), white spruce (*Picea glauca*) and jack pine (*Pinus banksiana*) dominate the area. The Cooking Lake Blackfoot recreation area is within the Beaver Hills Moraine (also called the Cooking Lake Moraine), a knob and kettle terrain. The Beaver Hills Moraine has a diverse landscape that is primarily dry mixed-wood boreal forest surrounded by aspen parkland. The Cooking Lake Blackfoot recreation area is a patch of aspen parkland within the Beaver Hills Moraine that is dominated by Grey Luvisolic soils.

Both sites are classified as Boreal Mixedwood, or more specifically, Dry Mixedwood (Beckingham and Archibald, 1996). Indicative tree species of this ecological area include: aspen (*Populus tremuloides*), balsam poplar (*Populus balsamifera*), white birch (*Betula papyrifera*), white spruce (*Picea glauca*) and balsam fir (*Abies balsamea*), with the understory commonly containing beaked hazelnut (*Corylus cornuta*), prickly rose (*Rosa acicularis*), Canada buffalo-berry (*Shepherdia canadensis*), twin-flower (*Linnaea borealis*), green alder (*Alnus crispa*), bunchberry (*Cornus canadensis*), wild sarsaparilla (*Aralia nudicaulis*) and dewberry (*Rubus pubescens*) (Beckingham and Archibald, 1996). The ecological area includes a variety of topography, ranging from flat-topped hill regions to lowland regions with shallow lakes (Beckingham and Archibald, 1996). Brunisols and Luvisols are common throughout the area (Beckingham and Archibald, 1996). Central Alberta's climate is characterized as cool continental, sub arid to sub humid, with long winters and warm summers. The annual mean temperature is 1.5 °C with a total precipitation of 389 mm. Seasonally, the mean summer temperature is 13.7 °C with a total precipitation of 238 mm, and the mean winter temperature is -11.9 °C with a total precipitation of 63.0 mm (Beckingham and Archibald, 1996).

Sample collection

Suberin distribution in soils have been found to closely follow rooting patterns (Spielvogel et al., 2014), which in turn may be influenced by factors such as soil texture. First, I determined whether rooting profiles varied significantly in soils of different textures to determine if soils should be sampled to different depths for organic matter. Towards this goal, five plots were selected at each of Cooking Lake and Woodbend Research Forest sites for a total of 10 plots. Within a site, plots were a minimum of 20 m apart. Both Cooking Lake and Woodbend are aspen-dominated parklands, with similar understories mainly comprised of hazelnut (*Corylus cornuta*) and rose (*Rosa acicularis*). While both sites support aspen stands, Cooking Lake has soils ranging from a silt loam to clay loam, while Woodbend soils range from sandy loam to a loamy sand (Table 1). Each of the ten plots were 10 x 10 m in

perimeter, with a transect running diagonally across the centre between two corners of the plot. Along this diagonal transect, cores were taken with a 5 cm x 18 cm (diameter x length) slide hammer (AMS, American Falls, Idaho, USA) every 2 m (for a total of seven coring locations per plot), to a depth of 40 cm separated into 10 cm increments. Cores were placed in plastic freezer bags and kept on ice for transport back to the University of Alberta, where they were stored at -23 °C. To assist in identification of roots to species using molecular tools (see, '*Species identification of roots using molecular techniques'),* shrubs and herbaceous plants within a 2 m radius of each core were identified, and percent cover was estimated within a 0.5 m radius around the centre of each coring location. The canopy height was measured with a clinometer, and the density of trees with a diameter greater than 10 cm at breast height (1.3 m) was calculated for each plot. Five trees were randomly selected at each plot and their diameter at breast height was measured. Canopy height was 15.3 m ± 0.9 and 16.9 m ± 1.3, mature tree density was 206 stems ha^{-1} ± 27 and 162 stems ha^{-1} ± 31, and diameter at breast height was 16.4 cm ± 1.6 and 17.8 cm ± 0.8 in Cooking Lake and Woodbend, respectively.

Three of the five plots at the Woodbend site were randomly selected to collect plant tissue and soil for cutin and suberin analysis. Within these plots, a smaller 6 x 6 m plot was established, and four to five of the most abundant tree and shrub species were identified. From multiple individuals of these species, I sampled approximately 5 g each of leaves, roots (roots were excavated to confirm they were connected to the individual of interest) and bark (when applicable). Approximately 30 g of the organic soil was sampled to the interface with the mineral soil (approximately 2 – 3 cm) from three randomly selected locations within each 6 x 6 m plot. These soil samples were air-dried (1 – 5 days) and the plant tissues were oven-dried at 60 °C for 24 hours and then kept in paper bags in the dark.

Soil processing

Roots contained in frozen soil samples were allowed to thaw overnight at 4 °C. The next day, cores were picked through for 20 minutes by two people to remove roots and stones. Roots were rinsed

with distilled water and stored at 4 °C for no longer than 10 days before they were lyophilized (Labcono FreeZone 2.5, Kansas City, USA) for approximately 72 hours. Once freeze-dried, roots were weighed based on root size (small roots: diameter <1 mm, medium roots: diameter 1-5 mm, large roots: >5 mm) and stored at -10 °C for subsequent molecular analysis. Due to the infrequent occurrence of large roots, they were not analysed on their own, and were only included in the total root biomass measurements. I used the air-dried soils to measure soil nutrients, texture and bulk density; these soils were weighed, and stored in paper bags in the dark. Of the seven soil cores collected from each plot, I randomly selected two for nutrient and texture analysis; these cores were dried at 35 °C, and each depth subsampled. Texture of mineral soils was measured by hydrometer (Bouyoucos, 1962, Richard, 1993; ASTM, 2007), available potassium and phosphorus by modified Kelowna extraction (Alberta Agriculture, 1995) and colorimetry (O'Dell, 1993); available nitrogen by 2M KCl extraction (Kalra and Maynard, 1991; Benton Jones Jr., 2001; Maynard, Kalra and Crumbaugh, 2008) and colorimetry (USEPA, 1978; Bower and Holm-Hansen, 1980; Jokinen et al. 2013); and pH by $CaCl_2$ followed by EC in water (McLean, 1982; Rhoades, 1982; Kalra, 1995; Hendershot, Lalande and Duguette, 2007; Miller and Curtin, 2007; Miller and Kissel, 2010). All analyses were performed by the Natural Resources Analytical Laboratory (NRAL) at the University of Alberta.

Bulk density of mineral soils was calculated by dividing the dry weight by the volume for each sample. Soils sent for nutrient analysis could not be dried above 40 °C, and to calculate bulk density, the oven-dried weight at 100 °C is required. To estimate the 100 °C oven-dried weight of the samples sent for nutrient analysis, three soil samples from each of the lower three depths (mineral soil) at each site were selected from the samples not sent for soil analysis. Using these samples, a correction factor was determined based on the relationship between their air-dried and 100 °C oven- dried weights (0.98; no significant difference between depth and/or sites). This correction factor was applied to the air-dried weights of the soils sent for nutrient testing to estimate their bulk density.

Species identification of roots using molecular techniques

We used fluorescent amplified-fragment length polymorphisms, a DNA-based analysis, to identify roots of species following methods of Metzler et al. (2019). In brief, freeze-dried roots were pulverized, and DNA extracted using 2% CTAB extraction and 5% CTAB cleaning. Three non-coding cpDNA regions were amplified (*trn*T-*trn*L intergenetic spacer, the *trn*L intron, and *trn*L-*trn*F intergenetic spacer) with the universal primer sets established by Taberlet et al. (1991). Amplified products were first resolved using capillary electrophoresis (ABI 3730 DNA analyzer; Applied Biosystems, Foster City, CA, USA) and then sized with GeneMapper (Applied Biosystems, Foster City, CA, USA) with GeneScan 1200 LIZ size standard (Applied Biosystems, Foster City, CA, USA). Fragment sizes read by the capillary sequencer were rounded to the nearest base pair. Combinations of resolved fragment lengths were compared to the database of known fragment length combinations compiled by Metzler et al. (2019) to produce a list of possible species present within each sample. Species returned by DNA analysis were compared to the list of species identified at the sampling site to confirm presence. Any species returned by DNA analysis that was not present at the site may be a remnant of previous vegetation, or, may be an artefact of some fragment length combinations representing more than one species.

Cutin and suberin analysis

Plant tissue samples of identical species were combined, to create a composite sample of individuals of the same species across all three plots. Plant tissue and soil samples for cutin and suberin analysis were pulverized using a mixer mill (Retsch M200, Germany) and kept in glass vials in the dark while awaiting processing.

Determination of cutin and suberin in soil samples was achieved using the methods described by Otto et al. (2005) and Mendez-Millan et al. (2010), with modifications made to allow for use of a Frontier EGA/PY-3030D multi-shot autosampler for lipids in-situ methylation using tetramethylammonium hydroxide (TMAH) with subsequent GCMS analysis. The modifications also

allowed for simplified separation (centrifugation instead of filtration as in Otto in (2005)), reflux (using water bath and centrifuge tubes instead of reflux using heaters, round-bottom flasks, condensers as in Otto (2005)), liquid-liquid extraction (using 50 ml glass vial and disposable glass Pasteur pipets instead of using separation funnels as in Otto (2005)), concentration (using drying with nitrogen instead of rotary evaporation as in Otto (2005)), and lipid methylation procedures (done using TMAH instead of using diazomethane in ether as in Otto (2005)).

All water-solubles were removed by sonication of samples with ultrapure deionised water until extracts remained clear. Solvent-extractable lipids were removed by rinsing with methanol, three extractions with a dicholoromethane:methanolmixture(1:1), followed by three rinses (or until clear liquid is observed) with dichloromethane (as described by Otto et al (2006)). All extracts were disposed of.

Lipid-free sample residues were dried under the fume-hood and hydrolyzed to break cutin and suberin polymers to their heavy acid and alcohol monomers. Before hydrolysis, 0.5—0.51 g of each remaining dry sample were transferred to 50 mL solvent-resistant centrifuge tubes and spiked with 250 µL C19:O PC surrogate recovery standard. Samples were then refluxed with 5 ml of 1N methylated KOH solution as described by Otto et al. (2006) in a hot water bath set to 70 °C for 3 hours. Samples were cooled, centrifuged and the extract was decanted into 50 mL glass vials and kept at 20 °C. Hydrolyzed residues were rinsed once with addition of 5.0 mL of methanol and 0.5 mL of ultrapure water (methanol/water (9:1) (V:V) (as per Dignac (2010)) in centrifuge tubes, sonicated for 15 minutes and centrifuged for 10 minutes at 4000 RPM and supernatants combined with previous extracts. The residues were then rinsed three more times with a mixture of 2.5 mL methanol and 2.5 mL DCM and the supernatants obtained from rinsing were added to the vials containing the extracts from the previous centrifugation steps (as described by Otto et al (2005). Combined extracts were acidified to pH=1 using

6M HCl (Otto et al (2006) and Dignac (2010)) converting lipids to their acidic form. An additional 10 mL of ultrapure water and 5 mL DCM was added to the vials, which were then vortexed for thirty seconds and left for phase separation in the dark overnight. The bottom dichloromethane (DCM) layer containing heavier acids and alcohols was transferred into a 10 mL clean glass vial and dried under nitrogen gas. Dried residues were transferred into 2 mL GC vials by re-dissolving in 0.5 mL DCM twice, and then dried under nitrogen gas before derivatization.

Ninety µL of N,O-Bis(trimethylsilyl)trifluoroacetamide (BSTFA) with 1% trimethylchlorosilane (TCMS; Sigma) and 10 µL pyridine (Mendez-Millan et al 2010) were added to each of the GC vials. Content was mixed before being placed in the oven at 70 °C for 3 hours to transform hydroxy acids and alcohols to their TMS forms (trimethylsilyl ether and ester derivatives). After cooling, 100 µL HPLC grade hexane was added to each vial (as per Otto (2005)) and sample mixed before 5 µL aliquot was injected into GCMS using Frontier AS-1020E auto-shot sampler.

Five µL of sample, 1 µL of 25% tetramethylammonium hydroxide (TMAH) in methanol and 4 µL of instrument response C17:0 acid ME internal standard were added into 80 µL aluminum cup. In open cups volatile solvents (hexane and pyridine) evaporated fast under the fume-hood and cups were loaded into the autosampler tray. One µL of 25% tetramethylammonium hydroxide (TMAH) in methanol was added to each sample to methylate any underivatized carboxylic groups in acids into their methyl-ester forms. Additionally, TMAH may methoxylate any underivatized hydroxy groups into their methoxy form.

Samples were separated with an Agilent Technologies 7890B gas chromatograph (GC) equipped with a 5977A MS detector and HP-5MS UI column (60 m x 0.25 mm x 0.25 µm). Injections using Frontier - EGA/PY-3030D pyrolyzer and SS1010E selective sampler were done in a single shot mode with pyrolyzer temperature set at 320 °C, interface and injector at 300 °C and 25:1 split ratio. The temperature program of the GC was kept at 100 °C for 2 minutes, then increased from 100 to 150 °C at

10 °C per minute, from 150 to 200 °C at 5°C per minute (Mendez-Millan et al 2010), from 200 to 320 °C at 2 °C per minute, and kept at 320 °C for 10 minutes. Compounds were identified on a 5977A MSD (Agilent Technologies) mass spectrometer run in EI mode at 70eV ionization energy and TIC acquisition scan with mass range 50 to 650.

Agilent Technologies Mass Hunter Qualitative Analysis B.07.00 software was used to extract TIC chromatogram, integrate and extract peak spectra using Agile 2 integrator, create compounds and search NIST14 and Wiley 17 reference libraries using MS search 2.2 v for compound identification. Final compound identification was done using compound mass spectra with comparison to library, spectra published in literature and retention times. Because I can only confidently report the number of unidentified monomers within a sample, and not across samples, I focus reporting on 'known' monomers, defined as those that returned a name when compared to reference libraries.

For data quantification, instrument response C17:0 internal standard response factor was used to calculate amount of each compound, including surrogate C19:0 PC standard, relative to C17:0 amount in the injected sample. Then all amounts were corrected for surrogate recovery and results for each compound expressed as $\mu g\ g^{-1}$ of hydrolysable soil (water and solvents solubles already removed). Compound classification was done as per Otto 2006 (Table 4 in Otto 2006).

Data analysis

ANOVA was used to test the effect of site, sample depth and their interaction on soil texture, N, P, K, pH, and root mass for each size class, separately (large roots were not tested separately due to sparse data and are only included in the total root mass measurements). When data did not meet assumptions of normality, data was log transformed (NH_4^+, K^+, pH, silt, small root weight and total root weight) before using ANOVA, and when normality could not be achieved, permutational ANOVA was

used (NO_3^-, PO_4^{3-}, bulk density, clay, sand). Significant differences with depth were further tested with Tukey's Test.

All statistics were done in R (R core team, 2013), using the packages plyr (Wickham, 2011), emmeans (Lenth, 2019), lmPerm (Wheeler and Torichiano, 2016), sciplot (Morales, 2017), multcompView (Graves et al., 2015), magrittr (Bache and Wickham, 2014), and tidyr (Wickham and Henry, 2019).

Monomers recovered from cutin/suberin analysis were reported as $\mu g\ g^{-1}$ hydrolysable sample for each plant tissue type and soil sample. Monomers were compared to those listed as cutin and/or suberin markers in by Otto and Simpson (2006). An additional table was constructed of the monomers recovered from only one tissue type, but that were not included in Otto and Simpson (2006). Monomers listed as cutin or suberin markers by Otto and Simpson (2006) were classified as 'known' markers. "Identified monomers" are defined as monomers identified by GCMS.

Monomers recovered from soil samples were compared to those listed as markers by Otto and Simpson (2006) as well as those found in only one tissue type in this study to look for the presence of tissue specific monomers/markers present in soils. The concentrations of monomers identified in soils samples were averaged across the three samples and compared to monomers found in plant tissue to determine possible origin.

Results

Soil properties and distribution of roots

As expected, texture differed between the two sites; Woodbend soils had a much higher sand (82.7% ± 0.74 (SE)) fraction than those from Cooking Lake (34.5% ± 1.8), and soils from Cooking Lake had a higher percentage of clay and silt than those from Woodbend (Table 1). Soil texture did not

significantly change with depth at either Woodbend or Cooking Lake (Tables 1 and S1). Bulk density increased with depth for both sites (Table 2). At the soil surface, bulk density was about 1 g cm^{-3} and at 40 cm depth, it was about 1.5 g cm^{-3}. NO_3^-, PO_4^{3-} and pH did not vary by site or sample depth (Table 2). There was a significant interaction between site and sample depth for NH_4^+ (Table S1). At Woodbend, ammonium decreased with depth from 10 cm to 30 cm, while at Cooking Lake there was no trend (Table 2). Potassium was higher in soils of Cooking Lake than those of Woodbend (Table 2). Site and sample depth significantly influenced the mass of roots (Tables 3 and A1). In particular, there was a significant interaction of site and sample depth for biomass of small diameter roots (Table A1). Biomass of small diameter roots steadily declined with soil depth from 0 – 40 cm at Woodbend, but at Cooking Lake, there was no significant decline beyond 30 cm in depth. For medium diameter roots and all diameters combined, the total amount of roots declined with depth at Woodbend. While a decline in the total amount of roots was observed at Cooking Lake, the mass of medium diameter roots did not change with depth.

Overall, DNA analysis returned 56 species (Table A2), (due to overlap between *Alnus alnobetula subsp. crispa* and *Alnus incana subsp. tenuifolia,* as well as *Symphyotrichum boreale*, and *Symphyotrichum lanceolatum*, these pairs were counted as a single species). Across both sites, the most common species identified belowground was *Typha latifolia*, which was found in 65 of 218 samples. The second most common species were *Symphyotrichum sp.*, which were found in 34 of 218 samples. The third most common species was *Populus tremuloides*, which was found in 32 of the 218 samples. These three species were found at both sites and across the entire range of sample depths. Species richness did not significantly differ by site, but significantly differed by depth (Table A1). While there was no significant trend with depth, Tukey's HSD test showed a significant difference between the species richness at the 10 -20 cm increment and the 30 – 40 cm increment (Figure 1).

Of the 160 root samples sent for DNA analysis, 31 samples did not amplify across all three regions, nine samples across two regions, and 17 samples in one region. Six samples returned no matching species, despite returning lengths for all three fragments. Of the 24 samples that were not amplified across 1—2 regions, nine returned with possible species identification. Of the 12 species reported from these samples, no values for the *trn* T-*trn* L region are available in Metzler et al (2019) for 11 of them, no *trn* L lengths available for four of them, and no *trn* F-*trn* L lengths available for three of them. Of the species returned for these samples, four were always returned together in the DNA analysis when only the *trn* L and *trn* F-*trn* L regions were resolved: *Fragaria virginiana*, *Symphyotrichum boreale*, *Symphyotrichum lanceolatum* and *Eurybia conspicua*. While the presence of *F. virginiana* and *E. conpsicua* are supported by reported values of their *Trn* L region, both *Symphyotrichum* speices only have lengths reported for their *trn* F- *trn* L regions, which are identical to each other as well as the *trn* F-*trn* L value of *E. conspicua*. The other 15 did not match any fragment lengths reported by Metzler et al. (2019).

Sourcing the origin of soil organic matter: cutin and suberin analysis

For reference monomers found in soils, I first characterized monomers present in leaves, roots and bark separately for species common to the sampled plots and compared these to published accounts of Otto and Simpson (2006; Table A3). A total of 61 known monomers were found in leaves across the eight species. Of these, 26 were unique to leaves, meaning that they were not identified in the root or bark samples of any of the tested species. Several of the monomers were present in leaves of only one species; three were found in *Rosa acicularis*, two in *Aralia nudicaulis*, two in *Corylus cornuta*, three in *Rubus idaeus*, two in *Populus tremuloides*, one in *Apocynum androsaemifolium*, one in *Prunus pensylvanica*, and two in *Symphoricarpos albus* leaves (Table A4). For roots, 64 known monomers were found across the eight plant species. Of these, 23 were unique to roots, and were not found in leaf or bark samples. Similar to leaves, many of the known monomers in roots occurred in a subset of species;

six in roots of *Aralia nudicaulis*, two in *Rubus idaeus*, six in *Populus tremuloides*, one in *Apocynum androsaemifolium*, and one in *Symphoricarpos albus* (Table A4). None of the identified monomers were isolated to *Corylus cornuta* or *Prunus pensylvanica* roots. Thirty-one known monomers were found in the bark of *Populus tremuloides* (Table A4), three of which were unique to this tissue.

A total of 44 monomers were identified by GCMS from the hydrolysis of cutin and suberin in the organic matter of soils. A large portion (45.5 %) of the monomers found in soil samples were present in all three tissue types, 11.4 % were present in leaf samples, 13.6 % in roots, 9.1 % in roots and leaves, 9.1 % in roots and bark, and 11.4 % of the compounds identified in soil samples were not found in any of the plant tissues tested. Eleven monomers classified as markers. as per Otto and Simpson (2006) were recovered from soil samples (Table 4). Two of these markers were cutin markers: 10,16-dixydroxy-hexadecanoate and 9,10-dihydroxy-1,16-hexadecanedioic acid, which were found only in the leaf samples of the tested species. Another marker, classified as a cutin marker, or a cutin and suberin marker, Methyl 16-Hydroxyhexadecanoate, was found in all three tested tissues. Three suberin markers were identified in the soil samples, two of which were found only in the roots and bark of the tested species: Eicosanebioic acid and Docosanedioic acid. The third suberin marker found in soil samples, Tetracosanedioic acid, was not identified in any of the plant tissues sampled. Six cutin and suberin markers were identified in the soil samples, five of which were found in all three tissue types (Table 4), one only in leaf tissues: Octadecanoic acid, 9,10-dihydroxy-, methyl ester, (R*,R*)-, and one only in root and bark samples: Octadecanedioic acid.

Ten compounds that did not meet the classification of a marker, but appeared only in cutin or suberin-containing tissues were identified in soil samples. Six monomers found only in roots, one monomer found only in roots and bark, and three monomers found only in leaves were recovered from

soils (Table 5). None of the compounds isolated to the bark of *Populus tremuloides* were recovered from soil samples.

In addition to the suberin marker recovered from soil that was not identified in any plant tissue samples (Tetracosanedioic acid), three monomers were also found in soil samples that were not recovered from plant tissues, nor can they be classified as markers: Methyl 14-hydroxytetradecanoate, Methyl 2-hydroxy-tetracosanoate, and Sarcosine, N-methoxycarbonyl-

There were 20 monomers recovered from plant tissue samples of the eight species tested that could be classified as markers as per Otto and Simpson (2006). Of these, three were cutin markers, four were suberin markers, 13 were cutin and suberin markers, two could be classified as either cutin markers or cutin and suberin markers, and one could be classified as either a suberin marker or a cutin and suberin marker (Table 6). All three of the markers that could be classified only as cutin markers were found only in the leaves of the tested species. Of the two that could be classified as cutin markers or cutin and suberin markers, one was found only in leaves, and one was found in all three tissue types. All four of the markers that could be classified only as suberin markers were found only in root and bark tissues of the plant species tested (Table 6). One marker, which could be classified as a suberin marker or a cutin and suberin marker was identified in leaves and roots of the tested plant tissues. Ten additional cutin and suberin markers were identified in plant tissue samples, not including those that could be classified as cutin markers or suberin markers mentioned above. Four of the markers that could be classified as cutin and suberin markers without overlap were found only in leaves, two were found only in roots or bark, and four were found in all three tissue types of the species tested (Table 6).

Discussion

In this study, I isolated monomers derived from the hydrolysis of cutin and suberin present in leaf, root and bark samples. These monomers were compared to published lists of cutin and suberin markers (Otto and Simpson, 2006). I was able to identify several monomers unique to a tissue type across multiple species, as well as several that appear to be specific to a single tissue type of only one of the species tested. Additionally, differences between monomers found in the bark and roots of *Populus tremuloides* were observed. Monomers were separated into their compound classes and compared to previous studies to investigate their use as potential tissue- or species-specific markers. Taken together, I found that while some of the published markers held true for the species tested in this study, others, while classified as cutin and suberin markers by Otto and Simpson (2006), were only identified in cutin, illustrating the importance of using markers appropriate for the species in the study area.

Phenols and benzyls

In my tissue samples, 13 benzyl/phenols or their derivatives were found, five of which were also identified in soil samples. The most abundant phenols were 2-propenoic acid, 3-(3,4-dihydroxy), methyl ester and its derivatives, and 2-propenoic acid, 3-hydroxy-, methyl ester and its derivatives. 2-propenoic acid, 3-(3,4-dihydroxy), methyl ester and its derivatives were found in concentrations of 3.39 $\mu g\ g^{-1}$ ± 2.04 (SE) in the leaves of four species, 11.00 $\mu g\ g^{-1}$ ± 2.22 (SE) in the roots of all eight species, and 82.55 $\mu g\ g^{-1}$ in *Populus tremuloides* bark. 2-propenoic acid, 3-hydroxy-, methyl ester and/or a derivative were found in concentrations of 6.72 $\mu g\ g^{-1}$ ± 2.03 (SE) in leaf tissues of seven species, 1.60 $\mu g\ g^{-1}$ ± 0.41 in the roots of three species and 40.97 $\mu g\ g^{-1}$ in *Populus tremuloides* bark. Two phenols were found only in the bark of Populus tremuloides, Benzene, 1-methoxy-2-(methoxymethyl)- at a concentration of 1.13 $\mu g\ g^{-1}$, and Vanillin, at a concentration of 1.70 $\mu g\ g^{-1}$. The higher number of occurrences of Benzyls in root samples compared to leaf samples (27 and 15 respectively), is likely the result of the aromatic domain of suberin.

Benzyls and phenols are not used as markers for biopolymers, as they can originate from multiple sources (Goñi et al., 2000). Phenols can also be derived from lignin, however, with the methods used in this study, the ether bonds of lignin are not cleaved, and instead, only the ester bonds of cutin and suberin are affected (Otto and Simpson, 2006). While phenols such as coumaryl, vannillyls and syringls are listed as common in suberin and not present in cutin in Otto and Simpson (2006), they are not listed as suberin markers, possibly due to their presence in the ligno-cellulose complex of the grasses in their study area. These phenols are also common constituents of lignin, rendering them unfitting as markers in a forest environment.

***n*-alkanols**

One of the components present in biopolymers are n-alkanols, which can make up as much as 11% of the aliphatic domain of suberin (Bernards, 2002). Suberin may contain *n*-alkanols, particularly those with chain lengths of C_{16} – C_{30} (Kolattukudy and Espelie, 1989; Bernards, 2005), which makes them appear as candidates for suberin markers. However, many *n*-alkanols are constituents of other plant components such as waxes (Bull et al. 2000, Kunst and Samuels, 2003) or are derived from microorganisms (Otto and Simpson 2005). Otto and Simpson (2005) compiled previous research on Alberta grassland and forest soils and reported cyclic and aliphatic biomarkers present. Aliphatic compounds listed as markers for microorganisms include primary *n*-alkanols with chain lengths of C_{16} – C_{18}, and secondary *n*-alkanoic acids with chain lengths C_{14} – C_{18} and iso-alkanoic acids with chain lengths of C_{16} and C_{18}. The *n*-alcohols from plant waxes have been found to be dominated by those with chain lengths of *n*-C_{26} and *n*-C_{28} (Bull et al, 2000; Kunst and Samuels, 2003). The extraction of soluble lipids before the hydrolysis of polymers used in this study would have removed any of these compounds from other sources, and as such, the compounds reported here should be strictly from cutin and/or suberin. The identification and concentration of *n*-alkanols present in plant tissues provides information on the chemical makeup of these compounds in different species. Unfortunately, the presence of many n-

alkanols in other tissues eliminates these monomers, such as the *n*-alkanol Eicosanol, which was found in the roots of seven species tested as a marker for suberin.

Alkanoic and alkenoic acids

Alkanoic acids have been reported as common components of suberin, however, like *n*-alkanols, they can also be derived from plant waxes and microbes (Otto and Simpson, 2006), and as such many are limited to investigating the differences in composition of suberin in cutin. In my study, I found that the majority of alkenoic acids $C_{>20:1}$ were found only in roots, while alkenoic acids $C_{16:1}$ acids were found only in leaves. Otto and Simpson (2006) suggest several categories of alkanoic and alkenoic acids that can be used as markers for cutin and/or suberin, which will be discussed in the following categories: mid-chain hydroxy acids, C_{18} epoxy acids, and ω-hydroxy acids.

Mid-chain hydroxy acids

While mid-chain hydroxy acids may be derived from belowground tissues (Mendez-Millan et al. 2010a), their contribution to the soil is so low (<2%) that Mendez-Millan et al. (2010a) has suggested they can be used as biomarkers for aboveground tissues. Otto and Simpson (2006) list mid-chain hydroxy acids of carbon lengths $C_{14:1}$, C_{15} and C_{17} as cutin markers. In my study, the mid-chain mono-hydroxy alkanoic acids 10-Hydroxypentadecanoic acid, methyl ester and 9-Hydroxypentadecanoic acid, methyl ester were found only in leaves, supporting their potential as aboveground biomarkers. However, they were not found in all the species tested, and were only present in *Prunus pensylvanica* leaves (10-Hydroxypentadecanoic acid, methyl ester) or *Populus tremuloides* and *Apocynum androsaemifolium* leaves (9-Hydroxypentadecanoic acid, methyl ester), which may limit their use as markers in certain environments.

C_{18} 9,10-epoxy acids

Short chain acids with mid-chain hydroxyl and epoxy groups have been found in abundance in base hydrolyzed cutin and suberin (Holloway, 1982; Kolattukudy and Espelie, 1989, Bernards, 2002),

with the distinction that suberin contains only C_{16} and C_{18} acids, while cutin contained a wider range of C_{14} – C_{18} acids; however, Otto and Simpson suggest only the use of C_{18} epoxy acids as markers. Unlike the mid-chain hydroxy acids discussed above that are considered markers for cutin, C_{18} 9,10-epoxy acids are a specific type of mid-chain dihydroxy acids that are classified as cutin and suberin markers by Otto and Simpson (2006). An epoxide group is a cyclical ether, or a triangular ring of two alkyl groups and an oxygen. Due to its triangular structure, this group is strained and highly reactive. Epoxide groups are readily hydrolysed in many solutions, including the base solution used in this study. When hydrolysed, an epoxy becomes two vicinial hydroxyl groups, which is the form they are in when passed through the GCMS.

In my samples, five compounds that fall into this category of cutin and suberin markers were identified: 9,10,18-trihydroxy octadecenoate, Methyl 18-hydroxy-cis-9,10-epoxyoctadecanoate, Octadecanoic acid, 9,10-dihydroxy-, methyl ester (R*,R*)- ,9,10-dihydroxy-1,18-octadecanedioc acid and 9,10-dihydroxy-1,16-hexadecanedioic acid, dimethyl ester. Otto and Simpson (2006) classify C_{18} 9,10-epoxy hydroxy acids as cutin and suberin markers, however, they classify C_{18} epoxy dioic acids as suberin markers – or more generally, a subcategory of C_{18} 9,10-epoxy acids can provide more specific information regarding their origin. Of the epoxy acids identified in this study, 9,10-dihydroxy-1,18-octadecanedioc acid, is a diacid, and is classified as a suberin marker, while the other four C_{18} 9,10 epoxy acids identified fall into the broader category of cutin and suberin markers.

ω-hydroxy alkanoic acids

ω-hydroxy acids have been reported as suberin-specific compounds (Nierop, 1998), and they can make up 13 – 61 % of the aliphatic domain of the suberin polymer (Bernards, 2002). Otto and Simpson (2006) listed ω-hydroxy alkanoic acids as cutin and suberin markers or as suberin markers, depending on the length of the carbon chain. Those with carbon chains of C_{16} and C_{18} (including ω-hydroxy alkeneoic acids with carbon chains of $C_{18:1}$ and $C_{18:2}$) are classified as more general cutin and

suberin markers, while long chain ω-hydroxy alkanoic acids are classified as more specific suberin markers (Otto and Simpson, 2006).

In my study, five C_{16} and C_{18} ω-hydroxy acids were identified as cutin and suberin markers. One of these monomers, 9 and 10, 16-dihydroxy-hexadecanoate and its derivatives, can be considered a short chain C_{16} ω-hydroxy alkanoic acids, however, it can be classified into the more specific category of a C_{16} mono- and di-hydroxy acid, which as per Otto and Simpson (2006) would qualify it as a cutin marker. This compound appeared exclusively in the leaves of all eight species tested (47.37 μg g^{-1} ± 15.55 (SE)) and was detectable in soil samples (2.64 μg g^{-1} ± 1.37(SE)) confirming it as a suitable marker in this study area. Another short-chain ω-hydroxy acid identified in this study was methyl 18-hydroxy-cis-9,10-epoxyoctadecanoate, which can be classified more specifically as an epoxy acid and is discussed in the sub-section "C_{18} 9,10-eopxy acids". The remaining short-chain ω-hydroxy acids identified in my study were the $C_{16:0}$ methyl 16-hexadecanoate, the $C_{18:0}$ 18-hydroxy octadecanoic acid and the $C_{18:1}$ methyl 18-hydroxy-9-octadecenoate. Methyl 16-hydroxy-hexadecanoate was found in similar concentrations in the leaves and roots of the tested species (15.17 μg g^{-1} ± 4.61 (SE) and 14.25 μg g^{-1} ± 4.03 (SE) respectively), as was methyl 18-hydroxy-9-octadecenoate (26.29 μg g^{-1} ± 11.13 (SE) in leaves and 34.97 μg g^{-1} ± 6.74 (SE) in roots). Both methyl 16-hydroxy-hexadecanoate and 18-hydroxy-9-octadecenoate were detectable in soil samples, in concentrations of 4.05 μg g^{-1} ± 1.23 (SE) and 13.64 μg g^{-1} ± 8.10 (SE) respectively. The presence of these two compounds in the soil as well as in the majority of the species tested suggest it is an appropriate marker for cutin and suberin derived carbon in the study area. Finally, 18-hydroxy octadecanoic acid was detected in the roots of six species (1.76 μg g^{-1} ± 0.25 (SE)), and the leaves of one species (1.50 μg g^{-1}), however it was not found in any of the soil samples, likely due to its already low concentration in plant tissues, and is not a suitable marker for the study area.

ω-hydroxy acids with chain lengths greater than 20 carbon atoms have often been considered as biomarkers for suberin and roots (Nierop, 1998 and Otto et al. 2005), however, while some studies have corroborated these findings, others have found contradictory results. In a study on maize and wheat by Mendez-Millan et al. (2010a), a large proportion of ω-hydroxy acids with $>C_{20}$ were derived from aboveground tissues, which seems to confirm their use as marker for suberin, however, in a similar study by Mendez-Millan et al. (2010b) $\omega C_{22:0}$ and $\omega C_{26:0}$ were found in both leaves and roots. ω-hydroxyalkanoic acids are also major products of the base hydrolysis of bark and roots and may be considered as derived from suberin when identified in soils (Kolattukudy and Espelie, 1989; Bernards, 2002). However, leaf waxes of grasses and conifers also contain polymers that may be broken down into ω-hydroxy alkanoic acids, such as estolides (von-Rudloff, 1959;, Bull et al. 2000), so the use of this group of chemicals as markers for suberin may not be appropriate in areas with high abundances of conifers and/or grasses. These conflicting findings highlight the importance of identifying the markers of the dominant species in the study area, as well as the challenge of finding consistent markers for cutin and suberin across species.

In my study, two monomers that could be classified as long-chain ω-hydroxy acids C_{20}-C_{32}, and therefore as suberin markers (Otto and Simpson, 2006) were identified. Methyl 22-hydroxydocosanoate was found in the roots of only three species (1.86 $\mu g\ g^{-1} \pm 0.77$ (SE)) and while methyl 20-eicosanoate was found in the roots of five species (1.11 $\mu g\ g^{-1} \pm 0.12$(SE)), neither were detectable in soil samples. While other long chain acids were identified in my samples, such as pentacosanoic acid and triacontanoic acid, they did not meet the specifications suggested by Otto and Simpson (2006) that would allow them to be considered a suberin marker. As a result of their conflicting results, Mendez-Millan et al. (2010a, 2010b) suggest the use of α,ω-alkanedioc acids as markers for below ground tissues, and mid-chain hydroxy acids as markers for aboveground tissues (Fig 3 in Mendez-Millan et al. (2010b)). Mid-chain hydroxy acids have already been discussed in the above sub-section “mid-chain

hydroxy acids" and showed some potential as reliable cutin markers. Alkanedioc acids are discussed below, in the sub-section "Diacids".

Diacids

Diacids show promise as markers for suberized tissues. α,ω-dioc acids make up 2 – 33 % of the aliphatic domain of suberin (Bernards, 2002), and Kögel-Knabner (2002) reported alkanedioic acids as important constituents of suberin. Studies have found α,ω-alkanedioic acids exclusively in roots (Mendez-Millan et al. 2010a, 2010b), with α,ω-alkanedioc acids of chain lengths C_{12}-C_{30} comprising up to 33% of suberin-derived hydrolysis products (Kolattukudy and Espelie, 1989; Bernards, 2002). Otto and Simpson (2006) suggest long chain α,ω-diacids (C_{20}-C_{32}) and 9,10-epoxy C_{18} diacids as suberin markers, C_{16} mono- and dihydroxy diacids as cutin markers, and short chain α,ω-diacids (C_{16}, C_{18} and $C_{18:1}$) as cutin and suberin markers.

In my study, I found three diacids that qualify as suberin markers, two of which fall under the category of long chain α,ω-diacids (C_{20}-C_{32}): eicosanedioic acid, and docosanedioic acid. Eicosanedioic acid was present in the roots of all species (1.97 μg g^{-1} ± 0.35(SE)) as well as in the bark of *Populus tremuloides* (1.34 μg g^{-1}) and was present in soil samples (1.10 μg g^{-1} ± 0.35 (SE)). Docosanedioic acid was present in the roots of seven species (it was not found in *Aralia nudicaulis* roots) and the bark of *Populus tremuloides* in similar concentrations (2.26 μg g^{-1} ± 0.37 (SE)) and was detectable in soil samples (1.92 μg g^{-1} ± 1.07 (SE)). Long chain α,ω-diacids were not identified in any leaf samples of the species tested, and as such, may be suitable markers for suberin in the study area. An additional long chain α,ω-diacid suberin marker, tetracosanedioic acid, was identified in soil samples (0.89 μg g^{-1} ± 0.44 (SE)), but was not identified in any plant tissue samples. It is likely that tetracosanedioic acid came from the suberin of other species present in the study area that were not analysed in this study, however, additional research needs to be done to confirm this. The third diacid suberin marker identified in my study was one that can be categorized as a 9,10-epoxy C_{18} diacid: 9,10-dihydroxy-1,18-octadecanedioic

acid, however; this diacid was only found in relatively low concentrations (2.82 μg g^{-1} ± 0.13 (SE)) in the roots of two of the tested species and was found in higher concentrations (7.26 μg g^{-1} ± 3.16 (SE)) in the leaves of three species. If classified more generally as a short-chain α,ω-diacid, 9,10-dihydroxy-1,18-octadecanedioic acid may be considered a cutin and suberin marker, which may be more suitable in this case, however, this diacid was not identified in soil samples, which limits its potential as a marker in the study area. As suggested by Mendez-Millan et al. (2010b), long chain α,ω-diacids may be a more reliable suberin marker than the long-chain ω-hydroxy acids discussed in the subsection "ω-hydroxy alkanoic acids".

Mono- and dihydroxy C_{16} diacids are listed as a cutin marker in Otto and Simpson (2006), one of which was identified in my study: 9,10-dihydroxy-1,16-hexadecanedioic acid. This diacid was found only in the leaf samples of one species, *Aralia nudicaulis*, at a concentration of 23.80 μg g^{-1}, but was detectable in concentrations of 3.55 μg g^{-1} ± 2.05 in soils. While this class of diacids may not be appropriate for a more general cutin marker, it may have some potential as an *A. nudicaulis* cutin marker in the study area, however, tests on additional species from the area would need to be conducted to confirm this.

Four monomers that can be classified as short-chain α,ω-diacids were identified in my samples. One of these, 9,10-dihydroxy-1,16-hexadecanedioic acid, can be classified into a more specific category, making it a cutin marker, as discussed in the previous paragraph. The other three monomers: hexadecanedioic acid, octadecanedioic acid, and methyl octadecene-1,18 dioate do not possesses any properties that would allow for more refined classification and as such, their use as a more specific marker. Hexadecanedioic acid was found in both leaves and roots of almost all species (it was not detected in the leaves of *Rubus ideaus*) in concentrations of 3.69 μg g^{-1} ± 0.86 (SE) in leaves and 7.78 μg g^{-1} ± 2.15 (SE) in roots. Methyl octadecene-1,18 dioate was present in the leaves of four species (3.63 μg

g^{-1} ± 0.76 (SE)) and the roots of all eight species (18.80 μg g^{-1} ± 3.11 (SE)). Interestingly, octadecanedioic acid was present in the roots of all eight species (4.74 μg g^{-1} ± 1.29 (SE)), but was not found in any leaf samples, despite its classification as a cutin and suberin marker. It is tempting to suggest that the specific subset of α,ω-alkanedioic acids that octadecanedioic acids belongs to may be better suited as a criteria for suberin markers, rather than a cutin and suberin marker, this study investigates eight species common to the study area, and it is likely that similar compounds may appear in the cutin of other species in the area. However, the seemingly unique presence of the C_{18} α,ω-alkanedioic acid in suberized tissues illustrates the interspecific variation of suberin when compared to the results of Otto and Simpson (2006). All three of hexadecanedioic acid, octadecanedioic acid and methyl octadecene-1,18 dioate were identified in soil samples in concentrations of 3.43 μg g^{-1} ± 0.92 (SE), 1.75 μg g^{-1} ± 0.75 (SE), and 7.67 μg g^{-1} ± 4.30 (SE) respectively, suggesting that they are good markers for the study area.

Suberin composition in bark and roots

There is little information regarding similarities or differences in the components of bark suberin compared to root suberin of the same species (Otto 2006). Only one tree species was included in my study (*Populus tremuloides*), however, I was able to identify compounds that were only found in suberin derived from bark or roots. Three compounds were identified only in bark that were not found in the roots or leaves of the other seven species in this study: Benzene, 1-methoxy-2-(methoxymethyl)-, Vanillin and Antiangor, however, Antiangor, is the closest match returned from the GCMS, but since I cannot be 100% confident in the identify of this compound, I will not discuss it further. The first two are aromatic compounds that are commonly derived from a variety of sources, including lignin, and cannot be used as markers. Six monomers were identified only in the roots of *P. tremuloides*, and not in bark tissues: Benzoic acid, 4-methoxy-, 4-(Methoxycarbonyl)phenol, Octanoic acid, 8-hydroxy-, Nonanoic acid, 9-hydroxy-, Eicosen-1-ol, cis-9-, and subric acid. If considering compounds that appeared in only *P. tremuloides* bark or roots but may also have appeared in other species, an additional three compounds

were present in bark suberin that were not found in root suberin, and nine were identified in root suberin that were not found in bark suberin (Table A4). Root and bark suberin of *P. tremuloides* shared the presence of 25 monomers. While these compounds do not meet any of the qualifications outlined by Otto and Simpson (2006) to be used as markers, they do provide insight into the differences in suberin composition within a single species.

Interspecific variation in cutin and suberin composition

In addition to little information on variation in components of suberin, there is perhaps less on plant group or species specificity (Otto and Simpson, 2006). In my study, I was able to identify a small number of compounds that were unique to a single species of the eight species tested. These include, but are not limited to, 9,10-dihydroxy-1,16-hexadecanedioic acid in *Aralia nudicaulis* leaves, Eicosen-1-ol, cis-9- in *Populus tremuloides* bark, 9,11-octadeadienoic acid in the roots and leaves of *Rubus idaeus*, and Hentriacontane and Tetratriacontane in *Rosa acicularis* leaves. Some of the apparently species-specific compounds fall into the categories for cutin or suberin markers as defined by Otto and Simpson (2006), such as 10-Hydroxypentadecanoic acid, which is a cutin marker, but was only identified in the leaves of *Prunus pensylvanica*. The majority of these "species specific" compounds are not markers for any specific tissue type, and may not be unique to cutin, suberin, or plants. Additionally, an investigation of more species may find compounds that appeared only in one of the eight species tested in this study in other species. Just as interesting as compounds that appear to be specific to the cutin or suberin of a single species, are the compounds that appear in the cutin or suberin of all but one or two species, especially when it is present in high concentrations. For example, Methyl 16-hydroxy-hexadecanoate was found in the leaves (15.17 $\mu g\ g^{-1} \pm 4.61$ (SE)) of all eight species and in the roots (14.28 $\mu g\ g^{-1} \pm 04.03$ (SE)) of all but one species, *Aralia nudicaulis*. The full list of compounds and their concentrations in tissue types of the eight species tested is available in table A4.

Proportion of cutin and suberin in soil samples

Otto and Simpson (2006) suggest a formula for calculating the suberin/cutin ratio. The formula they present is a revision of calculations used by other papers, is as follows: $(\sum S + \sum S\vee C)/(\sum C + \sum S\vee C)$. Where ∑S is the sum of the mass of suberin markers, ∑C is the sum of the mass of cutin markers, and ∑SvC is the sum of the mass of cutin and suberin markers (wherein 'markers' refer to those listed in Table 4 of Otto and Simpson (2006)).

In my study, only a small number of monomers classified as markers by Otto and Simpson were present in the soil. I found three suberin markers in the soils, one of which was not present in any tissue samples of the species tested (Tetracosanedioic acid, dimethyl ester). Additionally, three cutin markers were found in hydrolyzed samples, one of which could also be classified as a cutin and suberin marker. When selecting values for the calculation, I chose to exclude the suberin marker that was not present in any of my plant tissue samples. The ratio I calculated for these soil samples was 0.92. However, due to the limited monomers returned from soil samples, this ratio is likely to be unreliable.

Root identification by DNA-based methods

Based on aboveground plant surveys, the dominant understory species at the Woodbend Research Forest site were *Alnus*, *Corylus*, and *Rosa*. *Alnus* roots were only ever identified in samples from the 0 – 7.5 cm depth increment, *Rosa* roots were only identified in the 7.5 – 15 cm and 15 – 22.5 cm increments, and *Corylus* roots were not identified in any samples. PCR failed for all three regions for four samples in the 0 – 7.5 cm range, three regions in the 7.5 – 15 cm range, four samples in the 15 – 22.5 cm range, and seven samples in the 22.5 – 30 cm range. *Alnus* roots may have been present in any of the 14 failed samples from depths greater than 7.5 cm, however, this cannot be confirmed. There is also a possibility that *Corylus* roots may have been present in some of these unresolved samples, however, given its abundance within the plots, it was expected to show up in the majority of samples.

Interestingly, most of the species that resolved have relatively short *trn T-trn L* regions (less than 700), if they had reported *trn T-trn L* regions at all (there are several unknown fragment lengths in Metzler et al 2019). *Corylus* has a reported *trn T-trn L* region of 854 base pairs (Metzler et al 2019). Due to competition between fragments for primers during the PCR process, it may be that longer fragments cannot be replicated enough to be detected when smaller fragments are present (Karst, Chow and Landhäusser, 2015). It is extremely unlikely that hazelnut roots were not present in the samples, due to their sheer abundance at the site and the collection of several cores near the base of *Corylus* shrubs or within a dense cluster of *Corylus*. Another species that was never identified in root DNA samples at the Woodbend site was *Symphoricarpos albus*, which was present in moderate abundance within all plots, and also has a large *trn T-trn L* region (815 base pairs). The only species that was resolved within Woodbend plots with a *trn T-trn L* region greater than 800 base pairs was *Apocynum androsaemifolium*, with a length of 815 base pairs, which had a *trn L* region length 184 and 169 base pairs shorter than *Corylus* and *Symphoricarpos* respectively. Interestingly, *Symphoricarpos albus* was returned by DNA analysis in samples from Cooking Lake.

Other peculiarities in the DNA data included species that always appeared in pairs, and the high occurrence of *Typha latifolia*. *Symphyotrichum boreale* and *Symphyotrichum lanceolatum* subsp. *Hesperium* were always identified together, as both species only have one reported fragment length value in Metzler et al. (2019), a *Trn L-Trn* F region of 432 base pairs. With our current knowledge, we cannot differentiate these two species using FALP analysis alone, as both species will appear even if only one is present. *Typha latifolia* was identified in 67% of samples from Cooking Lake, and 51% of samples from Woodbend, and was not constrained to any depth at either site. Similar to the *Symphyotrichum* species previously mentioned, *T. latifolia* only has one fragment length reported in Metzler et al. (2019), a *Trn L-Trn F* region of 389 base pairs. *Typha latifolia* tends to grow in wetlands, and other moist soils. The Cooking Lake Recreation area has several low-lying depressions and wetlands, so while our plots did

not contain *T. latifolia*, it is possible that seed fibers have blown into the forested areas and been worked into soils, which then clung to roots during out extraction and washing procedures. While wet areas in the sandy Woodbend site were less common, a few were present, and *T. latifolia* DNA may have entered the soil in a similar manner. Alternatively, *T. latifolia* is sometimes temporarily present in young deciduous forests (ABMI, 2019).

Marker distributions in soil

One of the possible uses of cutin- and suberin-specific markers lies in the investigation of carbon contributions of below and aboveground tissues with varying depth in the soil. Spielvogel et al. (2014) investigated the distribution of cutin and suberin-specific markers in the soil beneath four different tree species and found that patterns of suberin distribution often closely followed rooting patterns. Not surprisingly Spielvogel et al. (2014) found that concentrations of suberin-derived compounds decreased with increasing depth, with the decrease becoming more rapid as distance from the tree increased. Spielvogel et al. (2014) also found a decrease in cutin-derived compounds with increasing depth, however, the rate of decrease was not the same beneath all tree species investigated. Additionally, no pattern of cutin-derived compounds was found with increasing horizontal distance from the tree. Inputs from understory species are also likely to influence the distribution of cutin-derived monomers with increasing soil depths and horizontal distances from the tree. In my study, the identification of what appear to be species-specific markers of common species in dry-mixed wood forests, may help future studies assess the influence of understory species in the distribution of cutin and suberin-derived compounds. These species-specific monomers may assist in separating cutin and suberin derived from a single species of interest, versus inputs from other species present in the area – such as in Spielvogel et al. (2014) where they suggest that understory species may have confounded any pattern of cutin input from their species of interest. Given the limited number of cutin or suberin markers found in soil

samples, as well as the overlap in cutin and suberin markers and monomers I found in my study, it is not possible to distinguish aboveground and belowground inputs with certainty based on these data alone.

Directions for future studies

All samples used for cutin and suberin analysis were collected from the Woodbend research forest. The soil at this site was predominantly sandy (Table 1) with a thin organic layer at the surface. Soil properties and root biomass were measured with depth to 40 cm at two sites of differing textures to aid in project design and sampling depths of future studies. At both the Woodbend and Cooking Lake sites, total and small diameter root biomass decreased with depth, with the decrease in small diameter root biomass being slightly more pronounced at the Woodbend site (Table 3). When sampling soils for cutin and suberin analysis to determine the extent of the rooting depth, should the study call for it, samples could be taken to similar maximum depths despite the significant difference in soil texture between the sites. Many of the species overlapped at these two sites, however, their abundances differed and there were a few species found only at one of the sites (*Mitella diphylla*, *Lonicera spp*. *Cornus stolonifera*, and *Heracleum spp*. Were only identified at the Cooking Lake site, while *Cirsium spp*. And *Achillea millefolium* were only identified at the Woodbend site), and as such the leaf and root specific compounds reported in this study should be useful.

Limitations

It should be noted that measuring the quantities of suberin and cutin markers may not provide accurate representations of plant litter inputs into the soil. The quantity of above *vs.* belowground litter may not be representative of their inputs to the soil (Crow et al., 2009). Some organic matter may take longer to break down than others. This may be due to the type of bonds present, and the energy of activation required to break them, or, the physical, three-dimensional structure of the compound that makes it difficult for enzymes to reach activation sites. The decomposition of complex polymers such as lignin may be limited by the presence of substrates, and not as a result of the polymers chemical

recalcitrance (Klotzbücher et al., 2011). Due to similarities between lignin and cutin and suberin, it is not unlikely that observations of the degradation of lignin may be similar to the degradation of cutin and suberin. Binkley and Fisher (2013) suggest the example wherein we assume equal mass of inputs from above and belowground sources. Even if litter mass is equal, the environments in which these inputs are deposited are different; the roots exist within the moist soil, while leaves are present in a dry environment. The differing environments of these litter inputs mean that soil microbes have different levels of access to these compounds. Also, the sizes of these inputs are different, with the fine roots that are turned over in the soil being much smaller than the larger leaves and twigs. In results from a study on wheat and maize, Mendez-Millan et al. (2010a) suggested that cutin and suberin may be degraded at different rates in the soil, complicating their use as biomarkers for studies with the aim to calculate the proportions of soil carbon derived from above and belowground sources. Studies have suggested that the presence of double bonds in root-indicator acids causes them to be preferentially degraded, leading to an underrepresentation of the root inputs into the soil (Goñi and Hedges, 1990; Feng and Simpson, 2007). Considering the limitations mentioned, cutin and suberin analysis may be limited to quantifying overall plant input to the stable SOM pool (vs. microbial input, animal input etc.), rather than leaf vs. root input to the stable SOM pool in some environments.

Conclusions

This study investigated the composition of cutin and suberin of eight species found in the boreal-mixed wood region of Alberta, Canada. While not all compounds identified can be used as markers (due to their presence in other plant tissues, or from other sources), this study, along with others before it, highlights the importance of using cutin and suberin biomarkers that are appropriate for the plant community present in the study area. Classes of chemicals reported as markers for root or leaf-derived tissues did not hold true for many of the species studied here, or, only held true for some of

the compounds within that chemical class. We identified what may be specific markers for some species, however, due to the limited number of species tested in this study, this cannot be certain. Additionally, this study provides some insight into the composition of suberin derived from bark and roots of a single species; *Populus tremuloides*. Future studies aimed at deriving the above- and belowground plant origins of soil organic matter should consider choosing markers from studies on the same or similar plant species to those that are dominant in their study area.

Tables

Table 1: Mean soil texture of mineral soils at the a) Woodbend Research Forest and b) Cooking Lake Recreation Area, Alberta, Canada. Texture is reported in 10 cm increments from 10 – 40 cm depth. Letters indicate significant differences (or lack thereof) with depth within each site; asterisks denote a significant difference of said soil increment between the two sites.

a) Woodbend Research Forest	Soil Texture		
Depth increment (cm below soil surface)	%Clay*	%Silt*	%Sand*
10-20 (n=5)	5.8±0.40a	11.9±0.44a	82.4±0.40a
20-30 (n=5)	6.2±0.74a	12±1.2a	82.1±1.45a
30-40 (n=5)	6.1±0.60a	10±1.8a	83.7±1.70a

b) Cooking Lake	Soil Texture		
	%Clay*	%Silt*	%Sand*
10-20 (n=4)	18.9±4.94a	45.9±3.67a	35.2±4.30a
20-30 (n=5)	22.5±5.28a	41.8±2.63a	35.8±3.41a
30-40 (n=5)	32.2±5.92a	35.0±6.50a	32.6±2.71a

Silt was log transformed; Permutational ANOVAs were used for clay and sand.

Table 2: Mean soil nutrients, pH, and bulk density (BD) at 10 cm depth increments at the a) Woodbend Research Forest, and b) Cooking Lake Recreation Area, Alberta, Canada. For each site, letters indicate significant differences (or lack thereof) with depth within each site. Asterisks indicate a significant difference in the levels of the nutrient between the two sites.

a) Woodbend Research Forest

Depth increment (cm below soil surface)	Soil properties					
	NH_4^+(mg kg^{-1})*	NO_3^-(mg kg^{-1})	PO_4^{3-}(mg kg^{-1})	K^+(mg kg^{-1})*	pH	BD (g cm^{-3})
10—20 (n=5)	5.74 ±0.67 a	0.96±0.072 a	20.42±6.03 a	134.53±24.66 a	5.2±0.38 a	1.0±0.04 a
20—30 (n=5)	2.67±0.22 b	0.94±0.059 a	16.58±5.12 a	75.93±8.00 a	5.40±0.25 a	1.4±0.08 ab
30—40 (n=5)	1.90±0.37 b	0.95±0.034 a	12.36±5.00 a	69.28±9.66 a	5.74±0.25 a	1.5±0.04 b

b) Cooking Lake

Depth increment (cm below soil surface)	NH_4^+(mg kg^{-1})*	NO_3^-(mg kg^{-1})	PO_4^{3-}(mg kg^{-1})	K^+(mg kg^{-1})*	pH	BD (g cm^{-3})
10—20 (n=4)	5.68±1.18 a	0.95±0.10 a	13.69±9.67 a	203.75±85.06 a	5.05±0.35 a	1.2±0.09 a
20—30 (n=5)	5.69±0.53 a	1.12±0.25 a	17.64±10.21 a	196.09±42.90 a	5.28±0.46 a	1.4±0.09 ab
30—40 (n=5)	4.84±0.99 a	1.20±0.29 a	11.52±6.70 a	191±40.22 a	5.36±0.54 a	1.5±0.07 b

ANOVA tests of logarithmic values were done for: NH_4^+, K^+, and pH; Permutational ANOVA tests were done for: NO_3^-, PO_4^{3-}, and bulk density (BD).

Table 3: Mean root biomass at a) Woodbend research forest, and b) Cooking Lake Recreation Area, Alberta, Canada. Values are reported for 10 cm increments from 0 – 40 cm. Small diameter roots: ≤ 5 mm diameter; Medium diameter roots: 5 – 10 mm diameter. Letters denote significant differences (or lack thereof) with depth within each site; there were no significant differences in the root biomass between the two sties.

a) Woodbend Research Forest

Depth increment (cm below soil surface)	Biomass (g) Total	Small diameter	Medium diameter
0-10 (n=20)	1.7+/-0.31 a	0.62+/-0.047 a	0.89+/-0.25 a
10-20 (n=20)	1.1+/-0.20 ab	0.39+/-0.030 b	0.55+/-0.081 ab
20-30 (n=20)	0.47+/-0.092 bc	0.20+/-0.028 c	0.22+/-0.057 b
30-40 (n=20)	0.26+/-0.061 c	0.085+/-0.014 d	0.17+/-0.061 b

b) Cooking Lake

	Total	Small	Medium
0-10 (n=20)	1.7+/-0.21 a	0.65+/-0.039 a	0.66+/-0.12 a
10-20 (n=20)	0.97+/-0.30 ab	0.28+/-0.031 b	0.57+/-0.21 a
20-30 (n=20)	0.40+/-0.10 b	0.11+/-0.015 c	0.25+/-0.082 a
30-40 (n=20)	0.39+/-0.12 b	0.11+/-0.032 c	0.26+/-0.11 a

Biomass for small diameter and total roots were log transformed for ANOVA.

Table 4: Compounds recovered in organic matter of soils of Woodbend Research Forest, Alberta Canada previously classified as markers by Otto and Simpson (2006). Reference tissue(s) in which these compounds were found is denoted by the presence of an "x".

Otto and Simpson 2006	Chemical Classification	Compound found in soil	Leaves	Roots	Bark
Suberin marker	ω-Hydroxy acids (C_{20}-C_{32})	absent			
	α,ω-Diacids (C_{20}-C_{32})	Docosanedioic acid, dimethyl ester		x	x
		Eicosanedioic acid, dimethyl ester		x	x
		Tetracosanedioic acid, dimethyl ester			
	Epoxy Dioc Acids (9,10-ep C_{18} DA)	absent			
Cutin marker	Mid-chain hydroxy C_{14}, C_{15}, C_{17} acids (*x*-OH $C_{14:1}$, *x*-OH C_{15}, *x*-OH C_{17})	absent			
	C_{16} Mono- and dihydroxy acids and diacids (*x*-OH C_{16}, *x*,ω-OH C_{16}, *x*-OH C_{16} DA)	9,10-dihydroxy-1,16-hexadecanedioic acid	x		
		9 and 10, 16-dihydroxy-hexadecanoate acid and derivatives	x		
Suberin or cutin marker	short chain ω-hydroxy acids	Methyl 16-Hydroxyhexadecanoate and derivatives	x	x	x

(C_{16}, C_{18}, $C_{18:1}$, $C_{18:2}$)				
	Methyl 18-hydroxy-9-octadecenoate and derivatives	x	x	x
C_{18} di- and trihydroxy acids (x,ω-OH C_{18}, 9,10,ω-OH C_{18})	Octadecanoic acid, 9,10-dihydroxy-, methyl ester, (R*,R*)- and derivatives	x		
C_{18} epoxy hydroxy acids (9,10-ep-ω-OH C_{18})	absent			
Short chain α,ω - diacids (C_{16}, C_{18}, $C_{18:1}$)	Octadecanedioic acid, dimethyl ester		x	x
	Hexadecanedioic acid	x	x	x
	Methyl octadecene-1,18 dioate and derivatives	x	x	x

Table 5: Monomers identified in specific tissue samples from eight species tested that were found in soil samples but not classified as markers by Otto and Simpson (2006). Reference tissue(s) in which these compounds were found are denoted by the presence of an "x".

Compound Class	Monomer	Leaves	Roots	Bark
Phenols	4-(Methoxycarbonyl)phenol		x	
	Benzoic acid, 4-methoxy-, methyl ester		x	
	Methylparaben		x	x
Alkanols	1-Hexadecanol	x		
Alkanoic Acids	Tetradecanoate	x		
	Octacosanoic acid	x		
Alkenes	Neophytadiene	x		
Alkenols	Eicosen-1-ol, cis-9-		x	
Alkenoic Acids	Oleic acid		x	
	12,15-Octadecadienoic acid		x	
Other	1,1,3-Triallyl-2-thiourea		x	

Table 6: Monomers classified as markers by Otto and Simpson (2006), and their presence in plant tissues analyzed in the current study. Reference tissue(s) in which these compounds were found are denoted by the presence of an "x". When compounds meet more than one classification criteria, they are listed under the more specific category, or, if the categories are equally specific, the compound is listed under both.

Otto and Simpson (2006)		Identified compound from current study	Leaves	Roots	Bark
Suberin markers	ω-Hydroxy acids (C_{20}-C_{32})	Methyl 20-Hydroxyeicosanoate and derivatives		x	
		Methyl 22-hydroxydocosanoate and derivatives		x	
	α,ω,-Diacids (C_{20}-C_{32})	Eicosanedioic acid, dimethyl ester		x	x
		Docosanedioic acid, dimethyl ester		x	x
	Epoxy Dioc Acids (9,10-ep C_{18} DA)	9,10-Dihydroxy-1,18- octadecanedioc acid and derivatives	x	x	
Cutin markers	Mid-chain hydroxy C_{14}, C_{15}, C_{17} acids (*x*-OH $C_{14:1}$, *x*-OH C_{15}, *x*-OH C_{17})	10-Hydroxypentadecanoic acid, methyl ester	x		
		9-Hydroxypentadecanoic acid, methyl ester	x		
	C_{16} Mono- and dihydroxy acids and diacids (*x*-OH C_{16}, *x*,ω-OH C_{16}, *x*-OH C_{16} DA)	9,10-dihydroxy-1,16-hexadecanedioic acid, dimethyl ester	x		
		9 and 10,16-dihydroxy-hexadecanoate and derivatives	x		
Suberin or cutin markers	short chain ω-hydroxy acids (C_{16}, C_{18}, $C_{18:1}$, $C_{18:2}$)	Methyl 16-hydroxy-hexadecanoate and derivatives	x	x	X
		18-hydroxy octadecanoic acid and derivatives	x	x	

	Methyl 18-hydroxy-9-octadecenaote and derivatives	x	x	x
C_{18} di- and trihydroxy acids (x,ω-OH C_{18}, 9,10,ω-OH C_{18})	Methyl 18-hydroxy-cis-9,10-epoxyoctadecanoate	x		
	Octadecanoic acid, 9,10-dihydroxy-, methyl ester (R*,R*)- and derivatives	x		
	9,10,18-trihydroxy octadecanoate	x		
C_{18} epoxy hydroxy acids (9,10-ep-ω-OH C_{18})	9,10-dihydroxy-1,18-octadecanedioc acid and derivatives	x	x	
Short chain α,ω -diacds (C_{16}, C_{18}, $C_{18:1}$)	Hexadecanedioic acid DME	x	x	x
	Octadecanedioic acid, dimethyl ester		x	x
	Methyl octadecene-1,18 dioate and derivatives	x	x	x

Figures

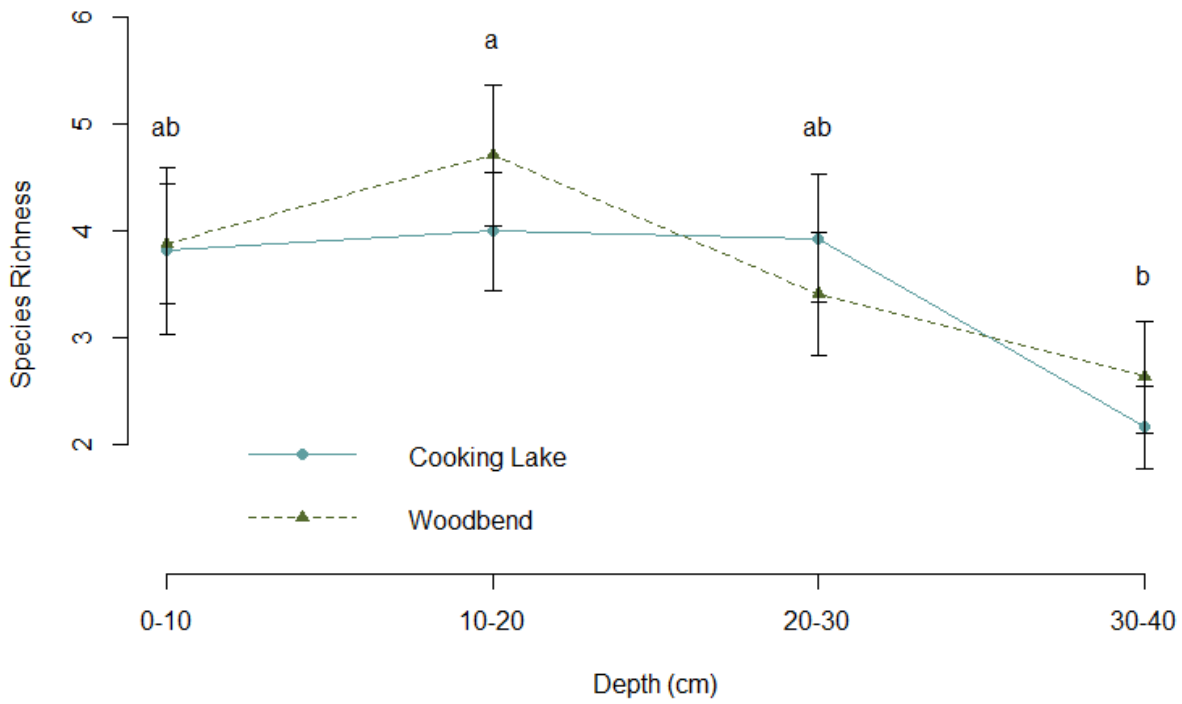

Figure 1: The number of species returned by DNA analysis of soil cores from Woodbend Research Forest and Cooking Lake Recreation Area near Edmonton Alberta, Canada. Bars represent standard error. Analysis showed no significant effect of site on species richness, however, there was a significant difference in the number of species found between the 10 – 20 cm increment and 30 – 40 cm increment.

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Appendix

Table A1: ANOVA and Permutational ANOVA tables of soil and root variables.

Response Variable: **NH_4^+**

Test: ANOVA(log(NH_4^+))	DF	Sum Sq	F Value	Pr (>F)
Depth	2	2.13	9.10	0.00
Site	1	2.28	19.41	0.00
Depth × Site	2	1.23	5.22	0.01
Residuals	23	2.70		

Response Variable: **NO_3^-**

Test: Permutational ANOVA	DF	Sum Sq	Iter	Prob
Depth	2	0.07	169	0.82
Site	1	0.13	63	0.62
Depth × Site	2	0.09	110	0.77
Residuals	23	3.32		

Response Variable: **K^+**

Test: ANOVA(log(K^+))	DF	Sum Sq	F Value	Pr (>F)
Depth	2	0.50	0.74	0.49
Site	1	3.21	9.55	0.01
Depth × Site	2	0.60	0.90	0.42
Residuals	23	7.72		

Response Variable: **PO_4^{3-}**

Test: Permutational ANOVA	DF	Sum Sq	Iter	Prob
Depth	2	173.1	173	0.55
Site	1	33.9	51	0.78
Depth × Site	2	76.3	74	0.74

Residuals	23	5871.0		
Response Variable: **pH**				
Test: ANOVA(log(pH))	DF	Sum Sq	F Value	Pr (>F)
Depth	2	0.02	0.44	0.65
Site	1	0.20	0.84	0.37
Depth × Site	2	0.00	0.077	0.93
Residuals	23	0.54		
Response Variable: **bulk density**				
Test: Permutational ANOVA	DF	Sum Sq	Iter	Prob
Depth	2	0.81	5000	<2e-16
Site	1	0.07	710	0.12
Depth×Site	2	0.05	224	0.34
Residuals	23	0.57		
Response Variable: **% clay**				
Test: Permutational ANOVA	DF	Sum Sq	Iter	Prob
Depth	2	242.98	700	0.23
Site	1	2484.22	5000	<2e-16
Depth × Site	2	228.57	842	0.19
Residuals	23	1572.88		
Response Variable**: % silt**				
Test: ANOVA(log(%Silt))	DF	Sum Sq	F Value	Pr (>F)
Depth	2	0.37	2.67	0.09
Site	1	11.92	174.11	3.249e-12
Depth × Site	2	0.01	0.10	0.91
Residuals	23	1.57		

Response Variable: **% sand**

Test: Permutational ANOVA	DF	Sum Sq	Iter	Prob
Depth	2	3.5	51	0.84
Site	1	16730.8	5000	<2e-16
Depth × Site	2	31.4	53	0.77
Residuals	23	701.3		

Response Variable: **Small diameter root biomass**

Test: ANOVA(log(Small diameter root biomass +1))	DF	Sum Sq	F Value	Pr (>F)
Depth	3	3.76	131.36	<2e-16
Site	1	0.03	3.26	0.07
Depth × Site	3	0.01	3.41	0.02
Residuals	152	1.45		

Response Variable: **Medium diameter root biomass**

Test: ANOVA(Medium diameter root biomass)	DF	Sum Sq	F Value	Pr (>F)
Depth	3	8.76	7.59	9.15e-05
Site	1	0.02	0.04	0.83
Depth × Site	3	0.58	0.50	0.68
Residuals	152	58.44		

Response Variable: **Total Root Biomass**

Test: ANOVA((log(Total root biomass))	DF	Sum Sq	F Value	Pr (>F)
Depth	3	130.51	39.38	<2e-16

Site	1	0.50	0.45	0.50
Depth × Site	3	3.83	1.16	0.33
Residuals	152	167.91		

Response Variable: **Species Richness**

Test: ANOVA (species richness)	DF	Sum Sq	F Value	Pr (>F)
Depth	3	51.74	3.60	0.02
Site	1	1.00	0.21	0.65
Depth × Site	3	5.99	0.42	0.74
Residuals	101	484.32		

Table A2: Belowground occurrence of species returned by DNA analysis, and their aboveground presence at the sampling site (note: field sampling was done in late August 2018, and plant identification was not always possible, especially to a species level)

Species of roots identified by DNA	Identified in soils from Cooking Lake	Identified within 10 x 10 m plots at Cooking lake	Identified in soils from Woodbend	Identified within 10 x 10 m plots at Woodbend
Abies balsamea (Linnaeus) Miller			yes	
Alnus alnobetula subsp. crispa (Aiton) Raus			yes	Alnus sp. present
Alnus incana subsp. tenuifolia (Nuttall) Breitung			yes	Alnus sp. present
Amelanchier alnifolia (Nuttall) Nuttall ex M. Roemer	yes	yes		
Apocynum androsaemifolium Linnaeus	yes		yes	yes
Arabis ssp. Linnaeus	yes		yes	
Aralia nudicaulis Linnaeus	yes	yes		
Betula glandulosa Michaux	yes			
Betula pumila Linnaeus	yes			
Botrypus virginianus (Linnaeus) Michaux	yes			
Carex concinna R. Brown	yes		yes	
Castilleja miniata Douglas ex Hooker	yes			
Corallorhiza trifida Chftelain	yes		yes	
Cornus Stolonifera Michaux	yes	yes		
Corydalis aurea Linnaeus	yes			

Equisetum arvense Linnaeus	yes	Equisetum sp. present		
Equisetum palustre Linnaeus	yes	Equisetum sp. present		
Equisetum pratense Ehrhart			yes	Equisetum sp. present
Eurybia conspicua (Lindley) G.L. Nesom	yes	Unidentified asters present	yes	
Fragaria virginiana Miller	yes	yes	yes	yes
Galeopsis tetrahit Linnaeus			yes	
Lathyrus ochroleucus Hooker	yes	yes	yes	yes
Lepidium densiflorum Schrader	yes	Several grass species present		
Leymus innovatus subsp. innovatus (Beal) Pilger	yes	Several grass species present		
Lonicera dioica Linnaeus	yes			
Lonicera involucrata (Richardson) Banks ex Sprengel	yes	yes	yes	
Maianthemum canadense Desfontaines	yes	yes	yes	yes
Myrica gale Linnaeus	yes			
Orthilia secunda (Linnaeus) House			yes	yes
Picea glauca (Moench) Voss	yes	yes	yes	
Picea mariana (Miller) Britton, Sterns & Poggenburgh	yes	yes	yes	yes
Pinus banksiana Lambert			yes	yes

Petasites frigidus var. palmatus (Aiton) Cronquist			yes	yes
Populus balsamifera Linnaeus	yes	yes	yes	yes
Populus tremuloides Michaux	yes	yes	yes	yes
Potentilla norvegica Linnaeus	yes		yes	
Rosa acicularis Lindley	yes	yes	yes	yes
Rosa woodsii Lindley	yes			
Rubus arcticus Linnaeus	yes			
Rubus chamaemorus Linnaeus	yes			
Salix myrtillifolia Andersson			yes	
Salix spp. Linnaeus	yes	yes	yes	yes
Sanicula marilandica Linnaeus	yes	yes		
Sibbaldia tridentata (Aiton) Paule & Soják			yes	
Solidago canadensis Linnaeus			yes	
Sorbus scopulina Greene	yes			
Streptopus amplexifolius (Linnaeus) de Candolle	yes		yes	
Symphoricarpos albus (Linnaeus) S.F. Blake	yes			
Symphyotrichum sp.	yes	yes	yes	yes
Symphyotrichum ciliolatum (Lindley) A. Love & D. Love	yes		yes	
Thlaspi arvense Linnaeus			yes	
Trifolium pratense Linnaeus	yes		yes	
Typha latifolia Linnaeus	yes		yes	

Vaccinium microcarpum (Turczaninow ex Ruprecht) Schmalhausen	yes	yes		
Vaccinium myrtilloides Michaux	yes			
Vaccinium vitis-idaea Linnaeus			yes	yes

Table A3: Tissue-specific monomers recovered from reference roots, leaves or bark not classified as markers as per Otto and Simpson (2006).

Compound Class	Compound name	Leaves	Roots	Bark
Phenols	Methylparaben		x	x
	Benzoic acid, 4-methoxy-, methyl ester		x	
	Benzene, 1-methoxy-2-(methoxymethyl)-			x
	Benzoic acid 3,4 dihydroxy, methyl ester and derivatives		x	x
	4-(Methoxycarbonyl)phenol		x	
	Ferulic acid, methyl ester		x	
	Vanillin			x
	2-Methoxy-4-vinylphenol		x	x
	Isoeugenol		x	
Alkanes				
	Nonacosane	x		
	Hentriacontane	x		
	Tetratriacontane	x		
Alkanols				
	8-Pentadecanol		x	
	1-Hexadecanol	x		
Alkanoic Acids				
	Octanoic acid, methyl ester		x	
	Octanoic acid, 8-hydroxy-, methyl ester		x	
	Nonanoic acid, 9-hydroxy-, methyl ester		x	
	Dodecanoic acid, methyl ester	x		
	Tetradecanoate and Derivatives	x		
	methyl-2-hydroxy-tricosanoate		x	
	Octacosanoic acid, methyl ester	x		
	Triacontanoic acid, methyl ester	x		
Alkenes				
	Neophytadiene	x		
Alkenols				
	Phytol	x		
	Eicosen-1-ol, cis-9-		x	
Alkenoic Acids				
	Pentadecanoic acid, methyl ester		x	
	11-Hexadecenoic acid, methyl ester	x		
	Methyl hexadec-9-enoate	x		
	Oleic acid and Derivatives		x	
	cis-13-Octadecenoic acid, methyl ester		x	

	(+-)-4-Ethoxy-5-methyl-2,5-dihydrofuran-2-one	x		
	12,15-Octadecadienoic acid, methyl ester		x	
	Methyl 9-eicosenoate		x	
	11-Eicosenoic acid, methyl ester		x	
	13-Docosenoic acid, methyl ester, (Z)-		x	
Alkynoic Acids				
	7,10,13-Hexadecatrienoic acid, methyl ester	x		
	Methyl 9.cis.,11.trans.t,13.trans.-octadecatrienoate	x		
Diacids				
	Subric acid and Derivatives		x	
Nitrogen Compounds				
	β-Alanine, N,N-diethyl-	x		
Other Acids				
	ω-3 Arachidonic Acid methyl ester	x		
Unclassified				
	Antiangor			x
	Dimethyl trans-1,2-Cyclopropanedicarboxylate		x	
	1,1,3-Triallyl-2-thiourea		x	

Table A4: Concentrations (µg g^{-1} of hydrolysable tissue) of monomers recovered by base hydrolysis of cutin and suberin found in tissues of different species. A green row header indicates it was only found in leaf tissues, a brown row header that it was only found in suberized tissues (roots or roots and bark), and a yellow row header indicates it was only found in bark samples from *Populus tremuloides*.

		ug g^{-1} of hydrolysable tissue																
compound name	**compound class**	*Aralia nudicaulis* leaves	*Aralia nudicaulis* roots	*Apocynum androsaemifolium* leaves	*Apocynum androsaemifolium* roots	*Corylus cornuta* leaves	*Corylus cornuta* roots	*Populus tremuloides* leaves	*Populus tremuloides* roots	*Populus tremuloides* bark	*Prunus pensylvanica* leaves	*Prunus pensylvanica* roots	*Rosa acicularis* leaves	*Rosa acicularis* roots	*Rubus idaeus* leaves	*Rubus idaeus* roots	*Symphoricarpos albus* leaves	*Symphoricarpos albus* roots
	Phenols																	
Benzoic acid + derivatives	B/Phenol							6.05	5.38	16.75	1.40	2.25						
Methylparaben	B/Phenol								10.32	2.51						0.52		
Benzoic acid, 4-methoxy-, methyl ester	B/Phenol								2.22									
Benzene, 1-methoxy-2-(methoxymethyl)-	B/Phenol									1.13								
Benzoic acid, 3,4-dihydroxy-, methyl ester + derivatives	B/Phenol		2.25						1.17	3.22						0.49		1.30
Benzoic acid, 3,4,5-trimethoxy-, methyl ester	B/Phenol					0.96	1.40							1.17	2.63			
2-propenoic acid, 3-hydroxy-, methyl ester + derivatives	Phenols	2.05	0.82	1.91		5.61			2.19	40.97	7.51		5.03		7.11		17.80	1.79
4-(Methoxycarbonyl)phenol, TMS derivative	B/Phenol								2.94									
2-Propenoic acid, 3-(3,4-dihydroxy), methyl ester + derivatives	B/Phenol		12.93		3.06		13.85		9.89	82.55	1.24	21.34	1.34	3.51	1.44	7.43	9.53	16.02
Ferulic acid, methyl ester, O-TMS	B/Phenol											3.14						1.05
Vanillin	B/Phenol									1.70								
2-Methoxy-4-vinylphenol	B/Phenol									2.68		1.18						
Isoeugenol, TMS derivative	B/Phenol-Vanillyl															0.49		
	Alkanes																	

Nonacosane	C29 Alkane			2.09														
Hentriacontane	C31 Alkane												2.48					
Tetratriacontane	C34 Alkane												2.54					
	Alkanols																	
8-Pentadecanol	C15 Alkanol-Mid Chain		0.84															
1-Hexadecanol	n-Alkan-1-ol C16							1.75										
1-Octadecanol + derivatives	1-Alkan-1-ol - C18		1.77		16.63			3.63	1.39					2.74		13.47		12.73
Eicosanol + derivatives	n-Alkan-1-ol C20		1.74		3.42		4.29	1.80	1.70	6.52		1.21				2.91		1.67
Behenic alcohol + derivatives	C22 Alkanol		6.03				5.87					4.19	2.20			4.60		31.28
Tetracosanol + derivatives	C24 Alkanol		1.31										4.01			1.04		13.59
Hexacosanol + derivatives	n-Alkan-1-ol C26												7.17			0.62		0.00
	Alkanoic Acids																	
Octanoic acid, methyl ester	C8:0 Alkanoic acid		1.05															
Octanoic acid, 8-hydroxy-, methyl ester	ω-C8:0-Hydroxy-Alkanoic acid								1.98									
Nonanoic acid, 9-hydroxy-, methyl ester	ω-C9:0 Hydroxy-Alkanoic acid								2.20									
Dodecanoic acid, methyl ester	n-Alkanoic acid C12:0														0.99			
Tetradecanoate + derivatives	n-Alkanoic acid C14	2.37		7.98		8.31		2.98			1.27		4.45		1.69		1.96	
10-Hydroxypentadecanoic acid, methyl ester	Mid chain C15:0 mono hydroxy Alkanoic acid										1.12							

9-Hydroxypentadecanoic acid, methyl ester	Mid chain C15:0 mono hydroxy Alkanoic acid			1.87				1.20										
Hexadecanoic acid + derivatives	n-Alkanoic acid C16:0	48.30	17.70	63.38	9.98	54.60	10.89	31.17	13.34	6.90	30.10	8.09	68.14	6.85	47.81	10.66	37.04	11.85
Methyl 16-hydroxy-hexadecanoate + derivatives	ω-C16:0-Hydroxy-Alkanoic acid	16.37		10.25	1.23	9.40	30.91	7.16	24.25	83.52	15.85	15.00	12.07	8.56	4.43	4.24	45.83	15.78
Heptadecanoic acid, methyl ester	C17:0 Alkanoic acid	10.93	13.23	13.86	9.30	12.42	13.11	6.96	11.28	14.27	9.26	10.94	12.44	13.68	9.67	12.06	18.26	13.31
Methyl stearate	n-Alkanoic acid C18:0 ME	4.91	3.45	8.64	2.66	3.69		5.48	1.74	1.93	7.85	1.32	8.68	3.25	20.30	4.83	4.11	1.32
Octadecanoic acid, 9,10-dihydroxy-, methyl ester (R*,R*)- + derivatives	C18:0 Dihydroxy Alkanoic acid			2.58		0.98							6.33		1.89			
Nonadecanoic acid, methyl ester	n-Alkanoic acid C19:0	1.24	1.25	1.25	1.22	1.24	1.23	1.25	1.25	1.24	1.24	1.25	1.24	1.21	1.24	1.22	1.25	1.23
Eicosanoic acid, methyl ester	n-Alkanoic acidC20:0	4.17	8.46	9.72	1.82	1.79	2.26	3.85	2.74	8.91	4.41	1.42	3.60	1.04	6.52	9.11	4.11	17.93
Docosanoic acid, methyl ester	n-Alkanoic acid C22:0	5.59	19.02	4.85	1.07	4.22	3.32	4.24	4.40	20.74	3.95	2.97	3.11	1.01	3.04	6.59	8.86	24.13
Tricosanoic acid, methyl ester	C23:0 Alkanoic acid	1.35	2.79	1.75	0.60	1.54	2.08	1.84	1.55	1.70					1.02		1.58	2.44
methyl-2-hydroxy-tricosanoate	α-Hydroxy Alkanoic acid C23:0		0.83															
Tetracosanoic acid, methyl ester	C24:0 Alkanoic acid	2.93	12.40	5.43	0.66	5.26	1.37	3.54	2.47	18.77	2.83	1.12	2.17	1.09	1.98		8.87	3.89
Pentacosanoic acid, methyl ester	n-Alkanoic acid C25:0		0.65			1.14		1.00										
Hexacosanoic acid, methyl ester	n-Alkanoic acid C26:0	2.00	0.87			1.50		3.54		6.90	1.56		4.71		1.45		1.83	
Octacosanoic acid, methyl ester	n-Alkanoic acid C:28	2.11				1.78		4.83			1.04		5.83		1.36		1.57	
Triacontanoic acid, methyl ester	n-Alkanoic acid C30	1.17		3.62		2.07					3.59				2.44		1.54	

9,10,18-trihydroxy octadecanoate, 3TMS derivative	ω-C18:0 triydroxy-Alkanoic acid	1.81						2.36										
18-hydroxy octadecanoic acid + derivatives	ω-C18:0 Hydroxy-Alkanoic acid					1.50	2.14		3.08			1.39		1.99		1.31		2.42
Methyl 18-hydroxy-cis-9,10-epoxyoctadecanoate, TMS ether	ω-C18:1 Hydroxy-Epoxy-Alkanoic acid	1.85						0.87										
Methyl 20-hydroxyeicosanoate + derivatives	Long chain-ω-C20:0 Hydroxy Alkanoic acid		0.82				1.53					1.18		0.91				1.12
Methyl 22-hydroxydocosanoate + derivatives	ω-C22:0 Hydroxy-Alkanoic acid		0.92				3.38					1.28						
9 and 10, 16-dihydroxy-hexadecanoate + derivatives	x,ω-C16:0 -Di-Hydroxy Alkanoic acid	2.30		38.00		34.50		18.92			32.02		114.77		20.68		117.78	
	Alkenes																	
Neophytadiene	C16 Alkene - Branched	1.70		1.48		1.40									0.89		2.18	
	Alkenols																	
Phytol + derivatives	C16 Alkenol	7.32		5.93		4.35		4.97			9.03		3.26		8.81		8.10	
Eicosen-1-ol, cis-9-	C20 Alkenol								10.46									
	Alkenoic Acids																	
Pentadecanoic acid, methyl ester	n-Alkenoic acid C15:0		1.26						1.31									
11-Hexadecenoic acid, methyl ester	C16:1 Alkenoic acid					1.19												
Methyl hexadec-9-enoate	C16:1 Alkenoic acid					12.59					1.38		13.59		2.57		17.44	

Oleic acid + derivatives	C18:1 Alkenoic acid		1.13				5.33					2.43		1.95		0.00		
cis-13-Octadecenoic acid, methyl ester	C18:1 Alkenoic acid				0.78													
6-Octadecenoic acid, methyl ester	C18:1 Alkenoic acid	14.89	7.79		3.80	21.02		24.99	3.29	4.33		4.28			32.24	6.42		6.39
(+-)-4-Ethoxy-5-methyl-2,5-dihydrofuran-2-one	C18:2 Alkenoic acid														1.25			
Methyl 10-trans,12-cis-octadecadienoate	C18:2 Alkenoic acid	2.68					3.96											
12,15-Octadecadienoic acid, methyl ester	C18:2 Alkenoic acid				5.57													4.72
9,12-Octadecadienoic acid, methyl ester + derivatives	C18:2 Alkenoic acid	16.29	5.97	9.83		8.17		8.71	8.11	10.11	5.63	3.07	8.78	4.63	29.16	4.55	11.44	
9,11-Octadecadienoic acid + derivatives	C18:2 Alkenoic acid														7.25	1.73		
Methyl 9-eicosenoate	C20:1 Alkenoic acid																	2.51
11-Eicosenoic acid, methyl ester	C20:1 Alkenoic acid		2.46															
13-Docosenoic acid, methyl ester, (Z)-	C22:1 Alkenoic acid		1.37															
Methyl 18-hydroxy-9-octadecenoate + derivatives	Short chain ω hydroxy Alkenoic acid C18:1	55.87	16.34	1.66	21.92		55.57	9.40	66.88	37.52	14.41	37.19		17.91		20.39	50.11	43.54
	Alkynoic Acids																	
7,10,13-Hexadecatrienoic acid, methyl ester	C16:3 Alkynoic acid	2.26																
9,12,15-Octadecatrienoic acid, methyl ester + derivatives	C18:3 Alkynoic acid	8.49		34.46	1.24	14.20		8.74	1.25		30.59		28.43	2.14	27.92		44.10	

Methyl 9.cis.,11.trans.t,13.trans.-octadecatrienoate	C18:3 Alkynoic acid													2.11				
	Diacids																	
2-Pentenedioic acid, dimethyl ester	C5:1 Dioic Alkenoic acid						1.28						1.44	2.06				
Subric acid + derivatives	Short chain-α,ω-C8:0-Diacid								3.03									
NONANEDIOC ACID + DERIVATIVES	C9:0 Dioic acid	4.40	7.66	1.73	2.70	2.65		1.42	8.40	5.79	2.13	3.73			2.44	0.62	3.53	2.30
9,10-dihydroxy-1,16-hexadecanedioic acid, dimethyl ester	C16:0 Di-Hydroxy Alkanoic Dioic acid, dimethyl ester	23.80																
Hexadecanedioic caid DME	Short chain C16:0 Dioic acid	1.59	1.53	2.75	1.72	1.89	13.60	2.20	4.86	28.44	6.54	17.12	3.72	5.69		3.98	7.17	13.74
Octadecanedioic acid, dimethyl ester	Short chain-α,ω-C18:0-Diacid		0.70		0.86		6.11		3.12	6.68		4.15		4.11		7.03		11.85
9,10-dihydroxy-1,18-Octadecanedioic acid + derivatives	C18:0 Dihydroxy Dioic acid	11.66	2.69				2.95	8.99									1.14	
Methyl Octadecene-1,18 dioate + derivatives	α, ω C18:1 Alkene Diacid	5.33	14.81		10.09		21.40	1.89	18.83	28.63	4.37	22.62		10.50		14.84	2.93	37.30
Methyl octadeca-9,12-diene-1,18 dioate (9,12 C18:2 DME)	C18:2 Dioic acid, Dimethyl ester							0.90										
Eicosanedioic acid, dimethyl ester	Long chain-α,ω-C20:0-Diacid		1.40		0.62		2.22		1.55	1.34		2.21		1.65		2.02		4.08
Docosanedioic acid, dimethyl ester	Long chain-α,ω-C20:0-Diacid C22:0				2.25		2.95		1.65	1.64		4.07		1.47		1.19		2.21
	Nitrogen Compounds																	
β-Alanine, N,N-diethyl-	Nitrogen compound												1.44					

Oxalic acid mono-(N-dimethyl)-amide, methyl ester	Nitrogen compound			9.00		5.67	8.11	3.89		7.77	4.58		6.30	8.57	1.96	9.14		4.97
	Other Acids																	
1,3,5-Triazine-2,4,6(1H,3H,5H)-trione, 1,3,5-trimethyl-	n-component isocyanuric acid		3.30			1.08		1.10				10.04			0.93			6.28
Nonanoic acid, 9-oxo-, methyl ester	C9:0 Oxo acid				0.86												1.15	
ω-3 Arachidonic Acid methyl ester	C20:4 Polyunsaturated aliphatic acid																9.05	
	Unclassified Monomers																	
Antiangor	Unknown									2.42								
Dimethyl trans-1,2-Cyclopropanedicarboxylate	Unknown															1.82		
1,1,3-Triallyl-2-thiourea	Unknown		1.16															