# LONG-TERM EFFECTS OF TIMBER HARVESTING ON HEMICELLULOLYTIC MICROBIAL COMMUNITIES IN NORTH AMERICAN CONIFEROUS FOREST SOILS

**by**

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## Abstract

Forests are essential for maintaining global climate and biodiversity, with industrial applications vital to the world economy. Forest soils are inhabited by a highly diverse community of macro- and microorganisms which are responsible for a variety of fundamental ecosystem services such as decomposition, and nutrient cycling. The effects of forest disturbance on soil microorganisms specific to these key processes have yet to be studied thoroughly. Bearing in mind the importance of forest soil organisms, I have identified and investigated the long-term effects of forest disturbance by timber harvesting on bacterial and fungal populations that degrade hemicellulose using molecular techniques coupled to stable-isotope probing (SIP) with $^{13}$C-hemicellulose. I identified 104 putatively hemicellulolytic bacterial operational taxonomic units (OTUs) and 52 putatively hemicellulolytic fungal OTUs. Based on analysis of $^{13}$C-enriched phospholipid fatty acids and DNA, harvesting resulted in long-term changes in relative abundances of putatively hemicellulolytic bacterial and fungal populations. Although harvesting resulted in long-term changes in these populations, no statistically significant differences in potential hemicellulolytic activity of the soils was observed, suggesting functional redundancy in this fundamental ecosystem process. Additionally, I identified *Methylibium*, a genus of facultative methylotrophs as a novel putative hemicellulose degrader. This study is the first to extensively survey both bacterial and fungal soil microorganisms specific to hemicellulose degradation using stable-isotope probing, and to provide evidence for long-term effects of timber harvesting on these populations. These results contribute towards the strategic management of forest ecosystems, and the identification of novel hemicellulolytic organisms in this study will pave new roads for industrial applications of cellulolytic and hemicellulolytic enzymes.

## Preface

The work described in this thesis is an original intellectual property, and based entirely on my unpublished experimental data. Dr. William W. Mohn was the principle investigator behind this project. Figures 1, 2, and 3 are from published sources. The $CO_2$ mineralization portion of this thesis was based on my own experiments. The SIP-PLFA portion described in this thesis was based on my own experiments, and my samples were analyzed using instrumentation at the Stable Isotope Facility at UC Davis. The $^{13}C$-enrichment measurements using UPLC-MS-MS is an unpublished method developed by Roland Wilhelm, and my experiments in that area were performed under his guidance. Identification of putative hemicellulolytic organisms described in the thesis were based on my SIP-pyrotag experiments using microcosms, and all subsequent community analysis was performed by myself on a pyrotag library generated by colleagues Dr. Kendra Maas, Dr. Martin Hartmann, and Roland Wilhelm from the Mohn Laboratory.

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# List of Abbreviations

**BS** – Black Spruce ecozone of Ontario

**CAP** – Canonical analysis of principal coordinates

**DNA** – Deoxyribonucleic acid

**IDF** – Interior Douglas Fir ecozone of British Columbia

**ITS** – Internal transcribed spacer

**JP** – Jack Pine ecozone of Ontario

**LTSP** – Long-term soil productivity

**LP** – Loblolly Pine ecozone of Texas

**NMS** – Nonmetric multidimensional scaling

**OM** – Organic matter (removal)

**OTU** – Operational taxonomic unit

**PCR** – Polymerase chain reaction

**perMANOVA** – Permutational multivariate analysis of variance

**PLFA** – Phospholipid fatty acid

**PP –** Ponderosa Pine ecozone of California

**SBS** – Sub-Boreal Spruce ecozone of British Columbia

**SIP** – Stable-isotope probing

**rRNA** – Ribonucleic acid

## Acknowledgements

I would like to thank my supervisor, Dr. William W. Mohn for his guidance, support, and unwavering patience throughout my time in graduate school. None of this would have been possible without the vision, wisdom and constant feedback of Dr. Mohn.

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## Dedication

To my family

# 1 Introduction

## 1.1 Forest Ecosystems and Soils

Forest ecosystems are fundamental aspects of the biosphere, playing a paramount role in maintenance of nutrient flows and global climate. Forests remove approximately 3 petagrams of anthropogenic carbon annually, which equates to roughly 30% of fossil fuel emissions (Canadell & Raupach, 2008). In addition to climate change mitigation, forest ecosystems are of critical importance in the global economy. According to Natural Resources Canada (NRC), the forest industry contributes towards $23.7 billion in Canadian economy, comprising of 1.9% of the total gross domestic product (GDP) (Canada, 2013). Forest productivity is defined as the rate of production of organic compounds through photosynthesis, and is generally measured as gross primary production, as a quantity of fixed carbon over a unit of area and time, however this is difficult to measure due to losses in carbon resulting from respiration and other means of allocation (R. Powers, 1999). Alternatively, productivity can be measured as “net primary productivity”, a measurement of biomass production over a unit of area and time, which has been accepted to be synonymous with the growth rate of the forest stand (R. Powers, 1999). Notably, this excludes a substantial proportion of fixed carbon that is incorporated into belowground microbial biomass. Forest management influences the availability of soil carbon and nitrogen, which are key variables determining soil fertility (Johnson & Curtis, 2001). Soils are essential components of the biosphere, as they are inhabited by a huge variety of microorganisms that are capable of degrading virtually all organic compounds, maintain biogeochemical cycles, and regulate productivity of terrestrial plants (Nannipieri et al., 2003). Taking into consideration the importance of forests with respect to economic demand, biodiversity, and global climate, it is imperative that forest resources must be managed strategically with sustainability in mind.

Soil ecosystems provide critical ecosystem services to forests. These ecosystems are heterogeneous and very biologically diverse, presumably due to micro-site and macro-site differences in physical, chemical, and biological aspects (Axelrood, Chow, Radomski, McDermott, & Davies, 2002). Soil microbes influence plant productivity, by either directly forming symbiotic relationships with plant roots or indirectly by ubiquitously altering nutrient availability as free-living microbes (Van Der Heijden, Bardgett, & Van Straalen, 2008). Nitrogen-fixing bacteria, for example, play a crucial role in regulating plant productivity, as plants alone are incapable of atmospheric nitrogen fixation (Van Der Heijden et al., 2008). Mycorrhizal fungi are another crucial group of plant symbionts that supply important nutrients in exchange for plant carbon, providing water, nitrogen, phosphorus, copper, iron, and zinc that would otherwise be inaccessible to plant roots (Van Der Heijden, Bakker, et al., 2006; Van Der Heijden et al., 2008; Van Der Heijden, Streitwolf-Engel, et al., 2006). Soil decomposition processes mediated by microbes play a predominant role in mineralization and cycling nutrients to plants and the atmosphere by breaking down complex polymers such as lignocellulose, proteins, nucleic acids, and chitin derived from dead organic matter (Doran, 2002; Van Der Heijden et al., 2008). These processes are critical in establishing a major interface between environment and plant productivity. With emerging global markets for forest matter as a source of alternative energy, a new paradigm for forest management practices is required, one which considers the long-term sustainability of soil. Understanding the relationships between soils, their microbial inhabitants and their role in carbon sequestration has become crucial for assessing forest sustainability and effects on global levels of atmospheric $CO_2$.

### 1.2 Timber Harvesting, Organic Matter Loss and Long-Term Productivity

Deforestation results in climate change and habitat loss, and hinders important ecosystem processes. Release of $CO_2$ into the atmosphere is amongst the major consequences of deforestation (Ramankutty et al., 2007; Woodwell et al., 1983). Inadequately

managed forests carry hazard of contributing towards greenhouse gas emissions (Kurz, Stinson, Rampley, Dymond, & Neilson, 2008). In effort to address the consequences of forest disturbance, the United States Department of Agriculture Forest Service established the Long-Term Soil Productivity Study (LTSP) (Robert F. Powers et al., 2005). This study continued as a cooperative effort to address these long term effects of pulse soil disturbance. Soil porosity and site organic matter are two essential properties directly influenced by forest management, affecting nutrient exchange and availability, physical restrictions on rooting, soil aggregate stability and microbial activity, all of which regulate fundamental biological processes of the site (Robert F. Powers et al., 2005). Timber harvesting has been reported to increase soil temperatures, diurnal fluctuations, expose large quantities of debris and dead roots for microbial degradation, and this disturbance to the forest floor affects both organic matter content and soil porosity (Robert F. Powers et al., 2005). Changes in vegetation, availability of nutrients, soil structure and microclimate, and soil litter in the short-term have been reported (Keenan & Kimmins, 1993), but over the long term, these properties are very resilient to current forest management practices (R. Powers, 1999; Sanchez, Scott, & Ludovici, 2006). LTSP study results to date demonstrated that disturbances from timber harvesting did not largely impair forest productivity, and long-term effects on the physical properties of the ecosystem were site-dependent (Robert F. Powers et al., 2005). Initial effects on seedling establishment were observed, with forest floor removal improving seedling establishment at Mediterranean climates while reducing it at warm-humid climates (Fleming et al., 2006). However, timber harvesting did not result in significant losses in total-stand biomass 10 years post harvesting for many of the 45 LTSP installments with the exception of aspen, further suggesting the species- and site-dependence of these long-term harvesting effects (Ponder Jr et al., 2012). Although these LTSP studies have yet to demonstrate consistent and significant effects of organic matter removal on forest productivity, it is crucial to bear in mind that these forest stands are at infancy, and continued monitoring of tree growth is necessary for any changes in trends that may occur.

Despite the initial lack of treatment effects associated with the physical and chemical characteristics reported in many LTSP studies, the microbial aspects of these disturbed ecosystems have yet to be investigated thoroughly. A recent study at LTSP study sites reported that the removal of organic matter (OM) significantly alters both bacterial and fungal communities in soil over the long term, suggesting that changes in microbial communities may serve as sensitive measures of environmental changes (Hartmann et al., 2012). A broad factorial experimental design of organic matter removal and soil compaction, with replicated LTSP treatment sites was examined. Findings from the study of Hartmann *et al.* had been unexpected, as previous surveys on soil chemistry, nitrogen mineralization and tree growth at similar LTSP sites have indicated no significant treatment effects 10-15 years post-harvest (Fleming et al., 2006; Kranabetter, Sanborn, Chapman, & Dube, 2006). These results justify further studies to investigate metabolic processes of these communities to determine any functional implications of the community composition changes observed.

### 1.3 Hemicellulose and Carbon Cycling

The process in which carbon is exchanged throughout the ecosystem is known as the carbon cycle. $CO_2$ emissions from soils are one of the largest sources of C flux involved in the global carbon cycle (Schlesinger & Andrews, 2000). Statistical models estimate an annual flux of 76.5 Pg C $year^{-1}$ of $CO_2$ from soils, constituting approximately 10% of atmospheric $CO_2$ carbon (J. W. Raich & Potter, 1995; Watson, Rodhe, Oeschger, & Siegenthaler, 1990). Soil respiration is directly correlated with net primary productivity (NPP), which is approximately 55 Pg C $year^{-1}$ globally (J. Raich & Schlesinger, 1992). Globally, soil organic matter reservoirs store approximately 1600 Pg of carbon, almost double that of atmospheric carbon (Eswaran, Van Den Berg, & Reich, 1993). With such a substantial reservoir of stored carbon, caution must be taken, as any large anthropogenic changes in global C pools may result in positive feedback in climate and potentially intensify rates of $CO_2$ production by soils (J. W. Raich & Potter, 1995).

Forest ecosystems exchange $CO_2$ with the atmosphere through photosynthesis which is then stored in the form of organic matter, in which a substantial amount of terrestrial carbon is stored in the form of woody biomass in forest ecosystems (Donnelly, Entry, Crawford, & Cromack, 1990). Various oxidative processes, such as autotrophic respiration by plants, heterotrophic respiration mediated by soil microbes, and combustion of organic matter by fires, recycle organic carbon back into the atmosphere (Falkowski et al., 2000). Recycling photosynthetically derived carbon, roughly half of which is lignocellulose, is an essential aspect of the carbon cycle, releasing $10^{11}$ tons of monosaccharides annually.

The main component of plant biomass is lignocellulose. Lignocellulosic biomass (**Figure 1**), consisting mainly of cellulose (35-50%), hemicellulose (25-30%), and lignin (25-30%), is derived from a variety of agricultural residues, deciduous and coniferous woods, municipal solid wastes from the pulp industry, and herbaceous energy crops (Rubin, 2008). Raw lignocelluosic biomass is highly recalcitrant and resistant to chemical treatments, thus the efficient harvesting of energy is a current obstacle in lignocellulose biofuel production. Particularly, in the absence of microbial and enzymatic intervention, the half-life of cellulose is estimated to be on the order of millions of years (Wilson, 2011). The microbial degradation of this carbon is mediated by a consortium of fungi and bacteria using a variety of hydrolytic and oxidative enzymes (Pérez, Muñoz-Dorado, de la Rubia, & Martínez, 2002), and is of global importance with regards to carbon turnover. The majority of the plant cell wall consists of lignocellulose, thus conferring its recalcitrance, and only a specialized minority of microorganisms are known to be capable of degrading this carbon. The degradation of lignocellulose is mediated cooperatively by microorganisms either synergistically or sequentially(Kubicek, 2012).

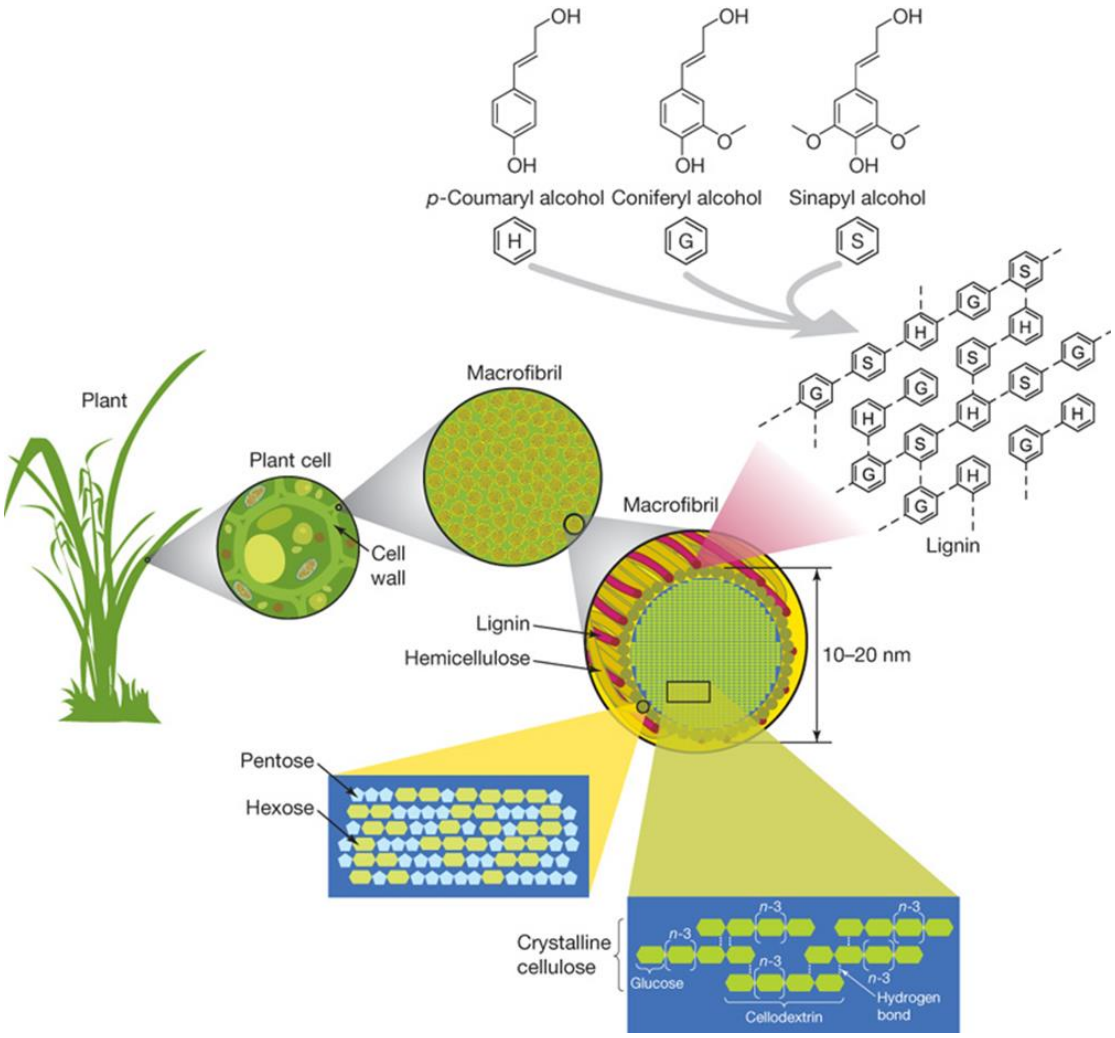



**Figure 1.** Structure of lignocellulose found in plant cell wall, illustrating subcomponents; cellulose, hemicellulose, and lignin which form heterostructures of microfibrils. Microfibrils are organized into macrofibrils which contribute towards cell wall structure. (Rubin, 2008)

Hemicelluloses play an integral role in adhering the components of lignocellullose. A robust network of crosslinked polymers is formed by covalently linking hemicellulose to lignin, and hydrogen bonding to cellulose microfibrils (Shallom & Shoham, 2003). Unlike cellulose, hemicelluloses are heterogeneous polymers consisting of furanoses, pyranoses and a variety of sugar acids, and they are branched with short lateral chains of varying saccharides having

easily hydrolysable bonds (**Figure 2**). The molecular weight of hemicellulose is typically lower than that of cellulose. Xylans, the main components of hemicellulose found in plant materials, are heteropolysaccharides with 1,4 β-linked backbones consisting of D-xylose, D-mannose, D-galactose, D-glucose, L-arabinose, 4-O-methyl glucuronic, D-galacturonic, and D-glucuronic acids (Pérez et al., 2002). In an additional report, the structure of hemicellulose included 4-O-methyl ether, acetic, ferulic, and ρ-coumaric acids (Saha, 2003). The exact composition of hemicellulose varies across plant species, with angiosperms (hardwoods) typically containing mostly xylans, while gymnosperms (softwoods) contain mostly glucomannans. Xylose units in hardwood hemicelluloses are typically highly acetylated (60-70%) and have a higher degree of polymerization, approximately 150-200, compared to 70-130 in softwood xylans (Jeffries, 1994).

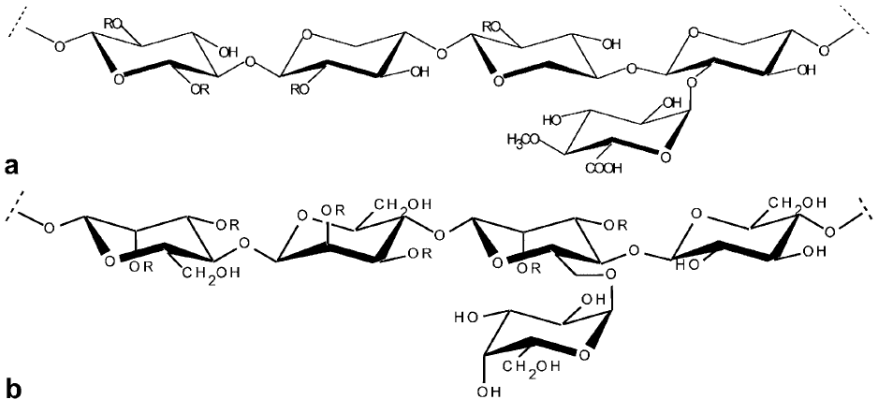



**Figure 2.** Structures of xylan heteropolymers derived from gymnosperm and angiosperms. **(A)** O-acetyl-4-O-methylglucuronoxylan from angiosperms. Gymnosperm hemicellulose has the same basic structure but lacks acetyl groups and α-1,3-1-arabinose residues. **(B)** O-acetyl-galactoglucomannan from gymnosperms. Angiosperms contain lesser amounts of glucomannan, which always lacks α-D-galactose. *R* represents H or acetyl group.
(Jeffries, 1994)

To process lignocellulose for biofuel production, a variety of chemical pre-treatments are used in order to solubilize, hydrolyze, and seperate hemicellulose, cellulose, and lignin. These treatments include sodium hydroxide, perchloric acid, peracetic acid, sulfuric and formic acid, ammonia freeze explosion, and organic solvents such as; *n*-propylamine, ethylenediamine, *n*-butylamine (Kumar, Singh, & Singh, 2008). In addition to chemical treatments, lignocellulosic biomass is subjected to physical treatments such as ball milling, compression milling, cryomilling, and steam explosion to reduce particle size, followed by an extensive series of enzymatic bioconversions(Kumar et al., 2008). Xylanases are of particular interest due to their applications in the bleach and biopulping industry (Pérez et al., 2002). Development of processes utilizing enzymes and biocatalysts to degrade hemicelluloses and lignocelluloses will result in a more sustainable and environmentally friendly means of biofuel production, more efficient pulp manufacturing and additional new markets for recycling of agricultural wastes.

### 1.4 Catabolic Potential in Soil Microorganisms

Decomposition of plant litter is essential for nutrient cycling in terrestrial ecosystems. The decomposition of plant detritus provides 69-87% of nutrients required for annual forest growth (Sinsabaugh et al., 1993; Swift, Heal, & Anderson, 1979). Due to its recalcitrance, woody biomass decomposes relatively slowly and remains unavailable for primary producers in the absence of microbial aid to liberate essential nutrients such as nitrogen and phosphorus (Boddy & Watkinson, 1995). Wood-rot fungi are the only organisms currently known to efficiently degrade cellulose and hemicellulose protected by lignin (Hatakka & Hammel, 2011), and thus play a critical role in decomposition of these woody materials. Certain “white-rot” fungi belonging to the Basidiomycete phylum are of great interest due to their ability to degrade and mineralize the most recalcitrant natural polymers (Hatakka & Hammel, 2011).

Hemicellulose decomposition is carried out by bacterial and fungal enzymes. A variety of enzymes, each acting on a specific bond such as; endo/exo-xylanase, β-xylosidase, α-arabinofuranosidase, α-glucoronidase, acetylxylan esterase, ferulic acid esterase, ρ-coumaric acid esterase act in concert for total biodegradation of xylan. (Saha, 2003). Filamentous fungi have been extensively researched for xylanase activity, as they secrete enzymes extracellularly, at much higher levels than that of bacteria and yeasts (Kubicek, 2012). Bacterial xylanases, on the other hand, have been reported to be produced at lower activity levels. Synergy and cooperation among hemicellulolytic enzymes more efficiently degrade the heteropolymer (Polizeli et al., 2005). Amongst mesophilic microorganisms, fungal genera *Apsergillus* and *Trichoderma* are prominent in xylanase production (Polizeli et al., 2005). *Talaromyces emersonii* is one of the commonly studied thermophilic species of fungus for xylan degradation, with particular commercial interest due to its thermal stability (Polizeli et al., 2005; Saha, 2003). White-rot fungus *Phanerochaete chrysosporium*, a species of Basidiomycota has also been shown to produce a variety of endoxylanases (Pérez et al., 2002). Much of the current understanding of microbial degradation of wood matter is based on the enzyme system derived from *Trichoderma reesei* (Dekker, 1983; Hatakka & Hammel, 2011; Martinez et al., 2008).

Historically, the inability to grow microorganisms in culture has limited our identification of organisms involved in these key catabolic roles. Traditional methods in identifying physiological roles of microorganisms requires isolation of the organism in pure culture, but the use of stable-isotope probing (SIP) permits a culture-independent method to link biological function with taxonomy by using stable isotopes to trace the organisms responsible for degradation of a labeled compound (Radajewski, Ineson, Parekh, & Murrell, 2000). Alternative culture-independent methods such as fluorescent *in situ* hybridization and radioactive isotope labeling have are applicable, but SIP offers the advantage of isolating entire copies of genomes and reliable DNA sequences of genes which may be assigned to taxonomy. By coupling stable-

isotope probing with techniques in molecular biology, it is possible to tease out the organisms involved in hemicellulose degradation by detecting the incorporation of an isotope with a low natural abundance, such as $^{13}C$, in a labeled compound into a biomarker of interest.

### 1.5 Research Question

In summary, forest ecosystems play a paramount role in climate regulation and sustaining terrestrial life, and increasing industrial demands for forest resources requires for careful consideration of forest management. Recent findings demonstrate the persistent impact of timber harvesting on the microbial residents in forest soils, raising the question of whether metabolic activities are influenced by changes in the community structure (Hartmann et al., 2012). In particular, microbially mediated degradation of woody biomass is essential for recycling of recalcitrant lignocellulose carbon back into the atmosphere. Most of what is currently known about forest ecosystems and their microbial communities is based on culture-dependent studies. Despite the growing collective knowledge of microbial communities in forest soils, the current understanding of the relationships between soil microbial communities, catabolic function, and forest productivity and sustainability is limited. Furthermore, degradation of hemicellulose is of particular importance, as the breakdown of plant cell walls increases accessibility of cellulose to enzymatic degradation, and releases monosaccharides for biofuel production.

The objective of this study was multi-fold: to establish a method to identify and characterize microbial communities that play a prominent role in the cycling of recalcitrant carbon in forest soils, and to determine whether these communities will experience any long-term changes resulting from timber harvesting. To elucidate such effects, a variety approaches were taken for community analysis of these systems. Metabolic processes in soil microorganisms are often used as a proxy for soil quality, which often depends on a complex consortium of physical, chemical, and biological properties (Kennedy & Papendick, 1995). By

measuring rates of hemicellulose substrate induced respiration, I estimated and compared rates of hemicellulose catabolism in timber harvested forest soils. In addition to activity measurements, I exploited recent developments in applications of stable-isotope probing (SIP) for its unprecedented ability to survey metabolically active microbial communities and connect taxonomy to function. Few studies have attempted to completely survey both bacterial and fungal communities capable of hemicellulose degradation in the context of long-term harvesting impacts on forest soils. This study is the first to utilize stable-isotope probing to massively sequence hemicellulolytic populations. For the first time, I have utilized the pre-existing LTSP experimental design to address the consequences of timber harvesting on a functional level and across a geographical scale.

# 2 Materials and Methods

## 2.1 Sample Collection

Detailed information on geographic and biological characteristics of the study sites can be obtained from LTSP establishment reports (Hannam et al., 2008; Robert F. Powers, 2006). Soils from British Columbia sites Dairy Creek, O'Connor Lake and Black Pines (**Figure 3**) were collected in June 2011, while soils from California sites Blodgett, Brandy City and Lowell Hill were collected in July 2011. Latitude and longitude coordinates are summarized in **Table 1** (Robert F. Powers, 2006; Teste, Simard, Durall, Guy, & Berch, 2010). Soils from forest stands of various levels of organic matter removal were collected and used for analysis in this study. The levels of OM removal were defined as; unharvested (OM0) reference soils, stem-only (OM1) harvesting, whole-tree (OM2) harvesting, and whole-tree plus forest floor (OM3) harvesting. It is important to note that the designations for levels of OM removal were used according to the study by Hartmann *et al.* (2012), and differ slightly from some LTSP reports (Ponder Jr et al., 2012; Robert F. Powers et al., 2005). Soils of organic and mineral layers were chosen from nine randomly selected sample points along the perimeter of the buffer zone of each rectangular plot and composited into a triplicate of three soils each. Organic soils were collected by scraping a circle of 10 cm diameter and retrieving entire depth of the layer avoiding mineral layer. A stoney auger was used to collect a soil core 20 cm deep into the mineral layer, avoiding any organic soil. All samples were mixed thoroughly and collected into a 50-ml conical tube for each treatment plot in triplicate. Excess soils were placed back into original sample plots. Soil samples were temporarily stored frozen at -20$^{o}$C and transported back to research facilities for processing and kept at -80$^{o}$C for long-term storage.

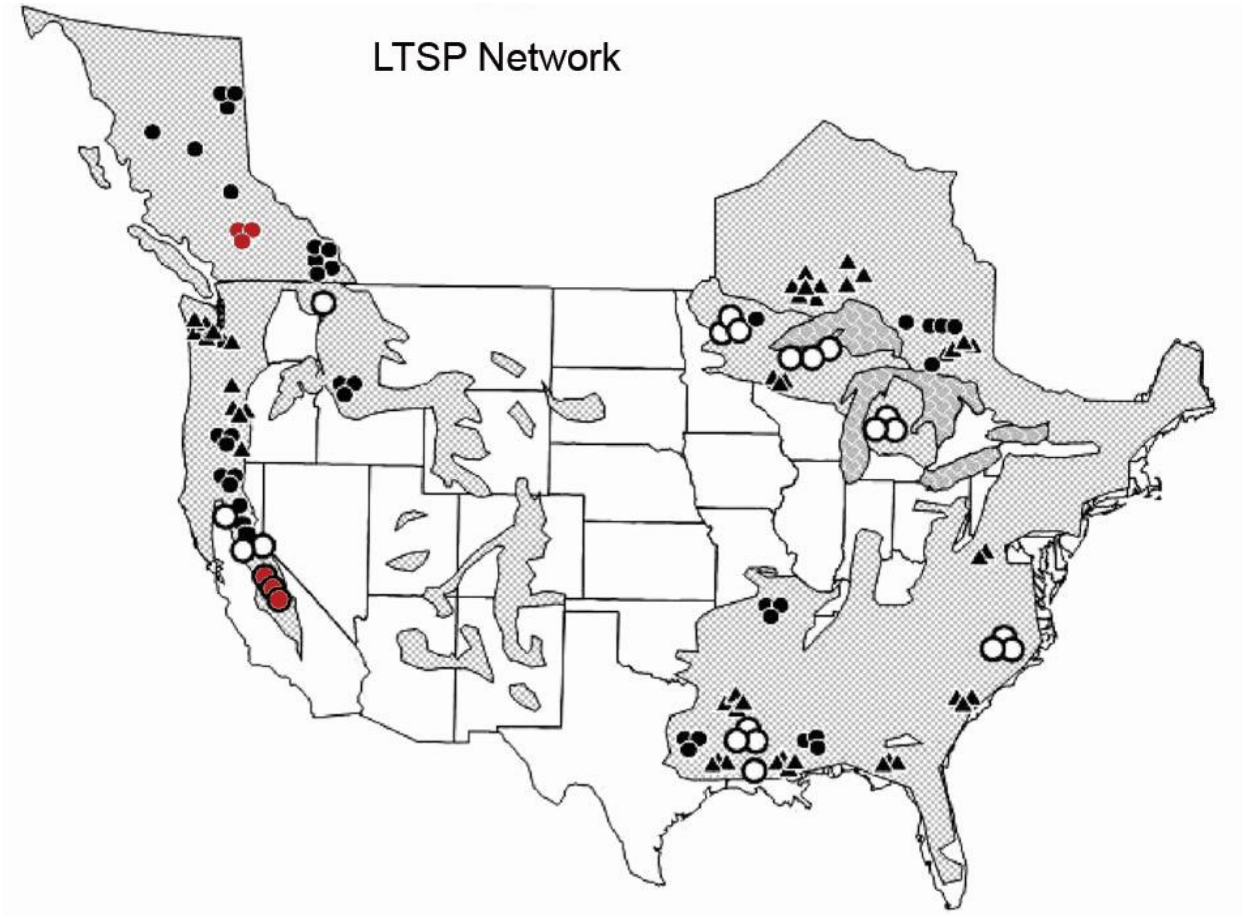



**Figure 3.** The Long-Term Soil Productivity study sites. Red markers indicate sampling sites for microcosm experiments. (Powers, *et al*., 2005)

**Table 1.** Environmental data at LTSP sites. Site information for soils used in microcosm experiments are indicated with blue and red shaded data for IDF and PP sites respectively.

| Ecozone | Location | Site | Lat. (°) | Long. (°) | Elevation (m) | Annual Precipitation (cm) | Precipitation, Warmest Quarter (mm) | Mean Annual Temperature (°C) |
|---|---|---|---|---|---|---|---|---|
| IDF | British Columbia | Dairy Creek | 50.51 | 102.25 | 1075 | 30 to 75 | n/a | 1.6 to 9.5 |
| | | O'Connor Lake | 50.53 | 120.21 | 1180 | | | |
| | | Black Pines | 50.56 | 120.17 | 1150 | | | |
| SBS | British Columbia | Log Lake | 38.88 | 122.61 | 785 | 62 | 146 to 193 | 2.2 |
| | | Skulow Lake | 52.32 | 121.92 | 1050 | 43 | | |
| | | Topley | 52.32 | 126.31 | 1100 | 53 | | |
| PP | California | Blodgett | 38.88 | 120.64 | 1320 | 165 | 51 to 55 | 11.2 |
| | | Brandy | 39.55 | 121.04 | 1130 | 190 | | |
| | | Lowell | 39.26 | 120.78 | 1270 | 173 | | |
| BS | Ontario | Fensom 1 | 89.41 | 49.07 | 442 | 61 | 266 | 0.4 |
| | | Fensom 2 | 89.38 | 49.08 | 450 | | | |
| | | Fensom 3 | 89.39 | 49.07 | 442 | | | |
| JP | Ontario | Superior 1 | 47.58 | 82.79 | 458 | 82 | 250 | 1.7 |
| | | Superior 2 | 47.58 | 82.81 | 461 | 83 | | |
| | | Superior 3 | 47.57 | 83.84 | 426 | 85 | | |
| LP | Texas | Kurth-1 | 31.11 | 95.15 | 88 | 109 | 253 | 19 |
| | | Kurth-2 | | | | | | |
| | | Kurth-3 | | | | | | |

California soil samples from Blodgett, Lowell Hill, and Brandy City (**Figure 3**) were collected in a similar fashion by collaborators under the direction of Dr. Matt Busse of USDA, Forest Services. California soils were transported on ice and immediately processed and aliquots were stored at both -20$^{o}$C and -80$^{o}$C.

### 2.2 Soil Processing and Storage

All soils were processed by removing non-soil debris through a 2.0-mm sieve and divided into aliquots of 2-mL cryotube vials for storage to avoid excessive freezing and thawing. Excess soils were stored in 50-mL conical tubes. Dry weight and water content measurements were taken gravimetrically by removing 1 g of each soil sample and evaporating all water content in a conventional oven overnight and weighing the following day. Soil samples were subsequently used for a variety of experiments described below (**Figure 4**).

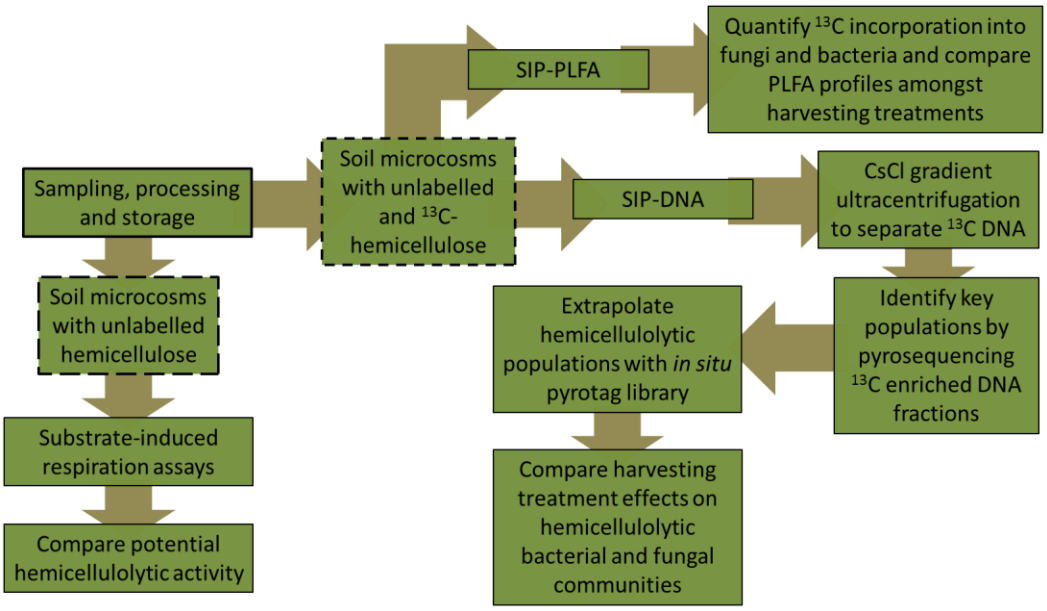



**Figure 4.** Experimental Design described in this thesis

### 2.3 Soil Microcosms

All glassware and equipment were autoclave sterilized prior to usage. Microcosms used for hemicellulose-induced respiration assays were prepared in 10-ml serum vials (Wheaton glass) with 0.25 g or 0.75 g dry weight of either organic or mineral soil, respectively. To each, 10 mg unlabeled ($^{12}$C) hemicellulose (IsoLife) was added and incorporated by mixing. The moisture content was adjusted to 60% of total weight. Soils were crimp sealed with a butyl rubber stopper and incubated for 24 hours at 20$^{o}$C in the dark.

Microcosms used for stable-isotope probing (SIP) experiments were prepared in 30-ml serum vials (Wheaton glass) with 0.75 g or 1.00 g dry weight of organic or mineral soil, respectively. The moisture content was adjusted to 60% of total weight. All microcosms were pre-incubated for 7 days at 20$^{o}$C in the dark, prior to addition of substrate. Following pre-incubation, 10 mg of substrate (maize-derived hemicellulose $^{12}$C and $^{13}$C-labeled, IsoLife) was added to microcosms, mixed thoroughly, and crimp sealed with a butyl rubber stopper and aluminum cap for a 2-day incubation at 20$^{o}$C in the dark. Hemicellulose composition (%w/w) is as follows; 29.7% xylan, 11.7% arabinan, 7.0% glucan, 4.5% galactan, 8.6% ash. Water is the main impurity, with traces of lignin and protein.

### 2.4 Substrate-induced Respiration Assays

Following 24 hour incubations of microcosms, $CO_2$ evolved was measured by withdrawing 0.2 ml of the headspace using a syringe and needle and injecting it in a gas chromatograph coupled to a thermal conductivity detector (Shimadzu, GC-8A) (**Figure 4**). The molar amount of $CO_2$ was calculated by subtracting hemicellulose negative controls and using the ideal gas law; $n = \frac{PV}{RT}$ and used to calculate respiration rates as µg of $CO_2$ mineralized per g of soil per day.

## 2.5 SIP-PLFA Analysis

### 2.5.1 Experimental setup

Soil microcosms were performed on soil samples from O'Connor Lake, Dairy Creek and Black Pines from the IDF ecozone, and Blodgett, Brandy City and Lowell Hill from the PP ecozone. All soils from OM0, OM1, and OM2 were used in both soil layers and OM3 exclusively in the mineral layer. Microcosms were incubated as described in section 2.3 (**Figure 4**).

### 2.5.2 PLFA extraction and detection

Select soil samples were incubated with $^{13}$C-labelled hemicellulose in microcosms, and fatty acid methyl esters were extracted as described in the literature (Bligh & Dyer, 1959; Å. Frostegård, Tunlid, & Bååth, 1991). Fatty acid methyl ester extracts were analyzed on a gas chromatograph isotope-ratio mass spectrometer (GC-IR-MS) instrument to determine incorporation of $^{13}$C-hemicellulose into biomass. This was performed on a total of 126 soil microcosms. All glassware used in PLFA extractions were thoroughly washed and rinsed in deionised water and burned at 450$^{o}$C for at least 4 hours prior to usage. Similarly, all Teflon-lined tubes were washed thoroughly with soap, followed by methanol and hexane rinses prior to usage. Rigorous glassware cleaning steps are necessary to avoid potential lipid contamination. Extraction was carried out with samples under minimal exposure of light, heat, and moisture. Frozen soils from soil microcosms were lyophilised to remove water content.

Freeze-dried soils were transferred to 50-mL Teflon-lined centrifuge tubes: 1.5 mL citrate buffer (0.15 M, pH 4.0), 1.9 mL CHCl3, 3.75 mL MeOH, and 2 mL of Bligh and Dyer reagent ($CHCl_3$:MeOH:citrate buffer, 1:2:0.8 v/v/v) was added in the order described. Samples were vortexed briefly, and shaken at 200 rpm at 20$^{o}$C for 1 hour, followed by a 30 min. centrifugation at 4000 rcf. Supernatant was collected in a clean 50-mL Teflon-lined centrifuge

tube, avoiding particulate matter. The soil pellet was washed with 2.5 mL of Bligh and Dyer reagent, followed by brief vortexing and another 30 min. centrifugation, and supernatant was added to the supernatant from the previous centrifugation. 3.1 mL citrate buffer and 3.1 mL $CHCl_3$ was added to the supernatant, vortexed for 20 sec., and kept at 4$^oC$ overnight for separation of solvent phases.

Following the overnight separation of phases, the top aqueous layer was removed, and residual water was removed by addition of MeOH droplets. Samples were dried down at room temperature under a stream of $N_2$ gas. 3 mL $CHCl_3$ was added to condition solid phase extraction cartridges (Agilent) suspended over glass tubes for waste collection. 500 µl $CHCl_3$ was added to samples to dissolve the lipids, which was then transferred to the solid phase extraction cartridge. An additional 500 µl $CHCl_3$ was added to wash sample vials and added to the solid phase extraction cartridges. Samples were allowed to drain, followed by addition of 4 mL $CHCl_3$ to the cartridge to elute neutral lipids and waxes. 10 mL of acetone was then added to elute glycolipids. 5 mL of MeOH was added to the solid phase extraction cartridges, in which glass waste tubes were replaced with 50-mL Teflon-lined centrifuge tubes to collect the fraction of interest. An additional 3 mL of MeOH was added and the cartridge was allowed to drain completely. 200 µl of C19:0 methyl ester internal standard was added to the samples, which were then allowed to evaporate at room temperature under $N_2$ gas.

Dried lipid samples were dissolved in 1 mL of a 1:1 MeOH:Toluene mixture, and 1 mL 0.2 M methanolic KOH was added. Samples were vortexed briefly and incubated in a 35$^oC$ water bath for 15 min., and allowed to cool to room temperature. 2 mL mixture of 4:1 hexane:$CHCl_3$, 300 µL of 1 M acetic acid, and 2 mL $dH_2O$ were added. Samples were vortexed for 20 sec. and centrifuged at 4000 rcf for 15 min. The upper organic layers were recovered in a small glass vials with Teflon-lined caps, while the bottom layer was washed with 2 mL of hexane:$CHCl_3$, followed by vortex and centrifugation. The new hexane fraction was added to

the previous and dried down under $N_2$ gas in the small glass vial. Samples were then dissolved thoroughly in 200 µL hexane and transferred to amber coated gas chromatography vials (Agilent), dried down and sealed in $N_2$ gas, stored at -80$^{o}$C until used for gas chromatography isotope ratio mass spectrometry (GC-IR-MS) analysis.

### 2.5.3 Data processing

PLFA content was calculated from raw measurements given injection volumes of each sample during GC-IR-MS analysis, and values were normalized to soil dry weight. Molar concentrations were calculated using an average PLFA molecular weight of 250 g mol$^{-1}$ as recommended in the literature (A. Frostegård & Bååth, 1996). Using the Shapiro-Wilk test, the PLFA data were shown to fail the assumptions of normality (P=0.0013 and 0.0002 for mineral and organic layers respectively), requiring the use of subsequent non-parametric statistical analyses.

## 2.6 SIP-Pyrotag Analysis

### 2.6.1 Experimental setup

A subset of soil microcosms were repeated identical to that of SIP-PLFA experiments (**Figure 4**). OM0, OM1, and select OM3 soils were incubated in parallel microcosms with $^{12}$C and $^{13}$C-hemicellulose. 35 microcosm pairs were incubated and analyzed.

### 2.6.2 Cesium-chloride gradient ultracentrifugation

Total genomic DNA was extracted from each parallel pair of $^{12}$C and $^{13}$C microcosms using MPBio FastDNA Spin Kit for Soil, as recommended by the kit manufacturer. DNA concentrations were quantified using the PicoGreen® fluorescent dye assay (Invitrogen), and 3 µg of DNA was used for each cesium chloride gradient ultracentrifugation. DNA was mixed with gradient buffer (0.1 M Tris, 0.1 M KCl, 1 mM EDTA, pH 8.0) for a final volume of 1.20 mL,

which is then added to 4.80 mL of 7.613 M CsCl stock solution for a desired volume of 6.0 mL in polyallomer Beckman centrifuge tubes, with a desired average density of 1.725 g $ml^{-1}$. Densities were estimated by measuring refractive indices of CsCl solutions using a digital handheld AR200 refractometer (Reichert), against a calibrated standard curve. Centrifuge tubes were heat sealed and placed in the Vti 65.2 vertical rotor. Ultracentrifugation conditions were set at 44100 rpm at 20$^{o}$C with vacuum, and samples were spun for 40 hours at max acceleration without brake.

### 2.6.3 Gradient fractionation

Gradients were quickly and carefully processed to minimize diffusion after centrifugation. Gradients were fractionated into 20 equal fractions using a single speed syringe pump (Razel Scientific, RE), and collected into 1.7-ml microfuge tubes.

### 2.6.4 DNA purification

DNA was precipitated from each fraction by adding 20 µg linear acrylamide (Ambion) and 2 volumes of PEG solution. Tubes were centrifuged at 13000 g for 30 min. at room temperature, and supernatant was removed. DNA pellets were washed with 500 µl 70% ethanol, centrifuged, and supernatant was removed once again. Pellets were dried under a SpeedVac and resuspended in 30 µl of TE buffer.

### 2.6.5 Measuring isotopic enrichment

Following gradient fractionation and purification of SIP-DNA samples, the selection of $^{13}$C-enriched DNA fractions was necessary, to confirm adequate separation and labeling of genomic DNA. This was achieved by hydrolysing a 5 µL aliquot of each of the 20 fractions of DNA in 100 µL of 88% formic acid at 70$^{o}$C for 1 hour. Following the incubation, fractions were

dried down under SpeedVac with an acid trap, and resuspended in 30 µL of 1% acetic acid (v/v). $^{13}C$ levels were determined by measuring the isotopic enrichment of individual adenine and guanine nucleotides on an ultra-performance liquid chromatography instrument coupled to a tandem mass spectrometer (UPLC-MS-MS). Fractions containing greater than 50% $^{13}C$ DNA (average densities 1.725-1.735 g $ml^{-1}$) was pooled.

### 2.6.6 PCR and sequencing preparation

Fraction DNA concentrations were quantified using the PicoGreen® fluorescent dye assay. Each sample of pooled fractions was diluted to a common concentration of 0.25 ng/µL DNA prior to barcoded amplification with polymerase chain reactions (PCR). Bacterial targets were amplified for the V1-V3 hypervariable region of the 16s rRNA gene using universal 27F and 519R barcoded bacterial primers, while fungal targets were using barcoded primers for the internal transcribed spacer (ITS2) region (**Table 2**) (Turner, Pryer, Miao, & Palmer, 1999; Weisburg, Barns, Pelletier, & Lane, 1991; White, Bruns, Lee, & Taylor, 1990). PCR reactions were performed in triplicate for each sample, and pooled prior to PCR cleanup.

**Table 2.** PCR primer pair sequences for universal bacteria 16s rRNA genes (27F, 519R) and fungal internal transcribed spacer (ITS) regions (ITS4F, ITS3R)

| Primer | Sequence (5'-3') | Reference |
|---|---|---|
| 27F | AGAGTTTGATCMTGGCTCAG | White et al. 1991 |
| 519R | GWATTACCGCGGCKGCTG | Turner et al. 1999 |
| ITS4F | TCCTCCGCTTATTGATATGC | Weisburg et al. 1990 |
| ITS3R | GCATCGATGAAGAACGCAGC | Weisburg et al. 1990 |

PCR reaction mixtures consisted of 2.5 ng of genomic DNA, 0.2 mM dNTPs, 200 nmol primers, 2 mM $MgCl_2$, 1x PCR Buffer, and 1.5 U of HotStar Taq DNA polymerase (Qiagen). Thermocycler program for PCR reactions for both bacterial and fungal targets consisted of initial denaturation at 95$^{o}$C for 1.5 min., followed by 35 cycles of denaturing at 95$^{o}$C for 40 sec., annealing at 59$^{o}$C for 40 sec., extension at 72$^{o}$C for 1 min., and a final extension at 72$^{o}$C for 10 min.

Agencourt AMPure XP PCR Purification kit was used to clean amplicons and remove primers. AMPure XP bead concentrations were calibrated with 0.9M NaCl 20% polyethylene glycol (PEG) 6000. 20 µL AMPure XP beads, 60 µL PEG solution, and 100 µL PCR product were mixed to achieve optimal recovery of PCR products 500 bp or greater in size. Bead and DNA suspensions were incubated for 5 min., followed by 5 min. incubation on a 96-well magnetic plate to form a bead pellet. Supernatant was discarded, and the magnetic bead pellet was washed with 200 µL 75% ethanol twice. Beads were allowed to dry for 2 min. and resuspended in 20 µL autoclaved deionized $H_2O$, followed by removal of resuspended DNA from beads. Purified PCR products were quantified for DNA concentration using the PicoGreen® fluorescent dye assay (Invitrogen), and each sample was normalized to 75 ng of DNA and pooled for 454- pyrosequencing using GS-FLX Titanium technology (Roche 454 Life Sciences, Branford, CT, USA). Bacterial and fungal amplicons were sequenced separately with a maximum of 40 samples per 454 half-plate.

### 2.6.7 Sequence processing and analysis

Bacterial sequences were processed using the MOTHUR standard operating procedure (Schloss, Gevers, & Westcott, 2011). This process entails an implementation of PyroNoise to reduce sequencing error, followed by trimming of barcoded sequencing primers, and setting a sequencing score cut-off of 350 flows. Sequences were de-replicated to lessen the computational burden for subsequent steps. Chimeric sequences were filtered out using the

uchime software implemented in MOTHUR. The SILVA 16s rRNA reference database was used for bacterial sequence alignment and taxonomy classification (Quast et al., 2013) , and operational taxonomic units (OTUs) were binned at a distance of 0.03, using the *k*-nearest neighbour (*k*-NN) algorithm implemented by MOTHUR (Schloss & Westcott, 2011). Similarly, fungal sequences were binned into OTUs using CrunchClust (Hartmann et al., 2012), against the fungal ITS UNITE database at a Levenshtein distance of 11 (Kõljalg et al., 2013). Both bacterial and fungal OTU matrices were normalized by subsampling to 1500 sequencing reads.

Statistical analyses were performed using select packages in R, and PRIMER 6 with the PERMANOVA+ add-on. All perMANOVA analyses utilized the permutation of residuals under a reduced model with 999 permutations. The R Boruta package was used for random forest analysis to select for important features in pyrosequencing data sets. Bubble plots were generated using a perl script developed by Charles Howes (Zaikova et al., 2010). Phylogenetic trees were generated using Silva-arb by a quick-parsimony addition of pyrotag sequences into a small subunit rRNA (SSU) tree provided by Silva (Quast et al., 2013), and visualized using the web-based Interactive Tree of Life (iTOL). Closest reference isolate strains were identified using the web-based RDP SeqMatch tool (Cole et al., 2009). Hemicellulolytic bacterial and fungal community richness and diversity estimations were calculated using MOTHUR.

# 3 Results

## 3.1 $CO_2$ Mineralization

Hemicellulose-induced respiration was measured in microcosms containing soil from the IDF sites. Respiration kinetics were significantly higher in organic versus mineral soil (**Figure 5**). However, the concentration of added hemicellulose was higher in the organic layer soil. Harvesting treatments did not significantly affect respiration rates; although there was a trend for higher rates in harvested versus the control treatments with organic layer soil (**Figure 6**).

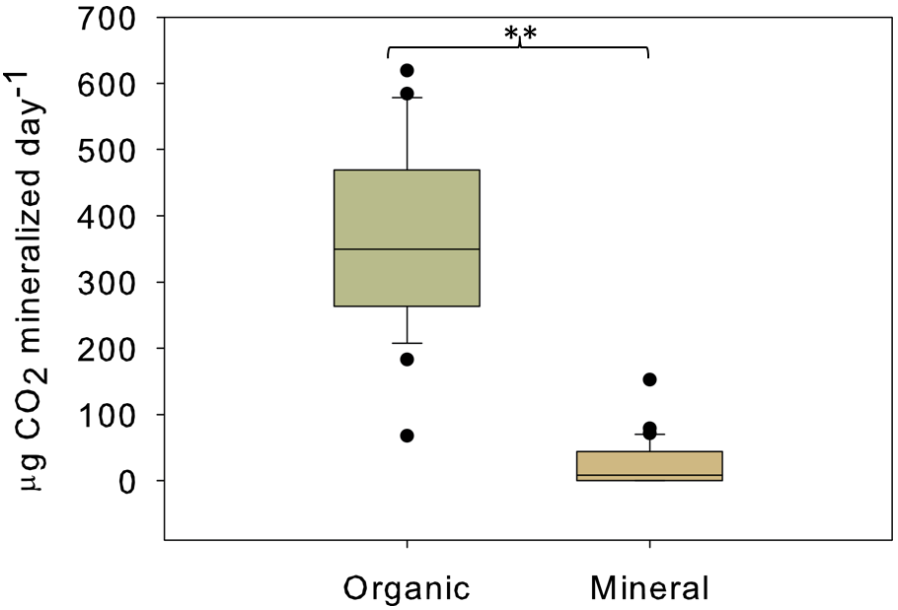



**Figure 5.** Box and whisker plots comparing respiration in organic and mineral layer soils from IDF. The Mann-Whitney rank sum test demonstrated statistical significance between $CO_2$ mineralization rates in the two soil layers (**P<0.001)

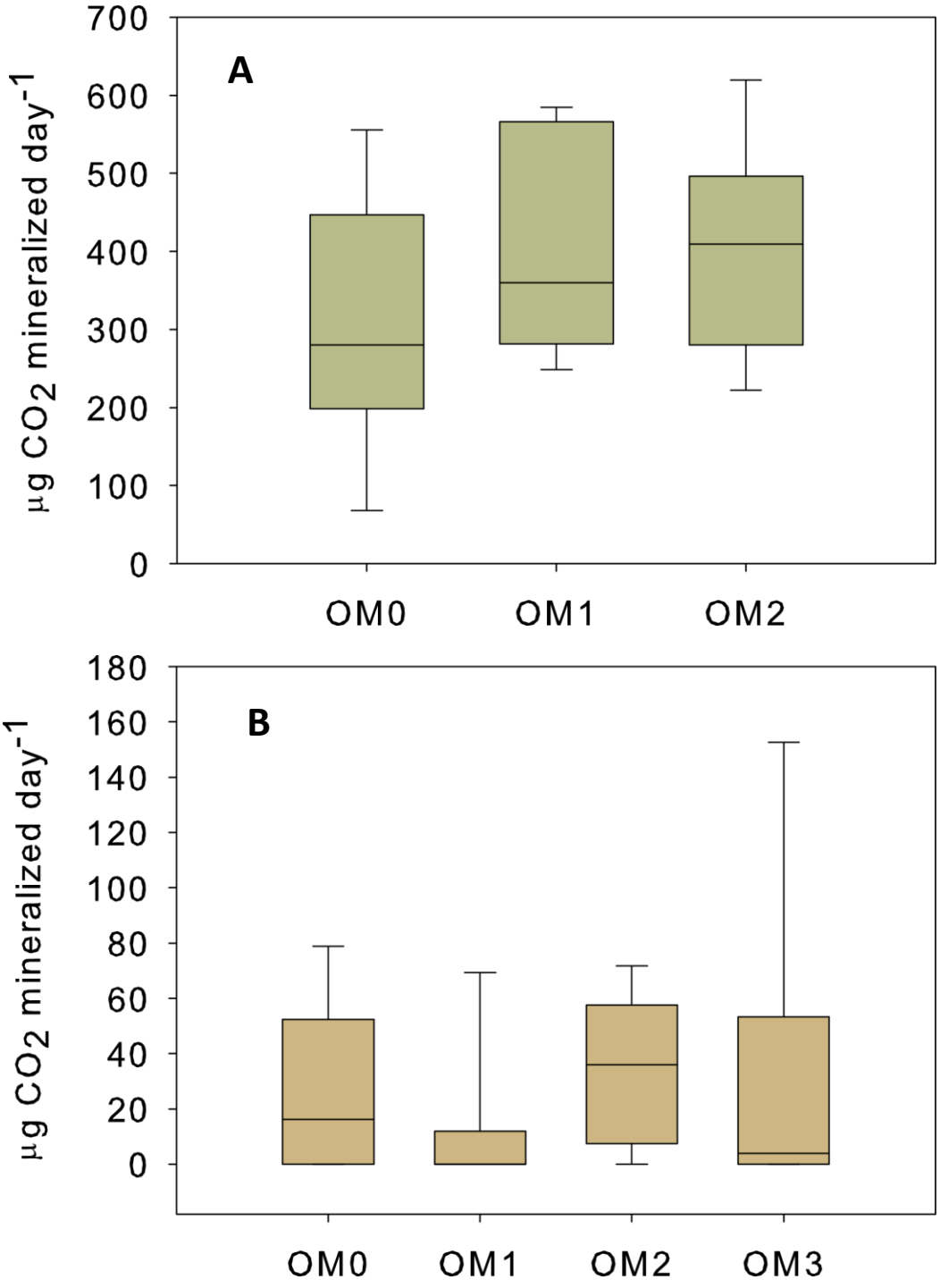



**Figure 6A&B.** Box and whisker plots comparing respiration amongst harvesting treatments in the (**A**) organic and (**B**) mineral layers.

## 3.2 Incorporation of Hemicellulose Carbon into PLFA

### 3.2.1 Total incorporation into microbial biomass

After incubation with $^{13}$C-hemicellulose, there was a strong correlation between total PLFA carbon and the amount of $^{13}$C incorporated into PLFAs. The relationship between total PLFAs and $^{13}$C incorporation was linear, and the slope of the line differed substantially between microcosms with soil from the organic versus mineral layer (**Figure 7**). Since total PLFAs are correlated with biomass, the total biomass at the end of the incubation is correlated with $^{13}$C incorporation. This relationship may be due to growth on hemicellulose. Alternatively or additionally, this relationship may reflect the biomass prior to incubation, with higher initial biomass causing more rapid hemicellulose degradation. The greater slope for microcosms with organic versus mineral layer soil suggests that a greater proportion of the organic layer community degrades hemicellulose.

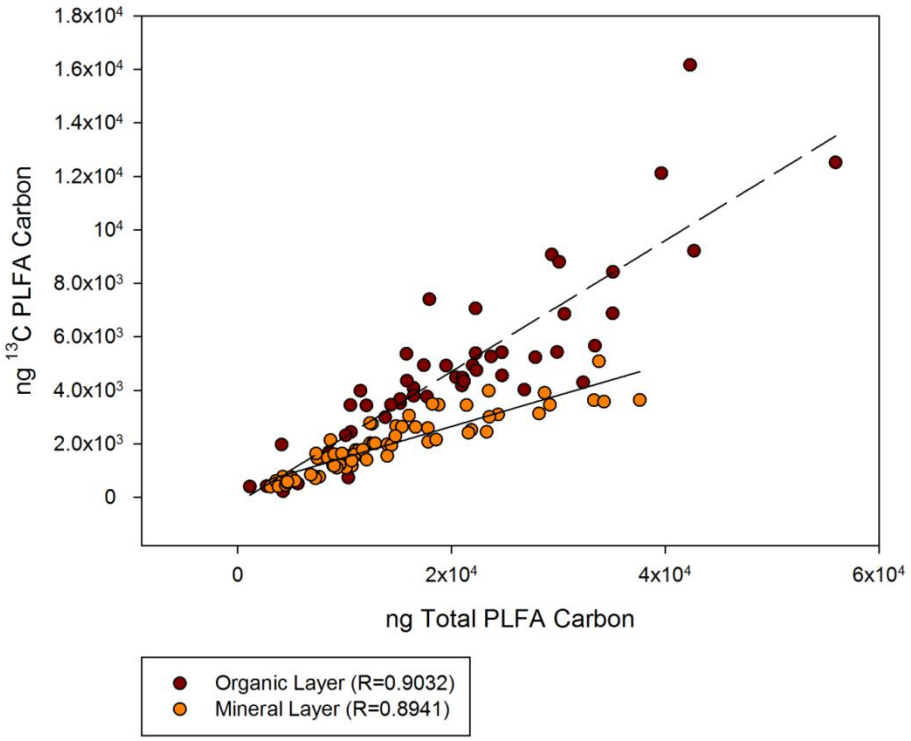
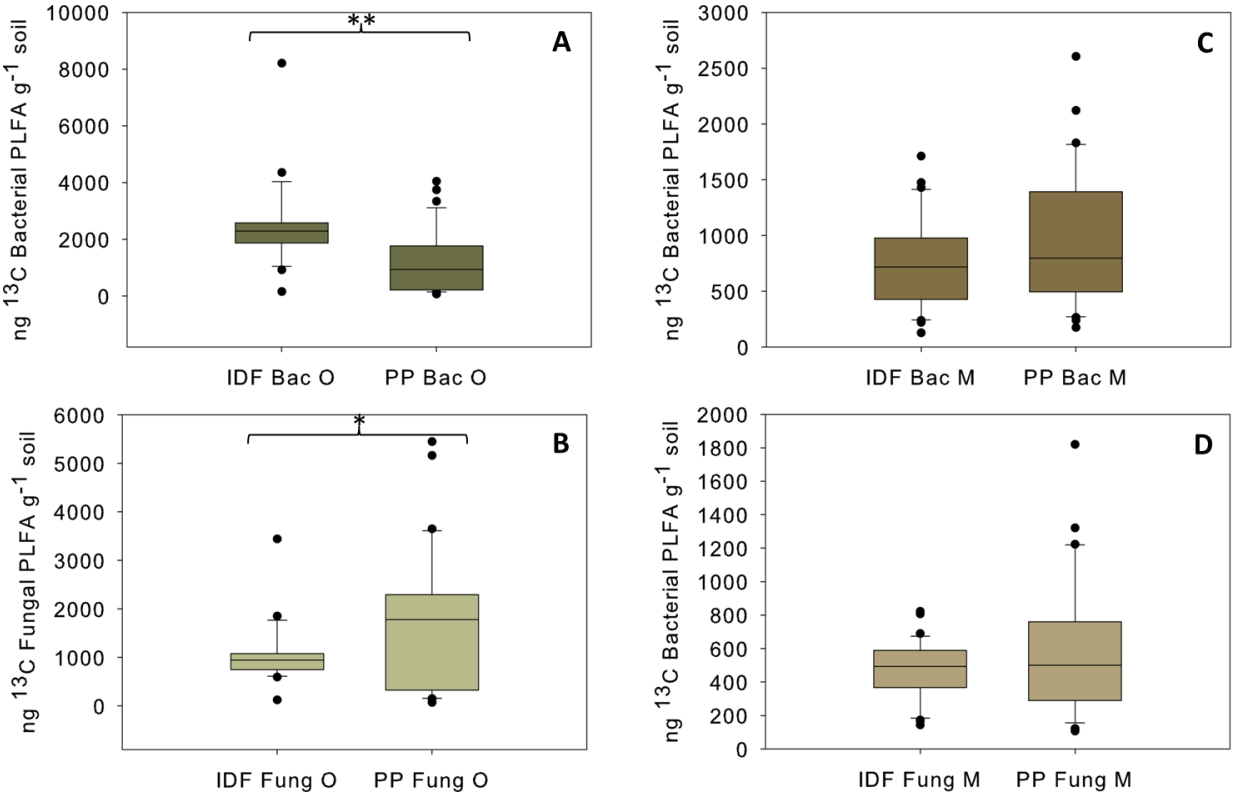
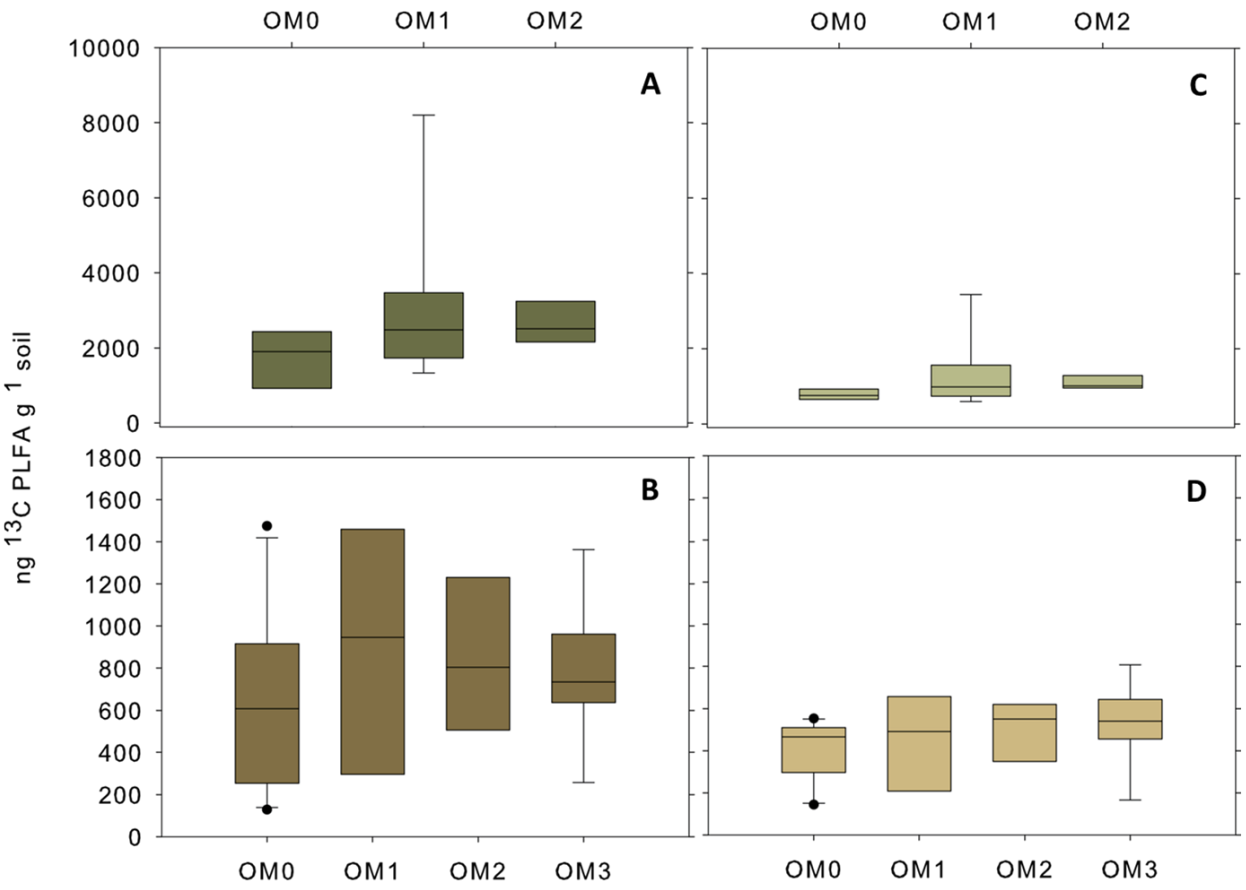



**Figure 7.** Scatter plot of ng of $^{13}$C PLFA carbon vs. ng of total PLFA carbon

Both bacterial and fungal incorporation of $^{13}C$ from hemicellulose differed between the interior Douglas fir (IDF) and ponderosa pine (PP) ecozones. The $^{13}C$-enrichment of total bacterial PLFAs was greater in organic soils from the IDF ecozone than those from the PP ecozone. On the other hand, $^{13}C$-enrichment of total fungal PLFAs was greater in organic soils from the PP ecozone than in those from the IDF ecozone (**Figures 8A&B**). There were no observable differences between ecozones in total $^{13}C$-enriched bacterial and fungal PLFA markers in the mineral layer (**Figure 8C&D**). Harvesting treatments did not have any significant effects on total $^{13}C$-enrichment of bacterial or fungal PLFAs in the mineral soils. Despite this, enrichment appeared to increase with increasing levels of OM removal in soils from the IDF ecozone (**Figure 9A-D**).




**Figures 8A-D.** Box and whisker plot comparing total $^{13}C$ incorporation into bacterial and fungal PLFA in IDF and PP ecozones in the organic (**A**&**B**) and mineral layers (**C**&**D**). Significant differences based on the Mann-Whitney rank sum test are shown (*P<0.05, **P<0.01).




**Figures 9A-D.** Box and whisker plot comparing total PLFA incorporation into (**A**&**B**) bacterial and (**C**&**D**) fungal biomass amongst harvesting treatments in the IDF ecozone, in both organic (top) and mineral (bottom) layers.

### 3.2.2 $^{13}$C-PLFA profiles

Profiles based on the amount of $^{13}$C in individual PLFAs were used as a general comparator of populations in soil communities that incorporated carbon from hemicellulose. Presumably, differences in profiles reflect variable incorporation of hemicellulose by different populations. The profiles were compared using the Bray-Curtis dissimilarity index. Populations differed significantly between soil layers, between ecozones and among OM removal treatments (**Table 3**). Soil layer and ecozone accounted for the vast majority of variability in the profiles. Ordination revealed obvious differences between profiles from organic and mineral

layers as well as between ecozones (**Figure 10**). The abundances of individual $^{13}C$-enriched PLFA markers are visualized in a heatmap, revealing soil layer and ecozone differences in levels of $^{13}C$-incorporation in bacterial and fungal markers. The c18:1n9c/3n3 and c18:2n6c fungal markers were more enriched in the PP ecozone of the organic layer, while c18:1n9t/7c was greater in IDF (**Figure 11**). In contrast, greater enrichment was found in Gram-positive and Gram-negative bacterial markers in IDF samples. The harvesting treatments accounted for a relatively small, yet significant component of the variability. Pairwise perMANOVA indicated significant differences between profiles of some of the harvesting treatments (**Table 4**). In the IDF ecozone, OM0 tended to differ from other treatments, while in the PP ecozone, OM3 tended to differ from other treatments. Random forest analysis with Boruta feature selection found that all measured PLFAs were predictive of soil layer and ecozone, indicating that all the PLFAs contributed to distinguishing the profiles from these sample groups. By contrast, only a subset of PLFAs (a15:0, a17:0, c18:2n6c and c16:1n5c/7t) were strongly predictive of OM removal treatments (Z-score greater than 4), indicating that these PLFAs were primarily responsible for distinguishing profiles from the different treatments (**Figure 12**).

**Table 3.** perMANOVA test indicating the effects of factors on $^{13}$C-PLFA profiles.

| Factor | P(perm) | % of Variance Explained |
|---|---|---|
| Soil Layer | 0.001 | 39.44 |
| Harvesting Treatment | 0.001 | 1.36 |
| Ecozone | 0.001 | 18.78 |
| Harvesting Treatment x Soil Layer | 0.021 | 1.43 |
| Ecozone x Soil Layer | 0.001 | 12.64 |
| Harvesting Treatment x Ecozone | 0.001 | 3.67 |
| Harvesting Treatment x Ecozone x Soil Layer | 0.001 | 7.39 |
| Residual | | 15.29 |

**Table 4.** Pairwise perMANOVA test comparing effects of harvesting treatment on PLFA profiles of $^{13}$C hemicellulose incorporation. Univariate t-statistics were indicated on significant tests with $P < 0.05$, while bolded t-statistics indicate $P < 0.01$.

| Layer | Treatments | IDF | PP |
|---|---|---|---|
| **Organic** | OM0 x OM1 | **3.34** | n.s. |
| | OM0 x OM2 | 2.02 | n.s. |
| | OM0 x OM3 | - | n.s. |
| | OM1 x OM2 | n.s. | n.s. |
| | OM1 x OM3 | - | 1.93 |
| | OM2 x OM3 | - | n.s. |
| **Mineral** | OM0 x OM1 | 1.88 | n.s. |
| | OM0 x OM2 | n.s. | n.s. |
| | OM0 x OM3 | **2.04** | 1.82 |
| | OM1 x OM2 | n.s. | n.s. |
| | OM1 x OM3 | n.s. | **2.48** |
| | OM2 x OM3 | n.s. | n.s. |

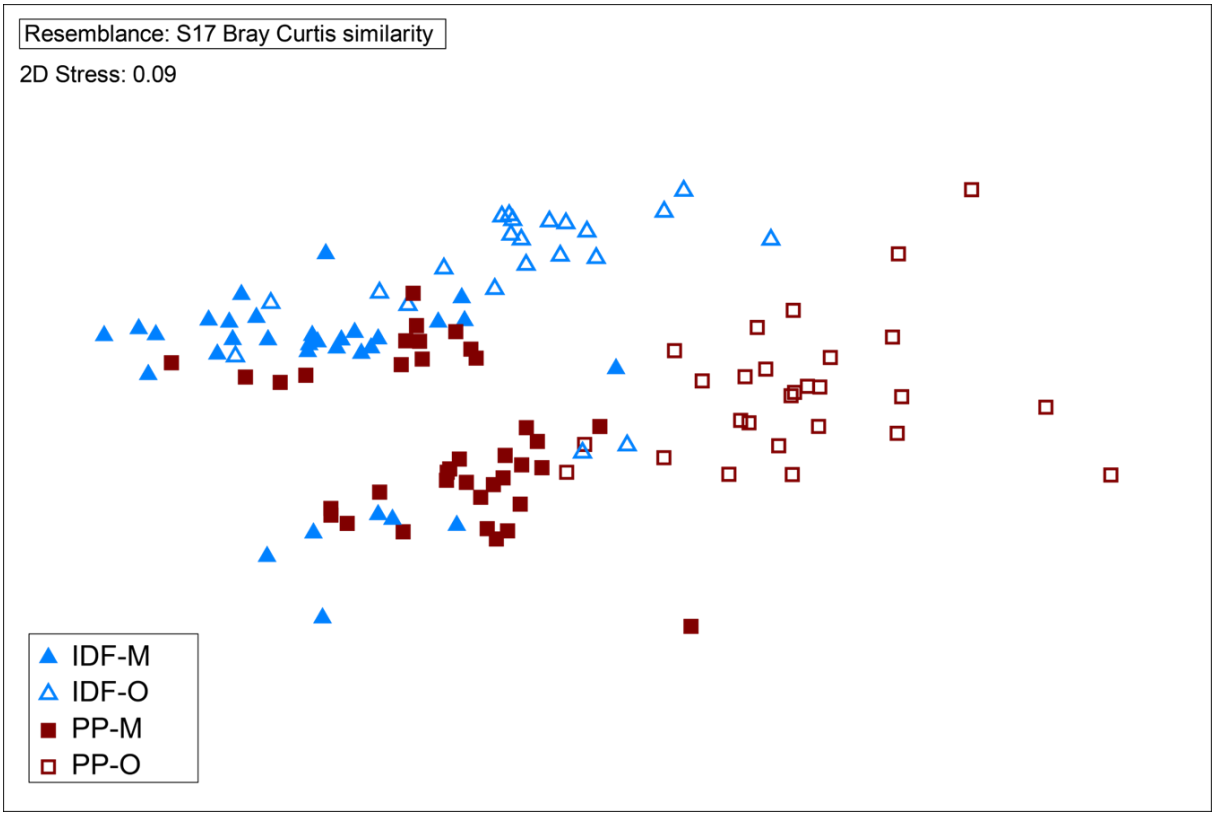



**Figure 10.** Non-metric multidimensional scaling (NMS) of $^{13}$C-profiles using Bray-Curtis dissimilarity.

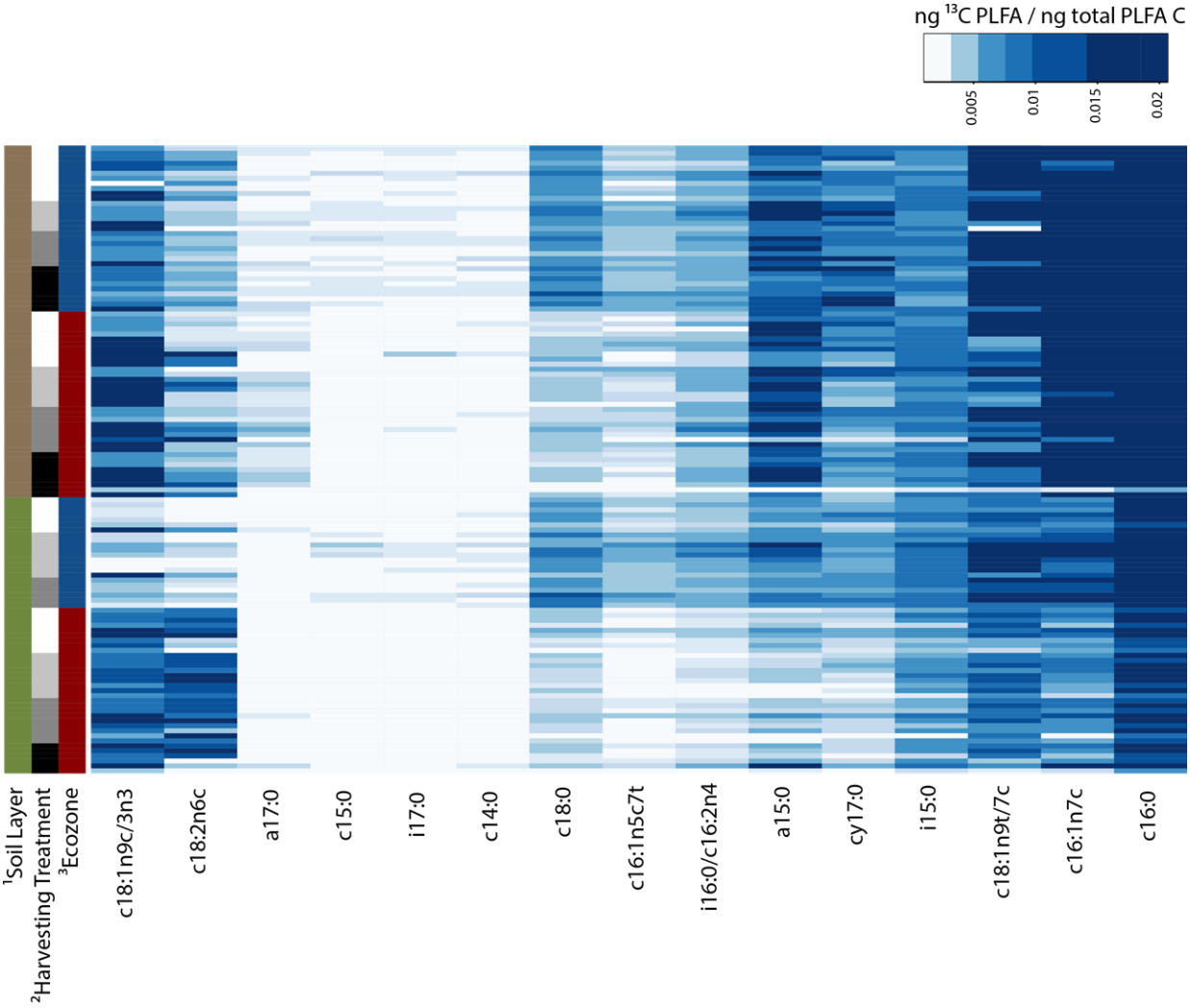



**Figure 11.** Heat map of hemicellulose incorporation into individual PLFAs.
[1]Green and brown annotations represent organic and mineral layers respectively,
[2]Greyscale annotations represent increasing severity of harvesting treatment, with white representing OM0, and black representing OM3.
[3]Blue and Red annotations represent IDF and PP eco-zones.

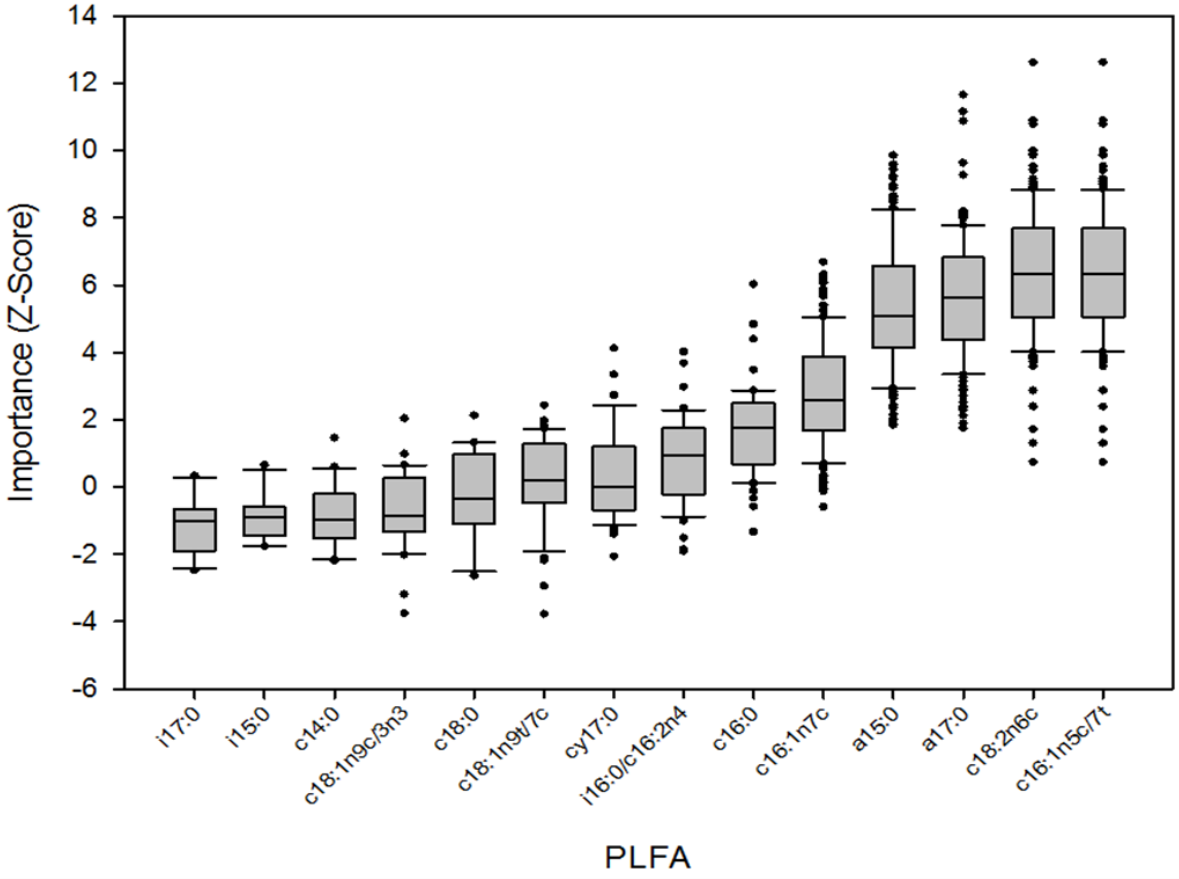



**Figure 12.** Random forest analysis using Boruta comparing importance of each PLFA for classifying levels of harvesting treatment.

### 3.3 Hemicellulolytic Microbial Populations *In Situ*

#### 3.3.1 Identification of putatively hemicellulolytic populations

DNA fractions containing high levels of $^{13}C$ were pooled and analyzed by pyrotag sequencing. Enrichment of $^{13}C$ in DNA from microcosms was determined using a combination of the PicoGreen assay and UPLC-MS-MS. Substantial $^{13}C$ enrichment was found in density gradient fractions with densities between 1.725 and 1.735 g $ml^{-1}$, corresponding to the first 5 to 6 fractions collected (**Figure 13**). These measurements were essential for determining the the $^{13}C$-enriched fractions, which were pooled for pyrotag analysis. OTUs having a 5-fold greater relative abundance in pooled fractions from $^{13}C$ microcosms versus in equivalent pooled fractions from $^{12}C$ control microcosms were identified as $^{13}C$-enriched OTUs, representing putatively hemicellulolytic populations. A total of 104 bacterial and 52 fungal putatively hemicellulolytic OTUs were identified in 223,989 bacterial and 97,180 fungal sequences.

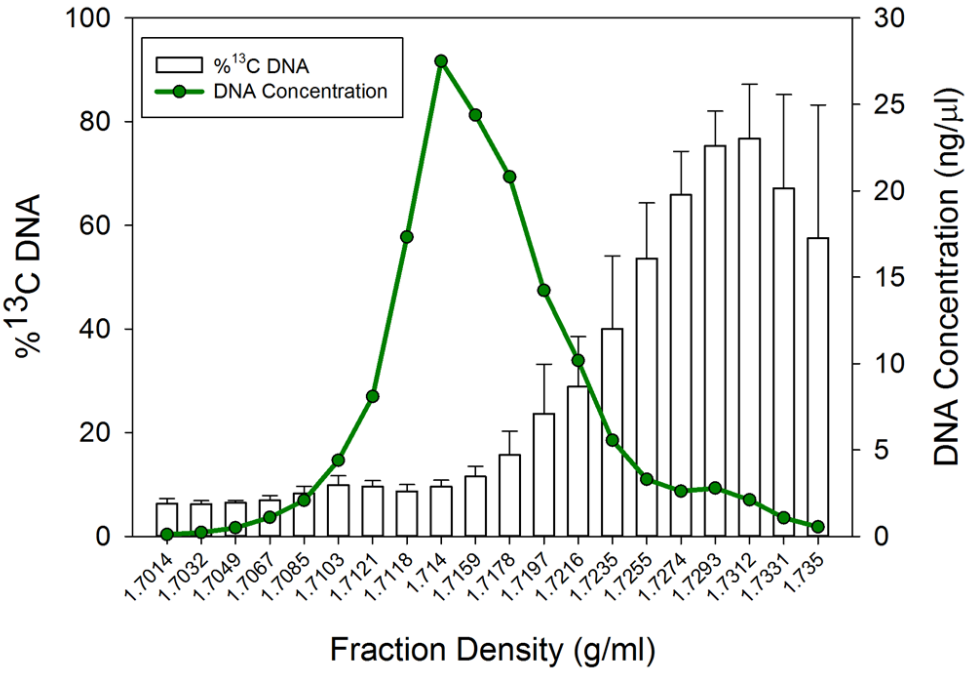



**Figure 13.** Distribution of DNA concentrations throughout CsCl gradient, indicating incorporation of $^{13}C$-hemicellulose into DNA.

Putative hemicellulose-degrading populations had a patchy distribution in microcosms. The $^{13}$C-enriched subset of bacterial microcosm communities show distinct ecozone and soil layer clusters, but harvesting treatments did not cluster due to the high variability in the data and a low number of replicates (n=3) (**Figures 14A&B**). Fungal microcosm communities exhibited an even greater degree of patchiness, showing no clustering in NMS. Thus, variability of putatively hemicellulose-degrading populations was too great in microcosms to allow assessment of harvesting treatment effects on these populations. To make statistical comparisons, relative abundances of the 104 bacterial and 52 fungal putatively hemicellulolytic OTUs were analyzed within a larger and highly replicated pyrotag dataset representing 691 soil samples from 18 forest sites in six ecozones. This pyrotag library, representing bacterial and fungal communities *in situ*, was provided by K. Maas (unpublished data). Communities from microcosms were significantly different from those found *in situ*. A substantial portion of the hemicellulolytic populations found in microcosms were among those of high relative abundance *in situ* (**Figure 15**).

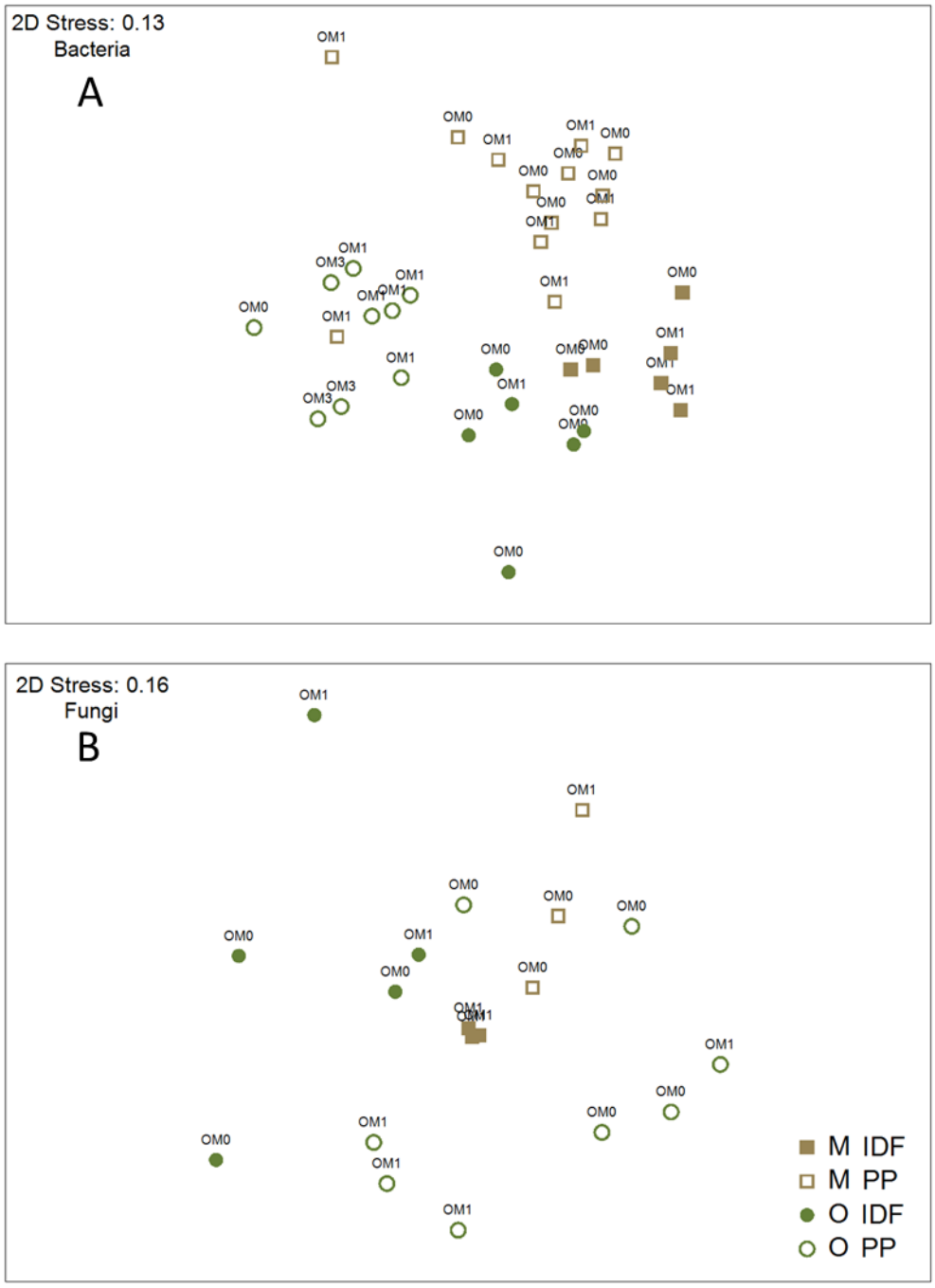



**Figure 14A&B.** Non-metric multidimensional scaling (NMS) illustrating putatively hemicellulolytic populations in (**A**) bacterial and (**B**) fungal pyrotags from microcosm experiments, using Bray Curtis dissimilarity.

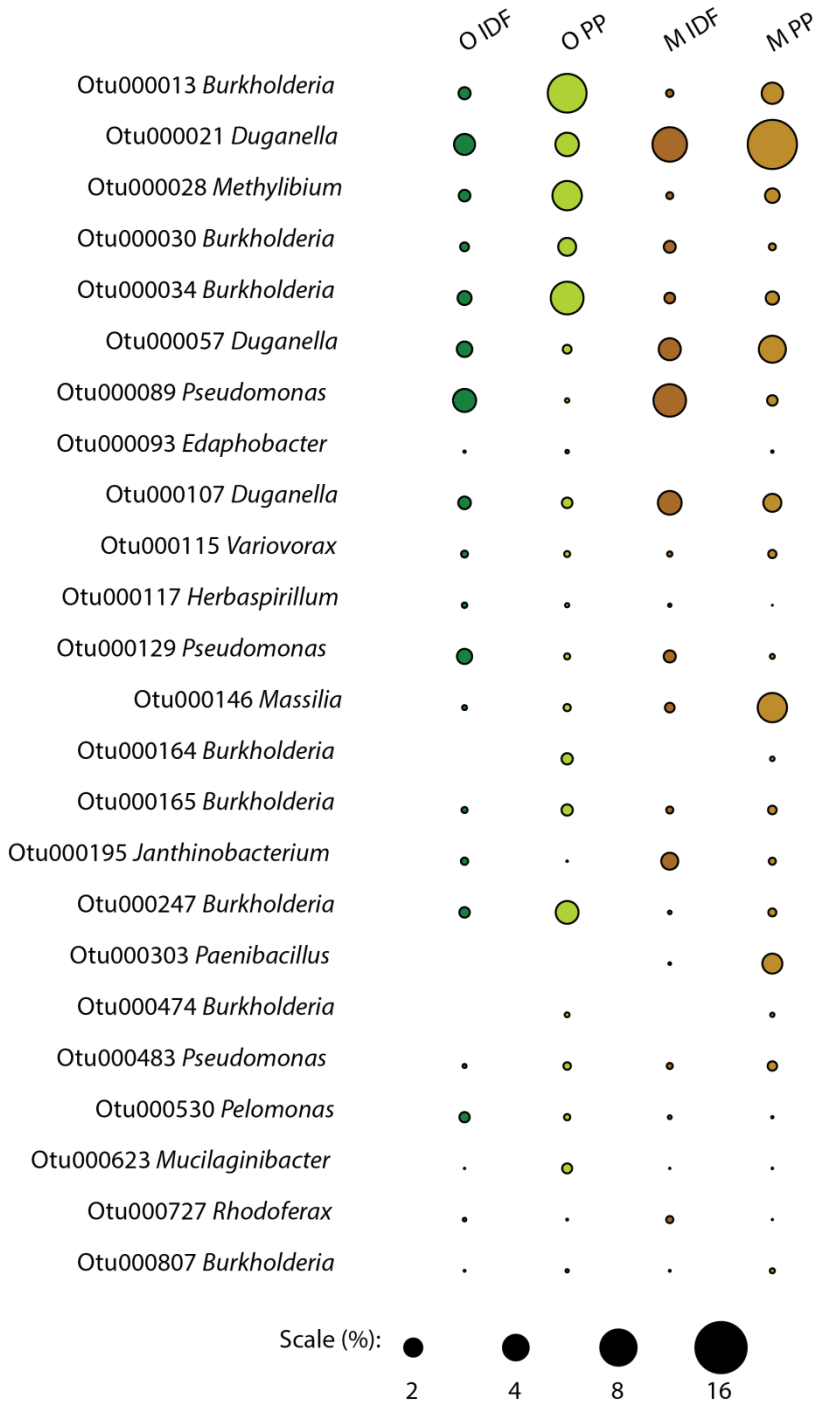



**Figure 15.** Percent relative abundance of putative hemicellulolytic bacterial OTUs in microcosms in organic (O) and mineral (M) soils from the IDF and PP ecozones.

### 3.3.2 Distribution of hemicellulolytic bacteria *in situ*

The putatively hemicellulolytic bacterial populations were found across all ecozones. These populations constituted an average of 1.96% of the total community across both organic and mineral soil layers in 6 ecozones (**Figure 16**). An average of 2.57% of the total bacterial *in situ* pyrotag community in the organic layer corresponded to hemicellulolytic OTUs, compared to an average of 1.47% in the mineral layer. The mineral layer of IDF soils had the lowest average in which 0.72% of its bacterial community were hemicellulolytic, while the organic layer of PP soils had the highest, with 5.06% of its bacterial community being potential hemicellulose degraders. **Figure 17** illustrates the mean relative abundance of the dominant hemicellulolytic bacterial taxa in each of the 6 ecozones, with the 8 of the 24 dominant hemicellulolytic OTUs being affiliated with the genus, *Burkholderia*. Of those 24 abundant putative hemicellulolytic bacterial OTUs, 18 belonged to β-Proteobacteria, 3 belonged to γ-Proteobacteria, and the remaining belonging to Firmicutes, Bacteroides, and Acidobacteria (**Figure 18**). Many of the hemicellulolytic OTUs are not closely related to existing isolates represented in GenBank.

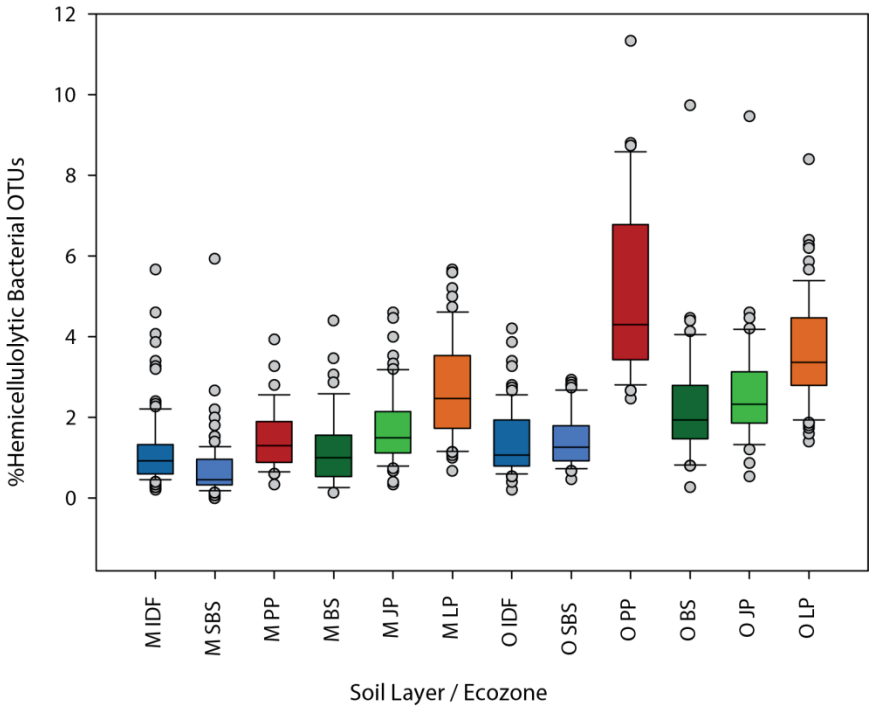



**Figure 16.** Box and whisker plots comparing the relative abundance of putatively hemicellulolytic populations *in situ*.

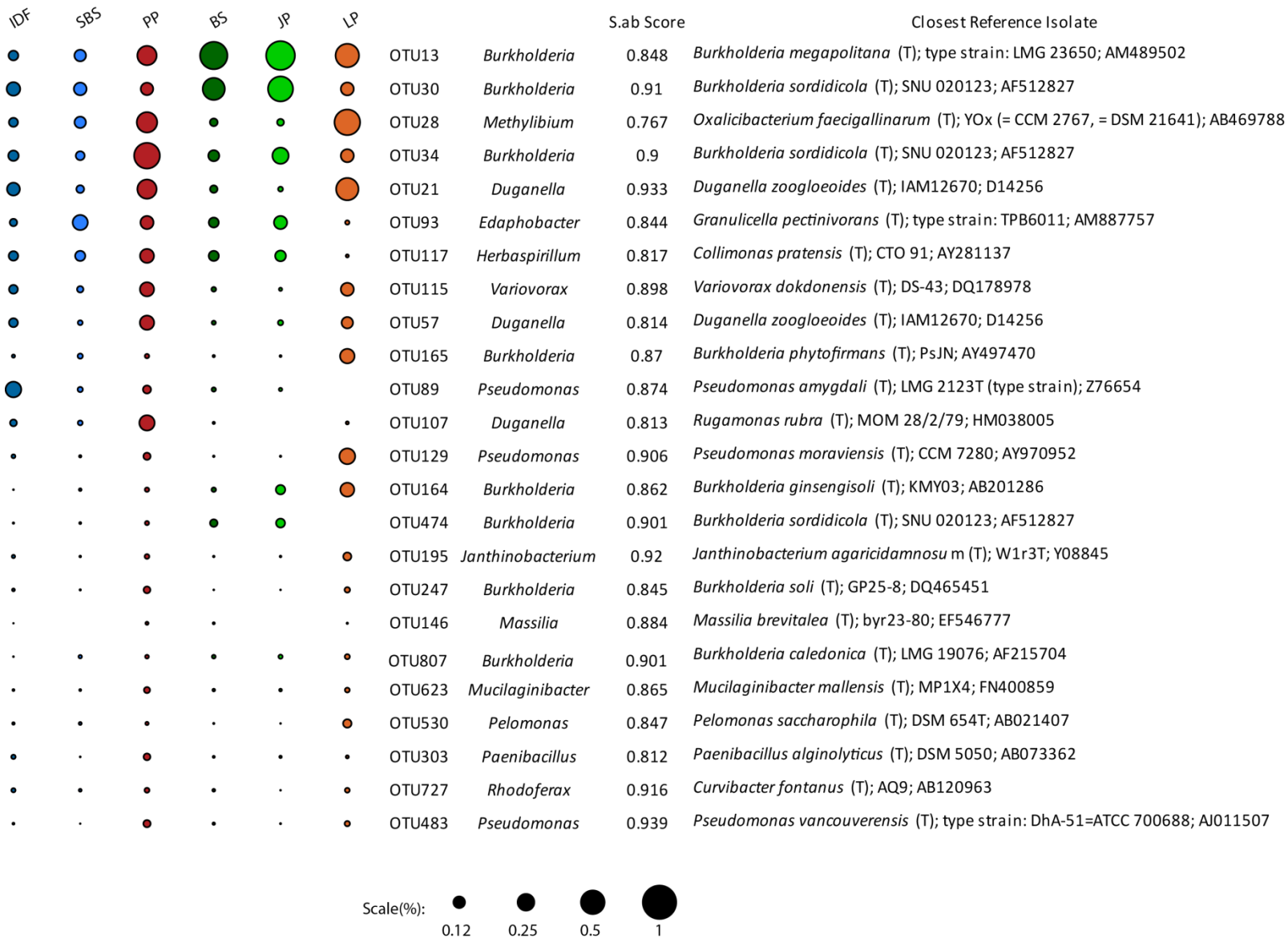



**Figure 17.** Hemicellulolytic bacterial OTUs of greater than 0.01% relative abundance *in situ* and their closest reference isolates.

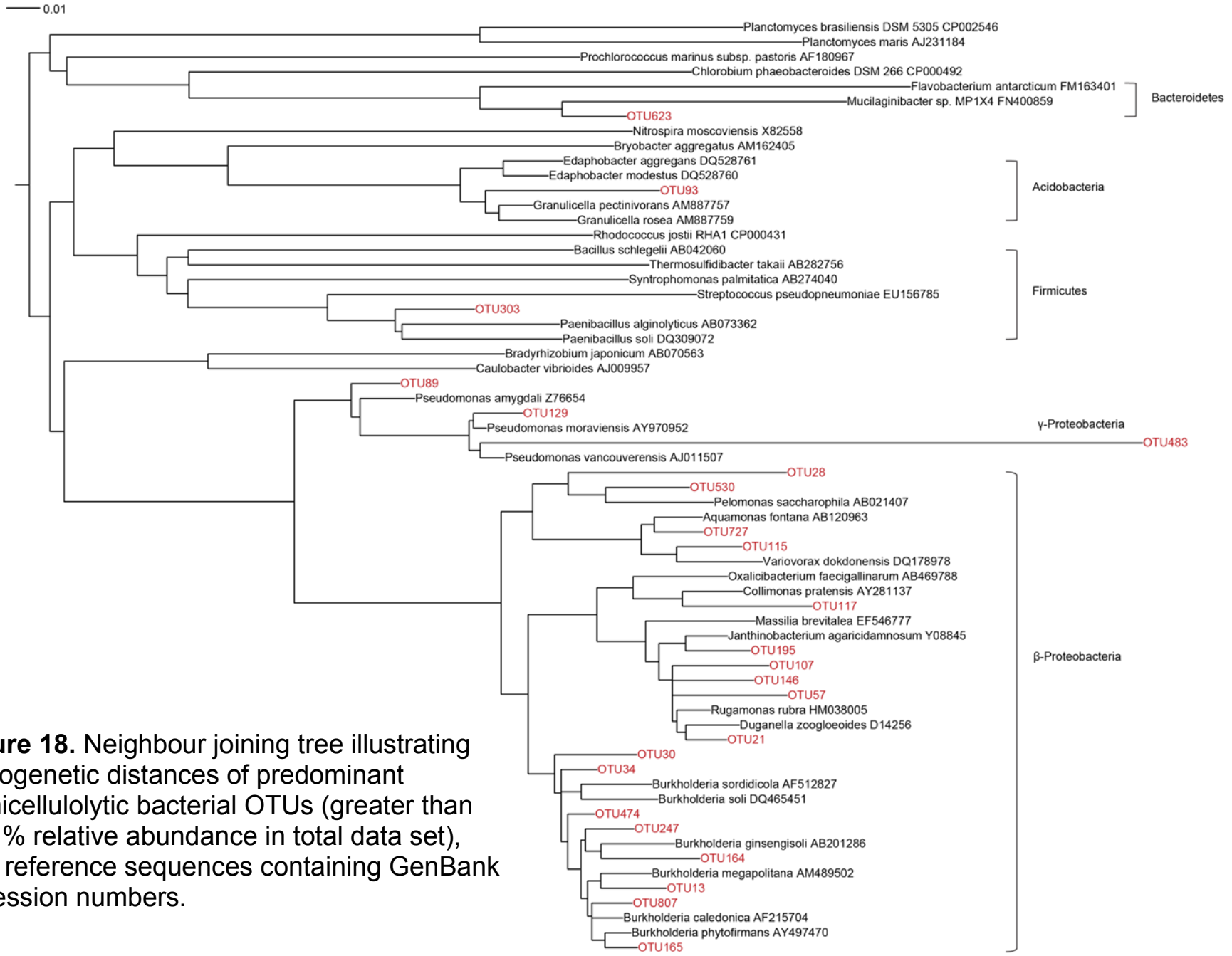



**Figure 18.** Neighbour joining tree illustrating phylogenetic distances of predominant hemicellulolytic bacterial OTUs (greater than 0.01% relative abundance in total data set), with reference sequences containing GenBank accession numbers.

### 3.3.3 Harvesting impacts on hemicellulolytic bacteria

Harvesting significantly altered the putatively hemicellulolytic bacterial populations in some ecozones. All experimental factors were found to be statistically significant, but ecozone differences explained most of the variation (19.2%) in the perMANOVA model (**Table 5**). When ecozones and soil layers were separately examined harvesting had the largest effect on hemicellulolytic populations in the organic layer of the PP ecozone (**Figure 19**). Generally the effect of harvesting was smaller in ecozones whose soils were not used in microcosm experiments. Ecozone differences were clearly illustrated in both layers using non-metric multidimensional scaling (NMS), and individual OTUs strongly correlated with ecozone clusters (**Figure 20A&B**). A 3-dimensional NMS was used to visualize the ordination, since the ordination stress (> 0.2) in 2 axes did not sufficiently explain species composition (Kruskal, 1964).

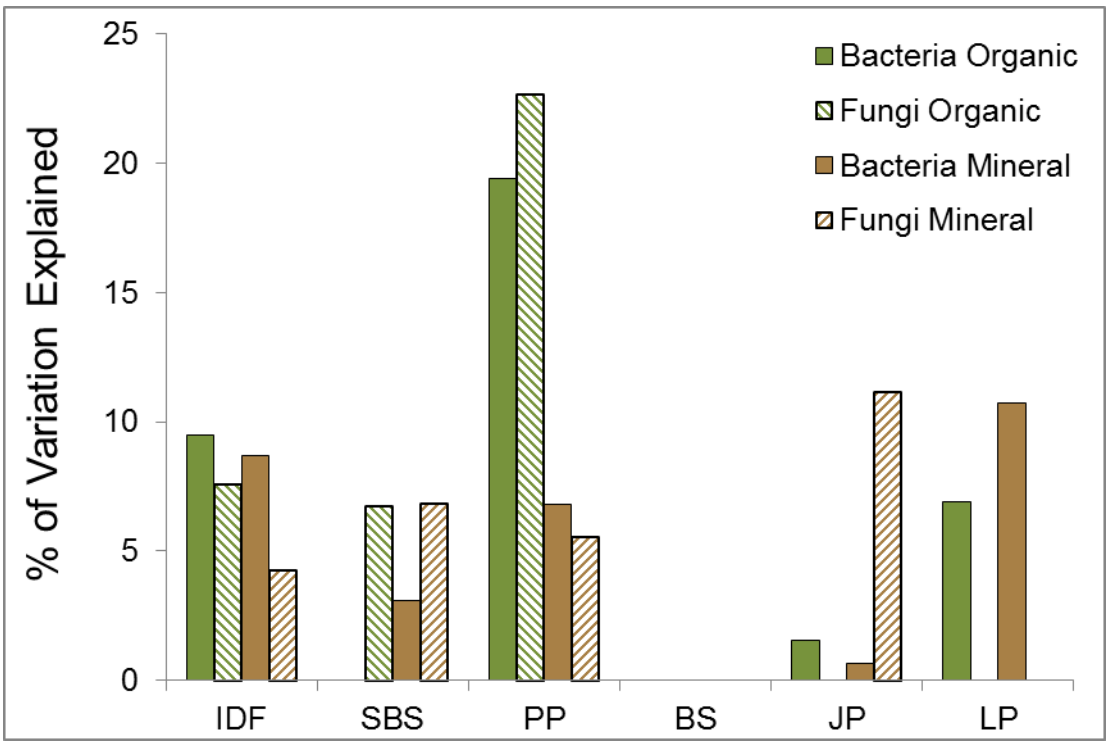



**Figure 19.** Effect size of harvesting treatment on putatively hemiellulolytic bacterial and fungal populations as estimated by components of variation in perMANOVA.

**Table 5.** perMANOVA test indicating effects of factors on relative abundances of hemicellulolytic bacterial OTUs, clustered at a 97% identity threshold and subsampled to 1500 reads.

| Factor | P(perm) | % of Variance Explained |
|---|---|---|
| Soil Layer | 0.001 | 12.42 |
| Ecozone | 0.001 | 19.22 |
| Harvesting Treatment | 0.001 | 0.85 |
| Ecozone x Soil Layer | 0.001 | 16.07 |
| Soil Layer x Harvesting Treatment | 0.001 | 1.05 |
| Ecozone x Harvesting Treatment | 0.001 | 1.93 |
| Ecozone x Soil Layer x Harvesting Treatment | 0.002 | 1.25 |
| Residual | | 47.20 |

**Table 6.** Pairwise perMANOVA test comparing the effects of harvesting treatment on relative abundances of hemicellulolytic bacterial OTUs, clustered at a 97% identity threshold and subsampled to 1500 reads. Univariate t-statistics were indicated on significant tests with $P < 0.05$, while bolded t-statistics indicate $P < 0.01$.

| Layer | Treatments | IDF | SBS | PP | BS | JP | LP |
|---|---|---|---|---|---|---|---|
| **Organic** | OM0 x OM1 | 1.35 | n.s. | n.s. | n.s. | n.s. | **2.34** |
| | OM0 x OM2 | **1.64** | n.s. | 1.58 | n.s. | 1.57 | **2.16** |
| | OM0 x OM3 | - | - | **2.30** | n.s. | n.s. | **2.13** |
| | OM1 x OM2 | n.s. | n.s. | n.s. | n.s. | n.s. | n.s. |
| | OM1 x OM3 | - | - | **2.19** | n.s. | n.s. | n.s. |
| | OM2 x OM3 | - | - | n.s. | n.s. | n.s. | 1.41 |
| **Mineral** | OM0 x OM1 | **2.32** | n.s. | n.s. | n.s. | n.s. | **2.50** |
| | OM0 x OM2 | **2.47** | n.s. | n.s. | n.s. | n.s. | **1.86** |
| | OM0 x OM3 | **2.18** | **1.73** | **1.45** | n.s. | **1.79** | **1.80** |
| | OM1 x OM2 | n.s. | n.s. | n.s. | n.s. | n.s. | **1.76** |
| | OM1 x OM3 | n.s. | 1.55 | **1.81** | n.s. | n.s. | **1.66** |
| | OM2 x OM3 | n.s. | n.s. | 1.62 | n.s. | **1.80** | n.s. |

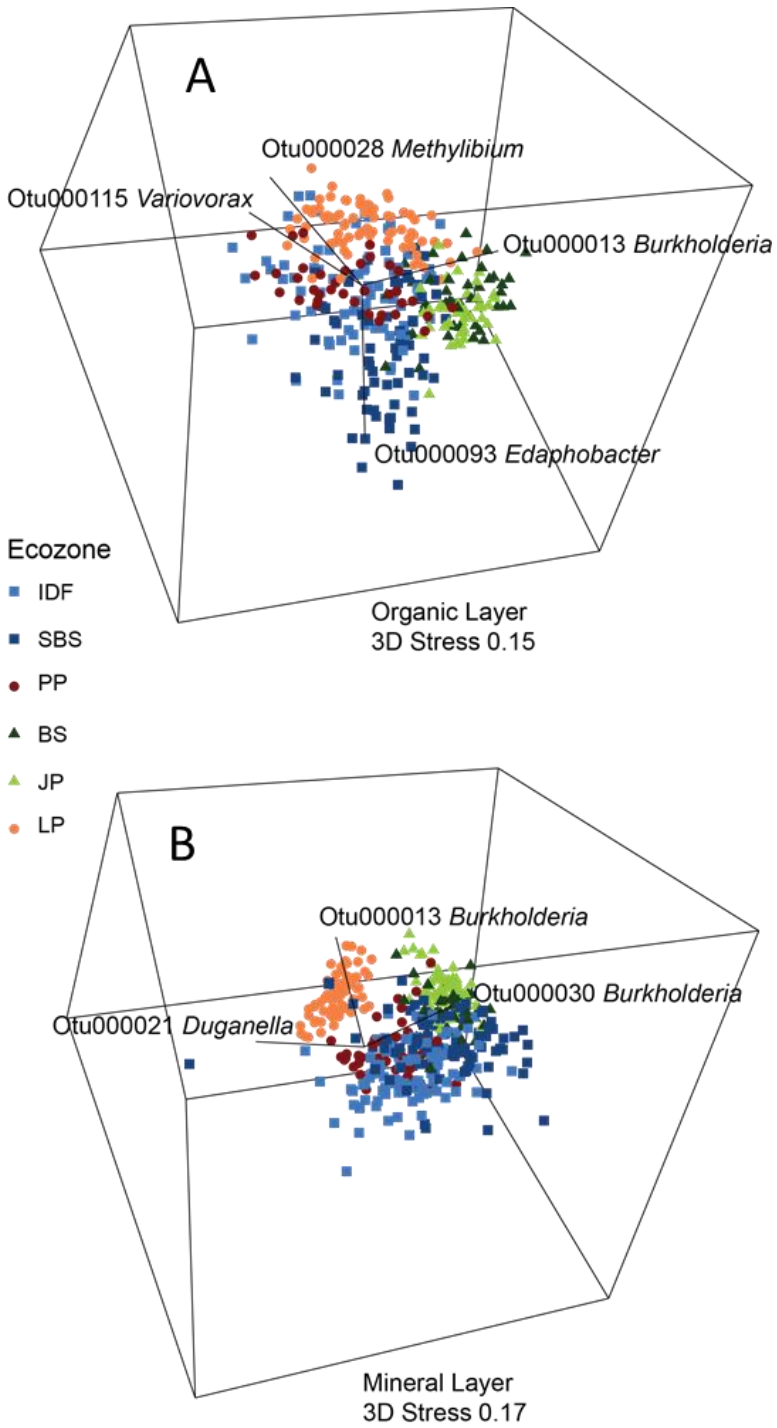



**Figure 20A&B.** 3D Non-metric multidimensional scaling (NMS) illustrating putatively hemicellulolytic bacterial populations across ecozones in the (**A**) organic and (**B**) mineral layers, using Bray Curtis dissimilarity. Vectors indicate OTUs with Pearson's correlation >0.6.

Harvesting significantly altered putatively hemicellulolytic bacterial populations (OM0 versus harvested treatments) in the ecozones where hemicellulolytic bacterial OTUs were originally identified in (IDF and PP). OM0 reference plots were significantly different from the harvesting treatments in both organic and mineral soil layers, but the different harvesting treatments (OM1, OM2, and OM3) showed no consistent differences amongst themselves in the IDF ecozone (**Table 6**). The effect of the harvesting treatments can be observed with the increasing univariate t-statistic as the severity of harvesting treatment goes from OM0 versus OM1 to OM0 versus OM3. **Figure 21** illustrates the clustering of different harvesting treatment groups in putatively hemicellulolytic bacterial populations at the IDF and PP ecozones in a canonical analysis of principal coordinates (CAP) ordination. Most misclassifications in the CAP analysis occurred in the OM1 and OM2 harvesting treatments (38% success rate for OM1 and 35% for OM2), indicating strong separation strengths in the reference OM0 and most extreme OM3 harvesting treatments, whereas the intermediate harvesting treatments show weaker separation.

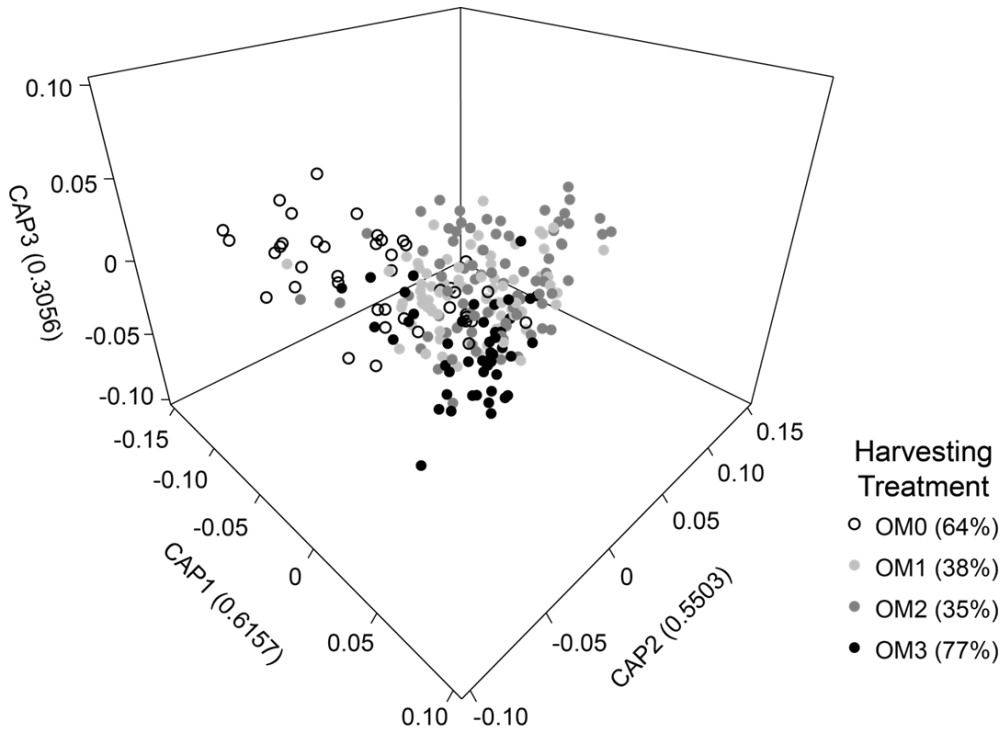



**Figure 21.** Canonical analysis of principal coordinates (CAP) ordination of relative abundances of putatively hemicellulolytic bacterial populations at IDF and PP ecozones showing maximized differences among harvesting treatments using Bray-Curtis dissimilarity. Classification success rates for each harvesting treatment are given in parentheses. The canonical correlation of each CAP axis is given in parentheses.

The putatively hemicellulolyitc subset of the bacterial community identified in soils from the IDF and PP ecozones was also examined in soil communities from the other ecozones represented by pyrotag libraries. Harvesting treatments had either no effect or significant effects only at the most extreme treatment pairs (OM0 versus OM3) on that subset of the community in the SBS, BS, and JP ecozones (whose soils were not used for microcosm experiments). By contrast, in the LP ecozone, that subset of the community differed between most harvesting treatment pairs. Although harvesting treatments affected the composition (beta diversity) of hemicellulolytic populations in some ecozones, the total relative abundance of these populations was unaffected by harvesting.

Individual putative hemicellulose degrading bacterial OTUs were examined for harvesting treatment effects. Random forest analysis selected 10 hemicellulolytic OTUs to be important features for classifying the harvesting treatments (**Figure 22**), whereas indicator species analysis was unsuccessful in identifying indicators for harvesting treatments. Of these important features, the relative abundance of OTU13 (*Burkholderia*) was significantly affected by harvesting in the IDF and LP ecozones (**Figure 23A-D**). Harvesting treatments did not result in any observable trends with the other important OTUs.

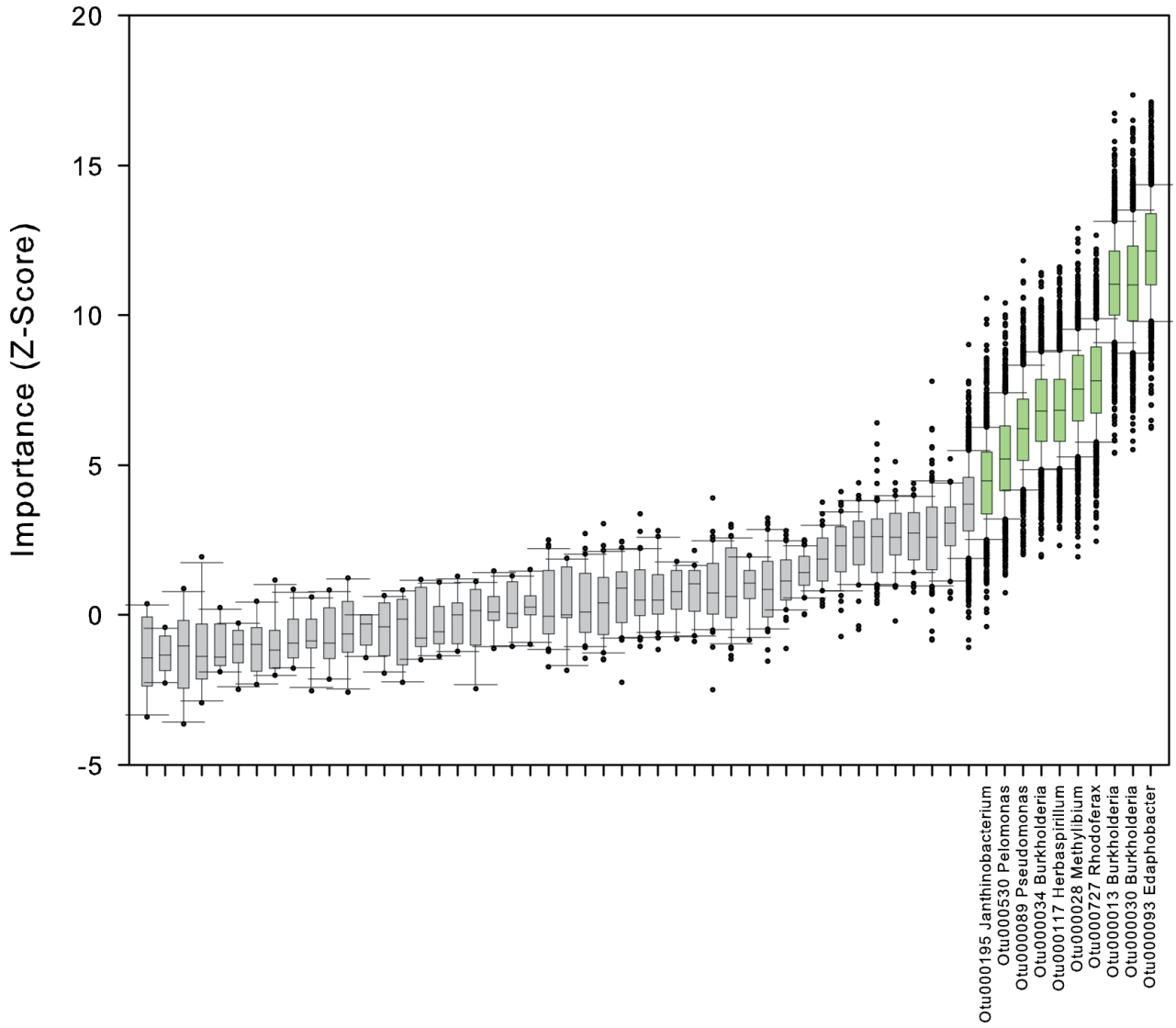



**Figure 22.** Random forest analysis with Boruta feature selection indicating the importance of putatively hemicellulolytic bacterial OTUs in classifying samples according to harvesting treatments.

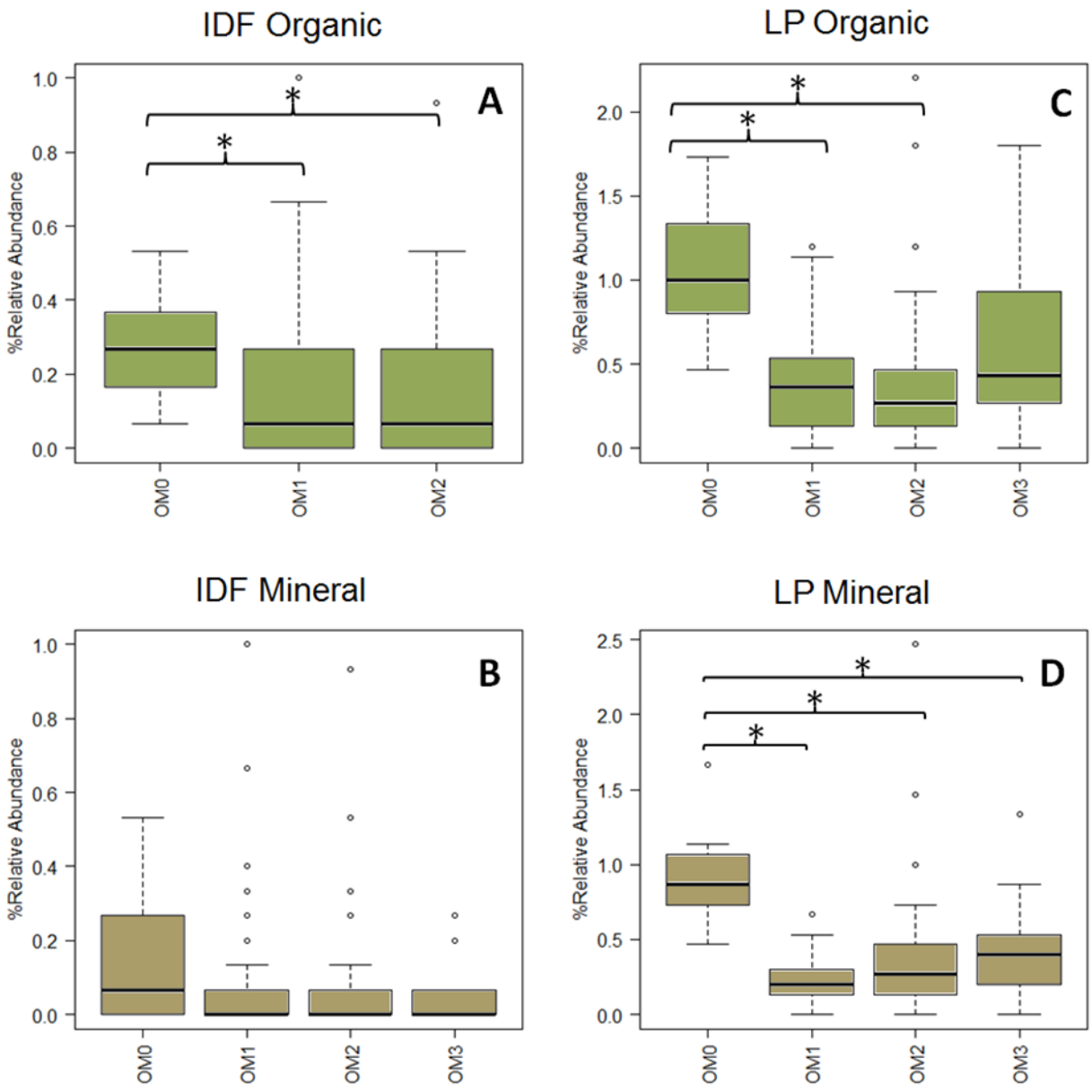



**Figure 23A-D.** Box and whisker plots illustrating the effect of harvesting treatment on relative abundances of OTU13 (*Burkholderia*) in the organic (**A**&**C**) and mineral (**B**&**D**) layers in IDF and LP ecozones. (*P<0.05)

### 3.3.4 Distribution of hemicellulolytic fungi *in situ*

Putatively hemicellulolytic fungal populations were unevenly distributed across ecozones. These populations constituted an average of 10.48% of the total *in situ* pyrotag community across both organic and mineral soil layers and 6 ecozones. An average 12.86% of the total fungal *in situ* pyrotag community in the organic layer corresponded to these OTUs, compared to an average of 7.93% in the mineral layer (**Figure 24**). The putatively hemicellulolytic fungi were most abundant in organic layer of the IDF ecozone, corresponding to an average of 32.77% of the *in situ* pyrotag community, and least abundant in the mineral layer of the JP ecozone corresponding to an average of 2.25% of the *in situ* community. **Figure 25** illustrates the mean relative abundance of the dominant putatively hemicellulolytic fungal taxa in each of the 6 ecozones. These populations show distinct ecozone clusters in both soil layers (**Figure 26A&B**). The separation of these ecozone clusters strongly correlated with individual fungal OTUs such as OTU00561 (*Wilcoxina*), OTU00041 (*M. clonocystis*), OTU00003 (*M. elongata*), and OTU00054 (*P. spinulosum*).

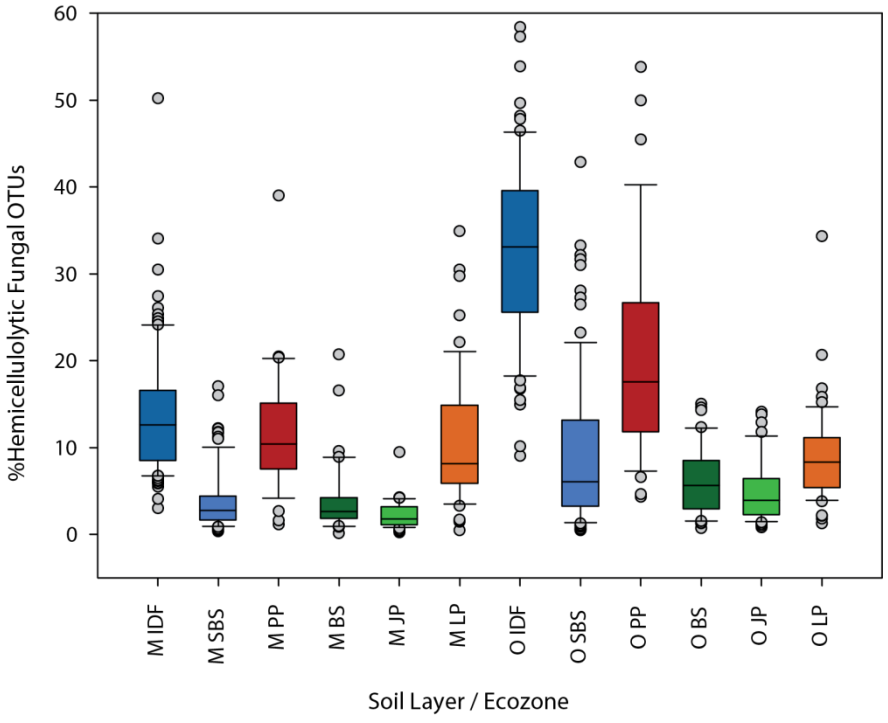



**Figure 24.** Box and whisker plots comparing the proportions of putatively hemicellulolytic fungal populations *in situ*.

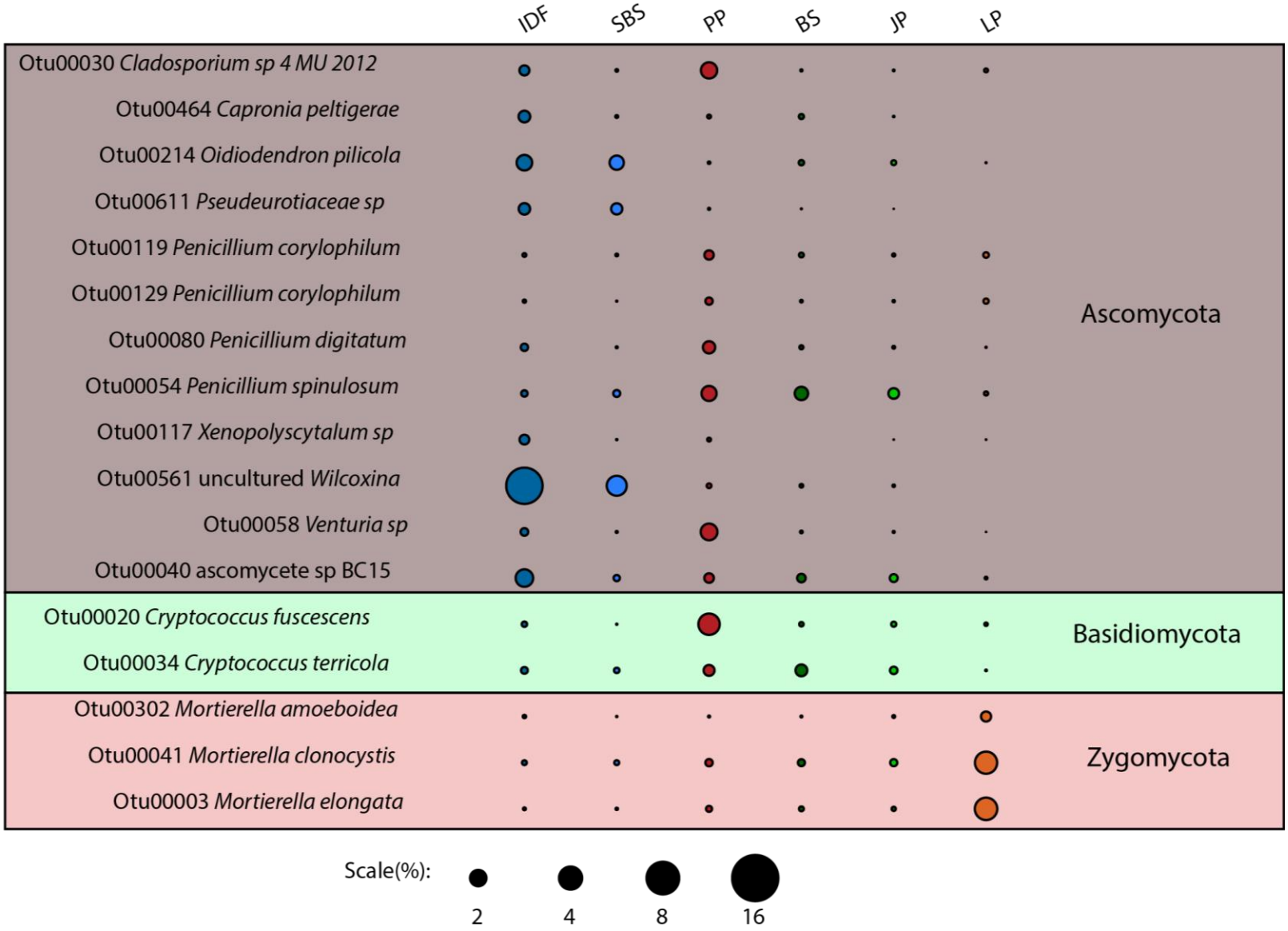



**Figure 25.** Hemicellulolytic fungal OTUs greater than 0.1% relative abundance *in situ* at each ecozone.

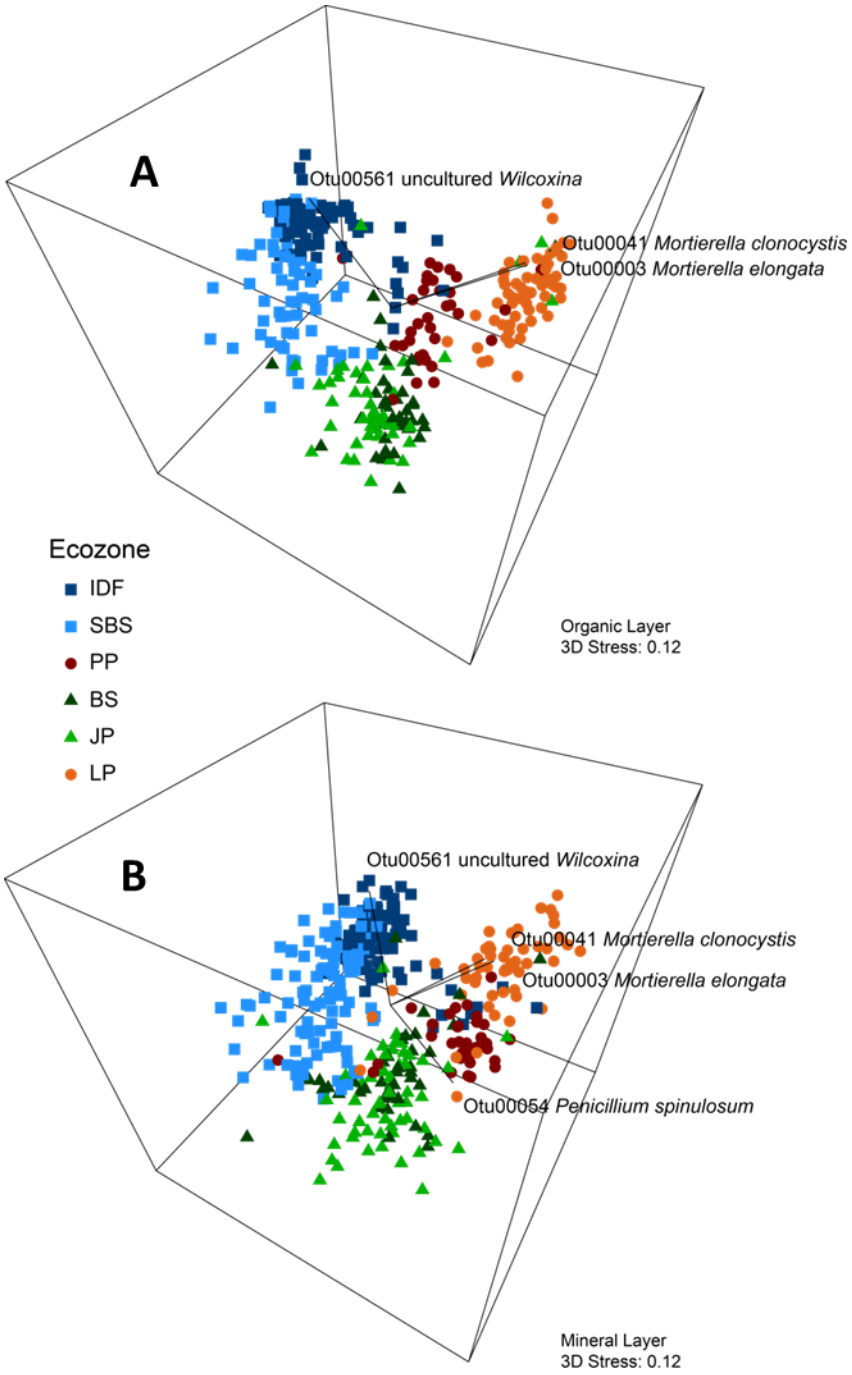



**Figure 26A&B.** Non-metric multidimensional scaling (NMS) illustrating putative hemicellulolytic fungal communities across ecozones in the (**A**) organic and (**B**) mineral layers, using Bray-Curtis dissimilarity. Vectors represent Pearson's Correlation (>0.6).

### 3.3.5 Harvesting impacts on hemicellulolytic fungi

Harvesting significantly altered the putatively hemicellulolyitc subset of the fungal community in some ecozones. Though the effects of harvesting treatments were significant, the harvesting treatment factor (0.73%) and all of its interactions accounted for less than 14.10% of the variance in the data (**Table 7**), while 45.22% of the variation in the data remains unexplained by perMANOVA. Harvesting had the largest effect on hemicellulolytic fungal populations in the organic layer of the PP ecozones (**Figure 19**). Generally, harvesting effects on fungal populations were larger in the organic layer. In contrast to bacterial populations, harvesting had no effect on fungal populations in the LP ecozone.

Harvesting treatments significantly altered putatively hemicellulolytic subset of the fungal community in the ecozones where the corresponding OTUs were originally identified in (IDF and PP), and additionally SBS (**Table 8**). Generally, harvesting had a significant effect (OM0 versus other treatments), but the three harvested treatments tended not to differ in pairwise comparisons. The organic layer was not sampled in the OM3 treatments at IDF and SBS ecozones. Some significant pairwise differences were observed in the mineral layer. Putatively hemicellulolytic fungal populations in the organic layer of the PP ecozone had greatest effect sizes by harvesting treatments ($P<0.01$ in OM1 vs OM3 treatments, univariate t-statistic = 2.85). The clustering of harvesting treatment groups were illustrated in a constrained ordination (**Figure 27**), and the lower misclassification of OM3 treatment groups support perMANOVA results, indicating the strong separation strength of the OM3 treatment group in the fungal populations. Most misclassifications occurred between OM1 and OM2 treatment, which can be attributed to the low separation strength, ergo high similarity between those two harvesting treatments.

**Table 7.** perMANOVA test comparing effects of factors on relative abundances of hemicellulolytic fungal OTUs, clustered at a Levenshtein distance of 11 and subsampled to 1500 reads.

| Factor | P(perm) | % of Variance Explained |
|---|---|---|
| Ecozone | 0.001 | 36.55 |
| Soil Layer | 0.001 | 3.35 |
| Harvesting Treatment | 0.001 | 0.73 |
| Ecozone x Soil Layer | 0.001 | 9.86 |
| Ecozone x Harvesting Treatment | 0.001 | 2.05 |
| Soil Layer x Harvesting Treatment | 0.002 | 0.79 |
| Ecozone x Soil Layer x Harvesting Treatment | 0.003 | 1.46 |
| Residual | | 45.22 |

**Table 8.** Pairwise perMANOVA test comparing the effects of Harvesting Treatment on relative abundances hemicellulolytic fungal OTUs, clustered at a Levenshtein distance of 11 and subsampled to 1500 reads. Univariate t-statistics were indicated on significant tests with $P < 0.05$, while bolded t-statistics indicate $P < 0.01$.

| Layer | Treatments | IDF | SBS | PP | BS | JP | LP |
|---|---|---|---|---|---|---|---|
| **Organic** | OM0 x OM1 | **1.95** | **1.92** | 1.32 | n.s. | n.s. | n.s. |
| | OM0 x OM2 | **2.02** | 1.81 | n.s. | n.s. | n.s. | n.s. |
| | OM0 x OM3 | - | - | **2.26** | n.s. | n.s. | n.s. |
| | OM1 x OM2 | n.s. | n.s. | **1.76** | n.s. | n.s. | n.s. |
| | OM1 x OM3 | - | - | **2.85** | n.s. | n.s. | n.s. |
| | OM2 x OM3 | - | - | **1.82** | n.s. | n.s. | n.s. |
| **Mineral** | OM0 x OM1 | n.s. | **2.36** | n.s. | n.s. | n.s. | n.s. |
| | OM0 x OM2 | n.s. | 1.70 | n.s. | n.s. | n.s. | n.s. |
| | OM0 x OM3 | 1.72 | **2.32** | n.s. | n.s. | **1.93** | n.s. |
| | OM1 x OM2 | n.s. | n.s. | n.s. | n.s. | n.s. | n.s. |
| | OM1 x OM3 | **1.92** | n.s. | 1.46 | n.s. | **2.15** | n.s. |
| | OM2 x OM3 | 1.70 | n.s. | n.s. | n.s. | **1.97** | n.s. |

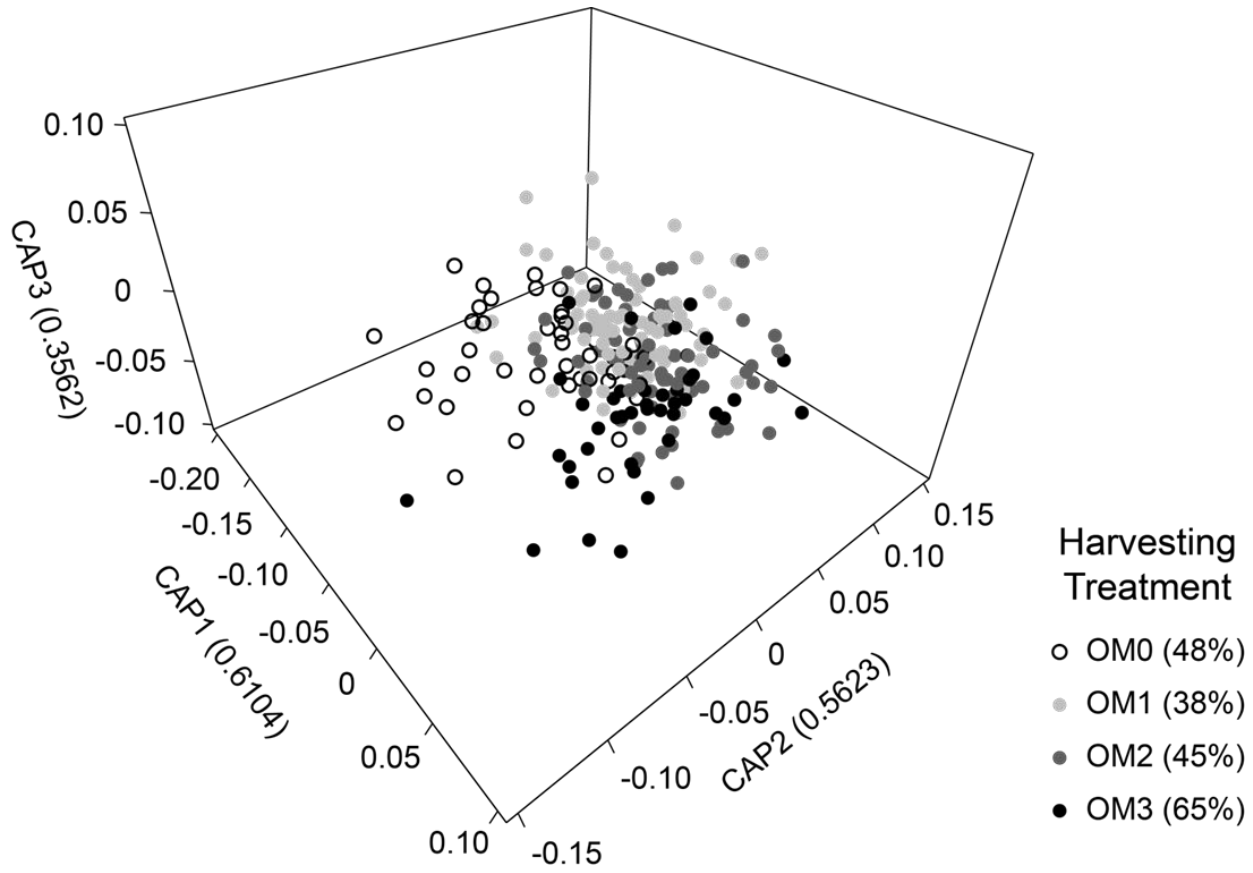



**Figure 27.** Canonical analysis of principal coordinates (CAP) ordination of relative abundances of putatively hemicellulolytic fungal populations at IDF and PP ecozones showing maximized differences among harvesting treatments using Bray-Curtis dissimilarity. Classification success rates for each harvesting treatment are given in parentheses. The canonical correlation of each CAP axis is given in parentheses.

The JP, BS, and LP ecozones, whose soils were not used in microcosm experiments, did not show any significant harvesting treatment effects (**Table 8**). The relative abundances of putatively hemicellulolytic fungi were also substantially lower in proportion at these ecozones (**Figures 24&25**).

Individual putative hemicellulose degrading fungal OTUs were examined for harvesting treatment effects. Indicator species analysis identified OTU00305 (Unclassified Ascomycota) and OTU00119 (*Penicillium corylophilum*) an indicators of harvesting treatment in the organic layers of the IDF and PP ecozones respectively (**Figure 28&29**). Harvesting resulted in an increase in the relative abundance of OTU00305 in the IDF ecozone, and a decrease in the relative abundance of OTU00119 in the PP ecozone. There were no consistent fungal indicators amongst ecozones and soil layers, which is in accordance with the patchy distribution of hemicellulolytic fungal populations.

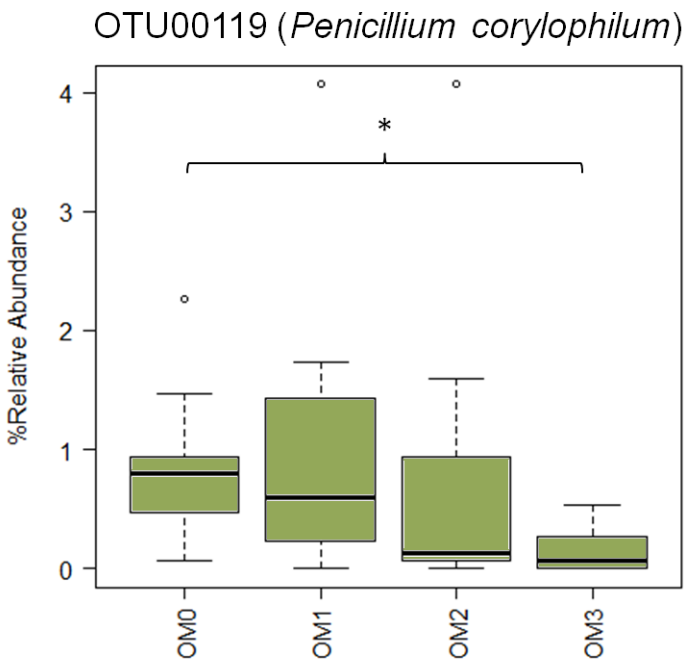



**Figure 28.** Box and whisker plot illustrating the effect of harvesting treatment on the relative abundance of OTU00305 in the organic layer of the IDF ecozone. (*P<0.05)

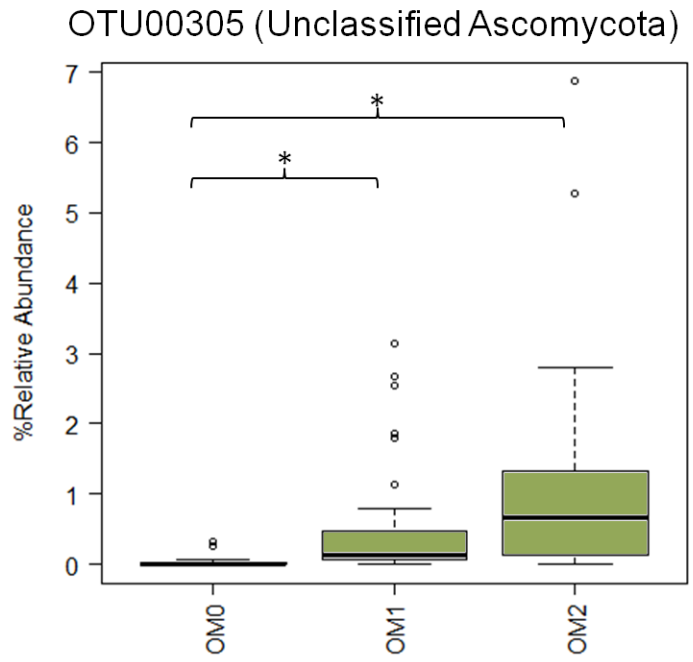



**Figure 29.** Box and whisker plot illustrating the effect of harvesting treatment on the relative abundance of OTU00119 (*P. corylophilum*) in the organic layer of the PP ecozone.

# 4 Discussion

The aims of this study were to address whether timber harvesting affects potential rates of hemicellulose catabolism in forest soils, to identify key bacterial and fungal populations responsible for hemicellulose degradation in these soils, and to determine whether timber harvesting results in significant long-term changes in these hemicellulolytic populations. This was achieved by tracing $^{13}C$ stable-isotope hemicellulose using molecular techniques PLFA analysis and 454-pyrosequencing.

## 4.1 SIP Methodology and its Use in Community Analysis

A key aspect to this study was the utilization of UPLC-MS-MS instrumentation to detect $^{13}C$-enrichment levels in nucleic acids resulting from the incorporation of $^{13}C$-hemicellulose. This method, developed by lab-mate Roland Wilhelm, accurately differentiates between $^{12}C$ and $^{13}C$-enriched adenine and guanine nucleotides by using multiple-reaction monitoring to track analytes with increases of 1-5 Da. To further validate the presence of $^{13}C$-DNA in the heavy fractions, I compared DNA concentrations of each $^{13}C$ fraction to a parallel $^{12}C$ control using the PicoGreen fluorescent dye assay. These validation steps are necessary, as environmental samples exposed to low concentrations of $^{13}C$ substrate often result in a “smear” of unlabelled DNA in the heavy fraction (Neufeld, Dumont, Vohra, & Murrell, 2007; J. D. Neufeld et al., 2007). Most studies involving stable-isotope probing have used a fingerprinting technique such as terminal restriction fragment length polymorphism or denaturing gradient gel electrophoresis to confirm the presence of $^{13}C$-label in high density fractions, but these methods are often qualitative, laborious, and time-consuming (Bell et al., 2011; Dumont, Pommerenke, Casper, & Conrad, 2011; Herrmann et al., 2010). Quantitative PCR-based approaches have also been used to verify enrichment in high density fractions by accurately measuring copy numbers of the 16s rRNA gene specific to certain groups of microbes (Martineau, Whyte, & Greer, 2010). While the use of qPCR is capable of accurately measuring and comparing copy numbers in $^{12}C$ and $^{13}C$ fractions with high sensitivity and is superior to

DNA quantification using a fluorescent dye, it does not serve as a direct method of differentiating between $^{12}C$ and $^{13}C$-DNA. Here, I was able to quickly and quantitatively verify the levels of substrate incorporation by directly measuring $^{13}C$ levels.

The use of 454-pyrosequencing with stable-isotope probing enabled this study to confidently identify populations that incorporate, and so likely degrade, hemicellulose in soil. Under the assumption that organisms enriched in microcosms are also present *in situ*, I was able to capitalize on our large *in situ* pyrotag library to extrapolate the relative abundance of these hemicellulolytic bacterial and fungal populations across all 6 ecozones. Though these hemicellulose catabolising microorganisms were identified in IDF and PP soils, the same organisms presumably retain a hemicellulolytic function across all ecozones. The correspondence of many of these populations in this study to known hemicellulose degraders (see below) supports this extrapolation.

One should be aware of biases in diversity associated with OTU binning in community analyses, particularly when studying populations with specific metabolic functions. While binning bacterial OTUs at a 97% identity of threshold runs the risk of grouping generalists with specialists involved in hemicellulose catabolism, binning sequences at a higher percentage level is likely to result in severe overestimations of diversity due to intragenomic variations in rRNA operons (Lopez-Perez et al., 2013; Sun, Jiang, Wu, & Zhou, 2013). Genomes harboring multiple different copies of rRNA will inevitably bias diversity estimations at higher identity threshold levels. In this study, the dominant hemicellulolytic bacterial phylum is the Proteobacteria, with an average of 3.94 copies of the rRNA gene amongst currently fully sequenced genomes, and with high copy numbers a higher level of intragenomic heterogeneity will be observed, thus leading to greater overestimations in 16s diversity (Lopez-Perez et al., 2013). The use of a 97% identity threshold results in an average of 8.9% overestimation in diversity compared to an overestimation of 82.6% using a unique threshold in the V1-V3 hypervariable regions of the 16s rRNA gene (Lopez-Perez et al., 2013). Having considered the

conundrum of the two opposing sources of bias; underestimating diversity by grouping generalists with specialists and overestimating diversity due to intragenomic heterogeneity of the 16s rRNA gene, a 97% sequence identity threshold is a common compromise.

### 4.2 Putative Bacterial Hemicellulose Degraders

Several phylogenetically diverse bacterial genera were identified as putative hemicellulose degraders in this study. By using the SILVA 16s rRNA database, I was able to determine the consensus taxonomy for each hemicelluloytic bacterial OTU (Quast et al., 2013). Interestingly, the hemicellulose degrading bacterial taxa were dominated by *Burkholderia* and *Pseudomonas*, both genera belonging to the Proteobacteria phylum, which were previously implicated in lignocellulose degradation *in vitro* (Cheng & Chang, 2011; Harazono et al., 2003; Mohana, Shah, Divecha, & Madamwar, 2008). Both genera are known for their metabolic diversity, and capability to degrade a wide range of organic compounds. Members of *Burkholderia* reside in a wide range of ecological niches, with roles in bioremediation and plant growth, as well as potential plant and animal opportunistic pathogens (Coenye & Vandamme, 2003; Salles, Van Veen, & Van Elsas, 2004). The hemicellulolytic *Burkholderia* identified here are affiliated with plant-associated saprophytes belonging to the Group A *Burkholderia* lineage, instead of the pathogenic species belonging to the Group B lineage, as determined by RDP SeqMatch (Cole et al., 2009; Estrada-de los Santos, Vinuesa, Martínez-Aguilar, Hirsch, & Caballero-Mellado, 2013). Hemicellulolytic OTU30 (*Burkholderia*) and OTU1367 (*Pseudomonas*) have pyrotag sequences matching the V1-V3 16S rRNA gene region of five bacterial strains isolated on the hemicellulose, xylan (unpublished data). These bacteria were isolated from soil samples from the PP, JP and LP ecozones. In addition, another seven isolates not yet tested for hemicellulytic activity match hemicellulolytic *Burkholderia* and *Pseudomonas* OTUs at a minimum of a 98% sequence identity (VanInsberghe, Hartmann,

Stewart, & Mohn, 2013). My results suggest that the genera *Burkholderia* and *Pseudomonas* include cosmopolitan members that are hemicellulolytic.

In addition to *Burkholderia* and *Pseudomonas*, I have identified putative hemicellulolytic bacteria from several other genera, including additional members of the β-Proteobacteria, Acidobacteria, Bacteroidetes, and Firmicutes. Interestingly, the third most abundant bacterial hemicellulose degrader (OTU28) was identified as *Methylibium* (β-Proteobacteria), a genus of facultative methylotrophs known to utilize both one-carbon substrates and aromatic compounds, but not previously known to use hemicellulose (Nakatsu et al., 2006; Song & Cho, 2007). Additional abundant hemicellulolytic OTUs belong to the genus, *Duganella,* which has previously been reported to contain members with a role in degradation of lignocellulosic biomass (Maki, Idrees, Leung, & Qin, 2012). Less abundant hemicellulolytic OTUs are members of the genera, *Paenibacillus* and *Variovorax,* which include members isolated from decaying wood with cellulolytic activity (Ghio et al., 2012; Schäfer et al., 1996). Metagenomic studies have also shown that members of *Variovorax* contain cellulolytic enzymes (Ghio et al., 2012; Talia et al., 2012). From the Bacteroidetes phylum, one abundant hemicellulolytic OTU (OTU623) was classified as a *Muciliginibacter*, which includes several members previously shown to degrade hemicellulose and cellulose (Khan, Chung, Jeon, & Chung, 2013; Khan, Chung, Kang, Jeon, & Chung, 2013; Štursová, Žifčáková, Leigh, Burgess, & Baldrian, 2012). Though many organisms identified in this study have been indicated to be capable of hemicellulose degradation in culture, little is known about their activity in soil communities, and for the first time, I have exploited stable-isotope probing in a microcosm to provide evidence for their hemicellulolytic activity in soil.

### 4.3 Putative Fungal Hemicellulose Degraders

Many of the putatively hemicellulolytic fungal taxa identified in this study correspond to species previously shown to be capable of hemicellulose degradation. Consensus taxonomy for putative hemicellulolytic fungal OTUs was determined by classification with the UNITE database (Kõljalg et al., 2013). Unlike the putatively hemicellulolytic bacterial populations, the distribution of fungal populations was much patchier across ecozones. Thus, it is difficult to extrapolate from microcosms with SBS and PP soils to forests in the other ecozones. The putatively hemicellulolytic fungal OTUs were largely dominated by *Mortierella*, *Cryptococcus*, and *Penicillium* species.

Members of the *Mortierella* genus are known to degrade a broad range of carbohydrates including hemicellulose. *M. elongata* has demonstrated single-cell oil production by growth on straw-derived hemicellulose (Varnaitė & Raudonienė, 2008; Zeng et al., 2013). This process has valuable commercial applications in production of polyunsaturated fatty acids. More expensive simple carbons such as glucose have traditionally been used as substrate for production of these microbial-derived lipids. The ability to utilize renewable materials such as lignocellulosic biomass will be invaluable to the emerging markets for renewable resources (Huang, Zong, Wu, & Liu, 2009). I additionally identified *M. clonocystis* and *M. amoeboidea* as putatively hemicellulolytic fungi in this study.

*Cryptococcus albidus* has been studied in the late 1970s for its ability to degrade hemicellulose in culture, and its xylanase gene has been cloned to produce xylan fermenting transformants for commercial applications (Biely, Krátký, Kocková-Kratochvílová, & Bauer, 1978; Morosoli, Zalce, & Durand, 1993). Additionally, *C. albidus* has demonstrated delignification ability and is used in the pulp and paper industry (Singhal, Jaiswal, Jha, Thapliyal, & Thakur, 2013). *C. terricola*, which I have identified as hemicellulolytic, is commonly found in decaying wood, but has not been reported to be hemicellulolytic (Buzzini, Branda, Goretti, & Turchetti, 2012).

Several *Penicillium* species including *P. corylophilum* identified in this study are both hemicelluolytic and cellulolytic (Chávez, Bull, & Eyzaguirre, 2006; El-Magraby & El-Maraghy, 1988; Yang, Xu, Wang, & Yang, 2005). *P. corylophilum* is known to produce various endo-xylanases, and has been utilized for various biotechnological applications (Chávez et al., 2006). This study provides the first evidence of hemicellulose degradation in *P. spinulosum*.

*Cladosporium* is commonly found on both living and dead plant matter, consistent with evidence from this study for its potential role in hemicellulose degradation. Though *Cladosporium sp. 4 MU-2012* has no previously demonstrated role in hemicellulose degradation, one study reports isolation and characterization of a xylanase in *C. cladosporioides* (Hong, Kim, Jung, Jo, & Choi, 2011). *C. cladosporioides*, *C. herbarum*, and *C. sphaerospermum*, are capable of cellulolytic activity in culture (El-Magraby & El-Maraghy, 1988).

*Wilcoxina*, a genus of ectendomycchorizal fungi (ectomycchorizal fungi with hyphae capable of penetrating plant cell wall) that are known symbionts of coniferous trees is abundant in the IDF ecozone (Scales & Peterson, 1991). Interestingly, several strains of *Wilcoxina* have been shown to be capable of degrading pectin and cellulose at relatively low rates, which is believed to be the mechanism allowing fungal hyphae to hydrolyze the cell wall and penetrate plant cells (Redlak, Dahm, Ciesielska, & Strzelczyk, 2001). Additionally, it is possible that ectendomycchorizae such as *Wilcoxina* may help facilitate seedling establishment by transferring hydrolyzed carbon compounds to plant seedlings prior to their development of autotrophic functions.

Little is currently known about the ability of the genera, *Venturia*, *Capronia*, *Xenopolyscytalum*, and *Pseudeurotiaceae,* to degrade hemicellulose. One study had investigated 14 known species of *Capronia*, all of which are unable to grow on cellulose or lignin, and are likely hypersaprobes (Untereiner & Malloch, 1999). It is possible that the some

or all of these genera are cross-feeding off primary hemicellulolytic organisms, which is a caveat inherent to the SIP methodology.

Well studied thermophilic hemicellulolytic fungal species *Talaromyces emersonii* and *Phanerochaete chrysosporium* were not identified in this study, likely due to a higher preference for these organisms to grow under different culture conditions (Singh & Chen, 2008; Waters, Murray, Ryan, Arendt, & Tuohy, 2010). In the case of *Phanerochaete chrysosproium*, wood-rot activity is strongly dependent on $O_2$ concentrations in culture, with complete absence of activity at concentrations below 5% (Kirk, Schultz, Connors, Lorenz, & Zeikus, 1978). The microaerobic and humid conditions in microcosms likely selected against these organisms. It is also possible that other important fungal hemicellulose degraders were neglected due to their exclusion from the DNA fractions submitted for sequencing, particularly slow-growing organisms. However, most studies on fungal degradation of plant matter such as lignin, cellulose, and hemicellulose are based on pure cultures, so this study advances our knowledge of hemicellulose decomposition in soil.

### 4.4 Timber Harvesting Had no Detectable Effect on Hemicellulolytic Activity

Harvesting treatments had no statistically significant effects on potential hemicellulolytic activity in soils. Though the soil respiration experiments did not yield statistically significant results, removal of organic matter resulted in an increasing trend in rates of $CO_2$ respiration in the presence of hemicellulose. Consistent with $CO_2$ respiration experiments, OM removal showed an increasing trend in $^{13}$C-hemicellulose incorporation in total bacterial and fungal PLFAs in the IDF ecozone, but not the PP ecozone. Although SIP results demonstrate significant changes in hemicellulolytic populations, respiration and PLFA experiments suggest that timber harvesting does not largely affect the potential rates of hemicellulose degradation of the forest stand as a whole. This might be due to functional redundancies among those populations. Alternatively, an effect of harvesting on activity may have occurred but been eclipsed by micro-site variability and heterogeneity. This result is not surprising, as many LTSP

sites lacked detectable changes in plant productivity between harvesting treatments after 10 years (Conlin & Driessche, 2000; Ponder Jr et al., 2012; Robert F. Powers et al., 2005). Micro-site variations in soil likely contributed to the large variations in the $CO_2$ respiration rates, and should be considered when interpreting the data (Buchmann, 2000). Furthermore, the extent to which these variations affect soil respiration remain difficult to quantify due to an information deficit in various biotic and abiotic aspects of the soil (Buchmann, 2000). Caution should be taken while interpreting respiration measurements in microcosms, as respiration rates are dependent on temperature, moisture and other factors, which fluctuate on diurnal and seasonal scales, unlike in microcosms (Robert F Powers, 2001). This study however, aimed to compare the differences in relative respiration potentials among harvesting treatments. The time scale of 10-15 years, in which these harvesting effects were monitored, is still relatively short, relative to forest regeneration. Thus, there should be continued surveying of these soil communities to determine any potential long term changes in activity.

### 4.5 Dominant Hemicellulolytic Populations Differ Geographically

Though this study is focused on the long-term effects of timber harvesting on populations that catabolise hemicellulose, it is important to note the differences in these populations among ecozones. Major variations were observed in both bacterial and fungal populations amongst different ecozones. This is particularly true with the patchy distribution of hemicellulolytic fungal populations (**Figures 20&26**). The ecozone factor accounts for 19.22% and 36.55% of the variability in the putative hemicellulolytic bacterial and fungal populations respectively. The high variability of hemicellulolytic fungal populations observed among ecozones is consistent with the little overlap that the total fungal communities share (unpublished data). The most notable example of this is, OTU00561 (*Wilcoxina*) whose relative abundance correlated strongly with the IDF ecozone, while OTUs 00041 and 00003 (*Mortierella*) correlated with the LP ecozone (**Figure 26**). Additionally, different levels of hemicellulose incorporation into bacterial and fungal PLFAs were observed between IDF and

PP ecozones. The relative incorporation of hemicellulose into fungal versus bacterial PLFAs was higher in the PP versus IDF ecozones (**Figures 8A-D**). Hemicellulolytic fungal OTUs identified in microcosms from IDF and PP soils had significantly lower *in situ* abundances at JP, BS, and LP ecozones compared to their native regions. These findings implicate the need to independently identify the hemicellulose catabolising fungal populations in these other ecozones in order to make accurate comparisons.

### 4.6 Timber Harvesting Alters Hemicellulolytic Populations

Removal of organic matter associated with timber harvesting causes significant long-term effects on microbial populations that catabolise hemicellulose. Though inter-site differences on a geographical scale account for most of the variability in both $^{13}$C-PLFA profiles and hemicellulolytic OTUs, harvesting significantly changed the composition of these hemicellulolytic populations, and this observation is most apparent at OM0 and OM3 levels. OM1 and OM2 harvesting treatments did not generally differ in their putatively bacterial or fungal hemicellulolytic populations at most ecozones. Removal of the forest floor (OM3) results in a significant change in both bacterial and fungal putatively hemicellulolytic populations in most ecozones, and this observation is extends to the mineral layer. This is not surprising, forest floor removal affects not only functionally important microbes, but the general soil microbiome as well, due to a loss of nutrients and an entire habitat (Hartmann et al., 2012; Simard et al., 2003). Ectomycorrhizal (ECM) fungi are known to be more affected by harvesting disturbances than bacteria (Hartmann et al., 2012).

Little or no significant effects of harvesting treatments on putatively hemicellulolytic populations were observed in BS, JP ecozones, and also in the LP ecozone for fungal populations. However, since the hemicellulolytic fungal OTUs identified in microcosm experiments from IDF and PP soils are likely not representative of the dominant hemicellulose

degraders in other ecozones *in situ* (**Figure 24**), effects may have been undetectable. While this study determined significant effects of harvesting treatments at IDF and PP ecozones, and some effects at SBS and LP ecozones, the findings at other ecozones are inconclusive and additional SIP experiments should be done to identify key hemicellulolytic microbial populations and test for significant harvesting treatment effects at these regions.

### 4.7 Conclusions, Implications and Recommendations for Future Work

The stable isotope method employed here was successful in identifying a subset of the soil microbial community that catabolizes hemicellulose and in determining the significant long term effects of timber harvesting on these bacterial and fungal populations. Timber harvesting significantly altered these functionally important bacterial and fungal populations, and these effects persisted a decade after harvesting. While the communities in stem-only harvesting (OM1) do not differ much from whole-tree harvesting (OM2), forest floor removal (OM3) had an effect on hemicellulolytic bacterial and fungal populations beyond that of tree harvesting in almost all ecozones. Forest floor removal does not represent a current harvesting practice, but it may be indicative of intensive organic matter harvesting over multiple rotations.

Although these experiments were conducted in a microcosm and may not entirely reflect hemicellulose degradation *in situ*, this study aimed to make clear treatment, soil layer, and ecozone comparisons on catabolic potential. Despite having identified putative hemicellulolytic organisms, these results are certainly an underestimation of the total active populations due to the nature of the microcosm conditions and the gradient ultracentrifugation technique. It is also likely that I have not identified organisms that grow slowly on hemicellulose, as microcosm incubations were limited to 48 hours. The dominant hemicellulolytic populations identified at one ecozone does not necessarily reflect those at another ecozone, as shown in the *in situ* fungal pyrotag communities. Additional SIP

experiments would need to be performed at other ecozones in order to accurately assess the effects of harvesting treatments on the hemicellulolytic populations at these regions.

The SIP approach is not perfect as with any other technique practiced in microbial ecology, and is subject to various sources of bias, but the experimental design combining SIP with PLFA analysis, high-throughput pyrosequencing, and a pre-existing *in situ* pyrotag library allowed for an unprecedented insight into the community structure of bacteria and fungi specific to hemicellulose catabolism, and its response to long-term effects of timber harvesting. An ideal but impractical experimental design would involve sequencing of all unpooled fractions to obtain a more comprehensive inventory of hemicellulolytic microorganisms. Lastly, exploiting the latest technology in single cell genomics to obtain full genomes of these hemicellulose catabolising organisms would be a valuable follow-up experiment to supplement this study.

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