**Indications of Shifting Microbial Communities Associated with Growing
Biomass Crops on Marginal Lands in Southern Ontario**

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To

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Abstract

In Canada, purpose grown biomass is derived from herbaceous species such as *Miscanthus* (*Miscanthus giganteus*) and Switchgrass (*Panicum virgatum*), as well as woody species such as Poplar (*Populus spp.*) and Willow (*Salix spp.*). Feedstock derived from these biomass crops are used in the production of biofuels. Soil microorganisms in biomass crop ecosystems play very important roles in soil fertility and cycling of nutrients. Their relevance to biomass crop productivity, soil health and sustainability cannot be over emphasized. This study assessed the distribution of soil microorganisms (bacteria and fungi) in biomass crops planted in nitrogen (N) fertilized and unfertilized plots. Four biomass crops: poplar, switchgrass, miscanthus and willow were planted in 2009 on a marginal land in Guelph Ontario Canada. Half the plots were fertilized in a split-plot design with 4 field replicates. In fall 2014 and spring 2015, soil samples were collected at a depth of 15 cm and transported back to the lab for organic carbon, total nitrogen and microbial analysis. DNA was extracted from fresh soil, and total bacterial (16S rRNA), fungal communities (ITS), and Arbuscular Mycorrhizal Fungi (AMF) were enumerated using the quantitative PCR (qPCR). Data was analyzed in SAS using ANOVA. Quantitative PCR revealed that total bacteria population did not differ across species of biomass crops; however, the bacterial community was significantly higher in N fertilized plots. Interestingly the fungal communities were significantly higher in the poplar and willow plots, resulting in higher fungal/bacterial ratios associated with poplar and willow compared to switchgrass and miscanthus. The abundance of the AMF was significantly higher in the perennial grasses (switchgrass and miscanthus) than in woody crops (poplar and willow).

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Chapter One: Introduction

The need to cultivate sustainable and high yielding biomass crops for bioenergy production has increased since biofuels obtained from these crops are suitable alternative to fossil fuels. Biofuels have become a preferred alternative because they are sustainable, economical, and renewable sources of energy with low environmental impact (Arnoult & Brancourt-Hulmel, 2014; Schmer et al., 2008). Recently, the Canadian government targeted increased biofuel production as a way to reduce the country's dependence on fossil fuels; this further increased the need for cultivation.

1.1 Purpose-Grown Biomass Crops

In Canada, herbaceous species such as miscanthus (*Miscanthus giganteus*) and switchgrass (*Panicum virgatum*) and woody species such as willow (*Salix* spp.) and poplar (*Populus* spp) are grown for their high biomass yields and also for their adaptability to marginal soil conditions. These crops, also referred to as purpose-grown biomass crops, are sustainable crops dedicated to bioenergy production (Schmer et al., 2008). The following characteristics make them suitable for this purpose:

- (i) They consist of cellulosic materials used as feedstock in the production of biofuels (Gelfand et al., 2013),
- (ii) They have the biomass composition that is appropriate for diverse bioenergy conversion processes,
- (iii) They require less nitrogen (N), phosphorus (P) and potassium (K) fertilization, and water to thrive, and
- (iv) They emit low greenhouse gases (GHG) (Arnoult & Brancourt-Hulmel, 2014).

Other characteristics also make them suitable for biofuel production, the short-rotation woody crops, poplar and willow, can be coppiced, (a process by which new shoots are regenerated from cut stump after harvest) to produce more stems per stool or stump (Hinchee et al., 2009). The perennial herbaceous species, switchgrass and miscanthus, are C4 grasses with high photosynthetic efficiency and don't have to be replanted yearly (Bouton, 2007).

Furthermore, the use of switchgrass and miscanthus to produce large biomass annually for biofuel has no negative influence on food crop production (Mao et al., 2011). These grasses and short-rotation tree crops can thrive on marginal lands because they can withstand drought and adapt to low-nutrient conditions found in such soil (Gelfand et al., 2013).

1.2 Marginal Lands

Currently, the increase in demand for renewable fuel production has placed pressure on agricultural lands to produce more food, fibre, feed and fuel; therefore, growing biomass crops on marginal land is an option to reduce pressure on agricultural lands, and also achieve productivity on marginal lands (Gelfand et al., 2013). Marginal lands in Canada are classified as grade 3 soils (*Canadian Land Inventory*), meaning that they are degraded lands. They are not used for agricultural purposes since they are inadequate to grow field crops due to natural climatic limitations, or because they are situated in areas that are susceptible to erosion or other environmental risks (Gelfand et al., 2013).

Economically, land is considered marginal if the cost of external inputs exceeds the revenue obtained from the sale of the economic product derived from that land (Dale et al., 2010). Also, using marginal lands is preferred to land-use change which results in loss of vegetation and soil disturbance and increasing CO₂ emission (Zimmermann et al., 2013). When

cellulosic crops are planted on marginal lands, indirect carbon cost as a result of displaced food and feed production is avoided and therefore can mitigate greenhouse gas (GHG) emissions (Searchinger et al., 2009).

1.3 Soil Microbial Community

To achieve sustainability and low-cost biomass production on marginal lands, soil microorganisms are crucial to enhance biomass production and maintain ecosystem services (Jesus et al., 2010). Soil microorganisms are a key component in a biomass crop ecosystem, and play important roles in organic matter decomposition and soil fertility. Microorganisms in soil drive several vital ecosystem processes such as nutrient cycling and availability, soil structure, suppression of root diseases, and plant growth and productivity (Jesus et al., 2010). One of the most important contributors to plant growth and productivity are the beneficial rhizosphere microorganisms that are found in and around the plant root. These naturally occurring microorganisms in the soil aggressively colonize the plant rhizosphere, and their interaction elicits beneficial effects by providing growth promotion (Saharan & Nehra, 2011).

Beneficial free-living soil bacteria are also called plant growth promoting rhizobacteria or PGPR (Kloepper et al., 1989). Plant growth-promoting rhizobacteria (PGPR) such as *Pseudomonas*, *Agrobacterium*, *Bacillus*, *Phyllobacterium*, and *Azospirillum* (Garbeva et al., 2004; Saharan & Nehra, 2011) and arbuscular mycorrhizal fungi (AMF) are among the most efficient plant growth-promoting rhizosphere microorganisms. Understanding the interaction between these microorganism and plant is essential to promote growth.

1.4 Plant Growth Mechanisms

Several mechanisms are used by PGPR to enhance growth, these mechanisms include biological N₂-fixation (BNF), solubilisation of nutrients, modulating production of ethylene through increased enzymatic activities, production of siderophores or antibiotics that decrease the growth of phytopathogens, and enhancement of other symbioses beneficial to the host (Ker et al., 2012). Arbuscular mycorrhizal fungi (AMF) increases soil microflora diversity and abundance and thereby improve soil quality. AMF forms symbiotic associations with about 80% of plants, especially herbaceous ones; this symbiosis promotes plant growth while substantially reducing fertilizer requirements (Agriculture and Agric-food Canada, 2013). Plant growth can also be enhanced through mechanisms such as biofertilization (Sadhana, 2014).

PGPR are used as biofertilizers, they are an environmentally sustainable choice to improve crop production and plant health as they have the potential to replace chemical fertilizers and pesticides (Altomare & Tringovska, 2011). In a study conducted by Ker et al. (2012), a 40% yield increase was reported in switchgrasses inoculated with PGPR when compared with those without PGPR. PGPR and AMF also regulate plant uptake by altering soil pH, and adjust the equilibrium of several chemical and biochemical reactions such as precipitation/dissolution, adsorption/desorption, complexation/dissociation, and oxidation reduction process of metal cations (Altomare & Tringovska, 2011).

1.5 Drivers of Soil Microbial Community Structure

Soil microbial communities in biofuel cropping systems are influenced by several factors that result in shifts in their communities. A major driver of microbial communities is the plant

species, as they significantly impact on the microbial communities (Garbeva et al., 2004; Mao et al., 2013). The above-ground vegetation is vital in determining soil microbial communities since carbon and nitrogen are supplied in large proportions by plants for soil microorganisms in the form of residues (such as litter and dead root) and root exudates (Mao et al., 2013). Different biofuel crops can choose diverse populations of bacteria and fungi, these have unrelated capabilities to make use of substrates and withstand stresses (Strickland & Rousk, 2010).

Interactions between plants and soil microorganisms are directed through root exudates. The release by plant roots of wide variety of compounds such as sugars, organic acids, polysaccharides, vitamins and enzymes into soil create exclusive environments for microbes living in association with plant roots in the rhizosphere. Root exudation supplies nutrients, which promotes the growth of microbes and results in the enrichment and abundance of microbial species in the rhizosphere (Mao et al., 2014). Root exudation has shown to increase the abundance of microbial species by 10–1000 times than that of the neighboring bulk soil (Lugtenberg & Kamilova, 2009). The composition of root exudates influence the distribution of rhizosphere communities, and likewise, a broad variety of bacteria in the rhizosphere influence plant growth via secretion of chemicals such as auxins, gibberellins, glycolipids, and cytokinins (Garbeva et al., 2004).

Agronomic practices such as addition of fertilizers also affects soil microbial communities (Mao et al., 2011). Different groups of N- cycling microorganisms such as nitrogen-fixing bacteria community respond to nitrogen fertilizers and pesticides (Wakelin et al., 2007). While the abundance and composition of ammonia-oxidizing bacteria (AOB), and denitrifying bacteria can be significantly changed by long-term fertilization, ammonia-oxidizing archaea (AOA) may be less responsive to fertilization (Wang et al., 2009). The soil microbial

communities also respond to environmental factors such as soil type (texture), soil chemistry pH, (carbon and nitrogen), and climate (temperature and precipitation) (Mao et al., 2013).

1.6 Shifts in Microbial Communities

Environmental impact on soil microbial communities can be assessed by the fungal/bacterial dominance. The fungal/bacterial ratio is a good indicator of environmental changes in the soil, and impacts on ecosystem function (Strickland & Rousk, 2010).

In grassland management, shifts in the fungal/ bacterial ratio (F/B) have been related to the quantity and quality of root exudates, changes in quality and quantity of litter, and plant composition and productivity. These factors are linked to nutrient availability; De Vries et al. (2006) reported that increasing inorganic nitrogen fertilizer led to decrease in F/B ratio, while the addition of organic matter with high C/N ratio resulted in higher F/B ratio. Substrate with a moderately high C: N ratio stimulates the growth of the fungal population, while fertilizer rich in nitrogen favors the bacterial community in a soil.

Chapter Two: Research Objective

In 2009, the University Of Guelph and Ontario Ministry of Agriculture, Food and Rural Affairs (OMAFRA) established a long-term research site to monitor biomass production for bioenergy on a marginal land in Guelph. Several studies have been conducted since 2010; for example, Mann between 2010 and 2012 compared yield, calorific value and ash content in woody and herbaceous biomass used for bioenergy production, likewise Guillot (2013), assessed factors influencing the sustainable production of biomass (feedstock) for bioenergy, and Marsal (2015), studied biomass production as influenced by fertilization or non-fertilization treatments over a period of time since 2009 to 2014. However, no study has been done to assess the microbial communities present and their influence on biomass yields in this site.

This study examined the abundance and distribution of bacterial, fungal and AMF communities in soils associated with poplar, switchgrass, miscanthus and willow stands, to understand the impact of nitrogen fertilization on the abundance of these microbial communities. Furthermore, the study explored the impact of different bioenergy cropping systems on soil microorganisms. The composition and quantities of 3 target genes were determined: the 16S rRNA gene for overall bacteria, the fungal internal transcribed spacer (ITS), and AMF small subunit rRNA (SSU rRNA) gene. To achieve these objectives, a molecular technique using real-time PCR (qPCR) was utilized. This technique, qPCR, allows for a rapid yet quantitative assessment of the abundance of specific phylogenetic groups of microorganisms in the soil (Fierer et al., 2005).

Chapter Three: Materials and Methods

3.1 Site Description and Layout

The experimental site is situated at the University of Guelph's agroforestry research station in Guelph, Ontario, latitude 43 32' 28" N, longitude 80 12' 32"W. The soil is a gray brown luvisol with a fine sandy loam texture. According to *Canadian Land Inventory (CLI)*, it is classified as class 3 soil and referred to as a marginal soil. In 2009, the experimental site was established with a total of 20 plots. The 20 plots consisted of 4 replicates of each of the 5 biomass crops specie (Figure 1). These crops consisted of poplar (*Populus* hybrid), switchgrass (*Panicum virgatum*), miscanthus (*Miscanthus giganteus*) var. (Nagara), willow (*Salix miyabeana*) and a polyculture (containing *Panicum virgatum*, *Sorghastrum nutans*, *Andropogon gerardii* and *Schizachyrium scoparium*).

The crops were planted in a randomized block design; each plot had a dimension of 10m by 10m with a 3m buffer separating each plot. Half of each of the plots was fertilized in a split-plot design. Fertilizer, N P K, in the proportion 75:42:62 kg / ha had been applied for 5 years, the application time was after harvest in May. Because the site is situated on a slope facing the Northeast, and because the plots were aligned perpendicular to the direction of the slope, lower portions of each plot were fertilized to avoid wash off of fertilizer into the unfertilized plot during rainfall (Figure 1). For this study, only 4 biomass crop species and their 4 replicates were sampled. Polyculture was excluded.

Fig 1. Photo and Schematic diagram showing randomized design of plots and the fertilized and unfertilized split-plots and biomass crops.

3.2 Field Sampling

Soil samples were collected in fall 2014 from poplar, switchgrass and miscanthus plots and in the spring of 2015 from those crops as well as willow from the split-plot (fertilized and unfertilized plots) and their 4 replicates. A total of 24 samples were collected in the fall and 32 samples in the spring. Soil was collected randomly at a depth of 0-15cm using a soil auger and kept in sterile, labelled sample bags. Ice packs were kept on the soil samples while they were transported from the site to the laboratory. Soil samples for microbial analysis were kept at 4°C, while samples needed for chemical analysis were stored in the freezer at -20°C.

3.3 Extraction of Soil DNA

Each soil sample was homogenized, and 0.5g was processed for DNA extraction using the PowerSoil DNA isolation kit ((Mo Bio Laboratories Inc., Carlsbad, CA, USA) following the manufacturer's instructions. A final volume of 100 μ L of DNA from each sample was stored in tubes and kept frozen at -20°C for further downstream applications.

3.4 Purification and Quantification of DNA

3.4.1 Nanodrop

The DNA was quantified with a Nanodrop ND-8000 spectrophotometer (Nanodrop Technology); concentration was measured by UV absorbance spectrometry. To obtain a blank reading, 1 μ L of deionized water was pipetted on the receiving fibre optic cable and the other source fibre was brought in contact with the water sample. The gap between the optic fibre ends was bridged and the blank reading was recorded. The step was repeated using 1 μ L of each template DNA, concentration of DNA was determined by measuring the optical density. The 260/280 ratio was used to obtain a measure of purity; a ratio of ~1.8 is generally accepted as “pure” for DNA.

3.4.2 Quantitative PCR

Targeted genes from the soil microbial community 16S rRNA (bacteria), ITS (fungi) and SSU rRNA (AMF) were separately estimated by qPCR on a Bio-Rad, CFX96 Real-Time System using FAM detection and primers from referenced articles. Because of the sensitivity of the qPCR, already prepared reagents were used for analysis to prevent contamination and interference with quantitative analysis. Each reaction in 20 μ L (Master Mix) contained the SsoFast EvaGreen supermix, a composition made up of PCR reaction buffer, MgCl₂, Taq

polymerase and dNTP. DNase-free water and primers are added to the supermix to obtain master mix for the reaction. Specific primer set used are as follows: primers targeting the 16S rRNA gene, 338F and 518R (Fierer et al., 2005), primer pair ITS1-F and ITS4 (White et al., 1990) for fungal Internal Transcribed Spacer (ITS), and AML1 and AML2 primers (Lee & Young, 2008) were used for the quantification of AMF SSU rRNA gene (See Table 1). 19µl of Master Mix was carefully pipette into 96-well plates and 1µl of template DNA was added to complete the reaction. The standard curves were created using 10-fold dilution series of plasmids containing the bacterial 16S rRNA gene, fungal ITS region, and SSU rRNA gene from pure and environmental samples for bacterial, fungal, and AMF communities, respectively. The quantity of the genes (copies per gram of dry soil) was estimated by the copy numbers of the gene per ng of DNA multiplied by the amount of DNA contained in each gram of dry soil assuming a DNA extraction efficiency of 100%.

3.4.2.1 Primer Temperature and Cycles

The Bio Rad CFX96 was used for real time PCR analysis of the extracted DNA. The template DNA and master mix reagent were mounted to a 96-well plate and analysed in the thermal cycler. For the 16S rRNA gene, primers 338F (5'-ACT CCT ACG GGA GGC AGC AG-3') and 518R (5'-ATT ACC GCG GCT GCT GG-3') were used for the amplification and had the following PCR conditions optimized for the samples: 2 min of initial denaturation at 98°C, followed by 35 cycles of 98°C for 0.05 s, 0.05 s for primers to anneal at 55°C, and 72°C for 0.5 min extension. ITS with primers ITS1 (5'-TCC GTA GGT GAA CCT GCG G-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') had the following PCR conditions: 98°C for 2 min, 40 cycles of 98°C for 0.15 s, 55°C for 0.30 s, 72°C for 0.15 s. AML1 (5'-ATC AAC TTT CGA TGG TAG GAT AGA-3') and AML2 (5'-GAA CCC AAA CAC TTT GGT TTC C-3'), PCR

conditions were 95°C for 3 min, 40 cycles of 95°C for 0.1 sec, 55°C for 0.30 s. The 96 well-plate had duplicate template DNA, appropriate 10 fold diluted standard in duplicate and 2 negative controls (NTC).

At the end of each assay, the melt curve analysis was performed to verify that the fluorescence signal was derived from specific PCR products and not from primer-dimers. Melt curve was at 55°C to 95°C, increment 0.5 for 0.05 s.

Table 1: Primers used for Assays and Sequences

Domain	Target	Primer Sequence(5-3)	Primer Name	References
Bacteria	All Groups	ACT CCT ACG GGA GGC AGC AG	Eub338 F	Fierer et al., 2005
	All Groups	ATT ACC GCG GCT GCT GG	Eub518 R	
Fungi	All Groups	CTTGGTCATTTAGAGGAAGTAA	ITS1-F	White et al., 1990
	All Groups	TCCTCCGCTTATTGATATGC	ITS 4	
Endofungi	AMF	ATC AAC TTT CGA TGG TAG GAT AGA	AML1	Lee & Young, 2008
	AMF	GAA CCC AAA CAC TTT GGT TTC C	AML2	

3.5 Soil Moisture Content Determination

Soil moisture was removed by oven-drying, the weight of empty tins were determined, and 10g of soil was weighed into each tin; the soils in tins were kept in the oven for 5 days at 120°C until constant weight was obtained. The weight of soil and tin was taken after drying. Moisture content (%) was determined from the weight before and weight after drying.

The calculation below was used to obtain the soil moisture content as a percentage of the dry soil weight.

$$MC\% = \frac{W_2 - W_3}{W_3 - W_1} \times 100$$

Where :

W_1 = weight of tin (g)

W_2 = weight of moist soil + tin (g)

W_3 = weight of dry soil +tin (g)

3.6 Determination of Total Nitrogen

Soil samples were thawed and homogenized; samples were spread out on individual trays till soil was completely air dried. Soil samples were ground with mortar and pestle, and sieved with 2mm sieve. Precisely 0.2000g of soil was measured into tin foil cups. The foil cups were carefully folded and rolled into balls, and were utilized in the Leco FP-428 nitrogen analyser. Total nitrogen was determined by the Technicon nitrogen analyser in % mass for each subsample.

3.7 Determination of Total Organic Carbon

Combustion method was used to determine the total organic carbon. To analyse the total carbon and inorganic carbon, approximately 0.300g of homogenized, dried and ground soil sample was placed into clean, carbon-free combustion boat. The Leco carbon determinator (LECO CR-12 carbon analyser, Model #781-700, Leco Instruments Ltd., St joseph, Michigan, USA) was used to analyse carbon following the dry combustion technique.

3.8 Statistical Analysis

The impact of plant types, treatment and season on abundance of the soil microbial community structure was determined by analysis of variance (ANOVA). The abundance was estimated and a Two-way analysis of variances was performed on the variables bacteria, fungi and AMF using the general linear model. Experimental analyses were carried out in duplicate. PostHoc was done for analysis with interaction effect. Statistically differences were considered significant when their probability by chance was less than 0.05, ($P \leq 0.05$). Results obtained were expressed as mean and standard deviation, and displayed as bar plots.

Chapter Four: Results and Discussion

4.1 Soil Microbial Community Structure

The cultivation of different biomass crops resulted in varied soil microbial communities. In this study, the abundance of key genes involved in soil microbial community supporting four different bioenergy crops, on split-plot of fertilized and unfertilized soils was quantified, and compared in both the fall and spring seasons. Quantitative PCR showed that the abundance of the 16S rRNA gene (overall bacterial community), ITS fungi community and SSU rRNA gene (AMF glomeromycota) was higher in the fall than in the spring (Figure 2 and 3). The variation observed in the seasons may have been due to several factors such as temperature, precipitation, and availability of substrates.

Studies done by Koranda et al. (2013) suggested that the functional response of microbial communities to addition of various substrates differed in the various seasons they studied. Also, the nutrients derived from organic waste (litter and debris) available from the crop in fall meant that the microbial communities could decompose and utilize more carbon and nitrogen. Nutrient availability is an important factor that influences the abundance of soil microbial community (De Vries et al., 2006).

Microbial abundance in soils containing perennial herbaceous species, including switchgrass and miscanthus, tended to be similar, and often significantly different than woody species, poplar and willow (Figure 2 and 3), suggesting different rhizosphere associations in these crop species. Vandenkoornhuyse *et al.* (2003) reported that microbial association transpiring in a woody crop species may differ from those in herbaceous species. The fungi community was greatly influenced by the above-ground vegetation, and the effect of plant

species on fungi distribution was consistent in both the fall and spring seasons. Mao et al. (2013) reported that plants supply large proportions of carbon and nitrogen for soil microorganisms in the form of residues and root exudates; therefore plants select the microbial community they host.

Interestingly, the drivers investigated (type of bioenergy crop and fertilizer treatment) showed a greater impact on the fungal community than the bacterial community. Wakelin et al. (2007) also made the same observation from the effect of agricultural management practice on microbial community.

4.1.1 Distribution and Abundance of the Bacterial Community

The overall bacterial community composition determined by 16S rRNA gene showed that main effect of treatment had a significant effect on the abundance of bacteria community ($P=0.0086$) in fall. The abundance of 16S rRNA was significantly higher in the fertilized plot than the unfertilized plot (Figure 2). This difference in abundance of bacteria may be as a result of available nutrients in the form of inorganic nitrogen fertilizer that stimulated the bacterial growth in the fertilized plot. De Vries et al. (2006) reported that increasing inorganic nitrogen fertilizer led to increase in bacterial community. However, the main effect of species showed no significant effect on the bacteria abundance of the 16S rRNA among the crop species ($P=0.9616$). In spring, the abundance and distribution of the bacterial community was not influenced by the crop species grown ($P = 0.4126$) and fertilizer treatment ($P = 0.2450$). This may be probably due to exhaustion of nutrients over the growing season.

4.1.2 Abundance of the Fungi Community

Statistically, the main effect of treatment and crop species in fall had a significant effect on abundance of the fungi community ($P = 0.0237$, 0.0001) respectively. The abundance of fungi was significantly higher in poplar (7.787 ± 0.091) than in switchgrass (7.185 ± 0.091), $P = 0.0002$, and also miscanthus (7.146 ± 0.091), $P = 0.0001$. There was no significant difference in the abundance of fungi in switchgrass and miscanthus ($P = 0.7676$). The distribution and abundance of the fungi community was affected by both the crop species and the treatment in fall (Table 2b).

From the spring samples, only the crop species had a significant effect on the abundance of the fungi communities they hosted ($P = 0.0001$). The abundance of fungi in poplar (5.711 ± 0.114) was significantly higher than in switchgrass (5.145 ± 0.114), $P = 0.0018$, and also miscanthus (5.219 ± 0.114), $P = 0.0055$. Also, the fungi abundance in willow (5.897 ± 0.114) was significantly higher than in switchgrass and miscanthus, $P < 0.0001$, 0.0003 respectively. Nonetheless, the abundance of fungi was not significantly different between poplar and willow ($P = 0.2599$), nor was it significantly different between switchgrass and miscanthus ($P = 0.6489$), showing that within the same crop species, the distribution and abundance of fungi was similar. Overall, in spring, the abundance of the fungi community was not affected by the treatment, but was influenced by the crop species (Table 3b).

4.1.3 Abundance of AMF Community

Quantitative PCR revealed that the abundance of the AML gene was significantly higher in the herbaceous perennial grasses (switchgrass and miscanthus) than in woody species (poplar and willow). Due to the chemistry of perennial grasses, the growth and activity of the AMF may have been stimulated by the root exudates (Wardle et al., 2004). The abundance of AMF in the biomass crops studied was highest in switchgrass (6.989 ± 0.124 and 4.729 ± 0.089) in both fall and spring respectively (Figure 4, Table 2c and 3c). This result can support the fact that biomass production of switchgrass may be enhanced because of its symbiosis with arbuscular mycorrhizal fungi (AMF), in particular when grown under abiotic stress conditions such as drought and low soil fertility (Ghimire et al., 2009).

Switchgrass mutualism with fungi in forming vesicular-arbuscular mycorrhizae has been linked to its adaptation to limiting soil conditions (Parrish & Fike, 2005). Furthermore, Mao *et al* (2014), also reported *Glomus* species among labelled DNA fractions isolated from switchgrass rhizosphere, and concluded that the root exudates from switchgrass supports the growth of beneficial AMF. Also, the rhizosphere is enriched with nutrients when a significant amount of net primary production is sent belowground by prairie grasses like switchgrass, and may be responsible for the abundance of microorganisms' recorded (Chapin *et al.*, 2002).

The crop species had a significant effect on the abundance of AMF in both fall and spring samples ($P = 0.0111$ and <0.0001) respectively. The abundance of AMF was significantly higher in switchgrass than in poplar and willow in fall ($P = 0.0042$) and ($P < 0.0001$) in spring. Miscanthus also had a similar trend, the abundance of AMF was significantly higher in miscanthus than in poplar ($P = 0.0227$, < 0.0001) in both seasons (Figure 2 and 3).

The effect of fertilizer on the abundance of AMF was not significant in both seasons. AMF population can be affected by the application of chemical fertilizers, and its diversity and abundance is reduced when fertilizer is applied to increase the levels of available phosphorus (Willis et al., 2013). In the study site however, the proportion of N P K utilized (75:42:62 kg/ha) did not have significant effect on the abundance of the AMF community.

Figure 2. The relative abundances of bacteria, fungi and AMF in different biomass crops (fertilized and unfertilized) in fall as estimated using the qPCR assays. Error bars are the standard errors of the mean for the four replicates.

Table 2. Mean Abundance of Bacteria, Fungi and AMF in Different Biomass Crops in fall 2014

Crop species	Mean (log gene copy/g of dry soil)
Polar	8.31275609 ± 0.1006
Switch grass	8.31065179 ± 0.1006
Miscanthus	8.27722486 ± 0.1006

(a) Bacteria LS Mean fall 2014

Crop species	Mean (log gene copy/g of dry soil)
Polar	7.78718438 $\pm$ 0.0916
Switchgrass	7.18451262 $\pm$ 0.0916
Miscanthus	7.14562911 $\pm$ 0.0916

(b) Fungi LS Mean fall 2014

Crop species	Mean (log gene copy/g of dry soil)
Polar	6.41576156 $\pm$ 0.1241
Switchgrass	6.98987268 $\pm$ 0.1241
Miscanthus	6.85300904 $\pm$ 0.1241

(c) AMF LS Mean fall 2014

Figure 3. The relative abundances of bacteria, fungi and AMF in different biomass crops (fertilized and unfertilized) in spring as estimated using the qPCR assays. Error bars are the standard errors of the mean for the four replicates.

Table 3. Mean abundance of bacteria, fungi and AMF in different biomass crops in spring 2015

Crop Species	Mean (log gene copy/g of dry soil)
Polar	7.96399320 $\pm$ 0.0995
Switchgrass	8.08834644 $\pm$ 0.0995
Miscanthus	8.18612543 $\pm$ 0.0995
Willow	8.15894828 $\pm$ 0.0995

(a) Bacteria LSMean spring 2015

Crop species	Mean (log gene copy/g of dry soil)
Polar	5.71089917 $\pm$ 0.1140
Switchgrass	5.14522134 $\pm$ 0.1140
Miscanthus	5.21952816 $\pm$ 0.1140
Willow	5.89704383 $\pm$ 0.1140

(b) Fungi LSMean Spring 2015

Crop species	Mean (log gene copy/g of dry soil)
Polar	4.01757307 $\pm$ 0.0888
Switchgrass	4.72915117 $\pm$ 0.0888
Miscanthus	4.65399772 $\pm$ 0.0888
Willow	4.01745723 $\pm$ 0.0888

(c) AMF LSMean spring 2015

4.2 Fungi/Bacteria ratio

Because of the extraordinary diversity of bacterial and fungal communities in the soil, quantifying and characterizing soil microbial communities can be an overwhelming task. However, quantitative PCR represents a rapid and quantifiable method to evaluate fungal: bacterial dominance in soils (Fierer et al., 2005). Shifts in microbial communities were established by the fungal/bacterial ratio (F/B) obtained from the quantification of targeted genes using qPCR. The Biomass crop types and seasons had a significant effect on the F/B ratio ($P <$

0.0001 and < 0.0001) respectively. The F/B ratio was significantly higher in the fall (0.1538 ± 1.1321) than in spring (0.0025 ± 1.1009). In addition, the fungal communities were significantly higher in the poplar and willow plots ($P < 0.0001$), resulting in higher F/B ratios associated with poplar and willow compared to switchgrass and miscanthus (Figure 5, Table 4). This was probably due to abundant litter and organic waste available as nutrients in the fall. In addition, bacteria are not as efficient as fungi at assimilating and storing nutrients (Lee & Schmidt., 2014).

Table 4: Mean abundance of Fungal/Bacterial ratio in popular, switchgrass, miscanthus, and willow plots

Crop Species	Log F/B ratio (gene copy/g dry soil)	F/B ratio (gene copy/g dry soil)
Poplar	-1.38929 ± 0.0590	0.04081 ± 1.1456
Switchgrass	-2.03465 ± 0.0590	0.00992 ± 1.1456
Miscanthus	-2.04913 ± 0.0590	0.00893 ± 1.1456
Willow	-1.36519 ± 0.0590	0.04313 ± 1.1456

High F/B ratios in the soil are indicative of more sustainable agricultural systems. A high F/B ratio is more favourable for agroecosystem because this ratio indicates availability of supplementary decomposed organic matter and N mineralization as nutrients for crop growth (De Vries et al., 2006). Most perennials, shrubs, and trees thrive better in soils with fungi proportion at ratios from 10:1 to 50:1, but taking into consideration that the soil is a less productive grade 3 soils, it is not surprising to see a low fungal/ bacterial ratio. Strickland and Rousk (2010) also suggested that qPCR may not estimate the abundance of microbial communities on a per biomass basis because of the physiological difference in fungi and bacteria.

Figure 4: AMF abundance as shown in both fertilized and unfertilized soils of poplar, switchgrass and miscanthus. Error bars are the standard error of the mean

Figure 5: F/B ratio in poplar, switchgrass, miscanthus and willow. Errors bars are standard error of means.

4.3 Effect of Nitrogen fertilization

In the fall season, the bacteria and fungi communities but not the AMF communities were influenced by fertilizer treatment (Figure 2). There was a higher abundance of bacteria and fungi in the fertilized plots, probably due to more nutrients available in these plots. The effect of treatment was however not significant for any of the microbial communities in the spring (Figure 3). This may have been due to low residual levels of fertilizer in the soil because it had been utilized the previous season. Ramirez et al. (2010) noted that with field studies, it is challenging to establish if shifts in microbial communities are absolutely a result of N addition or the shifts occur due to changes to plant community and their C contribution to soils. Chaplin et al. (2002) however stated that soil microbial communities should respond in ways that reflect lower N environment if perennial grasses are grown sustainably with minimal nitrogen fertilizer.

Previous studies done on the site noted that fertilization had no effect on biomass production of willow. Interestingly, the unfertilized plots of willow had higher abundance of bacteria, fungi and AMF than the fertilized plots (Figure 3). This result supports the postulation that willow thrives better without fertilizers. Quaye and Volk (2013) stated that lack of response to fertilization in willow may be due to internal nutrient cycling occurring, and because leaf litters on site usually contain high nutrients, these serve as main sources of plant and microbial nutrients reducing the need for external nutrients. This experiment did not investigate decomposition of litters and debris by the soil microbial communities which may have been responsible for nutrient availability in the willow plots.

4.4 Plant Species and Total Organic Carbon

Site preparation and disturbance during the first two years of establishment of the site for biomass production resulted in a decline of soil C. The total organic carbon (TOC) in the soil,

which is the carbon (C) stored in soil organic matter (SOM), showed no significant difference among the crop species and treatment in the fall of 2014 (Table 5). This may be due to the fact that these plots are still young. The average TOC of the site at inception in 2009 was 1.7%, the organic carbon was monitored each year, and all plants are gradually rebuilding the SOC after the disturbance. Currently, analysis of the 2015 soil samples showed that the soil C had slightly increased across all biomass crops, except poplar (Table 5). This result is consistent with the study done by Ceotto and Di Candilo (2011), where SOC was reported to have increased slightly after 7 years of soil conversion from arable soil to perennial crops. Moreover, their study also did not report significant differences in SOC among crop species.

Because of the short duration of this study, no significant impact was expected, or recorded from the total organic carbon on the microbial community. A longer duration between the sampling dates may have given a different result, as the organic carbon gradually builds up in the soil. Impact after a full growing season may have been more significant for the herbaceous perennials (switchgrass and miscanthus). Previous studies done on site observed that poplar plots for some reason did not show any decline in soil C since 2009 and it has been stable with no significant increase or decline. As mentioned earlier, probably the leave litters and debris from the tree is sufficient for the microbial communities present, and they contribute towards the rapid decomposition and release of nutrients to the poplar crop. It was also interesting to note that poplar had higher abundance of fungi than the perennial grasses in both seasons (Table 2b, 3b).

In perennial grasses cultivated for biomass production, the carbon input, that adds up to soil organic carbon (SOC) storage, mainly emanates from root production because the bulk of the aboveground biomass is harvested (Bransby et al., 1998; Richard et al., 2010). A significant amount of net primary production is sent belowground by prairie grasses like switchgrass, thus

enriching the rhizosphere with nutrients (Chapin et al., 2002). Increasing net primary production typically increases SOC. Also, the organic input of grasses to soils and microbial association is different from those of trees (Strickland et al., 2015). Furthermore, SOC serves as energy source for soil microorganisms, therefore agricultural practice that removes residues from the soil should be discouraged to prevent decrease in SOC.

Table 5: Total nitrogen and organic carbon content in percentage in fall 2014 and spring 2015

Plant specie	Fertilization	% C Fall	% C Spring	% N Fall	% N Spring
Poplar	Fertilized	1.530	1.580	0.085	0.078
	Unfertilized	1.568	1.497	0.082	0.073
Switchgrass	Fertilized	1.764	1.796	0.087	0.080
	Unfertilized	1.667	1.821	0.076	0.084
Miscanthus	Fertilized	1.755	1.767	0.084	0.075
	Unfertilized	1.699	1.679	0.081	0.132
Willow	Fertilized	ND	1.849	ND	0.137
	Unfertilized	ND	1.841	ND	0.140

ND = Not determined. (C = Carbon, N = Nitrogen)

4.5 Soil Sampling Methods

A significant limitation to this study could have been the sampling method used. Although all the soil samples were taken at the same depth (0-15cm), the number of soil samples taken in the fall (randomly taken at one point in each split-plot) was smaller than the number sampled in the spring (transept taken from 5 different points in each split-plot). However, comparing the results obtained from both seasons, the microbial distribution in each biomass crop had the same trend for both the herbaceous and woody crops. The spring sampling results were compared with the fall to assess the changes in microbial community due to sampling size used, but there was no difference in the distribution of the microbial communities in the biofuel crops. The only observation was the abundance of the soil

microbial community analysed was higher in the fall than in the spring and this result was not due to the sampling size used. Furthermore, the effect of fertilizer on the abundance bacteria and fungi was different for both seasons, but this was probably not due to sampling size as this observation was not recorded for AMF community.

Chapter Five: Conclusion

The microbial community in the biofuel cropping system examined showed that the abundance of total fungi, and AMF, but not total bacteria was influenced by the species of purpose grown biomass crop grown. Microbial abundance in soils containing herbaceous species, including switchgrass and miscanthus, tended to be similar, and often significantly different than woody species, poplar and willow, suggesting different rhizosphere associations in these crop species. This study showed that the abundance and distribution of microbial communities vary in different biomass crops, therefore to achieve a sustainable low-cost production of biofuel on marginal lands, understanding the interaction between the soil microbial community and different crop species is essential.

Fertilizer application at a minimal rate will not influence the microbial community negatively but will be a source of nutrient required to boost their abundance on marginal soils. The effect of fertilization was not conclusive because it was slightly different for bacteria and fungi in both seasons.

Future studies can investigate the effect of fertilization at different seasons, and assess the long-term effect of nitrogen fertilization on the soil microbial communities. In addition, microbial decomposition of plant biomass and what impact this has on microbial abundance and soil sustainability should also be determined.

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