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Mine reclamation using biofuel crops: Insights into the microbial ecology of the switchgrass (*Panicum virgatum*) microbiome

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Mine reclamation using biofuel crops: Insights into the microbial ecology of the switchgrass
(*Panicum virgatum*) microbiome

Brianna Mayfield

Thesis submitted to the Davis College of Agriculture, Natural Resources and Design at West
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Master of Science in
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metagenomics

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Abstract

Mine reclamation using biofuel crops: Insights into the microbial ecology of the switchgrass (*Panicum virgatum*) microbiome

Brianna Mayfield

Bioenergy crop production has steadily increased due to growing political support for renewable energy, thus initiating a demand to find alternative agricultural land. An innovative option is the use of marginal soils, such as reclaimed mine lands, to produce bioenergy crops. Switchgrass (*Panicum virgatum*) is a promising bioenergy crop that can be grown on marginal lands due to its robust growth in various soil types and climates. However, little is known regarding plant-microbe interactions among switchgrass systems within reclaimed mine lands. A study conducted in 2008 grew switchgrass on high- and low- quality reclaimed mine sites (Hampshire and Hobet, respectively) in West Virginia to examine the resilience of switchgrass as a reclamation-friendly bioenergy crop. Switchgrass yields at Hampshire were nearly an order of magnitude higher than Hobet (8.4 Mg ha⁻¹ vs 1.0 Mg ha⁻¹). Within Hampshire, the Cave-in-Rock cultivar yield was approximately 2-fold greater than that of Shawnee (12.9 Mg ha⁻¹ vs. 7.6 Mg ha⁻¹). Here, I sought to illuminate plant-microbial interactions that may account for this drastic shift in cultivar yield by assessing the soil microbial community's function and composition. I tested two hypotheses: i) that the microbial community's ability to acquire C, N, and P will be greatest in Hampshire soils compared to that of Hobet and ii) that there will be a cultivar-specific root-associated microbiome that may drive previously observed greater, but differential yields across switchgrass cultivars at Hampshire. I found that reclamation strategy substantially impacts the switchgrass microbiome's composition as well as its ability to acquire critical nutrients like carbon, nitrogen, and phosphorus. I also found that a functionally, but not necessarily compositionally, unique microbiome exists in the root-associated soils compared to that of the bulk soil. Additionally, there were indicators that organic amendments to the topsoil may induce cultivar-specific soil microbiomes that mediate or facilitate differential yields within Hampshire. Taken together, I suggest that organic amendments to the topsoil during reclamation selects for a cultivar-specific microbiome more adept to acquiring critical nutrients and thus, increases aboveground productivity.

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Table of Contents

Acknowledgements	iii
List of tables	vi
List of figures	vi
Chapter 1: Surface mine reclamation strategy alters the microbial community function and composition in switchgrass (<i>Panicum virgatum</i>) soils	1
Abstract.....	1
Introduction.....	2
Materials and methods.....	6
<i>Site Locations</i>	6
<i>Soil Properties</i>	7
<i>Extracellular Enzyme Activity</i>	7
<i>DNA Extraction and Sequencing</i>	8
<i>Bacterial and Fungal Community Composition</i>	9
<i>Functional Gene Identification</i>	10
<i>Statistical Analyses</i>	12
Results.....	13
<i>Soil Properties</i>	13
<i>Switchgrass microbiome extracellular enzyme activity</i>	14
<i>Bacterial and fungal taxonomy and composition</i>	14
<i>Metagenomic assessment of the switchgrass microbiome</i>	16
Discussion.....	18
Conclusions.....	28

Chapter 2: cultivar-specific microbiomes may modulate switchgrass (<i>Panicum virgatum</i>)	
biomass yields on reclaimed mine sites in West Virginia.....	29
Abstract.....	29
Introduction.....	30
Materials and methods.....	33
<i>Site Locations</i>	33
<i>Soil Properties</i>	34
<i>Extracellular Enzyme Activity</i>	35
<i>DNA Extraction and Sequencing</i>	35
<i>Bacterial and Fungal Community Composition</i>	36
<i>Statistical Analyses</i>	36
Results.....	37
<i>Soil Properties</i>	37
<i>Switchgrass microbiome extracellular enzyme activity</i>	38
<i>Bacterial and fungal taxonomy and composition</i>	38
Discussion.....	43
Conclusions.....	48
Tables and Figures.....	50
References.....	67

List of Tables

Chapter 1

Table 1: Site characteristics and soil chemical/physical properties at Hampshire and Hobet.....	48
Table 2: Summary of functional genes used in metagenomic analyses.....	49

Chapter 2

Table 3: Site characteristics and soil chemical/physical properties among switchgrass cultivars and soil types.....	50
Table 4: The relative abundance of bacterial taxa-associated P-values from a 3-way ANOVA...	51
Table 5: The relative abundance of fungal taxa-associated P-values from a 3-way ANOVA.....	52

List of Figures

Chapter 1

Figure 1: Extracellular enzyme activity responses of β -glucosidase, N-acetyl-glucosaminidase (NAGase), and acid phosphatase to differing reclamation techniques.....	53
Figure 2: The effect of reclamation strategy on the bacterial and fungal richness of the switchgrass microbiome.....	54
Figure 3: The effect of reclamation strategy on the bacterial and fungal community composition of the switchgrass microbiome.....	55
Figure 4: The effect of reclamation strategy on selected bacterial taxa.....	56
Figure 5: The effect of reclamation strategy on selected fungal taxa.....	57

Figure 6: The effect of reclamation technique on the relative abundance of C-, N-, and P-metabolism associated genes through KEGG Orthology level 3.....	58
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Figure 7: The effect of reclamation strategy on the relative abundances of C-, N-, and P-acquisition genes.....	59
---	----

Chapter 2

Figure 8: Extracellular enzyme activity responses of β -glucosidase, N-acetyl-glucosaminidase (NAGase), and acid phosphatase across switchgrass cultivars and soil types.....	60
---	----

Figure 9: The effect of reclamation strategy on the bacterial and fungal richness of the switchgrass microbiome.....	61
--	----

Figure 10: The effect of reclamation strategy on the bacterial and fungal community composition of the switchgrass microbiome.....	62
--	----

Figure 11: The effect of reclamation strategy on the relative abundance of bacterial taxa.....	63
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Figure 12: The effect of reclamation strategy on the relative abundance of fungal taxa.....	64
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Chapter 1: Surface mine reclamation strategy alters the microbial community function and composition in switchgrass (*Panicum virgatum*) soils.

Abstract

Since the 1970's, bioenergy crop production has steadily increased, initiating demand to find alternative growing land. An innovative option is the use of marginal lands, such as reclaimed mine lands, for bioenergy crop agriculture. Switchgrass (*Panicum virgatum*) is a promising bioenergy crop that can be grown on marginal lands due to its robust growth in various soil types and climates. However, little is known regarding plant-microbe interactions among switchgrass systems within reclaimed mine lands. A study conducted in 2008 grew switchgrass on high- and low- quality reclaimed mine sites (Hampshire and Hobet, respectively) in West Virginia to examine the feasibility of switchgrass as a reclamation-friendly bioenergy crop. Switchgrass yields at Hampshire were nearly an order of magnitude higher than Hobet (8.4 Mg ha⁻¹ vs 1.0 Mg ha⁻¹). Within Hampshire, the Cave-in-Rock cultivar yield was approximately 2-fold greater than that of Shawnee (12.9 Mg ha⁻¹ vs. 7.6 Mg ha⁻¹). Here, I sought to identify plant-microbial interactions that may account for this shift in cultivar yield by combining enzymatic activity analyses with shotgun metagenomics. I tested two hypotheses: i) that the microbial community's ability to acquire C, N, and P as well as the abundance of functional genes encoding enzymes associated with C, N, and P acquisition will be greatest in Hampshire soils compared to that of Hobet and ii) that there will be a cultivar-specific microbiome that may drive previously observed greater, but differential yields across switchgrass cultivars at Hampshire. Hampshire soils showed significant increases in extracellular enzyme activities associated with the mineralization and acquisition of C and N compared to Hobet, whereas the activity of an enzyme associated with P acquisition was variable across sites and cultivars. Metagenomic analyses revealed significant increases in carbon, nitrogen, and phosphorus metabolism-associated gene abundances within Hampshire's microbiome, but showed no differences in housekeeping genes across sites. Further, a taxonomically-unique bacterial community was found between sites but not cultivars, with Hampshire soils having a greater abundance of copiotrophic bacteria while Hobet soils had a greater abundance of oligotrophic bacteria. Similarly, a taxonomically-unique fungal community was found between sites but not cultivars, with Hampshire soils having a greater abundance of fungi that preferentially degrade labile C compounds while Hobet soils had a greater abundance of fungi that preferentially degrade recalcitrant C compounds. Throughout, there were no cultivar-specific microbiome differences observed. Together, these data suggest that differences in soil quality due to different reclamation strategies foster a compositionally and functionally unique soil microbiome but they do not suggest microbiome-influenced differences in yield across cultivars.

Introduction

In an effort to reduce the dependence, acquisition costs, and environmental implications of fossil fuel use, renewable energy production (i.e., bioenergy, solar, etc.) has gained substantial momentum in the past 30 years. To date, renewable energy sources comprise approximately 12% of the United States energy consumption market, with 44% sourced from plant biomass (U.S. Energy Information Administration 2019). With a forecasted 30% increase in global population (United Nations Department of Economic and Social Affairs 2017) and a 110% increase in global food and biomass crop production estimated by 2050 (Tilman et al. 2011), increased demands for productive growing land are inevitable. However, the environmental and economic sustainability of planting cellulosic or non-feedstock crops on agricultural land has been a highly debated matter in recent years (Koh and Ghazoul 2008; Rajagopal et al. 2007).

One possible means of producing biofuel crops is using marginal lands, that is, lands that have soils that harbor undesirable characteristics or are of little agricultural value. One type of marginal land that is particularly promising for the production of bioenergy crops are reclaimed mine lands (Skousen et al. 2013; Varvel et al. 2008). The Surface Mining and Reclamation Act (SMCRA) of 1977 requires that these lands be reclaimed to equal or better condition once mining ceases (U.S. Department of the Interior 1977), which can plausibly be achieved by productively farming marginal lands for bioenergy crops. One particularly favorable bioenergy crop is switchgrass (*Panicum virgatum*): a low-input high-yield bioenergy crop that can be grown in marginal soils due to its commercial scale growth in various soil types and climates as well as its ability to increase soil productivity and nutrient (namely carbon (C) and nitrogen (N)) cycling (McLaughlin and Adams Kszos 2005). More specifically, the large roots systems of switchgrass have been found to stabilize soil and aid in soil organic carbon (SOC) sequestration,

both of which are important for successful mine land reclamation (Liebig et al. 2005). While promising, several questions persist such as how to grow dedicated bioenergy crops like switchgrass on marginal lands, and whether these sites can produce abundant biomass while maintaining or improving ecosystem services (Blanco-Canqui 2016). Further, switchgrass cultivars have been found to respond to their environment differently (Casler and Boe 2003), making it imperative to gain insight into switchgrass cultivar-specific responses across sites of different soil qualities.

Microorganisms inhabiting the soil (i.e., soil microbiome) can associate with plants to confer various ecosystem services and are key components in establishing and maintaining soil fertility. For example, the soil microbiome is largely responsible for the mineralization and acquisition of critical nutrients, such as C, N, and P-containing compounds, that are needed for both plant and microbial success (Chapman et al. 2005; Fierer et al. 2012). Associations with beneficial soil bacteria and fungi, termed plant growth-promoting microbes, have been found to enhance switchgrass aboveground biomass yields (Kim-Dura et al. 2016; Xia et al. 2013; Xia, Amna, and Opiyo 2018). Notably, switchgrass form associations with endophytic fungi called arbuscular mycorrhizal (AM) fungi, which are responsible for converting soil P into plant-available forms (Clark et al. 1999; George et al. 1995) as well as enhancing aboveground biomass (Brejda, Moser, and Vogel 1998).

The diversity and functionality of the soil microbiome is largely shaped by the site's soil properties, such as moisture, pH, and C:N ratios (Van Horn et al. 2014; Rousk et al. 2010; Heuck et al. 2015). Nutrient availability, in particular, selects for a microbiome that exploits the resources available within the environment, a phenomenon called the copiotroph-oligotroph framework that aims to classify microorganisms based upon microbial life strategies (Fierer,

Bradford, and Jackson 2007; Ho et al. 2017). Within this framework, copiotrophic microorganisms are considered fast-growing taxa that thrive in environments with readily-available C compounds (Fierer et al. 2007). In contrast, oligotrophic microorganisms are considered slower-growing taxa that thrive under low C conditions (Ishida and Kadota 1981; Kuznetsov, Dubinina, and Lapteva 1979). The copiotroph-oligotroph framework has been utilized as a soil fertility indicator (Swedrzyńska et al. 2013), and may be a valuable tool for assessing the disturbance impacts and reclamation initiatives on reclaimed surface mines (Wolna-Maruwka et al. 2007).

Although land disturbance, such as surface mine reclamation, fundamentally alters both the physicochemical properties of soil and the soil microbiome, recent insights into productive farming potential on reclaimed lands have yielded promising results, especially for switchgrass (Mummey et al. 2002; Tang et al. 2010; Marra et al. 2013). Within the past decade, studies have described the soil microbiome of switchgrass grown on dedicated cropland (Bahulikar et al. 2014; Chaudhary et al. 2012; Jesus et al. 2016), but little is known regarding the cultivar-specific nutrient cycling and genomic capacities of the switchgrass microbiome on reclaimed mine lands. An improved understanding of the switchgrass microbiome will provide insight into bioenergy crop management strategies as well as reclamation impacts on critical soil biogeochemical processes in marginal soils.

In 2008, a field study was developed to quantify the aboveground productivity of three switchgrass cultivars on two surface mines, Hampshire Hill mine and Hobet 21 mine, that were experimentally reclaimed in different manners (Brown et al. 2016). Hampshire was reclaimed with topsoil and organic amendments; a technique historically found to promote aboveground biomass yields and increase soil productivity (Larney and Angers 2012). Hobet was reclaimed

without topsoil or any amendments; a technique historically found to limit productivity due to unfavorable changes to soil physicochemical properties (Haering, Daniels, and Roberts 1993). Ultimately, Hampshire's reclamation technique led to switchgrass yields 13 times higher compared to yields at Hobet. Further, yield of the Cave-in-Rock cultivar was 70% greater than that of the Shawnee cultivar at Hampshire, but similar differences between cultivar yield were not observed at Hobet (Brown et al. 2016). While this study quantified yield differences across two sites with different reclamation strategies, the potential influence of belowground microbial dynamics that may result in differential productivity between cultivars at Hampshire, and not at Hobet, are unknown.

Here, I assessed the function, composition, and metagenomic capacity of the soil microbiome beneath the two highest yielding cultivars of switchgrass, Cave-in-Rock and Shawnee, in long-term switchgrass plots. To do so, extracellular enzyme assays were performed in parallel with high-throughput ribosomal rRNA gene sequencing and shotgun metagenomics. Specifically, I tested two hypotheses: i) that the microbial community's ability to acquire C, N, and P as well as the abundance of functional genes encoding enzymes associated with C, N, and P acquisition will be greatest in Hampshire soils compared to that of Hobet and ii) that there will be a cultivar-specific microbiome that may drive previously observed greater, but differential yields across switchgrass cultivars at Hampshire. In this way, I obtained insight into the marginal land switchgrass microbiome, leading to a better understanding of the functional and genomic capacity attributable to differential reclamation strategies and yield response at Hampshire and Hobet.

Materials and Methods

Site Locations

To investigate the influence of mine reclamation strategy and switchgrass cultivar on the composition and metagenomic capacity of the soil microbiome, soils were sampled from long term switchgrass plots on reclaimed mine sites in West Virginia, USA (Table 1.). Experimental plots within each site were laid out in a completely randomized block design in 2008. Each site was reclaimed using a different reclamation strategy. Briefly, the Hampshire Hill mine, hereafter referred to as Hampshire, was reclaimed using topsoil amended with municipal sewage waste and paper pulp spread over the existing overburden. The Hobet 21 mine, hereafter referred to as Hobet, was reclaimed using crushed, unweathered overburden that was regraded to approximate original contour. Once reclaimed, three replicate 4,000 m² plots of each switchgrass cultivar (Cave-in-Rock and Shawnee) were broadcast seeded across each plot. Further detail regarding reclamation strategy can be found in Brown et al. (2016).

Soil sampling occurred in late May 2017, during a time at which ample moisture favored high rates of microbial activity and 9 years after the switchgrass stands were seeded. Within each plot, 10 soil cores were randomly collected at the base of a switchgrass plant to a depth of 15 cm (A horizon). Soil cores were homogenized and composited to yield one sample per plot. All samples were placed on ice and transported to West Virginia University where the soil was immediately passed through a 2 mm sieve to remove roots and rocks. A portion of each homogenized and sieved soil sample was stored at -4°C for enzyme and soil chemical analysis and the remainder was stored at -80°C for DNA extraction.

Soil Properties

Soil pH was determined using a 1:5 soil:0.01M CaCl₂ suspension with samples shaking at 60 rpm for 30 minutes (Rayment and Higginson 1992). Soil moisture was determined gravimetrically by drying soil samples at 105°C for 24 hours. Using this dried soil, organic matter content was assessed by combustion at 505°C for 6 hours via loss on ignition. To assess soil texture, soil samples were placed in a muffle furnace for 5 hours to remove organic matter at which point a hydrometer was used to quantify sand, silt, and clay proportions (American Society for Testing and Materials 1985). Microbial biomass was estimated by substrate-induced respiration (SIR), which measures induced microbial respiration when presented a labile carbon source (West and Sparling 1986). The average microbiome respiration rate (mg C-CO₂ g soil⁻¹ h⁻¹) was quantified by implementing adaptations to the West and Sparling protocol as described by Fierer, Schimel and Holden (2003). Soil total C, total N, and C:N ratios were determined by combusting approximately 250 mg air-dried, milled soils using a vario MAX cube (Elementar, Langenselbold, Germany).

Extracellular Enzyme Activity

To determine the microbial potential to mineralize C, N and P from organic matter, extracellular enzymatic activities associated with C-, N-, and P- acquisition were assessed, namely β -glucosidase (BG; EC 3.2.1.21), N-acetyl-glucosaminidase (NAGase; EC 3.2.1.50), and acid phosphatase (ACP; EC 3.1.3.2), respectively. Enzyme assays were performed according to the fluorometric protocol from Saiya-Cork, Sinsabaugh and Zak (2002) and read at 365 nm excitation and 450 nm emission on a Synergy HTX plate reader (Biotek, Winooski, VT). Enzymatic activity was expressed as $\mu\text{mol h}^{-1}$ of oxidized substrate per gram soil.

DNA Extraction and Sequencing

DNA was extracted in triplicate from 1.5 g total of each soil sample within 72 hours of sample collection. A PowerLyzer PowerSoil DNA isolation kit (Qiagen, Valencia, CA) was used following manufacturer's instructions in conjunction with a PowerLyzer 24 homogenizer (MoBio Laboratories, Carlsbad, CA). Purified DNA was quality checked on a ND8000 Nanodrop (Thermo Scientific, Waltham, MA) and quantified using a Qubit 4 Fluorometer (Thermo Scientific, Waltham, MA). Quantified DNA was submitted for high throughput sequencing of the bacterial 16S rRNA gene and the fungal internal transcribed spacer (ITS) region, as well as for the generation of shotgun metagenomes from total extracted DNA through the U.S. Department of Energy Joint Genome Institute (JGI) sequencing facility (JGI Project ID #1159680). Briefly, 10 ng of extracted DNA was used as template to amplify the V4-V5 region of the 16S rRNA gene using universal bacterial primers 515F (Parada, Needham, and Fuhrman 2016) and 805R (Apprill et al. 2015). The fungal ITS2 region was amplified using ITS9F and ITS4R universal fungal primers (Menkis et al. 2012; White et al. 1990). Unique i7 index barcode identifiers were included in the reverse primer. PCR reactions were performed in triplicate using the 5PRIME HotMasterMix (QuantaBio, Beverly, MA) under the following thermalcycling conditions: an initial 3 minutes at 94 °C, 30 cycles of 45 seconds at 94 °C, 60 seconds at 50 °C, and 90 seconds at 72 °C, a final 10 minutes at 72 °C once cycles were complete, then held at 4 °C. Reactions were pooled to yield a single 16S rRNA and ITS product per sample. Pooled amplicons were purified using 1.2X AMPure XP beads (Beckman Coulter, Brea, CA) prior sequencing. Purified barcoded amplicons were composited in equimolar concentrations for sequencing using an Illumina MiSeq 2x300 base read platform (Illumina, San Diego, CA). For

shotgun metagenome generation, total extracted DNA was barcoded by plot and composited in equimolar concentrations for sequencing using an Illumina HiSeq 2000 2x151 base read platform (Illumina, San Diego, CA). Ribosomal rRNA gene sequences are publicly available through the NCBI Sequence Read Archive (accession PRJNA533292) and metagenomic sequence data is publicly available on the Metagenomic Rapid Annotations using Subsystems Technology (MG-RAST) server (Meyer et al. 2008) under ID number mgm4776043.3.

Bacterial and Fungal Community Composition

For both 16S rRNA gene and ITS analyses, forward and reverse reads were joined using USEARCH (Edgar 2010) and the paired sequences were further processed in QIIME (version 1.9.1; Caporaso et al. 2010). Sequences were quality controlled by removing OTUs that were observed fewer than 5 times. Open reference operational taxonomic units (OTU) were picked using default parameters and the UCLUST (Edgar 2010) algorithm in QIIME. Sequences were rarified to 2,500 16S rRNA sequences per plot and 40,000 ITS sequences per plot. After rarifying, taxonomic assignments were performed at 90% similarity by comparing 16S rRNA OTUs against the Greengenes reference database (version 13.8; McDonald et al. 2012) using UCLUST and by comparing ITS OTUs against the UNITE reference database (version 8.0; UNITE Community 2019) using BLAST (Altschul et al. 1990). Initial ITS taxonomic assignments yielded a high proportion (41-66%) of “unassigned” phyla across all samples, so ITS taxonomic assignments were presented as the total hits of all identified phyla per sample and used for all subsequent taxonomic analyses (McGuire et al. 2013; Saravesi et al. 2015). Changes in bacterial and fungal taxonomy across sites and switchgrass cultivars were assessed at the phylum, order, and class level, with unassigned taxa or those under 3% in all samples binned as “other”. Fungal OTUs were classified into ecological guilds at the genus level using FUNGuild

(Nguyen et al. 2016a). After classification, any observation with a total of less than 3 hits were removed, binned into their primary functional guild, and summed by sample. Microbial community α - and β -diversity metrics were also calculated in QIIME using Chao1 richness (Chao 1984) and Bray-Curtis dissimilarity indices (Bray and Curtis 1957), respectively.

Functional Gene Identification

Metagenomic libraries were constructed, assembled, and annotated using the JGI's MAP v.4 sequencing pipeline, where adapter sequences were removed and contigs were assembled for each sample (n=12 metagenomes; Huntemann et al. 2016). First, raw sequences were trimmed, contaminants were removed, and reads containing 4 or more ambiguous bases, had an average quality score less than 3, or had a minimum length of ≤ 51 basepairs were removed using BBDuk within the BBTools software package (Bushnell n.d.). Reads were corrected for sequencing errors using BFC (version r181; Li 2015), dereplicated, and unassembled, quality controlled paired-end reads were saved in fasta format for analysis. Additionally, contigs were assembled from quality-controlled (QC) paired-end reads using the SPAdes assembler (version 3.1.1; Nurk et al. 2017) under the following options: -m 2000 --only-assembler -k 33,55,77,99,127 --meta -t 32. All filtered reads were mapped to the final contig assembly using default parameters, with addition of "ambiguous=random" switch, in bbmap (version 37.62) within the BBTools package. Protein-coding genes were identified using a combination of four *ab initio* gene prediction tools: prokaryotic GeneMark.hmm (version 2.8; Lukashin and Borodovsky 1998), MetaGeneAnnotator (version Aug 2008; Noguchi et al. 2008) , Prodigal (version 2.6.2; Hyatt et al. 2010), and FragGeneScan (version 1.16; Rho et al. 2010), and final gene callers were determined on a majority-rule basis.

The genetic potential of the soil microbial community was assessed using two complimentary approaches: i) by comparing post-QC metagenomic contigs for homology to the Kyoto Encyclopedia of Genes and Genomes (KEGG; Kanehisa and Goto 2000) pathways in MG-RAST and ii) by comparing post-QC metagenomic short reads to locally constructed functional gene databases using DIAMOND (Buchfink, Xie, and Huson 2014) and the blastn and blastx functions within BLAST (Altschul et al. 1990). For KEGG pathway classification, metagenome contigs were assigned to functional pathways using KEGG orthology (KO) under the “representative-hit classification” using the following default cutoffs: a maximum e-value of 10^{-5} , minimum percent identity of 60%, and a minimum alignment length of 15 bp. Homology to KEGG orthologous groups was assessed on level three classifications (herein termed functional pathways) that are associated with C-, N- and P-metabolism, namely starch and sucrose metabolism (herein referred to as C- metabolism; 29 genes included; PATH:ko00500), nitrogen metabolism (27 genes; PATH:ko00910), and phosphonate and phosphinate metabolism-associated (herein referred to as P-metabolism; 5 genes; PATH:ko00440) gene pathways, respectively. The effect of site and switchgrass cultivar on the abundance of functional pathways was assessed after summing the abundances of each gene within the gene pathway and normalizing the number of hits to the total number of predicted protein features for each sample. After classification, any pathway with a total of less than 3 hits were removed prior to analysis.

To delineate fungal from bacterial functional genes, local databases for functional genes associated with C-, N-, and P- acquisition were individually curated using the Carbohydrate Active Enzyme database (CAZy; Lombard et al. 2014) and the Functional Gene Repository (FunGene; Fish et al. 2013) *sensu* Freedman et al. (2016). Two bacterial reference genes (i.e., “housekeeping genes”), RNA polymerase (*rpoB*) and DNA recombinase (*recA*), and one fungal

reference gene, RNA polymerase II (*rpb2*), were included in the analysis to determine if site differences altered basic metabolic function in addition to C-, N- and P-associated functional genes. With the exception of the bacterial lignin decay-associated nucleotide-curated database, bacterial gene abundances were determined by comparing post-QC metagenomic short reads using DIAMOND's blastx function against each local functional gene database. To examine fungal functional genes, fungal-specific nucleotide functional gene databases were individually curated using the CAZy database, Peroxibase (Fawal et al. 2012), FunGene, and NCBI reference sequences (RefSeq; O'Leary et al. 2016) *sensu* Cline and Zak (2015). Databases for fungal functional genes and bacterial lignin-decay were determined by comparing filtered metagenomic short reads using NCBI's Magic-BLAST tool against each local functional gene database (version 1.4.0; Boratyn et al. 2018). Genes included in the functional databases are further detailed in Table 2.

Statistical Analyses

The effect of site, cultivar, and their interaction on soil physicochemical properties, microbial biomass, extracellular enzyme activities, richness, as well as the relative abundances of microbial taxa were determined using a two-way analysis of variance (ANOVA) using the integrated stats package in R (version 3.5.2; R Core Team 2013). β -diversity was determined using a Bray-Curtis distance matrix based on OTU abundance and analyzed through a two-way permutational multivariate analysis of variance (PerMANOVA; Anderson 2001), with site, cultivar, and their interaction as factors through the vegan package (version 2.5-4; Oksanen et al. 2019) in R. Significance was accepted at $\alpha=0.05$ and marginal significance was accepted at $\alpha=0.10$. Where applicable, means were compared using Tukey's Honestly Significant Difference post-hoc test (Tukey HSD; Tukey 1949).

Prior to analysis, each metagenome's functional gene assignments were normalized to the number of total predicted proteins for each sample (Glass et al. 2015; Warren et al. 2017). The impact of site and switchgrass cultivar on the relative abundance of KEGG pathways or functional genes were determined using a two-way ANOVA ($P < 0.05$) with site, cultivar, and their interaction as factors. When appropriate, means were compared by a Tukey HSD. The Benjamini & Hochberg false discovery rate correction (Benjamini and Hochberg 1995) was implemented to account for multiple comparisons; in this instance, an “adjusted P” value is listed.

Results

Soil Properties

The soil physical and chemical properties were distinctly different between Hampshire and Hobet (Table 1). Soil pH and moisture content at Hampshire were 23% and 135% greater than that at Hobet, respectively (site effect $P < 0.01$). Further, soils at Hampshire had greater soil organic matter (SOM) content (+149%) and microbial biomass (+55%) than Hobet soils (site effect $P < 0.01$). Soil texture was significantly different between sites; silt content was approximately double in Hampshire soils whereas clay content was over an order of magnitude higher in Hobet soils (site effect $P < 0.01$). Sand content was only marginally greater in Hobet soils compared to Hampshire (+29%; $P = 0.08$). Total soil C and N contents were both approximately three times higher in Hampshire soils compared to Hobet soils, respectively (site effect $P < 0.01$). Soil C:N ratios were 19% higher in Hampshire soils compared to Hobet soils (site effect $P = 0.03$). There were no differences in soil physical or chemical properties between cultivars.

Switchgrass microbiome extracellular enzyme activity

The activity of three extracellular enzymes associated with soil C-, N- and P-acquisition were assessed, namely β -glucosidase (cellobiose hydrolysis), N-acetyl-glucosaminidase (NAGase; chitin hydrolysis), and acid phosphatase (ACP; organic phosphate hydrolysis), respectively. β -glucosidase and NAGase enzymatic activities were both approximately triple in Hampshire compared to Hobet, respectively (Figure 1; site effect $P < 0.01$). No differences in β -glucosidase nor NAGase activity were observed across cultivars. Acid phosphatase activity was variable across sites and cultivars (Figure 1; site $\times$ cultivar $P = 0.05$). Post-hoc tests revealed acid phosphatase activity to be 78% higher in Hobet's Shawnee soils compared to Hobet's Cave-in-Rock soils as well as 274% and 96% higher than the Cave-in-Rock and Shawnee soils at Hampshire, respectively. No significant differences in enzymatic activity were observed across cultivars.

Bacterial and fungal taxonomy and composition

Sequencing of bacterial 16S rRNA gene amplicons yielded 3,616,272 post-QC sequences and prior to analysis, each sample was rarified to the sample with the lowest coverage (2,500 sequences). Bacterial richness, as estimated by Chao1, was 49% greater at Hobet compared to Hampshire (Figure 2A; site effect $P = 0.04$) and no cultivar effect was found. Similarly, the composition of the bacterial community differed across sites (Figure 3A; PerMANOVA site effect; $F = 3.38$, $P = 0.001$), but not across cultivars. Across sites, the most abundant bacterial phyla were Proteobacteria (42-43% relative abundance), Bacteroidetes (13-19%), and Acidobacteria (11-12%; Figure 4). Within the Proteobacteria, classes Alphaproteobacteria (11-18%) and Betaproteobacteria (11-15%) were most abundant across the two sites. The relative abundance of multiple bacterial taxa were different across sites. For example, Hobet's microbiome harbored a

greater relative abundance of Actinobacteria (+170%), Chloroflexi (+60%), and Alphaproteobacteria (+60%) as compared to Hampshire, while Hampshire's microbiome harbored a greater relative abundance of Firmicutes (+653%), Nitrospirae (+2,050%), Betaproteobacteria (+33%), and Gammaproteobacteria (+120%; site effect $P < 0.05$) as compared to Hobet. However, the relative abundance of these taxa did not change across cultivars. The relative abundance of Bacteroidetes was variable across sites and cultivars (site $\times$ cultivar $P < 0.05$). Post-hoc tests revealed that within Hampshire, the Shawnee cultivar harbored 25% greater relative abundance of Bacteroidetes compared to the Cave-in-Rock microbiome whereas the trend was reversed at Hobet, with the Cave-in-Rock cultivar harboring 25% greater relative abundance of Bacteroidetes compared to the Shawnee microbiome.

Sequencing of the fungal ITS region yielded 2,312,954 post-QC reads and prior to analysis, each sample was rarified to the sample with the lowest coverage (40,000 sequences). Fungal richness was 95% greater in Hobet as compared to Hampshire (Figure 2B; site effect $P < 0.01$), but there was no difference in richness across cultivars. Further, fungal composition also differed across sites (Figure 3B; PerMANOVA site effect; $F = 5.51$, $P = 0.001$), and no compositional differences between cultivars was observed. Initial fungal taxonomic assignments resulted in 42-66% of OTUs unassigned at the phylum level. For this reason, subsequent fungal taxonomic analyses were conducted by normalizing to the total classified to any given phylum. Of the 7 identified phyla, Ascomycota (53-67%) and Basidiomycota (16-35%) were most dominant across sites and cultivars. However, only members of the Basidiomycota were significantly different in abundance across sites, with the Hobet microbiome harboring 85% greater relative abundance of Basidiomycota compared to that of Hampshire (site effect $P = 0.04$). To consider the fungal community at a more resolved taxonomic level, fungal classes were

assessed as well. Of the 16 identified fungal classes, Agaricomycetes (9-28%), Dothideomycetes (11-18%), and Sordariomycetes (9-14%) dominated across sites and cultivars. Hampshire harbored a greater relative abundance of Microbotryomycetes (+1,505%), Chytridiomycetes (+709%), and Saccharomycetes (+4,414%) while Hobet harbored a greater relative abundance of Agaricomycetes (+246%) and Lecanoromycetes (Figure 5; +817%; site effect $P < 0.05$). Eurotiomycetes were variably affected across sites and cultivars (site $\times$ cultivar effect $P = 0.01$). Post-hoc tests revealed that the Shawnee microbiome harbored 63% and 136% greater relative abundance of Eurotiomycetes compared to the Cave-in-Rock and Shawnee microbiomes at Hampshire, respectively, but no differences were observed between microbiomes at Hobet. There were no significant differences in the relative abundance of Glomeromycetes across sites or cultivars. To achieve a deeper taxonomic resolution, fungal OTU genus-level classifications were cross-checked against the FUNGuild database to match fungal genera to their associated ecological guilds. Fungal OTUs assigned to the plant pathogen guild were two times greater in abundance at Hampshire compared to Hobet (site effect $P < 0.01$). The relative abundance of arbuscular mycorrhizae and saprotrophs were no different between sites or cultivars.

Metagenomic assessment of the switchgrass microbiome

The relative abundance of genes associated with C-, N-, and P- cycling were determined from shotgun metagenomes using two complimentary approaches: i) by comparing post-QC metagenomic contigs for homology to the KEGG pathways in MG-RAST and ii) by comparing post-QC metagenomic short reads to locally constructed functional gene databases using DIAMOND and BLAST queries (detailed in Table 2). Across the 12 metagenomes, 551,593 post-QC contigs were obtained (totaling 26.37 Mb). Contigs ranged from 5.44-66.76 Kb per contig, with a mean length of 16.14 Kb. The effects of site and switchgrass cultivar on the

abundance of KEGG-based C-, N- and P-metabolism functional pathways were examined to elucidate potential cultivar-specific differences across the two reclamation strategies. Genes associated with C-metabolism were marginally different in relative abundance across sites, with 19% greater abundance observed in Hampshire as compared to Hobet (Figure 6; adj $P=0.09$). Further, genes associated with N-metabolism and P-metabolism were 88% and 165% more abundant in Hampshire compared to Hobet, respectively (Figure 6; adj $P<0.01$).

To complement the KEGG pathway-based analysis, metagenomic short reads were compared to local functional gene databases curated from the CAZy, Peroxibase, FunGene, and NCBI RefSeq repositories. Across the 12 metagenomes, 296,465,928 post-QC short reads were obtained (totaling 44.3 Gb). Local functional gene databases were curated to include only high-quality sequences homologous to the functional gene of interest. In this way, local gene databases were built for both bacterial and fungal functional genes associated with C-, N-, and P-acquisition processes (summarized in Table 2). Bacterial genes associated with cellulose decay were marginally variably abundant across sites and cultivars (site $\times$ cultivar effect adj. $P=0.066$). Post-hoc tests revealed that Hobet's Cave-in-Rock and Shawnee microbiomes harbored 57% and 59% greater abundance of bacterial genes associated with cellulose decay compared to Hampshire's Shawnee microbiome, respectively, but Hampshire's Cave-in-Rock microbiome was not different between sites or cultivars. Hampshire soils harbored a greater relative abundance of bacterial phosphate mineralization-associated genes compared to Hobet soils (Figure 7; +18%, site effect adj. $P=0.001$). Bacterial genes associated with lignin and chitin decay were not significantly between sites (Figure 7; site effect adj. $P>0.05$). However, the abundance of bacterial genes associated with chitin decay were different across cultivars, with the Cave-in-Rock microbiome harboring a greater abundance of genes compared to that of Hobet

(+6%, cultivar effect adj. $P=0.02$). While there were significant differences in functional gene relative abundances, no differences were found in bacterial housekeeping genes associated with basic metabolic functions across sites (Figure 7; site effect adj. $P=0.7$).

Overall, the relative abundances of queried fungal functional genes were higher in Hobet compared to Hampshire. Fungal genes associated with cellulose and lignin decay were 663 and 21% higher in Hobet as compared to Hampshire (Figure 7; site effect adj. $P<0.001$). Similarly, fungal genes associated with chitin decay were 28% higher in Hobet compared to Hampshire (Figure 7; site effect $P<0.001$). Additionally, the fungal housekeeping gene was significantly greater in Hobet soils compared to Hampshire soils (Figure 7; +9%, site effect adj. $P<0.001$). There were no differences in the abundance of functional genes across cultivars.

Discussion

The enzymatic, taxonomic composition and metagenomic potential of the switchgrass soil microbiome was significantly different across sites, suggesting that the mine reclamation strategy employed at each site shaped both a functionally and taxonomically unique soil microbiome. However, I did not observe a functionally or compositionally unique microbiome across switchgrass cultivars. Soil physicochemical properties as well as microbial biomass, enzymatic, taxonomic, and metagenomic assessments were used to evaluate switchgrass soil microbiomes within two reclaimed mine sites. While I found that Hampshire's soil microbiome had a greater functional and genetic capacity to acquire critical nutrients than Hobet's microbiome, there was no indication that the microbiome influences differential yields between switchgrass cultivars at Hampshire.

Soil properties influence switchgrass microbiome activity across sites but not cultivars

It has been well established that soil properties (such as moisture, pH, and C:N ratios) play a substantial role in shaping the soil microbiome (Van Horn et al. 2014; Rousk et al. 2010; Heuck et al. 2015). Due to the drastically different reclamation strategies employed at Hampshire and Hobet, it can be postulated that soil enzymatic activity would be largely governed by the differences in soil properties between sites. Indeed, the differences in soil properties associated with distinct surface mine reclamation strategies significantly influenced the enzymatic capacity of the switchgrass microbiome to cycle critical nutrients like carbon, nitrogen, and phosphorus. Soil moisture has been positively linked to microbial respiration and activity (Barros et al. 1995; Greaves and Carter 1920). Low soil moisture levels disturb the physiological and metabolic integrity of microorganisms (Schimel, Balser, and Wallenstein 2007), which may have functional consequences (Manzoni, Schimel, and Porporato 2012; Stark and Firestone 1995). Foreseeably, the low soil moisture content in Hobet may have impeded microbial activity compared to that of Hampshire, which may elude to the substantial differences in enzymatic activity between sites.

Hampshire, the surface mine with a circumneutral pH and greater organic matter content as compared to Hobet, had higher activities of microbial β -glucosidase and NAGase (Fig 1). β -glucosidase catalyzes cellulose hydrolysis and in turn produces glucose, an important C source for the soil microbiome (Merino, Godoy, and Matus 2016). β -glucosidase activity is often positively related to SOM content (Stott et al. 2010; Zhang et al. 2011), similar to my findings. Additionally, NAGase impacts both C- and N-cycling due to its role in hydrolyzing the degradation of chitin into glucosamine. β -glucosidase and NAGase activities are positively linked to soil C and N contents, respectively (Cenini et al. 2016), which further supports the observation of higher C- and N-acquiring enzymatic activities at Hampshire compared to Hobet.

In contrast to β -glucosidase and NAGase activity increases observed at Hampshire, Hobet's microbiome exhibited overall greater acid phosphatase activity, an enzyme responsible for P-mineralization, with the greatest acid phosphatase activity seen in the Shawnee cultivar (Fig. 1). Hobet's soil was more acidic than that of Hampshire. Acid phosphatase activity is generally greater and the enzyme is more stable in acidic environments (Nguyen et al. 2016b; Tabatabai 1994), supporting the acid phosphatase response at Hobet. Further, plants require bioavailable P for the production of critical macromolecules like ATP and nucleic acids in order to grow (Schachtman et al. 1998). In particular, switchgrass yields have been found to increase as a function of P amendments to the soil (Kering et al. 2013; Sawyer et al. 2019). Under P-replete conditions, acid phosphatase activities are typically increased to mineralize organic P into inorganic, bioavailable forms (Ndakidemi 2006; R. and J. 2001; Tarafdar and Claassen 1988), which may elude to a bioavailable P-deficiency that is plausibly responsible for the low switchgrass yields at Hobet. This may be further substantiated by the 10-fold increase in clay content in Hobet, as phosphate ions have been found to adsorb to clay particles due to their differential charges (Edzwald et al. 1976; Anjembe et al. 2016).

Switchgrass plants form associations with arbuscular mycorrhizal fungi (Clark 2002; Jesus et al. 2016), which play a role in P acquisition (Smith et al. 2011). Glomeromycota, an exclusively arbuscular mycorrhizal fungal phylum (Helgason and Fitter 2009), harbors genes encoding acid phosphatase (Eivazi and Tabatabai 1976; Sato et al. 2015). It is possible that this mycorrhizal association may account for the observed increase in acid phosphatase activity at Hobet. While we found no differences in Glomeromycota between sites, it is possible that since this study assessed the bulk soil, differences in root-associated fungi such as Glomeromycota

(Treseder and Cross 2006) were less resolved. A more targeted analysis of the fungal community is required to ascertain differences in root-associated microbiota.

Reclamation strategy creates a unique soil microbiome across sites

Due to the differences in nutrient availability at each site, I expected that switchgrass plots at Hampshire would support a more diverse microbiome than the plots at Hobet, as soil pH and nutrient availability (namely C and N) largely shape microbial richness and diversity (Fierer and Jackson 2006; Rousk et al. 2010; Siciliano et al. 2014; Zhang et al. 2016). Counter to our predictions, the switchgrass soil-associated bacterial and fungal communities were 55 and 100% more rich in Hobet as compared to Hampshire (Fig. 2). While unexpected, one plausible explanation for these results could be explained by the intermediate disturbance hypothesis, which postulates that at intermediate levels of disturbance (i.e., heavy metal contamination in soils or surface mining), richness is highest due to the reduction of competitive exclusion for niche spaces (Connell 1978). Disturbance events, especially as a result of mining, have been found to increase species richness and diversity in both macro- and micro-environments (Armesto and Pickett 1985; Engelmann and Weaks 1985; Galand et al. 2016; Hong et al. 2015). My findings of increased microbial richness at Hobet, the poor-quality mine site, may suggest that disturbance effects continue to affect the microbiome at Hobet.

Switchgrass biomass yields across cultivars can be variable under different soil physicochemical conditions, which has been reported in these plots (Brown et al. 2016) as well as other studies (Casler et al. 2017; Fike et al. 2006). For this reason, I hypothesized that distinct microbiomes would exist across cultivars that may contribute to differences in crop yield at Hampshire. In this study, the diversity of the switchgrass soil microbiome differed across sites, but surprisingly no cultivar-specific differences were observed (Fig 3). Moreover, multiple

bacterial and fungal taxa were significantly different in abundance across sites (Fig. 4,5). I expected a greater abundance of copiotrophic microbial taxa, or taxa that thrive in environments with readily-available C compounds (Fierer et al. 2007) within Hampshire due to the increased amount of soil C and N compared to Hobet. Indeed, greater abundances of the bacterial phyla Bacteroidetes, Betaproteobacteria, and Firmicutes, all of which are typically considered copiotrophic due to their nutrient acquisition strategies (Fierer et al. 2007; Ho et al. 2017; De León-Lorezana et al. 2018; Mueller, Belnap, and Kuske 2015) were observed at Hampshire compared to Hobet. In contrast, Hobet's reclamation technique involved the application of crushed overburden with no topsoil amendment; thus I expected to observe a greater abundance of oligotrophic taxa, or taxa that thrive under low C conditions (Ishida and Kadota 1981; Kuznetsov et al. 1979). Again, similar to my expectations, abundances of Actinobacteria, Alphaproteobacteria, and Chloroflexi, all of which are considered oligotrophic taxa (Fierer et al. 2007; Ho et al. 2017; De León-Lorezana et al. 2018; Mueller et al. 2015), were of greater abundance in Hobet as compared to Hampshire. These results suggest that each sites' soil microbiome is selected for based upon the sites nutrient availability and the propensity of microbial taxa to exploit available nutrients.

When discussing microbial life strategies, soil fungi are considered generally more oligotrophic compared to bacteria due to their ability to acquire nutrients in more recalcitrant C environments (Hammer et al. 2011; Rinnan and Baath 2009). However, there were differences within the fungal community that also align with the copiotroph and oligotroph framework among sites. I found greater abundances of Saccharomycetes and Microbotryomycetes, or yeasts that readily degrade labile substrates like cellulose (i.e., a copiotrophic life strategy), in Hampshire compared to Hobet, (van der Klei et al. 2011; van der Wal et al. 2013). While the

fungus class Agaricomycetes are broad in their substrate usage, they are primarily considered wood-decay fungi that degrade recalcitrant substrates like lignin (Lundell et al. 2014). In congruency with previous research, the Hobet soil microbiome harbored a significantly greater abundance of Agaricomycetes compared to that of Hampshire, further substantiating that the employed reclamation strategy selects for a compositionally unique microbiome.

By classifying the fungal community into functional guilds using FUNGuild, I found no significant differences in arbuscular mycorrhizal fungi or saprotrophs between sites, but there was a greater relative abundance of plant pathogen-associated fungi at Hampshire compared to Hobet. These results were surprising, as organic amendments such as paper pulp and municipal waste have been historically found to suppress soil-borne plant pathogens (Abawi and Widmer 2000; Lazarovits 2001; Stone et al. 2003). However, an interesting dichotomy existed where the relative abundances of Firmicutes and Gammaproteobacteria, which include many bacterial taxa accountable to plant disease suppression (Köberl et al. 2017; Mendes et al. 2011), were also greater in Hampshire. Regardless, these results suggest that the reclamation strategy impacts soil microbiome composition, and is largely modulated by nutrient availability.

Functional genes associated with nutrient cycling differ across reclamation strategies

Previous studies on reclaimed mine sites have found that the organic amendments increase soil nutrient availability and cycling (Bendfeldt, Burger, and Daniels 2001; Shrestha, Lal, and Jacinthe 2009), so I hypothesized that Hampshire would have a greater genomic capacity to cycle C, N, and P compared to Hobet. The response of both functional gene pathways and selected functional genes associated with critical nutrient cycling differed across sites, suggesting that reclamation technique impacts the genomic capacity for the soil microbiome to cycle C, N and P (Fig. 6,7). The relative abundances of fungal cellulose, lignin, and chitin decay-

associated genes as well as overall DNA metabolism-associated genes were greater in Hobet compared to Hampshire, likely due to their increased ability to scavenge N and P in nutrient-limiting and recalcitrant C environments (Behie and Bidochka 2014; Ho et al. 2017). KEGG orthology genes associated with C-metabolism were marginally more abundant in Hampshire, but the relative abundances of genes associated with bacterial and fungal cellulose decay were both significantly greater in Hobet's microbiome compared to Hampshire. While it was expected that Hobet's microbiome would have a greater abundance of fungal-associated functional genes for aforementioned reasons, a greater abundance of bacterial cellulose decay-associated genes was unexpected. However, while the KEGG functional pathway for C metabolism included genes involved in cellulose decay (i.e., cellobiosidase and β -glucosidase), there are multiple genes not directly related to cellulose decay, such as maltose phosphatase and trehalose synthase. Additionally, an increase in bacterial cellulose decay-associated genes at Hobet may be modulated by an increase in plant root exudation of labile C compounds (Rovira 1969), a phenomenon found to increase as nutrient (namely N and P) availability decreases (Carvalhais et al. 2011; Edayilam et al. 2018). However, a more thorough analysis of switchgrass metabolites on reclaimed mine lands is warranted to further elucidate the influence of root exudation in shaping the microbiome composition.

Bacterial genes associated with chitin decay were not different across sites, but fungal genes associated with chitin decay were 22% greater in Hobet compared to Hampshire. Chitin is the second most abundant polysaccharide in nature, and is primarily derived from fungal cell walls that are especially chitin-rich (Bowman and Free 2006; Tharanathan and Kittur 2003). Thus, the increase in chitin decay-associated genes may suggest greater amounts of fungal necromass at Hobet (Freedman et al. 2016). With this knowledge, it is to be expected that there

was an increase in fungal chitin decay-associated genes at Hobet, as soil fungi are able to degrade complex compounds such as chitin (Dighton 2016) and the abundance of fungal chitin decay-associated genes have been found to increase in chitin-rich environments (Deng et al. 2007).

Bacterial genes associated with lignin decay were not significantly different across sites, but fungal genes associated with lignin decay were 17% greater in Hobet compared to Hampshire. Decomposition has been well documented to be rate-limited by lignin decay (Fog 1988; Meentemeyer 1978), and lignin decay-associated enzymes have been found to be constrained by lower soil pH (Sinsabaugh et al. 2008). While I did not assess lignin decay-associated enzymes in this study, lignin decay-associated enzymes can be positively correlated to lignin decay-associated genes (Chen et al. 2018). Surprisingly, Hobet, the site with the lower pH, had a greater relative abundance of lignin decay-associated genes. However, labile C sources are primarily preferentially degraded before recalcitrant compounds, such as lignin, in soils (Berg 2000; Rinkes et al. 2011), and fungi are able to more readily degrade recalcitrant compounds than most bacteria (Boer et al. 2005; Ho et al. 2017), so it is to be expected to observe a greater abundance of fungal lignin decay-associated genes within Hobet. With this knowledge, these results suggest that different reclamation strategies select for a functionally unique microbiome that is influenced by nutrient availability, particularly C.

The relative abundance of genes associated with N-metabolism was 61% greater in Hampshire compared to Hobet, respectively. This was to be expected, as municipal waste and paper pulp amendments are typically N-rich (Abdullah et al. 2015; Wolkowski 2003), and N-rich amendments can increase the abundance of genes involved in N cycling (i.e., *nirK*, *nirS*, *nosZ*; Ouyang et al. 2018; Wang et al. 2018). Indeed, I found that Hampshire had 195% higher total

soil N compared to Hobet. Previous studies have found that organic amendments increased plant N uptake between 30-60% (Ma, Dwyer, and Gregorich 1999). Switchgrass yield is N-limited (Hong et al. 2014; Owens et al. 2013) and responds positively to N amendments in otherwise low N conditions (Kering et al. 2013; Pedroso et al. 2013). Taken together, this may suggest that Hampshire has a greater functional capacity to cycle N compared to Hobet, which plausibly modulated higher yields in Hampshire that were reported by Brown et al. (2016).

A similar effect was found for genes associated with P-cycling, where Hampshire's soil microbiome harbored 90% greater relative abundance of genes associated with P-metabolism via the KEGG pathway and 18% greater relative abundance of genes associated with bacterial phosphate mineralization. Organic amendments such as municipal waste and paper pulp typically have higher P due to the treatment processes employed (Abdullah et al. 2015; Sokka, Antikainen, and Kauppi 2004), and an increase in soil P has been found to increase the abundance of genes associated with P acquisition (Fraser et al. 2015; Xue et al. 2018). With this knowledge, it was to be expected that Hampshire had greater abundances of genes associated with P-metabolism and phosphate mineralization. In addition, phosphatases are primarily excreted by root-associated mycorrhizal fungi (Sato et al. 2015; Smith et al. 2011). Unfortunately, this study was unable to assess fungal phosphate mineralization-associated genes due to currently unresolved records of fungal genes regulating P acquisition. However, further characterization of fungal P acquisition in a switchgrass system is warranted, as switchgrass closely associate with arbuscular mycorrhizal fungi for P acquisition (Clark, Baligar, and Zobel 2005; Kering et al. 2012). Arbuscular mycorrhizal fungi can secrete organic acids to solubilize P from rock phosphate (Antunes et al. 2007; Javaid 2009), so it is possible that further studies could incorporate an

assessment of fungal organic acid-associated genes to elucidate differences in fungal P acquisition across sites.

Potential contributors influencing differential yield responses

It has been well established that switchgrass cultivars are genetically and morphologically unique, even within upland and lowland ecotypes, and that these differences cause differential yield responses (Boe and Casler 2005; Casler and Boe 2003; Lemus et al. 2002; Ma, Wood, and Bransby 2000). For this reason, I hypothesized that there will also be a functionally and compositionally unique microbiome amongst switchgrass cultivars, specifically within Hampshire. Counter to my predictions, there were no strong indicators of Cave-in-Rock or Shawnee cultivar-specific microbiomes. While this is the only known study examining the switchgrass cultivar-specific microbiome on reclaimed mine soils, multiple studies have reported differences in the soil microbiome across switchgrass cultivars, potentially due to reported differences in root exudation strategies across cultivars (de Graaff et al. 2014; Mao et al. 2014; Roosendaal et al. 2016; Sawyer et al. 2019). Studies across a myriad of plants have concluded that root exudates shape the root-associated microbiome (Hu et al. 2018; Huang et al. 2014; Walker et al. 2003). It is possible that the bulk microbial community may not be modulating the differential yield response seen at Hampshire, but rather cultivar-unique secondary metabolites may increase productivity and nutrient cycling efficiency via the root-associated microbiome (Chomel et al. 2016). Regardless, additional analyses are required to understand the extent of the plant-microbial dynamics associated with switchgrass cultivars.

Conclusions

Here, I determined that the switchgrass microbiome does not exhibit cultivar-specificity, but does change based on differences in soil chemical and physical properties across two reclaimed mine lands in WV, USA. These data suggest that amended topsoil reclamation increases the propensity for a functionally unique soil microbial community compared to non-amended overburden, wherein Hampshire's microbiome acquires C and N more readily than Hobet's microbiome. Although I am currently unable to determine if the switchgrass soil microbiome mediated differential yield responses seen at Hampshire, further examination into switchgrass cultivar-specific metabolites and the root-associated microbiome is warranted. Together, results presented in this study suggest that reclamation technique alters the microbial potential to acquire C, N, and P in a marginal switchgrass agroecosystem, which occurred in parallel with differences in the abundance of functionally important bacterial and fungal taxa as well as the abundance of functional genes associated with C, N, and P cycling. As such, differences in the bulk soil microbiome may not influence differential switchgrass yield across sites, but further study is necessary to draw a causative link between soil microbiome dynamics and switchgrass yield.

Chapter 2: Cultivar-specific microbiomes may modulate switchgrass (*Panicum virgatum*) biomass yields on reclaimed mine sites in West Virginia.

Abstract

Switchgrass (*Panicum virgatum*) has been supported by the U.S. Department of Energy as a “model bioenergy crop” due to its well-characterized growth in various soil types and climates. Its ability to withstand harsh environmental conditions and increase soil stability has incentivized the use of switchgrass as a reclamation crop in recent years as well. However, little is known regarding plant-microbe interactions among switchgrass systems within reclaimed mine lands. A study conducted in 2008 grew switchgrass on high- and low- quality reclaimed mine sites (Hampshire and Hobet, respectively) in West Virginia to examine the resilience of switchgrass as a reclamation-friendly bioenergy crop. Switchgrass yields at Hampshire were nearly an order of magnitude higher than Hobet (8.4 Mg ha⁻¹ vs 1.0 Mg ha⁻¹). Within Hampshire, the Cave-in-Rock cultivar yield was 70% greater than that of Shawnee (12.9 Mg ha⁻¹ vs. 7.6 Mg ha⁻¹) and 187% greater than that of Carthage (12.9 Mg ha⁻¹ vs. 4.5 Mg ha⁻¹). Here, I sought to illuminate plant-microbial interactions that may account for this drastic shift in cultivar yield by examining the root-associated and bulk soils underneath three switchgrass cultivars using enzymatic activity analyses and microbiome taxonomic identification. Specifically, I tested two hypotheses: (i) that the root-associated microbiome will have a functionally and compositionally unique microbiome compared to that of the bulk soil and (ii) that there will be a cultivar-specific root-associated microbiome that may drive previously observed greater, but differential yields across switchgrass cultivars at Hampshire. Hampshire soils showed significant increases in extracellular enzymes associated with the acquisition of C, N, and P compared to Hobet. Additionally, the root-associated microbiome had a greater activities associated with the acquisition of C, N, and P compared to the bulk soil. Further, diverse and taxonomically-unique microbiomes were found between sites and cultivars. While Together, this data suggests that the root-microbiome may contribute to overall aboveground biomass yields between sites, and cultivar-specific microbiomes were observed that may substantiate differential yield responses within Hampshire.

Introduction

Plant productivity responds to a diverse array of environmental, biochemical, and microbial stimuli in the soil; many processes of which are still not well characterized. With a rapidly increasing global population and a comparable demand for food (United Nations Department of Economic and Social Affairs 2017), understanding plant-soil dynamics that lead to productivity is of utmost importance to an agriculturally sustainable future. Additionally, to reduce our dependence on fossil fuels, obtaining renewable energy sourced from plant biomass (i.e., cellulosic biofuel) has been nationally supported through the Energy Policy Act of 1992, the Renewable Fuel Standard of 2007, among other environmental statutes (Environmental Protection Agency 2007; United States 102d Congress 1992). In 1991, the United States Department of Energy deemed switchgrass (*Panicum virgatum*) a “model” bioenergy crop due to its ability to withstand nutrient-poor soil and unfavorable climates as well as its ability to increase soil productivity and nutrient (namely carbon and nitrogen) cycling (McLaughlin and Adams Kszos 2005). Moreover, switchgrass’ large roots systems have been found to stabilize soil and aid in soil organic carbon (SOC) sequestration, both of which are important in marginal soil environments (Liebig et al. 2005).

Since switchgrass has been found to not only survive in marginal lands, but actually promote healthy soils, substantial interest has advocated for farming switchgrass in marginal soils, like reclaimed surface mines (Skousen et al. 2013; Varvel et al. 2008). The Surface Mining and Reclamation Act (SMCRA) of 1977 requires that these lands be reclaimed to equal or better condition once mining ceases (U.S. Department of the Interior 1977), which switchgrass could plausibly help achieve. Despite this growing interest in farming switchgrass on reclaimed mine

lands, little is known about above- and belowground dynamics leading to productive yields on marginal soils.

Many root-associated bacterial and fungal taxa (i.e., plant growth-promoting bacteria and fungi) play an important role in the mineralization and immobilization of nutrients required for both plant and microbial growth (Badalucco and Kuikman 2001; Darrah 1993; Mendes et al. 2013). Previous studies in agricultural lands have found that the switchgrass soil microbiome may mediate aboveground productivity, especially within root-associated soils (Brejda et al. 1998; de Graaff et al. 2014; Roosendaal et al. 2016). Root-associated plant growth-promoting bacteria (PGPB) have been linked to upwards of 17% increased biomass in potato plants (Kloepper 1980) and 18% in alfalfa (Liu et al. 2019). The switchgrass rhizosphere has also been found to play a crucial role in promoting aboveground productivity, with studies observing 40-49% greater yields with the colonization of PGPBs (Ker et al. 2012; Kim et al. 2012). In addition, switchgrass form associations with arbuscular mycorrhizal (AM) fungi, which are responsible for converting soil P into bioavailable forms (Clark et al. 1999; George et al. 1995) and enhancing aboveground biomass (Brejda et al. 1998).

Switchgrass cultivars have been found to respond to their environment differently (Casler and Boe 2003), as in the case of differences in root structures and biomass yields documented between switchgrass cultivars (Brown et al. 2016; de Graaff et al. 2013). Switchgrass cultivars also secrete unique labile organic compounds from the roots to act on the root-associated microbiome (de Graaff et al. 2014), a process termed priming (Dalenberg and Jager 1989). Foreseeably, these differences in switchgrass cultivars may select for a unique microbiome that facilitate yield responses on reclaimed mine lands. However, there are currently no known

studies that have examined the influence of the switchgrass root-associated microbiome on aboveground productivity in reclaimed mine lands.

In 2008, a field study was developed to quantify the aboveground productivity of three switchgrass cultivars on two surface mines, Hampshire Hill mine and Hobet 21 mine, that were experimentally reclaimed in different manners (Brown et al. 2016). Hampshire's reclamation strategy that employed topsoil and organic amendments has historically been found to promote aboveground biomass yields and increase soil productivity (Larney and Angers 2012). In contrast, Hobet's reclamation strategy that employed no topsoil or amendments has been historically linked to reduced productivity due to unfavorable changes to soil physicochemical properties (Haering et al. 1993). Ultimately, Hampshire's reclamation technique led to switchgrass yields 13 times higher compared to yields at Hobet. Further, yield of the Cave-in-Rock cultivar was +70% greater than that of the Shawnee cultivar and 187% greater than that of the Carthage cultivar at Hampshire, but similar differences between cultivar yield were not observed at Hobet (Brown et al. 2016). While this study quantified yield differences across two sites with different reclamation strategies, the potential influence of belowground microbial dynamics that may result in differential productivity between cultivars at Hampshire, and not at Hobet, are unknown.

Here, I assessed the function and composition of the soil microbiome beneath three cultivars of switchgrass, Carthage, Cave-in-Rock, and Shawnee, in long-term switchgrass plots. Additionally, I sought to identify unique differences in the root-associated microbiome that may account for differential yield responses. To do so, extracellular enzyme assays were performed in parallel with high-throughput ribosomal rRNA gene sequencing. Specifically, I tested two hypotheses: (i) that the root-associated microbiome will have a functionally and compositionally

unique microbiome compared to that of the bulk soil and (ii) that there will be a cultivar-specific root-associated microbiome that may drive previously observed greater, but differential yields across switchgrass cultivars at Hampshire. In this way, I obtained insight into the root-associated switchgrass microbiome in marginal lands, leading to a better understanding of the functional capacity attributable to differential reclamation strategies and yield response at Hampshire and Hobet.

Materials and Methods

Site Locations

To investigate the influence of mine reclamation strategy, switchgrass cultivar, and soil type on the function and composition of the soil microbiome, soils were sampled from long term switchgrass plots on reclaimed mine sites in West Virginia, USA (Table 3.). Experimental plots within each site were laid out in a completely randomized block design in 2008. Each site was reclaimed using a different reclamation strategy. Briefly, the Hampshire Hill mine (herein referred to as Hampshire) was reclaimed using topsoil amended with municipal sewage waste and paper pulp spread over the existing overburden. The Hobet 21 mine, hereafter referred to as Hobet, was reclaimed using crushed, unweathered overburden that was regraded to approximate original contour. Once reclaimed, three replicate 4,000 m² plots of each switchgrass cultivar (Carthage, Cave-in-Rock, and Shawnee) were broadcast seeded across each plot. Further detail regarding reclamation strategy can be found in Brown et al. (2016). A particularly noteworthy difference in site management occurred in 2018, when Hampshire was amended with municipal sewage waste and paper pulp prior to sampling while Hobet remained unamended.

Soil sampling occurred in late May 2018, during a time at which ample moisture favored high rates of microbial activity and 10 years after the switchgrass stands were seeded. Within each plot, 10 soil cores were randomly collected at the base of a switchgrass plant to a depth of 15 cm (A horizon) and further referred to as bulk soil samples. Root-associated soil samples were obtained via removing a portion of the switchgrass roots and manually collecting the soil within the root system. Soil samples were homogenized and composited to yield one bulk soil and one root-associated sample per plot. All samples were placed on ice and transported to West Virginia University where the soil was immediately passed through a 0.2 mm sieve to remove roots and rocks. A portion of each homogenized and sieved soil sample was stored at -4°C for enzyme and soil chemical analysis and the remainder was stored at -80°C for DNA extraction.

Soil Properties

Soil pH was determined using a 1:5 soil:0.01M CaCl₂ suspension with samples shaking at 60 rpm for 30 minutes (Rayment and Higginson 1992). Soil moisture was determined gravimetrically by drying soil samples at 105°C for 24 hours. Using this dried soil, organic matter content was assessed by combustion at 505°C for 6 hours via loss on ignition. Microbial biomass was estimated by substrate-induced respiration (SIR), which measures induced microbial respiration when presented a labile carbon source (West and Sparling 1986). The average microbiome respiration rate (mg C-CO₂ g soil⁻¹ h⁻¹) was quantified by implementing adaptations to the West and Sparling protocol as described by Fierer, Schimel and Holden (2003).

Extracellular Enzyme Activity

To determine the microbial potential within switchgrass root-associated and bulk soils to mineralize critical soil nutrients from organic matter, extracellular enzymatic activities associated with C-, N-, and P- acquisition were conducted as described in Chapter 1 (page 6).

DNA Extraction and Sequencing

DNA was extracted in triplicate from 1.5 g total of each soil sample within 72 hours of sample collection. A PowerLyzer PowerSoil DNA isolation kit (Qiagen, Valencia, CA) was used following manufacturer's instructions in conjunction with a PowerLyzer 24 homogenizer (MoBio Laboratories, Carlsbad, CA). Purified DNA was quality checked on a ND8000 Nanodrop (Thermo Scientific, Waltham, MA) and quantified using a Qubit 4 Fluorometer (Thermo Scientific, Waltham, MA). Quantified DNA was submitted to the University of Minnesota Genomics Center for high throughput sequencing of the bacterial 16S rRNA gene and the fungal internal transcribed spacer (ITS) region. Briefly, 10 ng of extracted DNA was used as template to amplify the V4-V5 region of the 16S rRNA gene using universal bacterial primers 515F (Parada et al. 2016) and 805R (Apprill et al. 2015). The fungal ITS2 region was amplified using ITS9F and ITS4R universal fungal primers (Menkis et al. 2012; White et al. 1990). Unique i7 index barcode identifiers were included in the reverse primer. PCR reactions were performed in triplicate under the following thermalcycling conditions: an initial 3 minutes at 94 °C, 30 cycles of 45 seconds at 94 °C, 60 seconds at 50 °C, and 90 seconds at 72 °C, a final 10 minutes at 72 °C once cycles were complete, then held at 4 °C. Reactions were pooled to yield a single 16S rRNA and ITS product per sample. Pooled amplicons were purified using 1.2X AMPure XP beads (Beckman Coulter, Brea, CA) prior sequencing. Purified barcoded amplicons were

composited in equimolar concentrations for sequencing using an Illumina MiSeq 2x300 base read platform (Illumina, San Diego, CA) and a dual-indexing approach (Gohl et al. 2016). Ribosomal rRNA gene sequences are publicly available through the NCBI Sequence Read Archive (accession PRJNA533308).

Bacterial and Fungal Community Composition

For both 16S rRNA gene and ITS analyses, forward and reverse reads were joined using USEARCH (Edgar 2010) and the paired sequences were further processed in QIIME (version 1.9.1; Caporaso et al. 2010). Sequences were quality controlled by removing OTUs that were observed fewer than 5 times. Open reference operational taxonomic units (OTU) were picked using default parameters and the UCLUST (Edgar 2010) algorithm in QIIME. Sequences were rarified to 8,000 16S rRNA sequences per plot and 5,500 ITS sequences per plot. After rarifying, taxonomic assignments were performed at 90% similarity by comparing 16S rRNA OTUs against the Greengenes reference database (version 13.8; McDonald et al. 2012) using UCLUST and by comparing ITS OTUs against the UNITE reference database (version 8.0; UNITE Community 2019) using BLAST (Altschul et al. 1990). Changes in bacterial and fungal taxonomy across sites, switchgrass cultivars, and soil types were assessed at the phylum, order, and class level, with unassigned taxa or those under 3% in all samples binned as “other”. Microbial community α - and β -diversity metrics were also calculated in QIIME using Chao1 richness (Chao 1984) and Bray-Curtis dissimilarity indices (Bray and Curtis 1957), respectively.

Statistical Analyses

The effect of site, cultivar, soil type, and their interaction on soil physicochemical properties, microbial biomass, extracellular enzyme activities, richness, as well as the relative

abundances of microbial taxa were determined using a 3-way analysis of variance (ANOVA) using the integrated stats package in R (version 3.5.2; R Core Team 2013). β -diversity was determined using a Bray-Curtis distance matrix based on OTU abundance and analyzed through a three-way permutational multivariate analysis of variance (PerMANOVA; Anderson 2001), with site, cultivar, soil type, and their interaction as factors in PRIMER (version 7; Primer-E Ltd., Plymouth, UK; Clarke and Gorley 2015). Where interactions were observed for multivariate analyses, a pairwise PerMANOVA was conducted in PRIMER to parse statistical dissimilarities. Significance was accepted at $\alpha=0.05$ and marginal significance was accepted at $\alpha=0.10$. Where applicable, means were compared using Tukey's Honestly Significant Difference post-hoc test (Tukey HSD; Tukey 1949).

Results

Soil Properties

The soil physical and chemical properties were distinctly different between Hampshire and Hobet (Table 3). Soil pH and moisture content at Hampshire were 10% and 271% greater than that at Hobet, respectively (site effect $P<0.01$). A marginal increase in soil pH was observed in root-associated soils compared to bulk soils ($P=0.08$). SOM content was overall approximately 4 times greater in Hampshire soils compared to Hobet soils, but was variable across sites and cultivars (site $\times$ cultivar effect $P<0.01$). Post-hoc tests revealed that SOM content within the Carthage and Shawnee cultivar soils were 39 and 44% higher than that of Cave-in-Rock soils at Hampshire, respectively, while there were no differences observed between cultivars at Hobet. Overall, Hampshire soils had approximately 5 times greater microbial biomass than Hobet soils. Though, microbial biomass was marginally variable across sites and cultivars (site $\times$ cultivar $P=0.06$), wherein post-hoc tests revealed that the Shawnee

cultivar soils exhibited 58% and 75% greater microbial biomass than that of Cave-in-Rock and Carthage in Hampshire, respectively. There were no differences in microbial biomass observed between cultivars at Hobet.

Switchgrass microbiome extracellular enzyme activity

The activity of three extracellular enzymes associated with soil C-, N- and P- acquisition were assessed, namely β -glucosidase (cellobiose hydrolysis), N-acetyl-glucosaminidase (NAGase; chitin hydrolysis), and acid phosphatase (organic phosphate hydrolysis), respectively. β -glucosidase and NAGase enzymatic activities were both approximately an order of magnitude higher in Hampshire compared to Hobet (Figure 8; site effect $P < 0.01$). In addition to site differences, β -glucosidase and NAGase activities were higher in root-associated soils, although NAGase was only marginally higher (+27%, soil type effect $P = 0.01$; +27%, soil type effect $P = 0.09$). Acid phosphatase activity was also higher in Hampshire soils compared to Hobet soils (Figure 8; +178%, site effect $P < 0.001$). I also found a significant increase in acid phosphatase activity in root-associated soils compared to bulk soils (+31%, soil effect $P = 0.009$). For all examined enzyme activities, no differences were observed across cultivars.

Bacterial and fungal taxonomy and composition

Sequencing of bacterial 16S rRNA gene amplicons yielded 972,904 post-QC sequences and prior to analysis, each sample was rarified to the sample with the lowest coverage (8,000 sequences). Bacterial richness, as estimated by Chao1, was not significantly different between sites, cultivars, or soil types (Figure 9A). However, the composition of the bacterial community was variable across sites and cultivars (Figure 10A; site $\times$ cultivar PerMANOVA $F = 1.43$, $P = 0.01$). Pairwise tests revealed that the Cave-in-Rock microbiome was dissimilar from the

Shawnee microbiome ($t=1.4$; $P=0.03$) and marginally dissimilar from the Carthage microbiome within Hampshire ($t=1.4$; $P=0.06$), but no dissimilarities were observed between the Carthage and Shawnee microbiomes at Hampshire or between cultivars at Hobet ($P>0.05$).

Across sites, the most abundant bacterial phyla were Proteobacteria (38-39%), Actinobacteria (12-17%), and Acidobacteria (12-17%; Figure 11). Within Proteobacteria, orders Alphaproteobacteria (15-20%) and Betaproteobacteria (9-10%) were most abundant across the two sites. There were multiple differences in bacterial taxonomic abundance across sites, detailed in Table 4. The Hampshire microbiome had greater abundances of Firmicutes (+1,096%), Nitrospirae (+181%), Verrucomicrobia (+10%), and Deltaproteobacteria (+34%; site effect $P<0.01$). The Hobet microbiome harbored greater abundances of Acidobacteria (+32%), Armatimonadetes (+73%), Chlorobi (+47%), Cyanobacteria (+74%), Fibrobacteres (+59%), and Alphaproteobacteria (+28%; site effect $P<0.01$).

Significant, but few, differences in bacterial taxonomic abundances were observed between soil types. Specifically, Gemmatimonadetes were 26% and Nitrospirae were 44% more abundant in the bulk soil microbiome compared to root-associated microbiome (soil type effect $P<0.05$).

There were also cultivar-specific differences in microbiome composition observed for bacteria, namely Acidobacteria and Deltaproteobacteria (cultivar effect $P<0.05$). Post-hoc tests concluded that the Carthage microbiome harbored 25% greater relative abundance of Acidobacteria compared to the Cave-in-Rock microbiome, but the relative abundance of Acidobacteria in the Shawnee microbiome was not significantly different amongst cultivars. Post-hoc tests also revealed that Deltaproteobacteria were 30 and 31% greater in the Cave-in-Rock microbiome compared to that of Carthage and Shawnee, respectively.

While Actinobacteria and Gammaproteobacteria were more abundant in Hampshire, differential responses across sites and cultivars were observed for both (site $\times$ cultivar $P < 0.05$). Post-hoc tests revealed that while there were no differences in Actinobacteria relative abundance between cultivars within sites, the Hampshire Cave-in-Rock microbiome harbored a greater relative abundance of Actinobacteria compared to all cultivars at Hobet (+56-82%). For Gammaproteobacteria, post-hoc tests revealed that Hampshire's Carthage and Shawnee microbiomes were 85 and 75% greater in relative abundance compared to Hobet's Carthage and Shawnee microbiomes, respectively, but no differences were observed within the Cave-in-Rock microbiome between sites. While Bacteroidetes was marginally variable across sites and cultivars (site $\times$ cultivar $P = 0.09$), post-hoc tests concluded there were only site differences, with the Hampshire microbiome harboring 88% greater relative abundance of Bacteroidetes compared to Hobet's microbiome.

Two site $\times$ cultivar $\times$ soil type interactions were found for bacterial microbiome composition: Chloroflexi (site $\times$ cultivar $\times$ soil type effect $P = 0.03$) and a marginal interaction was observed for Elusimicrobia (site $\times$ cultivar $\times$ soil type effect $P = 0.07$). While multiple post-hoc differences existed between sites, cultivars, and soil types for Chloroflexi, many comparisons were not significant. However, post-hoc tests did reveal a 64% increase in Chloroflexi relative abundance in Hobet's Cave-in-Rock microbiome compared to Hampshire's Cave-in-Rock microbiome. Multiple post-hoc differences were also observed for Elusimicrobia, but it was revealed that Hobet's Cave-in-Rock bulk soil microbiome harbored 1,250% greater relative abundance of Elusimicrobia compared to Hampshire's Cave-in-Rock bulk soil microbiome.

Sequencing of the fungal ITS region yielded 946,465 post-QC reads, and each sample was rarified to the sample with the lowest coverage (5,500 sequences) prior to analysis. Fungal richness was 34% greater in Hampshire as compared to Hobet (Figure 9B; site effect; $P < 0.001$), but there was no difference in richness across cultivars or soil types. Fungal community composition was variable across sites and cultivars (Figure 10B; site $\times$ cultivar PerMANOVA $F = 1.7$, $P < 0.01$). Pairwise tests revealed that the Cave-in-Rock microbiome was marginally dissimilar from the Shawnee ($t = 1.4$; $P = 0.08$) and Carthage ($t = 1.4$; $P = 0.07$) microbiomes at Hampshire, but no dissimilarities were observed between Hampshire's Carthage and Shawnee microbiomes ($t = 1.3$; $P = 0.12$). Additionally, pairwise tests also revealed that the Cave-in-Rock microbiome was marginally dissimilar from the Shawnee microbiome at Hobet ($t = 1.3$; $P = 0.052$), but no other dissimilarities were observed at Hobet ($P > 0.05$).

Of the 21 fungal classes identified, Dothideomycetes (23-27%), Sordariomycetes (13-32%), and Agaricomycetes (12-32%) dominated across sites, cultivars, and soil types. There were multiple differences in fungal taxonomic abundance across sites, detailed in Table 5. The Hampshire microbiome had greater abundances of Basidiobolomycetes (+10,333%), Leotiomycetes (+200%), Olpidiomyces (+3,83%), and Sordariomycetes (+139%; site effect $P < 0.01$). The Hobet microbiome harbored greater abundances of Agaricomycetes (+64%), Eurotiomycetes (+64%), Glomeromycetes (+78%), Kickxellomycetes (+84%), and Paraglomeromycetes (+94%; site effect $P < 0.01$).

There were also cultivar-specific differences in microbiome composition observed for Eurotiomycetes (cultivar effect $P = 0.06$). The Cave-in-Rock and Shawnee microbiomes harbored 45 and 36% greater relative abundance of Eurotiomycetes compared to the Carthage microbiome, respectively.

Pezizomycetes and Rhizophydiomycetes relative abundances were variable across sites and cultivars (site $\times$ cultivar $P < 0.05$) while the relative abundance of Orbiliomycetes was marginally variable across sites and cultivars (site $\times$ cultivar $P = 0.07$). Post-hoc tests revealed that Hampshire's Carthage microbiome harbored % greater relative abundance of Pezizomycetes compared to Hobet. Post-hoc tests revealed that Hampshire's Carthage microbiome harbored 4,493% greater relative abundance of Pezizomycetes compared to Hobet's Carthage microbiome, while no differences were observed for the Shawnee or Cave-in-Rock microbiomes across sites. Additionally, post-hoc tests revealed that Hampshire's Cave-in-Rock and Shawnee microbiomes harbored 332 and 537% greater relative abundance of Rhizophydiomycetes compared to Hampshire's Carthage microbiome, respectively, and no differences were observed between cultivars within Hobet. A marginal site $\times$ cultivar effect was observed for Orbiliomycetes (site $\times$ cultivar $P = 0.07$), wherein post-hoc tests revealed that Hobet's Shawnee microbiome had 244% greater relative abundance of Orbiliomycetes compared to Hampshire's Shawnee microbiome, but no differences were observed for the Cave-in-Rock or Carthage microbiomes across sites.

The relative abundance of Microbotryomycetes was variable across sites and soil types (site $\times$ soil type $P = 0.016$) while the relative abundance of Rhizophydiomycetes and Spizellomycetes were marginally variable across sites and soil types (site $\times$ soil types $P < 0.1$). Post-hoc test revealed that Hampshire's root-associated microbiome harbored 167% greater relative abundance of Microbotryomycetes, but there were no differences between soil types within Hobet. While a marginally significant site $\times$ soil type existed for Rhizophydiomycetes (site $\times$ soil type $P = 0.088$), post-hoc tests revealed that there were only site differences, with the Hampshire microbiome harboring 573% greater relative abundance of Rhizophydiomycetes

compared to Hobet's microbiome. Additionally, post-hoc tests on the relative abundance of Spizellomycetes concluded that there were no significant differences between sites or soil types.

Discussion

This research highlights that a unique microbiome exists between switchgrass cultivars on two reclaimed mine sites in West Virginia, USA. The results herein are suggestive of a cultivar-specific microbiome that may influence differential plant productivity within Hampshire. Soil physicochemical properties as well as microbial biomass, enzymatic, and taxonomic assessments were used to evaluate switchgrass soil microbiomes grown within the Hampshire and Hobet mines. I found that Hampshire's soil microbiome had an overall greater enzymatic activity associated with C, N, and P acquisition than Hobet's microbiome. In addition, I found that the root-associated microbiomes have greater enzymatic activity associated with C-, N-, and P-acquisition compared to the bulk soil microbiome across sites, but no compositional differences were observed between soil types. Taken together, a compositionally unique microbiome exists between sites and cultivars, suggestive of a microbial influence on aboveground biomass yields.

Extracellular enzymatic activity is highest in switchgrass root-associated soils

Similar to Chapter 1, I expected the differences in soil properties to foster a unique microbiome across sites, since soil microbial activity is largely mediated by soil physicochemical properties like soil pH and OM content (Van Horn et al. 2014; Rousk et al. 2010; Xue et al. 2018). For this reason, I also expected that while the Hampshire microbiome would foreseeably have greater enzymatic activities associated with C-, N-, and P-acquisition compared to Hobet, particularly within the root-associated microbiome. Further supporting the results obtained in

Chapter 1, differences in the soil physicochemical factors associated with distinct surface mine reclamation strategies significantly influenced the enzymatic capacity of the switchgrass microbiome to cycle critical nutrients such as carbon, nitrogen, and phosphorus (Fig 8). In particular, β -glucosidase, N-acetyl-glucosaminidase, and phosphatases all have been found to increase with substrate availability, microbial nutrient demand stoichiometry, and soil pH (Sinsabaugh et al. 2008). Indeed, Hampshire, the site with circumneutral pH, had greater enzymatic activities associated with all three enzymes. Together, this knowledge suggests that increased enzymatic activity at Hampshire suggests that Hampshire had greater nutrient availability than that of Hobet.

It has been found that root-associated soils have higher enzymatic activity compared to that of the bulk soil, likely due to the influence of plant root exudates on the microbiome (Dai et al. 2016). For this reason, I hypothesized that the root-associated soils would have greater enzymatic activity associated with C, N, and P acquisition in root-associated soils. Indeed, an overall increase in β -glucosidase and acid phosphatase as well as a marginal increase in NAGase activities were observed within the root-associated soils within both Hampshire and Hobet (Fig 8). A unique difference between these sites recent management strategies is that Hampshire was amended with organic wastes shortly before sampling while Hobet remained unamended. It is likely that the organic amendments at Hampshire further stimulated the root-associated microbiome to cycle C, N, and P, similar to the findings from (Fang et al. 2010). However, it has been well established that plant root exudates stimulate soil enzymatic activity, especially in AM-fungi-associated systems like switchgrass (Brzostek et al. 2013; Egamberdieva et al. 2011), so it is to be expected that the root-associated microbiome had an increased propensity to acquire C, N, and P compared to the bulk soil within Hampshire and Hobet. While there were no

significant interactions that elude to cultivar-specific enzymatic activity differences, my data does suggest that the root-associated microbiome acquires C, N, and P more readily than that of the bulk soil.

While Chapter 1 observed the greatest acid phosphatase activity in Hobet soils, I found that Hampshire had the greatest acid phosphatase activity after the addition of organic wastes. Although acid phosphatase activity is most stable at a lower pH (Eivazi and Tabatabai 1976), activities increase as a function of P limitation (Bragazza et al. 2006). This study did not quantify C/N ratios within Hampshire and Hobet, but the addition of organic amendments has been linked to increased mineralizable C and N (Goyal et al. 1999; Ryals et al. 2014). While these results may arise as an artifact of one sampling date, it is more likely that the addition of organic amendments caused a stoichiometric nutrient imbalance towards greater amounts of C and N which may have limited P acquisition at Hampshire, thus causing an increase in phosphate-acquiring enzymes (Chang, Chung, and Tsai 2007).

Organic amendments increase switchgrass soil microbiome diversity between cultivars

In concordance with previous studies that have found that soil pH and nutrient availability largely shape microbial richness and diversity (Fierer and Jackson 2006; Rousk et al. 2010; Siciliano et al. 2014), I expected that switchgrass plots at Hampshire would support a more diverse microbiome than the plots at Hobet. Additionally, due to plant-influenced selection on the root-associated microbiome, the rhizosphere is typically more biodiverse (Campbell and Greaves 1990; Gilbert et al. 1996). Although I assessed root-associated soils and not the rhizosphere, I expected similar increases in biodiversity to be apparent in the root-associated soils within Hampshire and Hobet. Indeed, I observed greater fungal richness in Hampshire compared to Hobet (Fig. 9). For fungi, richness has been found to increase as a function of

organic matter content (Siciliano et al. 2014), which is supported here with increased organic matter content in Hampshire compared to Hobet. However, I found no differences in richness between sites, cultivars, or soil types for bacteria. In addition, there were no differences in the root-associated and bulk soil richness for either bacteria or fungi. While these results were surprising, richness metrics may be useful to identify trends on a large scale, while community composition via taxonomic identification may better to elucidate differences on smaller scales (Hartmann et al. 2015; Hartmann and Widmer 2006). Moreover, bacterial richness is largely variable across temporal and spatial scales (Naveed et al. 2016; Terrat et al. 2017), so direct taxonomic and functional assessments may better elude to compositional differences in the microbiome.

While richness examines diversity within a given site, β -diversity examines the heterogeneity of microbial composition between sites (Barwell, Isaac, and Kunin 2015). β -diversity indices concluded that the cultivars within Hampshire had greater diversity amongst each other than cultivars within Hobet (Fig. 10). Previous studies have reported that increases in nutrient availability increases the significance of diversity within both bacteria and fungi (Maron et al. 2018), which compliment results found in this study. Interestingly, previous studies have positively linked β -diversity indices with differences in aboveground plant biomass (Li et al. 2018). Taken together, these results suggest that Hampshire's reclamation strategy of organic amendments foster a unique and biodiverse microbiome across cultivars that may substantiate differential yields within Hampshire.

Cultivar-specific microbiomes may link to aboveground switchgrass productivity

Similar to the results observed in Chapter 1, reclamation strategy selected for a unique soil microbiome across sites, likely due to differences in nutrient availability. Similar site-

specific trends existed, where Hampshire harbored generally greater relative abundances of Bacteroidetes, Betaproteobacteria, and Firmicutes, all of which are copiotrophic bacteria that preferentially degrade labile C compounds (Fierer et al. 2007; Ho et al. 2017; De León-Lorenzana et al. 2018; Mueller et al. 2015). Additionally, Hobet harbored generally greater relative abundances of Alphaproteobacteria and Chloroflexi, all of which are oligotrophic bacteria that typically degrade recalcitrant C substances (Ishida and Kadota 1981; Kuznetsov, Dubinina, and Lapteva 1979).

However, in contrast to Chapter 1, I also found multiple interactions between cultivars that may indicate a cultivar-specialized microbiome potentially influencing differential yields observed within Hampshire. A notable site $\times$ cultivar interaction was observed for Actinobacteria where the greatest abundance was found in Hampshire's Cave-in-Rock and Shawnee root-associated microbiomes (Fig. 11). Interestingly, these two cultivars had the greatest aboveground biomass at Hampshire (Brown et al. 2016). While Actinobacteria are broad in their nutrient acquisition and substrate usage schemes, they have been considered plant growth-promoting bacteria due to their influence in nitrogen fixation, phosphate mineralization, and contribution to plant pathogen suppression via secondary metabolites (Sathya, Vijayabharathi, and Gopalakrishnan 2017). Previous studies have found that increased abundances of Actinobacteria increases plant biomass (Lasudee et al. 2018). Although Actinobacteria alone may not modulate the differential yield response observed at Hampshire, this may be a contributory link to cultivar-specific microbiome-induced aboveground productivity.

Fungal community composition also was substantially different across sites, following similar trends to Chapter 1 with generally greater abundances of copiotrophic taxa (i.e., Microbotryomycetes; van der Klei et al. 2011; van der Wal et al. 2013) observed in Hampshire

while generally greater abundances of oligotrophic taxa (i.e., Agaricomycetes; Lundell et al. 2014) were observed in Hobet. Notably dissimilar from Chapter 1, I found that Glomeromycetes were significantly greater in Hobet compared to Hampshire. This was expected since Glomeromycetes, an arbuscular mycorrhizal clade, have been seen to promote plant growth under environmentally stressful conditions, such as water and nutrient deficiencies (Hashem et al. 2018). An interesting site $\times$ cultivar was also observed for Rhizophydiomycetes where there was a greater relative abundance within the Cave-in-Rock and Shawnee microbiomes, or the cultivars with the highest reported yields by Brown et al. (2016). Due to their low abundance in soil, this fungal class has been substantially understudied. However, taxa within this class have been identified as saprobes, which may aid in nutrient acquisition within the system (Letcher et al. 2006). It is possible that these bacterial taxa may serve as an indicator species for plant productivity; however, additional research is warranted to support a plant-microbe interaction for Rhizophydiomycetes. To date, this is the only known study that has observed Rhizophydiomycetes differences as a function of plant aboveground productivity.

Conclusions

Here, I determined that there are switchgrass cultivar-specific microbiomes that may modulate differential aboveground yield responses across two reclaimed mine lands in WV, USA. This data suggests that amended topsoil reclamation increases enzymatic activities associated with C, N, and P acquisition in soil microbiome compared to non-amended overburden, wherein Hampshire's microbiome acquires C, N, and P more readily than Hobet's microbiome. Additionally, functionally relevant microbial taxa were positively connected to differential aboveground yields within Hampshire and Hobet. More specifically, Actinobacteria and Rhizophydiomycetes were highest in abundance within the microbiomes of the two highest-

yielding sites at Hampshire. Together, results presented in this study suggest that reclamation technique alters the microbial potential to acquire C, N, and P in a marginal switchgrass agroecosystem, and that cultivar-specific microbiomes may promote aboveground switchgrass yields in marginal soils. However, a more thorough analysis of plant influence via metabolites and root exudates on the microbial community is warranted to foster a causative link between the soil microbiome and switchgrass yields.

Tables

Table 1: Site characteristics and soil chemical/physical properties at Hampshire and Hobet.

Site Characteristics				
Site	Hampshire		Hobet	
Latitude (N), Longitude (W)	39.4°, -79.1°		38.1°, -81.6°	
Elevation (m)	599		203	
Mean annual temp. (°C)	11.3		13.2	
Mean annual precip. (cm)	101		112	
Soil Properties (0-10 cm)				
Site	Hampshire		Hobet	
Cultivar	Cave-in-Rock	Shawnee	Cave-in-Rock	Shawnee
pH (1:5 soil/CaCl ₂)	7.0 (0.1) ^a	7.2 (0.1) ^a	5.9 (0.01) ^b	5.7 (0.2) ^b
Gravimetric moisture content	61.4 (3.1) ^a	69.2 (2.3) ^a	17.7 (2.8) ^b	22.9 (1.3) ^b
Organic matter (% dry weight)	15.1 (1.7) ^a	16.7 (0.7) ^a	6.2 (1.9) ^b	6.6 (0.7) ^b
Microbial biomass (mg C-CO ₂ g soil ⁻¹ h ⁻¹)	72.0 (2.0) ^a	77.3 (3.6) ^a	43.7 (6.9) ^b	52.8 (1.2) ^b
Soil C (%)	6.7 (1.2) ^a	7.6 (0.9) ^a	2.0 (0.5) ^b	2.1 (0.3) ^b
Soil N (%)	0.37 (0.04) ^a	0.43 (0.04) ^a	0.13 (0.02) ^b	0.15 (0.02) ^b
C:N Ratio	17.6 (1.3) ^a	17.5 (0.5) ^a	14.8 (0.2) ^b	14.8 (0.2) ^b
Soil texture class	sandy loam	silt loam	sandy loam	sandy loam
% sand	56 ^a	38 ^a	60 ^a	61 ^a
% silt	44 ^a	61 ^a	29 ^a	26 ^a
% clay	0 ^a	1 ^a	11 ^b	12 ^b

Values represent means ± SEM (n = 3). Different letters indicate significant differences between groups (P<0.05).

Table 2: Summary of functional genes used in metagenomic analyses.

Bacterial enzyme/gene name	EC Number	Function Category
β -glucosidase	3.2.1.21	Cellulose
Endocellulase	3.2.1.4	Cellulose
Alpha-N-acetylglucosaminidase	3.2.1.50	Chitin
Chitin deacetylase	3.5.1.41	Chitin
Endochitinase	3.2.1.14	Chitin
β -N-acetylhexosaminidase	3.2.1.52	Chitin
Laccase	1.10.3.2	Lignin
Alkaline phosphatase (ALP)	3.1.3.1	Phosphate
RNA Polymerase II (<i>rpoB</i>)	2.7.7.6	Reference
Protein recombinase A (<i>recA</i>)	3.4.99.37	Reference
Fungal enzyme/gene name	EC Number	Function Category
β -glucosidase	3.2.1.21	Cellulose
Cellulose 1,4-beta-cellobiosidase (<i>cbhI</i>)	3.2.1.91	Cellulose
α -N-acetylglucosaminidase	3.2.1.50	Chitin
β -N-acetylhexosaminidase	3.2.1.52	Chitin
Laccase (<i>lcc</i>)	1.10.3.2	Lignin
Lignin peroxidase (<i>lip</i>)	1.11.1.14	Lignin
Manganese peroxidase (<i>mnp</i>)	1.11.1.13	Lignin
Versatile peroxidase (<i>vp</i>)	1.11.1.16	Lignin
RNA polymerase II (<i>rpb2</i>)	2.7.7.6	Reference

Table 3: Site characteristics and soil chemical/physical properties at Hampshire and Hobet among switchgrass cultivars and soil types.

Site Characteristics									
Site	Hampshire				Hobet				
Lat. (N), Long. (W)	39.4°, -79.1°				38.1°, -81.6°				
Elevation (m)	599				203				
Mean ann. temp. (°C)	11.3				13.2				
Mean ann. precip. (cm)	101				112				

Soil Properties (0-10 cm)									
Site	Hampshire				Hobet				
Cultivar	Shawnee				Shawnee				
Soil Type	Cave-in-Rock		Carthage		Cave-in-Rock		Carthage		
	Root	Bulk	Root	Bulk	Root	Bulk	Root	Bulk	
pH (1:5 soil/CaCl ₂)	6.9±0.0 ^a	6.8±0.0 ^b	6.9±0.0 ^a	6.9±0.1 ^b	6.0±0.2 ^c	6.5±0.1 ^d	6.1±0.1 ^c	6.2±0.1 ^d	6.3±0.3 ^c 6.4±0.2 ^d
Moisture (%)	47±13 ^a	43±7 ^a	55±13 ^a	45±8 ^a	14±0 ^b	12±0 ^b	12±1 ^b	11±1 ^b	14±1 ^b 12±1 ^b
Organic matter (%)	13±2 ^a	12±1 ^a	19±2 ^b	17±1 ^b	4±1 ^c	4±1 ^c	4±0 ^c	4±0 ^c	4±0 ^c 3±1 ^c
Microbial biomass (mg C-CO ₂ g soil ⁻¹ h ⁻¹)	44±9 ^a	37±7 ^a	71±16 ^b	57±10 ^b	39±7 ^a	34±11 ^a	9±1 ^c	7±0 ^c	6±1 ^c 3±1 ^c

Values represent means ± SEM (n = 3). Different letters indicate significant differences between means (P<0.05). ^ after letters indicates marginal significance (P<0.1).

Table 4: The relative abundance of bacterial taxa-associated P-values from a 3-way ANOVA with site, cultivar, and soil type as effects. Yellow highlight indicates significance at $P < 0.05$ and orange highlight indicates marginal significance at $P < 0.1$.

	Site	Cultivar	Soil	Site × Cultivar	Cultivar × Soil	Site × Soil	3 Factor
Acidobacteria	<0.01	0.04	0.82	0.51	0.18	0.64	0.27
Actinobacteria	<0.01	0.33	0.27	0.04	0.10	0.76	0.12
Armatimonadetes	<0.01	0.47	0.18	0.67	0.83	0.94	0.51
Bacteroidetes	<0.01	0.11	0.85	0.09	0.50	0.74	0.60
Chlamydiae	<0.01	0.60	0.05	0.35	0.17	0.07	0.78
Chlorobi	<0.01	0.73	0.86	0.49	0.41	0.51	0.60
Chloroflexi	<0.01	0.17	0.64	0.88	0.12	0.31	0.03
Cyanobacteria	<0.01	0.64	0.68	0.11	0.44	0.83	0.65
Elusimicrobia	<0.01	0.19	0.71	<0.01	0.05	0.09	0.07
Fibrobacteres	<0.01	0.57	0.42	0.15	0.59	0.53	0.71
Firmicutes	<0.01	0.14	0.31	0.12	0.26	0.20	0.26
Gemmatimonadetes	0.01	0.25	0.03	0.12	0.83	0.48	0.45
Nitrospirae	<0.01	0.30	0.04	0.12	0.76	0.46	0.32
Planctomycetes	0.22	0.10	0.03	0.01	0.11	0.39	0.37
Alphaproteobacteria	<0.01	0.79	0.90	0.40	0.90	0.42	0.48
Betaproteobacteria	0.77	0.38	0.85	0.66	0.33	0.58	0.38
Deltaproteobacteria	<0.01	<0.01	0.59	0.30	0.45	0.49	0.90
Gammaproteobacteria	<0.01	0.86	0.47	0.03	0.18	0.63	0.57
Verrucomicrobia	0.20	0.11	0.28	0.14	0.49	0.93	0.56

Table 5: The relative abundance of fungal taxa-associated P-values from a 3-way ANOVA with site, cultivar, and soil type as effects. Yellow highlight indicates significance at $P < 0.05$ and orange highlight indicates marginal significance at $P < 0.1$.

	Site	Cultivar	Soil	Site $\times$ Cultivar	Cultivar $\times$ Soil	Site $\times$ Soil	3 Factor
Agaricomycetes	<0.01	0.34	0.868	0.942	0.469	0.985	0.877
Archaeorhizomycetes	0.133	0.518	0.262	0.519	0.636	0.264	0.635
Basidiobolomycetes	<0.01	0.191	0.838	0.212	0.604	0.866	0.56
Blastocladiomycetes	0.316	0.39	0.316	0.39	0.385	0.323	0.385
Dothideomycetes	0.075	0.298	0.62	0.96	0.2	0.19	0.96
Eurotiomycetes	<0.01	0.061	0.934	0.412	0.837	0.777	0.429
Exobasidiomycetes	0.071	0.218	0.743	0.257	0.99	0.773	0.996
Glomeromycetes	0.003	0.796	0.909	0.254	0.708	0.424	0.425
Kickxellomycetes	<0.01	0.176	0.502	0.718	0.553	0.902	0.924
Lecanoromycetes	0.063	0.596	0.898	0.235	0.275	0.259	0.495
Leotiomycetes	0.013	0.262	0.386	0.707	0.867	0.378	0.895
Microbotryomycetes	<0.01	0.242	0.02	0.216	0.68	0.016	0.703
Mortierellomycetes	0.085	0.966	0.795	0.714	0.244	0.934	0.9
Olpidiomycetes	0.044	0.5	0.463	0.42	0.31	0.41	0.34
Orbiliomycetes	<0.01	<0.01	0.271	0.073	0.685	0.147	0.573
Paraglomeromycetes	<0.01	0.665	0.906	0.749	0.884	0.71	0.866
Pezizomycetes	<0.01	0.258	0.659	0.027	0.64	0.766	0.62
Rhizophydiomycetes	<0.01	<0.01	0.134	<0.01	0.492	0.088	0.736
Sordariomycetes	<0.01	0.179	0.276	0.288	0.527	0.633	0.338
Spizellomycetes	0.49	0.043	0.991	0.727	0.987	0.079	0.504
Tremellomycetes	0.066	0.417	0.895	0.406	0.871	0.924	0.704

Figure 1: Extracellular enzyme activity responses of β -glucosidase, N-acetyl-glucosaminidase (NAGase), and acid phosphatase (ACP) to differing reclamation techniques. Values expressed as mean $\pm$ standard error ($n=3$) with eight analytical replicates. Different letters indicate significant differences in means at $P=0.05$.

Figure 2: The effect of reclamation strategy on the (A) bacterial and (B) fungal richness of the switchgrass microbiome, as measured by Chao1 index. Values expressed as mean $\pm$ standard error (n=3). Different letters indicate significant differences in means at P=0.05.

Figure 3: The effect of reclamation strategy on the (A) bacterial and (B) fungal community composition of the switchgrass microbiome. A principal coordinates analysis was conducted using the Bray-Curtis dissimilarity metric on OTU relative abundances.

Figure 4: The effect of reclamation strategy on selected bacterial taxa. All included bacterial phyla, with Proteobacteria divided into class, were significantly different between sites ($P < 0.05$). Bacteroidetes was the only phyla that exhibited a site $\times$ cultivar interaction ($P = 0.01$). CIR indicates the Cave-in-Rock cultivar and Shaw indicates the Shawnee cultivar.

Figure 5: The effect of reclamation strategy on selected fungal taxa. Listed fungal classes were significantly different between sites ($P < 0.05$). Eurotiomycetes was the only phyla that exhibited a site $\times$ cultivar interaction ($P = 0.01$). CIR indicates the Cave-in-Rock cultivar and Shaw indicates the Shawnee cultivar.

Figure 6: The effect of reclamation technique on the relative abundance of C-, N-, and P- metabolism associated genes through KEGG Orthology level 3. (A) The relative abundance of starch and sucrose metabolism-associated genes [PATH:ko00500]. (B) The relative abundance of nitrogen metabolism-associated genes [PATH:ko00910]. (C) The relative abundance of phosphonate and phosphinate metabolism-associated genes [PATH:ko00440]. Different capital and lowercase letters indicate statistically significant and marginally significant differences in means, respectively. Hits were normalized to total predicted proteins per sample and visualized as hits per 1,000 sequences.

Figure 7: The effect of reclamation strategy on the relative abundances of C-, N-, and P- acquisition genes. Values are expressed as percent change, where positive values indicate a greater relative gene abundance at Hampshire and a negative value indicates greater relative gene abundance at Hobet. * indicates significance at $P < 0.05$. Substrate classes with a superscript 1 indicate nucleotide-curated functional gene databases instead of amino acid-curated databases.

Figure 8: Extracellular enzyme activity responses of β -glucosidase, N-acetyl-glucosaminidase (NAGase), and acid phosphatase (ACP) across switchgrass cultivars and soil types. Values expressed as mean $\pm$ standard error ($n=3$) with eight analytical replicates. Different letters indicate significant differences in means at $P < 0.05$. ^ indicates marginal differences across soil types at $P < 0.1$. Orange boxes indicate bulk soil and gray boxes indicate root-associated soil.

Figure 9: The effect of reclamation strategy on the (A) bacterial and (B) fungal richness of the switchgrass microbiome, as measured by Chao1 index. Values expressed as mean $\pm$ standard error (n=3). Different letters indicate significant differences in means at $P < 0.05$. Orange boxes indicate bulk soil and gray boxes indicate root-associated soil.

Figure 10: The effect of reclamation strategy on the (A) bacterial and (B) fungal community composition of the switchgrass microbiome. A principal coordinates analysis was conducted using the Bray-Curtis dissimilarity metric on OTU relative abundances.

Figure 11: The effect of reclamation strategy on the relative abundance of bacterial taxa. Stacked bar charts include bacterial phyla, with Proteobacteria divided into class, and stacked from most abundant to least abundant. Statistical results using a 3-way ANOVA for each bacterial phylum are detailed in Table 4.

Figure 12: The effect of reclamation strategy on the relative abundance of fungal taxa. Stacked bar charts include fungal classes, stacked from most abundant to least abundant. Statistical results using a 3-way ANOVA for each fungal phylum are detailed in Table 5.

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