Determining Microbial Bioindicators of Phosphorus Limitation in an Eastern Deciduous Forest

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ABSTRACT

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Determining Microbial Bioindicators of Phosphorus Limitation in an Eastern Deciduous Forest

Nitrogen deposition has increased the nitrogen content in soils in the unglaciated Allegheny plateau maybe to the point of saturation, which can limit the availability of phosphorus in the acidic, low-phosphorus soils of this region. The soil microbial community plays a critical role in increasing phosphorus availability through the secretion of phosphorus-acquiring extracellular enzymes to "mine" for organic phosphorus. However, there is a lack of fine-scale data on the community members that are directly involved in the mining of phosphorus, *e.g.* the microbial bioindicators of low-phosphorus soils. In this study, I used data generated in 2014 from the pyrosequencing of the soil microbial community present in an Eastern deciduous forest to determine if there are any observable microbial bioindicators of low-phosphorus availability. Conversely, if there are no candidate bioindicators, I hypothesized that phosphorus mining is a common trait among the soil microbial community. To test these hypotheses, fungal and bacterial DNA was extracted from a long-term research site in the unglaciated Allegheny Plateau. Since 2009, naturally acidic, low-phosphorus soils were fertilized with a phosphate fertilizer to directly increase phosphorus availability and lime to raise pH and indirectly increase phosphorus availability. Metagenomic community analysis revealed several candidate bioindicators of low-phosphorus availability and potential miners of recalcitrant phosphorus, as well as candidate bioindicators of phosphorus abundance.

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INTRODUCTION

The soil microbial community is a critical component of forested ecosystems and is largely responsible for the cycling of carbon, nitrogen, and phosphorus (Baldrian et al., 2012). Certain microbial guilds may specialize in obtaining different resources, and these guilds may act as the drivers of various nutrient cycles, mediating nutrient limitations, and increasing nutrient availability to plants (Baldrian et al., 2012). Determining the link between nutrient limitation and microbial response is essential for understanding the bottom-up control of ecosystems by the soil microbial community (Baldrian, 2017; Moorhead and Sinsabaugh, 2006). In this study, I focus on the members of the microbial community that mitigate phosphorus limitations in an Eastern deciduous forest with acidic, low-phosphorus soils.

Although nitrogen is traditionally considered the most limiting nutrient in many ecosystems (Elser et al., 2007), the use of fossil fuels and fertilizers have increased nitrogen inputs in forests in Eastern North America, which as shifted the balance of nutrients in in these ecosystems towards nitrogen saturation (Galloway et al., 2008; Lovett and Goodale, 2011). As nitrogen reaches saturation, the availability of phosphorus in the ecosystem becomes the limiting nutrient, and its availability controls ecosystem function (Davidson and Howarth, 2007). Acid deposition is associated with nitrogen saturation and has the potential to reduce phosphorus availability when elements like aluminum more readily bind with phosphorus as pH drops, making the phosphorus unavailable for uptake by plants (Deforest and Scott, 2010; Kishore et al., 2015; Rousk et al., 2010). Unlike nitrogen, when phosphorus leaches out of an ecosystem, there are very

few ways to renew the supply (Walker and Syers, 1976). In short, the highly weathered, acidic soils common to Eastern deciduous forests tend to be naturally limited by phosphorus availability, and the addition of nitrogen and increased acidity further reduces the amount of phosphorus available to plants and microbes (Güsewell, 2004; Vitousek et al., 2010).

The soil microbial community responds to and regulates phosphorus availability through the secretion of extracellular enzymes (Burns et al., 2013). Inorganic phosphorus, the kind of phosphorus that is readily available for uptake by the plant and microbial communities, easily binds to organic molecules becomes largely unavailable for life. Generally speaking, this recalcitrant pool of organic phosphorus can come in two forms. One is easily broken down by extracellular enzymes through a process called solubilization, and another, larger pool that is far more recalcitrant. This more recalcitrant pool can be accessed, or "mined", through the secretion of extracellular enzymes by the microbial community. Under conditions of phosphorus limitation, potentially specialized members of the soil microbial community can excrete extracellular enzymes to obtain phosphorus from this larger, more recalcitrant pool (Johnson et al., 2003; Turner, 2008). Here, these organisms are termed the phosphorus "miners". Although some plants can produce required extracellular phosphorus-acquiring enzymes from their roots (Asmar, 1997; Lung and Lim, 2006), the microorganisms that can access the more recalcitrant pool are considered the primary miners because plant roots have a limited capacity to explore soil aggregates that are not physically available to plants (Rodríguez and Fraga, 1999; Wiseman and Wells, 2005).

It has been observed that the addition of phosphorus suppresses the production of phosphorus-acquiring enzymes as these are no longer necessary to ensure availability of the nutrient (DeForest et al., 2012; Ragot et al., 2015; Turner, 2008; Turner and Blackwell, 2013). This effect is especially noticeable in soils that naturally have a limited amount of phosphorus available to plants and microbes. These naturally low-phosphorus soils include those that are acidic or highly weathered, such as unglaciated regions in the U.S.

However, there is still the question of which microbes are most affected by changes in nutrient availability and the role these particular members play in mitigating phosphorus limitation (Baldrian, 2017; Escobar-Zepeda et al., 2015). Advances in metagenomics allow for detailed observation of microbial communities. Researchers use biomarkers to selectively amplify DNA of communities and bioinformatics to detect members of that community (Escobar-Zepeda et al., 2015). The Internally Transcribed Spacer Region (ITS) is a commonly used biomarker for metagenomic analysis of the fungal community. This segment of ribosomal DNA has both conserved and variable regions that allow, in many cases, for the detection of individual genera or species by amplification and DNA sequencing (White, 1990). The 16S rDNA region is a commonly used biomarker for bacterial community analysis, and similar to the ITS region in the fungal kingdom, its conserved and variable regions allow for fine-scale detection of bacterial community members (Weisburg et al., 1991). The sequencing platform used in this study, Roche 454 pyrosequencing, works by adding nucleotides in solution to a DNA sample. If the ‘correct’ nucleotide in the solution attaches to the DNA in the sample, a

pyrophosphate molecule is released, and a light is emitted. A sensor reads the light and its intensity and translates that information into one nucleotide base of a DNA sequence (Ronaghi, 2001). Researchers have used pyrosequencing has been used in many previous studies to measure changes in community composition with treatment, sometimes in conjunction with more traditional molecular techniques such as cloning and quantitative PCR (Escobar-Zepeda et al., 2015).

Some previous studies have shown that phosphorus “miners”, those that can access the large, recalcitrant pool of soil phosphorus, include members bacterial phyla Acidobacteria and Actinobacteria (Bergkemper et al., 2016). These bacterial taxa may be bioindicators of acidic soil conditions (Bergkemper et al., 2015, 2016; Jenkins et al., 2009). The genomes many members of these phyla contain genes related to phosphorus-acquisition in acidic, low-phosphorus soils (Bergkemper et al., 2015; Kielak et al., 2016).

Phosphorus solubilizers, the microorganisms that are able to access the smaller and less recalcitrant pool of phosphorus in soils, tend to be members of the orders Pseudomonodales, Rhizobiales, and Bacilliales (Rodríguez and Fraga, 1999). Although these taxa may indicate the cycling of phosphorus through the solubilization of the less recalcitrant phosphorus pool, their presence is typically indicative of soils with a circumneutral pH and a potentially greater amount of phosphorus. Many bacterial taxa in soils live in close association with their plant host, as a rhizosphere species or as a endophyte inside the plant cell, and the metabolic needs of one partner affect the metabolic needs of the other (Medo et al., 2017; Santoyo et al., 2016; Soussi et al., 2016).

Ectomycorrhizal fungi play a role in carbon and nutrient cycling in forest ecosystems (Baldrian, 2017; Courty et al., 2010). These fungi form a symbiotic association with plant roots by entering the root and inhabiting the intracellular space. Ectomycorrhizal fungi aid in the uptake of phosphorus and nitrogen in exchange for carbon, which the plant exudes from its roots (Baldrian, 2017) and tend to tolerate a wider range of pH than bacteria, making them ubiquitous in acidic forest soils (Rousk et al., 2010). In many studies, abundant ectomycorrhizal taxa include Russulales, Sebacinales, Agaricales, Thelephorales, and Atheliales (Bueé et al., 2007; Carrino-Kyker et al., 2016; Kluber et al., 2012; Phillips et al., 2012).

Fungi that exhibit saprotrophic, or decomposer activity, are also common in forests, although there is not much information on saprotrophic contribution to the phosphorus cycle; most of the research on these organisms is conducted on their ability to breakdown organic matter as part of the cycling of carbon or nitrogen (Zak et al., 2011) or their role as plant pathogens (Hibbett et al., 2011; Ohm et al., 2012). Saprotrophs are directly involved in decomposition of organic matter and do not form an association with a host (Hodge, 2006). Members of the phyla Sordariomycetes, Leotiomycetes, and Dothideomycetes are examples fungal saprotrophs commonly found in forest soils (Freedman et al., 2015; Kellner et al., 2010).

Understanding microbial community response and mitigation on the ecosystem level is critical to understanding the consequences of chemical changes within the environment because these changes can alter the composition and function of the microbial community (Crawford et al., 2012; Freedman et al., 2015). In this study, I aim

to determine the microbial bioindicators of phosphorus limitation in soils and miners of phosphorus in low-phosphorus soils, and to observe changes in the microbial community with the experimental elevation of phosphorus. If there is no observable bioindicator of low phosphorus or community shift with treatment, then it can be assumed that the phosphorus mining is ubiquitous among the members of the soil microbial community.

I tested these hypotheses using a long-term research site on the unglaciated Allegheny Plateau. Study sites were established in 2009 on naturally acidic, low-phosphorus soils in an Eastern deciduous forest. The plots have been fertilized each year with phosphorus (Triple Super Phosphate, TSP) to directly increase phosphorus availability and with lime to raise pH to decrease exchangeable aluminum and thereby indirectly increasing phosphorus availability. These results will act as preliminary data for additional “genes-to-ecosystem” exploration in this ecosystem and will help fill in the gaps in the field’s knowledge of phosphorus mining by the microbial community in Eastern deciduous forests.

MATERIALS & METHODS

Site Design & Sampling Procedures

To observe the effects of phosphorus availability and pH on the microbial community, I used a long-term field manipulation experiment in a mixed mesophytic deciduous forest on the unglaciated Allegheny plateau near Athens, Ohio. The study site is comprised of three forests with three distinct soil types: an Alfisol (developed and weathered), an Inceptisol (undeveloped, but weakly weathered), and an Ultisol (well developed and heavily weathered). These soil types represent soil composition typical of the unglaciated Allegheny Plateau. The average annual temperature near these sites is 10.7 ˚C, and the average annual precipitation is 100 cm, and the sites are dominated by *Quercus spp. (60%)*, *Acer spp. (13%)*, and *Fagus grandifolia (8%)* (DeForest et al., 2012).

In 2009, 36 plots were established in a complete randomized block design. The plots are 20m by 40m and spaced at least 10 meters apart within a replication unit. Each of the three forest sites (i.e. blocks) had three replicates the four treatments - ambient soil conditions (as control), elevated phosphorus (with the addition of Triple Super Phosphate, TSP), elevated pH (with the addition of lime), and elevated phosphorus + pH (DeForest et al., 2012) (*Figure 1*). TSP was applied yearly, and at the time of sampling, the plots had received about 9.85 kg P/m^2. Lime was added to reach the desired pH of 6.5. This treatment also served to decreased exchangeable aluminum, which increased the amount of available phosphorus (*Table 1*).

In the summer of 2014, 12 soil cores were haphazardly sampled from each plot using a 2 cm soil core to a depth of 5 cm. This sampling method provided a composite sample for each plot which was later subsampled for DNA extraction, soil chemistry, and enzyme analysis. All samples were labeled, placed in a cooler with ice packs for transport, and homogenized through a 2mm sieve within 8 hours of sampling. After homogenization, subsamples for DNA extraction was stored at -80˚C until the time of the extraction. All other samples were stored overnight at 4˚C for the next day's enzyme analyses and subsequently sampled for soil chemical analysis.

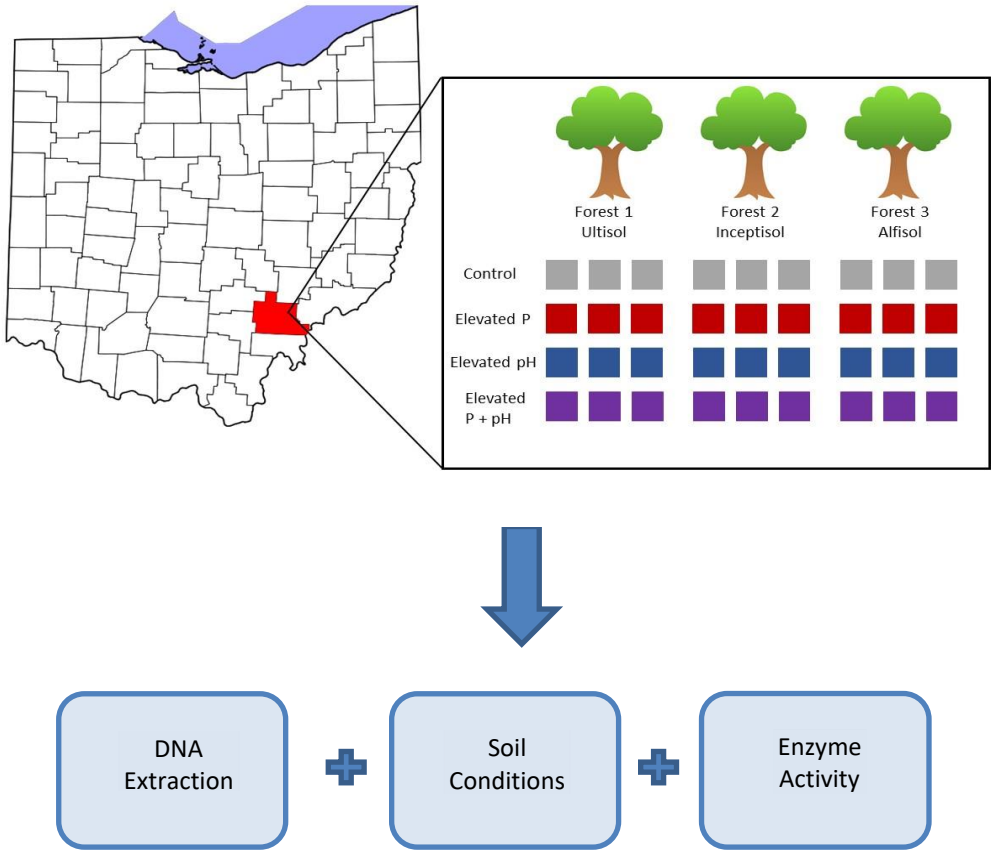


Figure 1. Experimental Design. Technical replicates of each treatment: control (ambient soil conditions), phosphorus elevation with the addition of fertilizer, pH elevation with the addition of lime, and phosphorus + pH elevation) are dispersed across three forests with diverse soil types near Athens, OH. Composite samples were taken in Summer 2014 for DNA extraction and molecular analyses, analysis of soil conditions, and extracellular enzyme analyses. Sites were established in 2009 and have been maintained since.

Table 1

Soil Chemical Characteristics

	Available P (mg P/kg)	Carbon (g C/ kg)	Nitrogen (g N/kg)
Control	1.0 ± 0.01	30.4 ± 0.33	1.7 ± 0.01
Elevated P	13.0± 0.28*	31.7 ± 0.10	2.0 ± 0.00
Elevated pH	1.3 ± 0.06	35.4 ± 0.04*	2.0 ± 0.00
Elevated P+pH	4.4 ± 0.27	32.6 ± 0.10	1.8 ± 0.00

	Exchangeable Aluminum ($cmol_c/kg$)	pH
Control	4.6 ± 0.08	4.7 ± 0.02
Elevated P	0.30 ± 0.05	5.1 ± 0.02
Elevated pH	3.0 ± 0.00	6.6 ± 0.03
Elevated P+pH	0.30 ± 0.05	6.5 ± 0.04

Table1. Soil chemical characteristics measured in Summer of 2014.

Soil Chemistry & Enzyme Activity

Because phosphorus binds easily to soil fractions after disturbance (Turner et al., 2005), available phosphorus was measured within eight hours of sieving using anion exchange membranes (AEMs). AEMs were placed in a 0.5 M solution of sodium bicarbonate and agitated to charge (Shaw and Deforest, 2013). AEMs were then rinsed with DI water, placed in a polyethylene centrifuge tube with a 3:1 slurry of DI water and soil, and shook for 4 hours. AEMs are then removed from solution, rinsed with deionized water to remove debris, and shaken with 0.5 M hydrochloric acid for 18 hours. Per Shaw and DeForest (2013), I used the Murphy-Riley ascorbic acid method to analyze the hydrochloric acid extract for available phosphorus (Murphy and Riley, 1962). Absorbance was measured on microplate reader at 880 nm. Soil pH was measured in a 1:2 dilution of deionized water within 24 hours of sampling, and exchangeable aluminum, which represents exchangeable acidity, through titration with potassium fluoride (DeForest et al., 2012). Total soil carbon and nitrogen were determined using an ECS 4010 CHNSO elemental analyzer (DeForest et al., 2012).

In 2014, researchers measured the activities of phosphomonoesterase, phosphodiesterase, β-glucosidase and xylosidase, leucine aminopeptidase, and N-acetyl glucosaminidase (NAGase). Phosphomonoesterase and phosphodiesterase acquire phosphorus in soils by depolymerizing organic phosphate sugars and phospholipids, respectively (Turner, 2008). To acquire nitrogen, leucine aminopeptidase hydrolyzes amino acids from polypeptides and NAGase catalyzes the hydrolysis of chitin (Sinsabaugh and Shah, 2012). Finally, β-glucosidase and xylosidase degrade cellulose

into monomers that can be assimilated by the microbial community (Bosetto and Justo, 2016; Dan et al., 2000). Due to the predictable activities of soil enzymes on substrates, it is common to use the activities of one or two enzymes as a metric for the activity of all extracellular enzymes that work on a substrate (Moorhead et al., 2013). Therefore, the activities of phosphomonoesterase and phosphodiesterase were used as a metric of phosphorus-acquiring enzymes; β-glucosidase and xylosidase were used as a metric for carbon-acquiring enzyme activity, and NAGase and leucine aminopeptidase activities were used as a metric for all nitrogen-acquiring enzyme activates. To simplify analyses, enzyme activities of each type were summed and will be referred to as "carbon-acquiring" (β-glucosidase + xylosidase), "nitrogen-acquiring" (leucine Aminopeptidase + NAGase), and "phosphorus-acquiring" (phosphodiesterase + phosphomonoesterase).

To measure extracellular enzyme activities of the soil microbial community, one gram of soil was mixed with 50 mM sodium acetate trihydrate buffer (pH 5.0). Fluorogenic substrates were prepared prior to analysis and stored at 4˚C. The substrates bind to the enzyme of choice and fluoresce when the enzyme is active. The substrates include 4-methylumelliferone (MUB), (4-MUB) phosphate (binds to phosphomonoesterase), bis-(4-MUB) phosphate (binds to phosphodiesterase, L-leucine 7 AMC (binds to leucine aminopeptidase), 4-MUB- N-acetyl-β-D-glucosaminide (binds NAGase), 4-MUB- β-D-glucopyranoside (binds β-glucosidase), and 4-MUB- β-D-cellobiose (binds to xylosidase) (DeForest et al., 2012). Samples were prepared and dispensed into black polystyrene 300 µl 96-well microplates (Whatman Inc., Florham Park, NJ). with blanks (sodium acetate buffer), reference standards (MUB + Buffer),

sample controls (soil slurry + buffer), quench (MUB + soil slurry), assay (soil slurry + substrate), and negative controls (substrate + buffer) in each plate. Because soil conditions can affect enzyme activity, each plate was measured four times to provide a more robust measure of enzyme activity than a single measurement (Shaw and Deforest, 2013). Enzyme activities were calculated by the slope of the product formed at each of the four time points at 450 nm emission using a microplate fluorometer (Syngery HT, BioTek, Winooksi, VT, USA) (DeForest et al., 2012). Enzyme activity is expressed as nM g^{-1} soil h^{-1}.

DNA Extraction & Amplification

To evaluate community composition in soil samples, DNA was extracted from the microbial community and ribosomal biomarkers were amplified by PCR prior to high-throughput pyrosequencing. The biomarkers used in this study were the 16S gene for bacteria and the ITS gene for fungi. For each sample, two analytical replicates of 250 mg soil were used for DNA extraction with the MoBio PowerSoil DNA extraction kit per Feinstein et al 2009). Briefly, manufacturer's instructions were modified to increase yield, and regions of the ITS and the 16S genes were then separately amplified by PCR using tagged primers already used in the Blackwood lab at Kent State University. A ~ 400 base pair region was selected from the ITS region using primers ITS1F (5'- CTT GGT CAT TTA GAG GAA GTA A – 3') and ITS2 (5' – GCT GCG TTC TTC ATC ATC GAT GC – 3'), and a ~600 base pair region was amplified from the 16S rDNA gene using primers 530F (5' – GTG CCA GCM GCN GCG G – 3') and 1100R (5' – GGG TTN CGN TCG TTG – 3') (Acosta-Martínez et al., 2008; Jumpponen and Jones, 2010).

This large-scale sequencing run was followed by a smaller sequencing run on the GS Junior system at Kent State University to correct any uneven sampling common to large-scale pyrosequencing

Bioinformatics & Statistical Analysis

I imported sequence data from the fungal community into Qiime1 software (Caparoso et al 2010) to be demultiplexed. The demultiplexed sequences were imported into Qiime2 and were dereplicated using the software VSEARCH. Closed-reference OTU clustering to 97% with the UNITE database (version 7) was performed using VSEARCH (Kõljalg et al., 2013; Rognes et al., 2016). De novo chimera filtering was performed in VSEARCH. Sequences were then filtered for singletons and doubletons (Bokulich et al., 2013), and taxonomy was assigned using the UNITE database (Appendix 1)(Kõljalg et al., 2013). OTU tables and taxonomy were then exported for Nonmetric Multidimensional Scaling analyses in the Vegan package in R (version 3.5.0), after within-sample normalization (Mcmurdie and Holmes 2014; Oksanen et al. 2018; R Core Team 2013). Bray-Curtis distances were used because the solution provided the least amount of stress; this arrangement of data ensured that the software put forth the least amount of effort to connect data points on a non-dimensional plane, placing statistically similar points close together. Pearson's correlations were then performed to determine the relationships between fungal taxa and environmental variables and extracellular enzyme activity.

Data from 16S sequencing was demultiplexed at the sequencing facility, and the reads were imported directly into Qiime2 using the Cassava 1.8 pipeline. The paired ends

were joined in VSEARCH (Rognes et al 2016). The samples were then dereplicated and de novo OTU clustering to 97% was performed in VSEARCH. Chimeras were filtered out as well as singletons and doubletons (Bokulich et al., 2013). A Naïve-Bayes classifier was trained to the SILVA 132 database at 97% similarity, and taxonomy was assigned to this database. The R package Phyloseq was used to perform Hellinger's transformation (Bienhold et al., 2011; Oksanen et al., 2018) (Appendix 2). Data was then exported for Non-metric Multidimensional Scaling in the Vegan package in R (version 3.5.0) using Bray-Curtis dissimilarity matrix (Bienhold et al., 2011; Mcmurdie and Holmes, 2014; Oksanen et al., 2018). Pearson's correlations were performed in R on orders that ordinated closely with significant ($P < 0.05$) environmental variables.

A linear mixed effects model was used in the nlme package in R (version 3.5.0) to determine if select taxa differed in relative abundance as well as enzyme activities between treatments (Pinheiro et al. 2018). I designated site as the random effect and treatment as the fixed effect for all analyses.

RESULTS

Soil Chemical Characteristics & Enzyme Activity

On average, adding lime raised soil pH from 4.7 in the native soils to about 6.5 in the elevated pH sites and decreased exchangeable aluminum (*Table 1*). As pH increases, the amount of exchangeable aluminum decreases; the variables are autocorrelated (*Figure 2*). Changes in carbon and available phosphorus with the elevation of pH or phosphorus were also observed (*Table 1*). The elevation of pH decreased the amount of exchangeable aluminum by 94%, increased the amount of available phosphorus by 20% and increased the total carbon content by 17%. The elevation of phosphorus decreased the amount of exchangeable aluminum by 36% and increased the amount of available phosphorus by 1146%. The elevation of phosphorus + pH decreased the amount of exchangeable aluminum by 94%, increased the amount of available phosphorus by 327%.

Both the elevation of phosphorus and pH suppressed the activity of phosphorus-acquiring enzymes and increased the activity of carbon-acquiring enzymes (*Table 2*). The elevation of pH increased carbon-acquiring enzyme activities by 91% and decreased phosphorus-acquiring enzyme activities by 34%. The elevation of phosphorus increased carbon-acquiring enzyme activities by 25% and decreased phosphorus-acquiring enzyme activities by 33%. The elevation of phosphorus + pH increased carbon-acquiring enzyme activities by 70% and decreased phosphorus-acquiring enzyme activities by 48%.

Relationship between pH and Exchangeable Aluminum

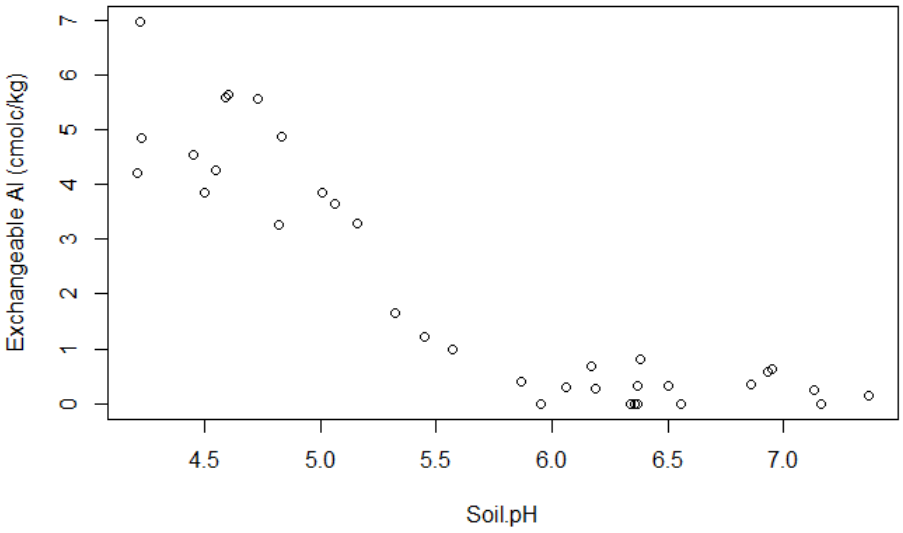


Figure 2. As soils become more acidic, exchangeable aluminum increases in concentration until a buffering point of about pH 4.2. This is a commonly observed phenomenon (DeForest & Scott, 2010; Kishore et al. 2015, Rousk et al 2010).

Table 2

Rate of Enzyme Activity

	Carbon-acquiring
Control	101.07
Elevated P	192.73
Elevated pH	126.58*
Elevated P + pH	171.86*
	Nitrogen-acquiring
Control	204.81
Elevated P	260.61
Elevated pH	246.07
Elevated P + pH	223.33
	Phosphorus-acquiring
Control	661.27
Elevated P	434.85*
Elevated pH	442.16*
Elevated P + pH	346.15*

Table 2. Rate of enzyme activity for the phosphorus-, nitrogen- , and carbon-acquiring enzymes measured in this study. Enzyme activity is expressed as nM g^{-1} soil h^{-1}.
* indicates significance ($P < 0.05$). To simplify analyses, enzyme activities of each type were summed and will be referred to as "carbon-acquiring" (β-glucosidase + xylosidase), "nitrogen-acquiring" (leucine Aminopeptidase + NAGase), and "phosphorus-acquiring" (phosphodiesterase + phosphomonoesterase).

Fungal Community Analysis

Analysis yielded 5,786 fungal OTUs from 171,565 reads. These were grouped into 196 taxa. On average, native soils are dominated by Russulales (27%), Thelephorales (21%), Agaricales (20%) and Sebacinales (10%). Overall, the elevation of pH appeared to have greater control on relative abundance of taxa than the elevation of phosphorus or the elevation of both, although many taxa, such as Thelephorales, were insensitive to the treatments (*Figure 3*). Most notably, the relative abundance of Russulales decreased significantly with the elevation of pH (-10%) ($P < 0.05$), a large significant drop in abundance that could have serious implications for ecosystem function. The relative abundance of Sebacinales increased significantly with the elevation of pH (+18%) ($P < 0.05$). The relative abundance of Agaricales tended to decrease with the elevation of pH (-5%) ($P < 0.1$). The relative abundance of Mytilinidales, 1% in native soils, increased significantly with the elevation phosphorus + pH (2%) ($P < 0.05$).

The soil chemical characteristics that controlled the composition of the fungal community are exchangeable aluminum and pH. These included orders Boletales, Capnodiales, Lecanorales, Onygenales, Thelebolales, and unidentified members of Eurotiomycetes, Sordariomycetes, Tremellomycetes and Dothideomycetes (*Figure 4*).

The relative abundance of several of these fungal orders significantly correlated with soil conditions that relate to native soils including low pH, high exchangeable aluminum, and high phosphorus acquiring enzyme activity ($P < 0.05$). The relative abundance of Agaricales had a negative relationship with pH ($r = -0.58$) and a positive relationship with exchangeable aluminum ($r = 0.54$). Russulales had a strong negative

relationship with pH (r = -0.71), a positive relationship with exchangeable aluminum (r = 0.68), and a positive relationship with phosphorus acquiring enzyme activities (r = 0.58). Sebacinales had a positive relationship with pH (r = 0.63), a negative relationship with exchangeable aluminum (r = -0.58).

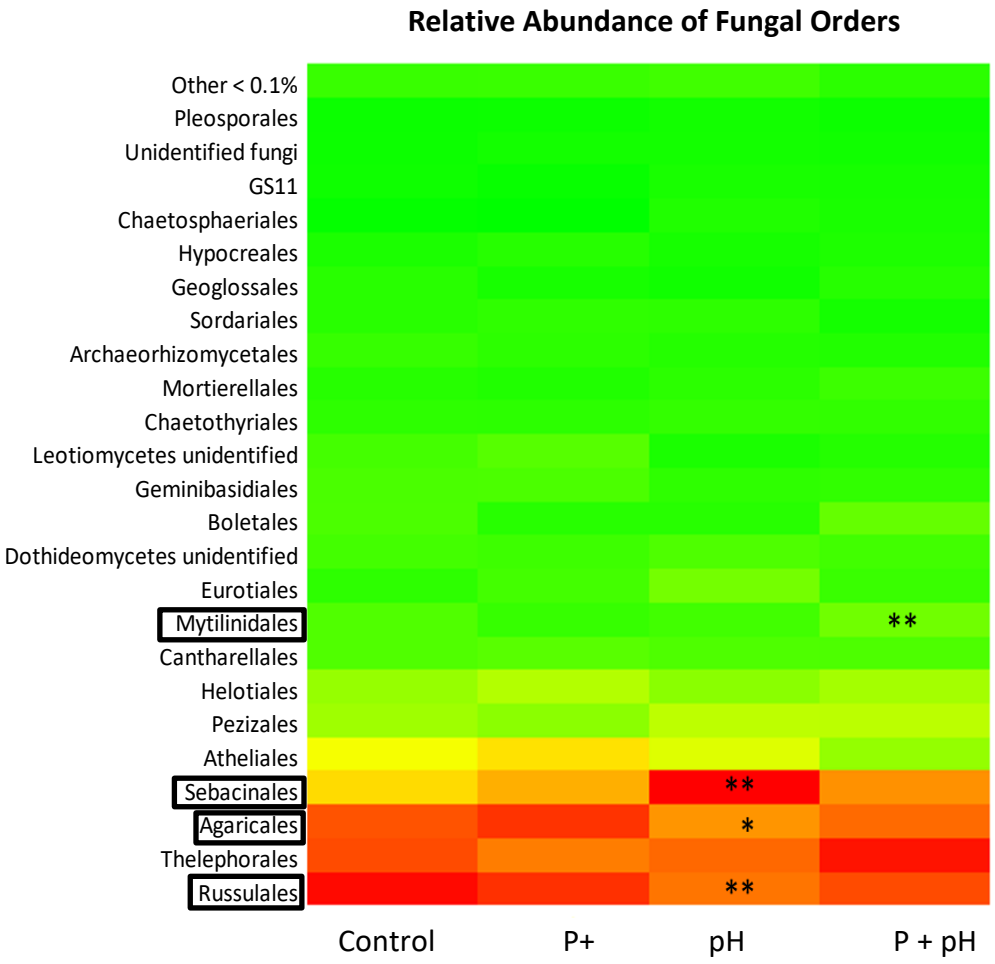


Figure 3. Fungal community composition by site. Green indicates low relative abundance, and red indicates high relative abundance. ** shows significant changes in relative abundance ($P < 0.05$). * indicates shows biologically significant changes ($P< 0.1$). Orders with a relative abundance of < 0.1% were summed and are included in this figure as "Other < 0.1%".

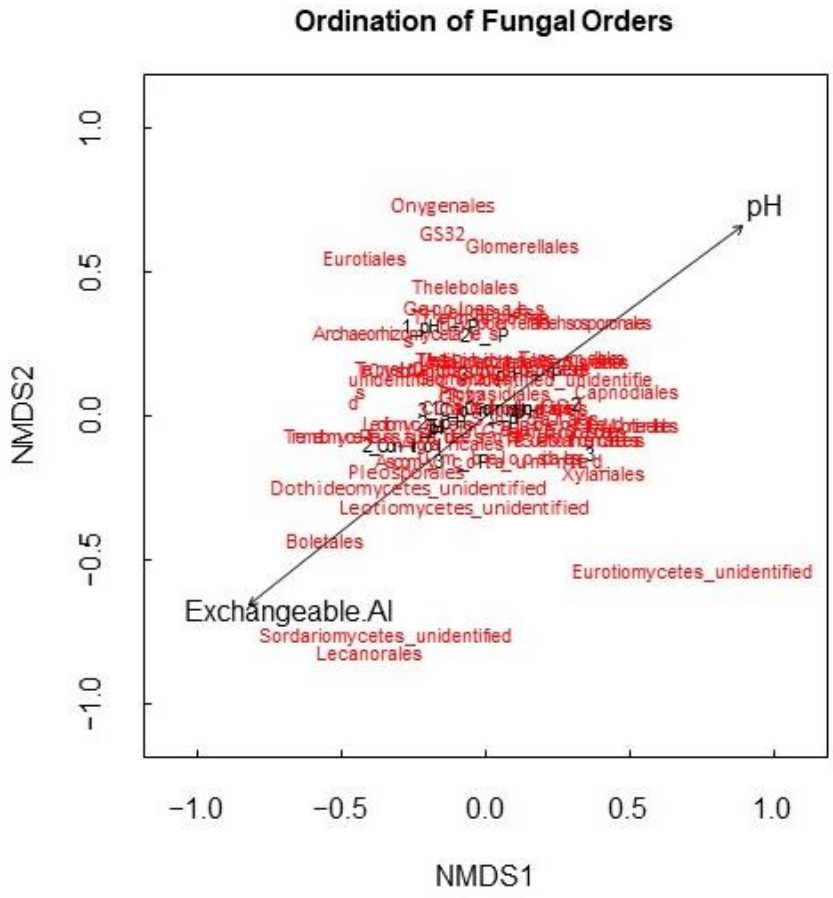
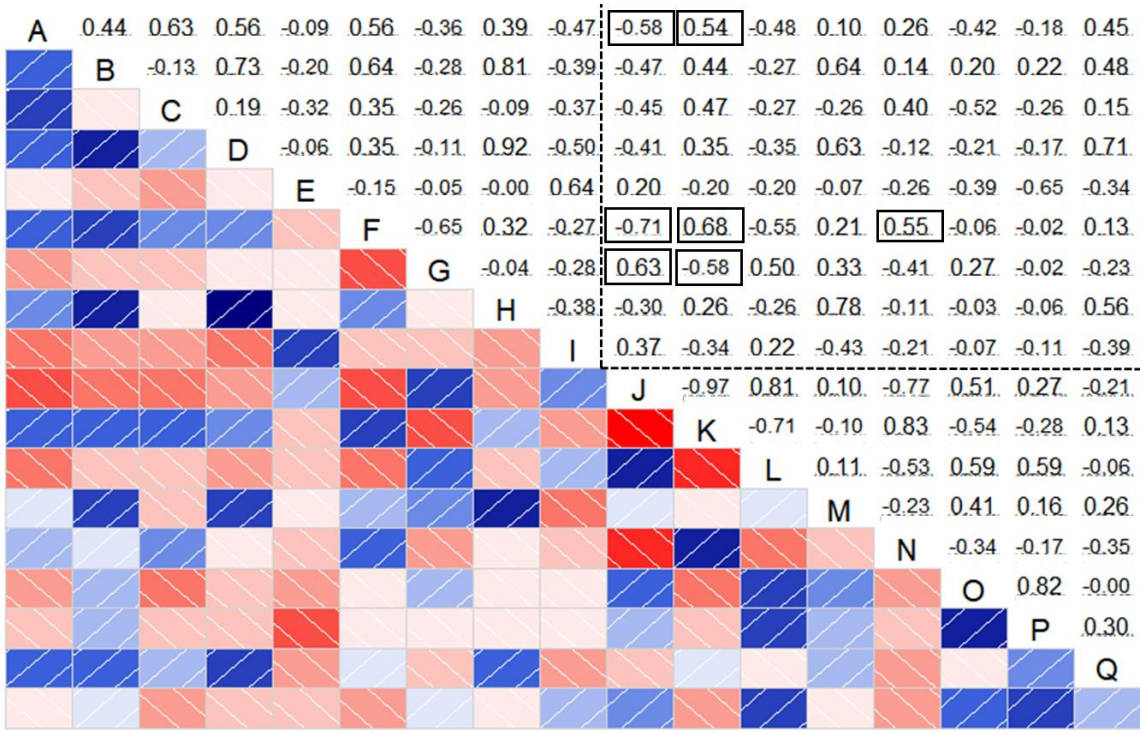


Figure 4. NMDS reveals that the fungal community ordinates with pH and exchangeable aluminum. In this system, pH is responsible for 54% of community diversity and exchangeable aluminum is responsible for 48% of community diversity. Bray-Curtis distances were used to minimize stress on the solution (0.123).

Correlations between Fungal Orders, Soil Chemistry, and Enzyme Activities



Order				Environmental			
A	Agaricales	F	Russulales	J	pH	O	Total C
B	Boletales	G	Sebacinales	K	Exchangeable Al	P	Total N
C	Dothideomycetes unidentified	H	Sordariomycetes unidentified	L	C-acquiring Enzyme Activity	Q	Available P
D	Lecanorales	I	Thelephorales	M	N-acquiring Enzyme Activity		
E	Mytilinidales			N	P-acquiring Enzyme Activity		

Figure 5. Correlogram of Pearson's correlations between fungal orders and soil conditions or enzyme activities. Red indicates a negative relationship, and blue indicates a positive relationship. Intensity of color indicates strength of correlation. Boxes around values indicate significant correlation between the fungal order (A – I) and the soil condition or enzyme activity (J – Q) ($P < 0.05$).

Bacterial Community Analysis

Molecular analysis of 16S rDNA yielded 875 OTUs from 6,710 reads. Two samples, one replicate from an elevated phosphorus site and one from native soil site, were discarded in upstream processing due to very low yield (one and two sequences per sample, respectively). Native soils were dominated by Acidobacteria, Subgroup 2 (17%), Acidobacteriales (16%), Solibacterales (10%), and Rhizobiales (6%). The relative abundance of Aciobacteria, Subgroup 2 decreased significantly with the elevation of phosphorus (-5%), elevation of pH (-11%), and the elevation of phosphorus + pH (-10%) ($P < 0.05$). The relative abundance of Acidobacteriales decreased significantly with the elevation of pH (-4%), the elevation of pH (-12%), and the elevation of phosphorus + pH (-11%) ($P < 0.05$). The relative abundance of Solibacterales decreased significantly with the elevation of phosphorus (-4%), the elevation of pH (-6%), and the elevation of phosphorus + pH (-7%) (*Figure 6*) (*$P < 0.05$)*.

Other rarer orders were affected by the elevation of phosphorus, pH or both. The order Chitinophagales had a relative abundance of 4% in native soils, and significantly increased in relative abundance with the elevation of phosphorus (+3%), pH (+11%), and phosphorus + pH (12%). Pseudomonodales was not present in native soils but tended to increase with the elevation of phosphorus + pH (0.3%) ($P < 0.1$). The relative abundance of Cytophagales increased significantly from 0.3% relative abundance in the native soils to 8% with the elevation of pH and the elevation of phosphorus + pH ($P < 0.05$). The relative abundance of uncultured Acidobacterium A increased significantly from 0% native soil to 1% with the elevation phosphorus and pH 0.4% (*Figure 6*).

The soil chemical characteristics that control the bacterial community composition are pH, carbon-acquiring enzyme activities, exchangeable aluminum, and phosphorus-acquiring enzyme activities. Orders whose relative abundance ordinated with the amount of exchangeable aluminum and phosphorus-acquiring enzyme activity include uncultured Acidobacteria A, Obscuribacterales, Solibacterales, and members of the order dubbed soil group S-BQ2-57 from the class *Verrucomicrobiae*. Taxa whose relative abundance ordinated with carbon-acquiring enzymes activities include Cellvibrionales, Nitrospirales, Rhodospirales, and Oceanospirales (*Figure 7*).

The relative abundance of several of these bacterial orders significantly correlated with soil conditions that relate to acidic, native soils: low pH, high exchangeable aluminum, and high phosphorus acquiring enzyme activity ($P < 0.05$). The relative abundance of both Acidobacteria, Subgroup 2 and Solibacterales had positive relationships with exchangeable aluminum (r = 0.93 and 0.75) and phosphorus acquiring enzyme activity (r = 0.72 and 0.82) and negative relationships with pH (r = -0.91 and -0.65, respectively). The relative abundance of Obscuribacterales had significant positive relationship with phosphatase and exchangeable aluminum and a negative relationship with pH. Cytophagales had a negative relationship with pH (r = -.75) and positive relationships with exchangeable aluminum and phosphorus-acquiring enzyme activity (r = 0.74 and 0.52, respectively). The relative abundance of Chitinophagales had a significant positive relationship with pH (r = 0.88) and a significant negative relationship with exchangeable aluminum and phosphorus-acquiring enzyme activities (r = -0.92 and 0.80, respectively) (*Figure 8*).

See *Table 3* for a summary of both fungal and bacterial candidate bioindicators of phosphorus limitation and abundance. The presence of candidate bioindicators of low-pH, low-phosphorus soils or the absence of candidate bioindicators for circumneutral, high-phosphorus soils indicate that the ecosystem is under conditions of phosphorus limitation. Candidates that exhibit phosphorus-acquiring abilities in low-phosphorus, acidic environments can be considered the “miners” of phosphorus in those ecosystems.

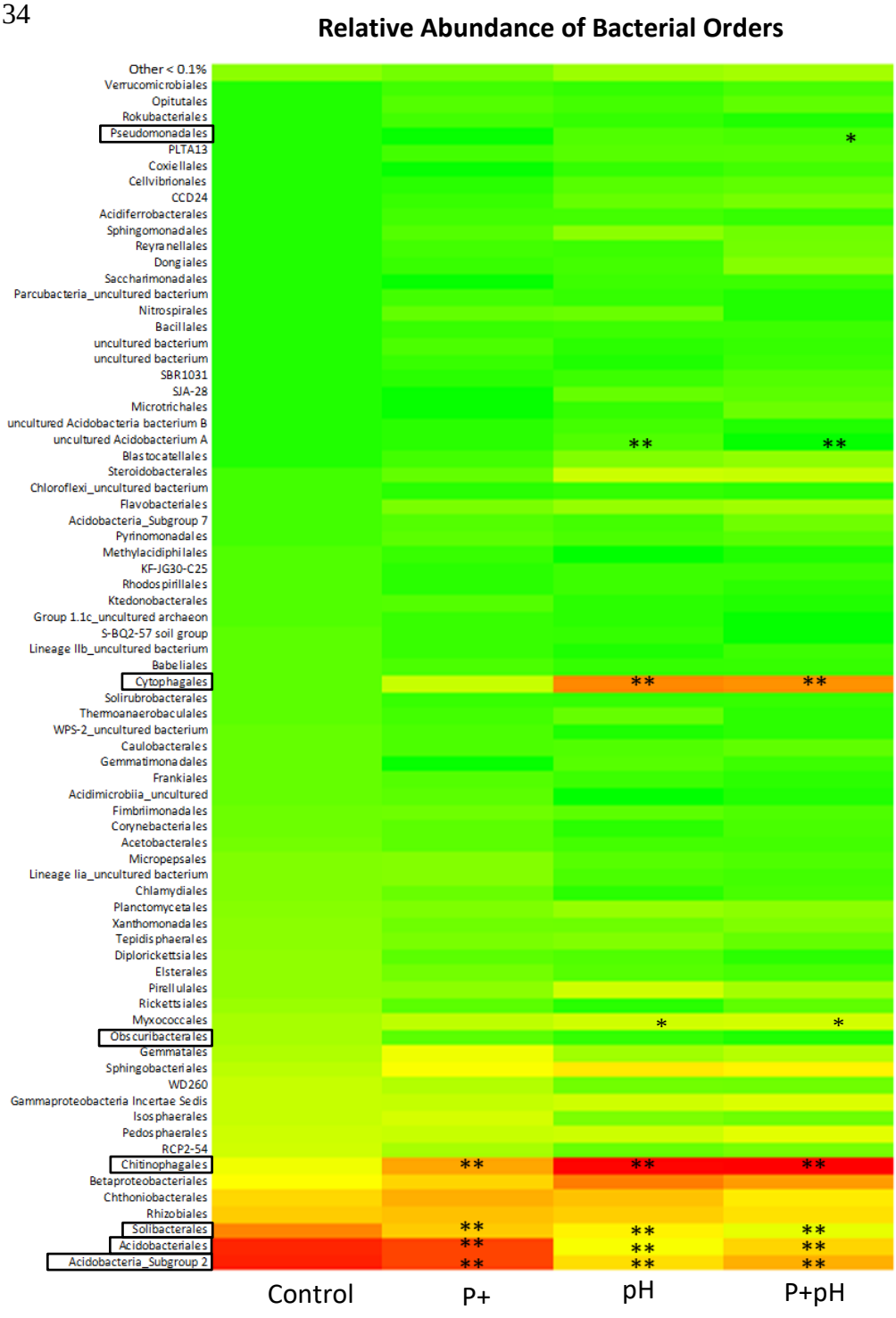


Figure 6. Bacterial community composition by site. Green indicates low relative abundance, and red indicates high relative abundance. ** shows significant changes in relative abundance ($P < 0.05$), and * shows changes in relative abundance significant at $P < 0.1$. All orders with a relative abundance of < 0.1% were summed and are referred to as "Other < 0.1%"

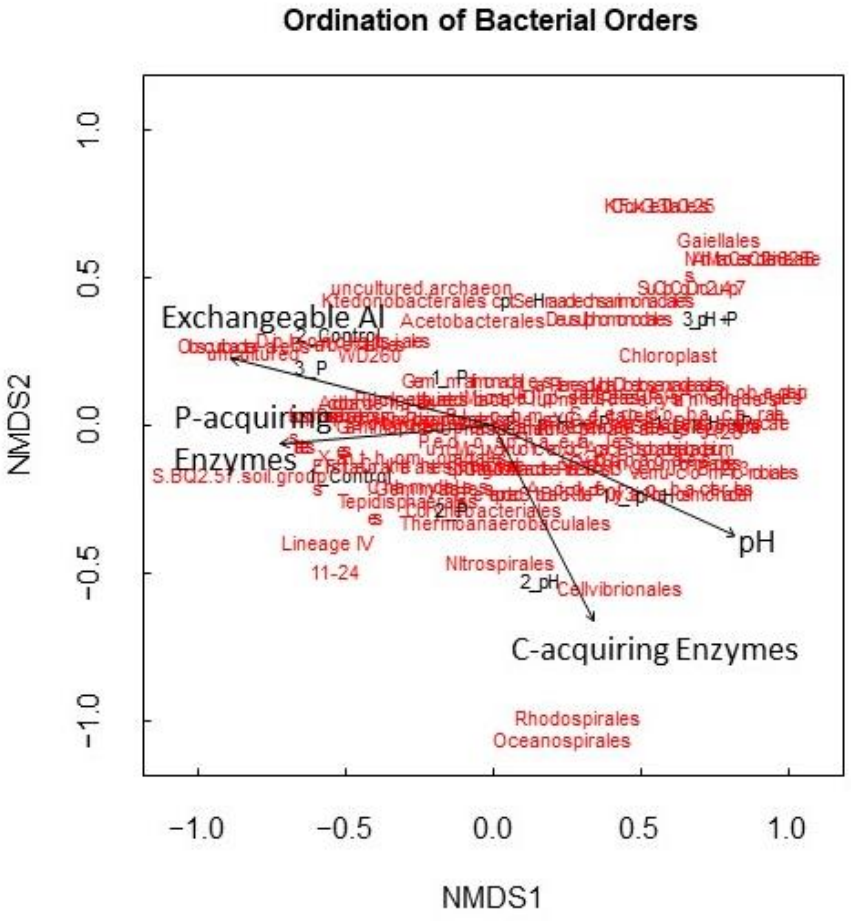
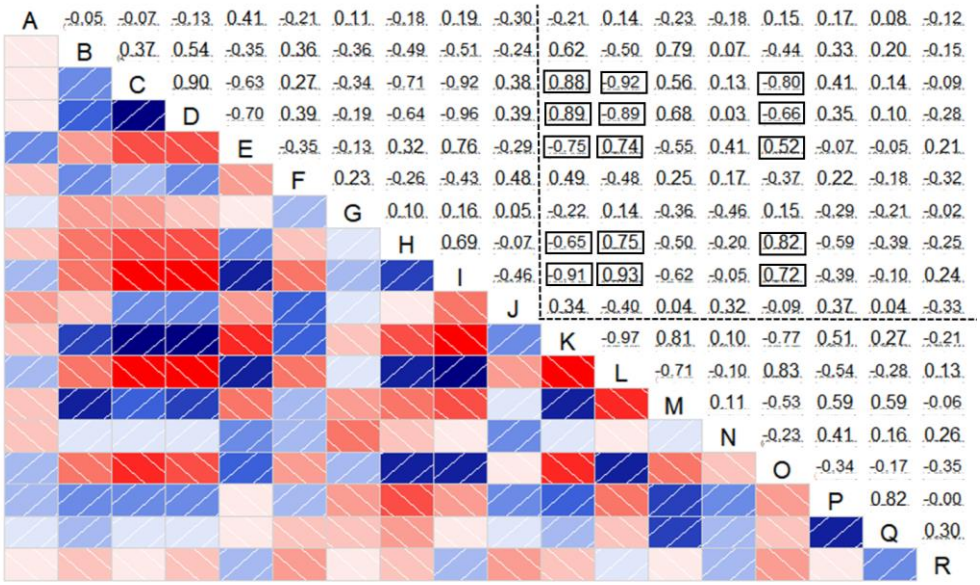


Figure 7. NMDS reveals the bacterial community ordinates with exchangeable aluminum (89%), pH (85%), carbon-acquiring enzyme activities (58%), and phosphorus-acquiring enzyme activity (55%). Bray Curtis distances were used to minimize the stress on the solution (0.105).

Correlations between Bacterial Orders, Soil Chemistry, and Enzyme Activities



	Order				Environmental		
A	Acidobacteriales	F	Pseudomonodales	K	pH	P	Total C
B	Cellvibrionales	G	Rhizobiales	L	Exchangeable Al	Q	Total N
C	Chitinophagales	H	Solibacteriales	M	C-acquiring Enzyme Activity	R	Available P
D	Cytophagales	I	Subgroup 2	N	N-acquiring Enzyme Activity		
E	Obscuribacterales	J	Uncultured Acidobacterium A	O	P-acquiring Enzyme Activity		

Figure 8. Correlogram of Pearson's correlations between bacterial orders and soil chemical conditions or enzyme activities. Red indicates a negative relationship, and blue indicates a positive relationship. Intensity of color indicates strength of correlation. Boxes around values indicate significant correlation between the bacterial order (A – J) and the soil chemical characteristic or enzyme activity (K – R)) ($P<$ 0.05).

Table 3

Candidate Bioindicators of Phosphorus Limitation and Abundance

Low pH	**Low phosphorus**
Russulales	Russulales
Agaricales	-
Acidobacteria, Subgroup 2	Acidobacteria, Subgroup 2
Acidobacteriales	Acidobacteriales
Obscuribacterales	-
Solibacterales	Solibacterales
Circumneutral pH	**Elevated Phosphorus**
Sebacinales	-
Mytilinidiales	Mytilinidiales
Uncultured Acidobacterium A	Uncultured Acidobacterium A
Cytophagales	-
Chitinophagles	Chitinophagles
Pseudomonodales	Pseudomonodales

Table 3. Candidate bioindicators of low-pH, low-phosphorus soils and candidate bioindicators of circumneutral-pH, high-phosphorus soils.

DISCUSSION & CONCLUSIONS

Soil Chemistry & Extracellular Enzyme Activity

The activities of extracellular enzymes can be roughly classified as nutrient acquiring (in this study, phosphorus- and nitrogen-acquiring) and energy acquiring (carbon-acquiring). It has been observed that as a nutrient becomes less biologically available the activity of associated extracellular enzymes increases (DeForest et al 2012). Conversely, as organic matter increases in soils, the activity of carbon-acquiring enzymes typically increases (Sinsabaugh et al., 2008). I observed the same trend in this study. The activities of phosphorus-acquiring enzymes significantly decreased with both the indirect and direct elevation of phosphorus (*Table 2*). There also is evidence that the treatments are increasing the growth of certain trees that contribute to belowground carbon through fine-root turnover, and an increase in total carbon was observed in the elevated pH sites (*Table 1*) (Dorkoski, 2016).

Fungal Community Analysis

Native soils were dominated by the orders Russulales (27%), Thelephorales (21%), Sebacinales (10%), and Agaricales (20%) *(Figure 3*), all of which are ectomycorrhizal fungi. The relative abundance of Russulales decreased significantly with the elevation of pH, indicating that members of this order can be considered candidate bioindicators of acidic, low-phosphorus soils (*Figure 3*). Kluber et al (2012) observed a similar trend in the relative abundance of Russulales in the same ecosystem, and this order is known to form associations with tree in acidic, low-phosphorus soils in tropical forests (Kettle, 2010). Courty et al. (2005), Maghnia et al. (2007), and Pritsch and

Garbaye (2011) have observed enzyme activities from the around a pH of 4.5, indicating that in general ectomycorrhizal fungi are more effective at obtaining nutrients at lower pH (Courty et al., 2005; Maghnia et al., 2017; Pritsch and Garbaye, 2011). In this study, Russulales was associated with characteristics of acidic soils and the activities of phosphorus-acquiring enzyme activities, implying its ability to mine phosphorus from recalcitrant sources in these low-phosphorus ecosystems.

Members of Russulales, like most ectomycorrhizal fungi, play an important role in obtaining phosphorus for plant hosts when nutrient availability is low, and this contributes to the cycling and storage of that nutrient within the ecosystem (Courty et al., 2010). A decrease in the abundance of a dominant order indicates that the plant host no longer needs to rely on its mycorrhizal association to obtain nutrients (Heijden et al. 2008). The effect of this compositional shift has been observed previously among saprotrophic fungi (Zak et al., 2011), and it can be reasonably assumed that a significant decrease in the relative abundance of the common ectomycorrhizal order Russulales marks a change in how the ecosystem processes phosphorus and others.

Similarly, the order Agaricales should be considered a bioindicator of acidic, low-phosphorus soils. Its relative abundance tended to decrease 14% with the elevation of phosphorus + pH. This order's relative abundance was positively associated with acidic soil conditions, such as decreasing pH and increasing exchangeable aluminum (*Figure 5*). A study by Carrino-Kyker et al (2016) found members from the order Agaricales were bioindicators of the low-pH, low-phosphorus soils that characterize this region. Much like Russulales, the relative absence of this order may indicate a substantial

change in ecosystem function with the elevation of phosphorus or pH. Unlike Russulales, Agaricales did not associate with phosphorus acquiring enzyme activity (*Figure 6*) and should not necessarily be considered a phosphorus miner.

Other fungi reacted more positively to these changes. The relative abundance of the order Sebacinales increased with the elevation of pH (28%) but did not change with the elevation of phosphorus or phosphorus + pH (*Figure 5*). This suggests that members of this order are sensitive to pH, but not to phosphorus; another experiment performed on these sites showed similar results (Carrino-Kyker et al., 2016). Another research group reported members of Sebacinales were observed to improve the uptake of phosphorus by *Dendrobium chrysanthum*, a species of orchid, through the secretion of phosphorus-acquiring enzymes (Hajong et al., 2013). It should be noted that this study was performed *in vitro*, at a pH of 5.8, which is roughly in the middle of the range of pH values measured in this study (*Table 1*).

Sebacinales, a highly diverse group, has been described elsewhere as containing members that can efficiently colonize soils after a disturbance (Weiß et al., 2016). In this study, Sebacinales may have filled a niche left open by the decrease of Russulales and Agaricales with the elevation of phosphorus or pH. Both Russulales and Agaricales are acid-tolerant, and their relative abundance decreased with the elevation of pH when their association is no longer necessary for host nutrient uptake (Heijden et al., 2008). If conditions are no longer favorable to an abundant group, another group may replace it by producing the necessary enzymes or by using a carbon source that was not by the original colonizers, or by physically moving into an area that is chemically favorable (Dickie et

al., 2002; Dumbrell et al., 2010; Pritsch and Garbaye, 2011). The latter has been observed in arbuscular mycorrhizal fungi (Dumbrell et al., 2010; Lekberg et al., 2007), and because the members of the order Sebacinales can form a variety of associations with plant hosts, it is possible that the diverse members of Sebacinales were allowed to flourish in the niche that opened with the decrease in relative abundance of Russulales and Agaricales (Weiß et al., 2016). The shift in community composition would then alter the ability of the ecosystem to cycle carbon and other nutrients, making Sebacinales an impactful group for soil function as well as a bioindicator of circumneutral soils (*Table 3*).

The saprotrophic fungal community also contributes to the cycling of nutrients and may contain members associated with the cycling of phosphorus as well. A well-described saprotrophic order, Capnodiales, ordinated towards exchangeable aluminum and had a positive relationship with carbon-acquiring enzyme activity (*Figure 4*, *Figure 5*), but was relatively rare in all treatments (< 0.1%) (*Figure 3*). This order is a common food crop pathogen, so it is not surprising that this order was found in low abundance in forest soils (Hibbett et al., 2011b; Ohm et al., 2012). A seldom-described Dothideomycetes order, Mytilinidales, was the eighth most abundant order in this ecosystem, ranging from 0.5% in the native soils to 1.7% in the phosphorus + pH sites. From an extensive literature search, it appears that little research has been conducted on this order beyond phylogenetic placement compared with other members of its class (Ohm et al., 2012; Ruggiero et al., 2015). Mytilinidales may serve as a bioindicator of soils with circumneutral pH and high nutrients as its relative abundance increases significantly with the elevation of phosphorus + pH (*Figure 3*).

Bacterial Community Analysis

Native soils were dominated by members of the phylum Acidobacteria. Members of this phylum are common in forest and agricultural soils, and have previously been found to contribute to phosphorus cycling in forest soils (Bergkemper et al., 2015; Janssen et al., 2002; Orwin et al., 2018; Smit et al., 2001). Acidobacteria is common in many soil types and is functionally diverse (Ward et al., 2009), and so it is not surprising that I found members of this phylum that could be considered candidate bioindicators of low phosphorus and other members that could be considered candidate bioindicators for increased phosphorus availability. Currently, research points to this group's ability to degrade cellulose, although the extracellular enzyme activities are unclear at more specific taxonomic levels, with some members of this phylum secreting high levels of carbon-acquiring enzymes and others not (Kielak et al., 2016).

The most abundant order in the native soils in this ecosystem, and a well-studied group within Acidobacteria, Aciobacteria Subgroup 2, (17%) decreased significantly with the elevation of phosphorus (-5%), the elevation of pH (-12%), and the elevation of phosphorus + pH (-10%) (*Figure 6*). Subgroup 2 also was associated with characteristics of acidic, low-nutrient forest soils, like decreased pH, high exchangeable aluminum, and phosphorus-acquiring enzyme activities (*Figure 8*). Therefore, Acidobacteria, Subgroup 2 should be a candidate bioindicator for low-pH, low-phosphorus soils, and may have the ability to mine recalcitrant phosphorus.

Two abundant orders in the phylum Acidobacteria, Acidobacteriales and Solibacterales, seemed to be negatively affected by the elevation of phosphorus, pH, and

both, and may be another bioindicator of low-phosphorus or acidic soils. Solibacterales is part of class *Acidobacterii*, Subgroup 1, flourishes in low-pH, low-nutrient soils (Kielak et al. 2016). The results of this study corroborated these findings as the relative abundance of this order decreased from 10% in the native sites to 3% in the elevated phosphorus + pH sites (*Figure 6*). The order is associated with phosphorus-acquiring enzyme activities (*Figure 8*), and other research groups have observed the expression of genes related to the acquisition and turnover of phosphorus in forest soils (Bergkemper et al., 2015, 2016; Newsham et al., 2010). Solibacterales is therefore another candidate bioindicator for acidic, low-phosphorus soils, and a potential miner of phosphorus in this ecosystem.

Acidobacteriales may also be a candidate bioindicator of acidic, low-phosphorus soils, but its ability to mine phosphorus is less certain. Like Solibacterales, Acidobacteriales experienced a significant decrease in relative abundance with the elevation of phosphorus (-4%), pH (-12%) and phosphorus+ pH (-11%) (*Figure 6*) but was not associated with phosphorus-acquiring enzyme activities (*Figure 8*). Although there is some evidence that this order may be found in acid forest soils, and that it may have some ability to acquire phosphorus in phosphorus limited conditions (Bergkemper et al., 2015, 2016; Kielak et al., 2016), I did not find evidence to suggest that this organism is capable of mining recalcitrant phosphorus.

An example of a functionally divergent, but ecologically relevant, member of Acidobacteria is the uncultured Acidobacterium A from Subgroup 22. This taxon may be a bioindicator of a higher pH environment, as it is relative abundance increases with the

elevation of pH (*Figure 6*). This uncultured order was associated with acidic soil conditions, such as decreasing pH and increasing exchangeable aluminum (*Figure 7*). Other studies have found that other members of Subgroup 22 appear to be more abundant in circumneutral pH, high- phosphorus soils (Pessoa-Filho et al., 2015)**.** The order uncultured Acidobacterium was not highly abundant (< 1%), which indicated that it may have had a disproportionate effect on the cycling of nutrients in circumneutral soils (Mikkelson et al., 2016).

The well-known phosphorus solubilizing taxa, Pseudomonodales and Rhizobiales, may be bioindicators of soils with some available phosphorus and a circumneutral pH and was only found at elevated pH and elevated phosphorus + pH sites. Other studies have shown that members of this order are effective phosphorus solubilizers in agricultural soils and laboratory experiments (Collavino et al., 2010; Rodríguez and Fraga, 1999), but because the relative abundance of Psuedomonodales is unaffected by the elevation of pH alone, I cannot say that I observed its ability to mineralize lowly available phosphorus. Another known phosphorus solubilizer, Rhizobiales, appeared insensitive to treatments. Rhizobiales was the fourth most abundant order, comprising 6% of the native soil community, but its relative abundance did not significantly change with the elevation of phosphorus or pH (*Figure 3*). However, other research groups have found that members of Rhizobiales contribute to phosphorus solubilization in agricultural soils and are commonly found in higher pH soils than those found in this study site (Rodríguez and Fraga, 1999; Tsukui et al., 2013), and so is somewhat surprising that the

relative abundance of Rhizobiales was not affected by the elevation of pH and phosphorus + pH in this ecosystem.

The endophytic bacterial order Obscuibacterales should be considered a candidate bioindicator for low-pH, low-phosphorus soils. The order makes up 2% of the native soil community and had a tendency to decrease in relative abundance with the elevation of pH (*Figure 6*, $P = 0.08$), and its relative abundance corresponded with acidic soil conditions (*Figure 7*). A non-photosynthetic member of Cyanobacteria, Obscuribacterales has been found more frequently than other orders of its phylum to express genes for phosphorus acquisition, and may therefore have the ability to mine phosphorus (Soo et al., 2014).

The relative abundance of another endophytic order, Cytophagales, increased significantly with the elevation of pH (8%) and elevation of phosphorus + pH (8%) when compared to native soils (0.2%), making it another candidate bioindicator for circumneutral, high nutrient soils. This endophytic order has been observed to grow in the roots of plants in the absence of a mycorrhizal association in high nutrient soils (Angel et al., 2016). Another potential explanation for the increase in these orders' relative abundances could be the significant decrease in abundant, acid-tolerant orders, such as members of Acidobacteria, with the elevation of phosphorus and pH (*Figure 8*).

Endophtyic colonization has been shown to increase with pH, potentially explaining the increase in the relative abundances of Obscuribacterales and Cytophagales (Rondon et al., 2000). It is important to note that although both Obscuribacterales and Cytophagales are endophytic orders, they indicate differing environmental characteristics, implying that their function in the community may be determined by their hosts' differing

metabolic needs. The effect of the host's metabolic needs on the endophyte has been observed in laboratory and agricultural studies settings, although not in forest settings or with phosphorus availability (Medo et al., 2017; Zgadzaj et al., 2016).

The relative abundance of Chitinophagales also increased from the elevation of phosphorus and pH and may be considered a candidate bioindicator of high-phosphorus soils with circumneutral pH. Its relative abundance increased with the elevation of pH (15.4%) and the elevation of phosphorus + pH (16.3%) when compared with native soils (3.7%), which may be explained by the slight increase in nitrogen with this treatment, as well as an increase in phosphorus availability and an increase in pH. Like Cytophagales, Chitinophagales may have replaced abundant, acid-tolerant organisms like Acidobacteriales and Solibacterales in elevated pH conditions. Members of this order were shown to be negatively correlated with exchangeable aluminum in another study, emphasizing its preference for a circumneutral pH (Hermans et al., 2016; Lekberg et al., 2007). Other research groups have observed an increase in relative abundance of the family Chitinophagaceae within Chitinophagales with the addition of phosphorus in agricultural soils (Wang et al., 2017a).

Caveats

It should be noted that the relative abundance of several taxa changed significantly with treatment but was not high overall; many taxa made up less than 1% of the soil microbial community. Other studies have assigned taxa as "rare" that comprise less than 20%, or even less than 0.1%, of the ecosystem's total abundance. These lowly abundant groups may have important roles in nutrient cycling, as even one gram of soil

could contain millions of microorganisms (Mikkelson et al., 2016; Wang et al., 2017b). Per Mikkelson, Bokman, and Sharp (2016), several taxa such as Sebacinales, Mytilinidales, Uncultured Acidobacterium A seemed to be ecologically relevant and so were included in this discussion. However, some microbial taxa including Lecanorales, Cellvibrionales, Rhodospirales, Oceanospirales, and S-BQ2-57 from *Verrucomicrobiae* are only found in one or two replicates of the same treatments, and their abundance is not a function of treatment but of plot location.

It is important to note that many bacterial taxa, especially in soils, are unculturable, and we lack the information on their metabolism and genomes that is widely available for more culturable strains. This makes it challenging to definitively discern unculturable strains' function in ecosystems. Further, more research on the metabolisms or transcriptomes of the proposed phosphorus miners is required to confirm their role in the mining of recalcitrant phosphorus.

Conclusions

I found several fungal and bacterial taxa that I believe are good candidate bioindicators of acidic, low-phosphorus soils (*Table 3*). The order Russulales is a candidate bioindicator for acidic soils. The relative abundance of this order decreased significantly with the elevation of pH, which may have detrimental effects on the ecosystem. Ectomycorrhizal fungi can be responsible for delivering up to 75% of phosphorus to plants (Baldrian, 2017). A decrease in the abundance of a very common organism, like members of the order Russulales, could represent a change in an ecosystem's ability to cycle phosphorus when soil chemical characteristics change and

could indicate poor soil health. Russulales may also be considered a miner of recalcitrant phosphorus.

Members of the order Sebacinales are candidate bioindicators of circumneutral pH, high-nutrient soils. Similarly, the saprotrophic order Mytilinidales is a bioindicator of circumneutral pH in this ecosystem, and this finding contributes to the limited body of work on the function and ecology on this Dothideomycetes (*Table 3*).

I also found several bacterial orders that should be considered candidate bioindicators for low-pH, low-phosphorus soils. These include three members from the phylum Acidobacteria: Subgroup 2, Acidobacteriales, and Solibacterales. Subgroup 2, in particular is associated with the activities of phosphorus-acquiring enzymes, and may also have the ability to mine phosphorus. Finally, the endophytic bacterial order Obscuribacterales should be considered a candidate bioindicator of low-pH, low-phosphorus soils as well as a potential miner of recalcitrant phosphorus (*Table 3*).

Uncultured Acidobacterium A, another order from the phylum Acidobacteria, and members of the order Cytophagales should be considered a bioindicator of circumneutral pH, high nutrient soils (*Table 3*). Chitinophagales another bacterial order that should be considered a bioindicator of circumneutral pH, high-nutrient soils, based on the findings in this and other studies (Hermans et al., 2016; Wang et al., 2017a). Psuedomonodales can also be considered a bioindicator of circumneutral soils with some available phosphorus and is known in literature as a phosphorus solubilizer, although that was not observed in this study (Rodríguez and Fraga, 1999).

In conclusion, there is evidence of bacterial and fungal clades that are candidate bioindicators of phosphorus limitation in forest soils, as well as their abilities to mine phosphorus from recalcitrant sources. This research shows the connection between microbially mediated ecosystem processes and individual microbial orders, and it can be used as a stepping stone into further investigation of the relationships between microbial and ecosystem function.

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APPENDIX 1

ITS analysis workflow in QIIME1 and QIIME2

1) I have 454 .fna data without quality scores

2) Concatenate & Demultiplex in QIIME1

3) Import into QIIME2 as Sample data[sequences]

4) OTU picking with VSEARCH

5) Chimera filtering (VSEARCH)

6) Removed low-abundance reads

7) Assign Taxonomy

8) Export data into text files

Code

2) cat *.fna>seqs_noDemux.fna

split_libraries.py -m map_file.tsv -f seqs_noDemux.fna --barcode_type 6 -o split_library_output

#Split library output contains demultiplexed reads

3) import into QIIME2

in split_library_output/:

qiime tools import --input-path seqs.fna --output-path seqs.qza --type SampleData[Sequences]

4) OTU picking with VSEARCH

4a) Dereplicate sequences:

qiime vsearch dereplicate-sequences --i-sequences seqs.qza --o-dereplicated-table table.qza --o-dereplicated-sequences rep-seqs.qza

4b) Perform OTU clustering at 97% using the UNITE database as above

#4b.2 Import UNITE

qiime tools import --input-path /Users/lauramason/UNITE_database/sh_refs_qiime_ver7_97_s_01.12.2017.fasta --output-path /Users/lauramason/UNITE_database/UNITE_97.qza --type 'FeatureData[Sequence]'

qiime vsearch cluster-features-closed-reference --i-table table.qza --i-sequences rep-seqs.qza --i-reference-sequences /Users/lauramason/UNITE_database/UNITE_97.qza --p-perc-identity 0.97 --o-clustered-table table-UNITE-97.qza --o-unmatched-sequences unmatched_UNITE_97.qza --o-clustered-sequences sequences-UNITE-97.qza

5) filter for Chimeras

#5a) Denovo Chimera picking

qiime vsearch uchime-denovo --i-table table-UNITE-97.qza --i-sequences rep-seqs.qza --output-dir uchime-dn-out

#5b) Generate chimera checking summary visualization

qiime metadata tabulate --m-input-file uchime-dn-out/stats.qza --o-visualization uchime-dn-out/stats.qzv

```
# 5c) Filter the table and sequences for chimeras and “borderline” chimeras
qiime feature-table filter-features --m-metadata-file uchime-dn-out/nonchimeras.qza --i-table table.qza --o-filtered-table uchime-dn-out/table-nonchimeric.qza

qiime feature-table filter-seqs --i-data rep-seqs.qza --m-metadata-file uchime-dn-out/nonchimeras.qza --o-filtered-data uchime-dn-out/rep-seqs-nonchimeric.qza

qiime feature-table summarize --i-table uchime-dn-out/table-nonchimeric.qza --o-visualization uchime-dn-out/table-nonchimeric.qzv
```

6) Filter low abundance reads from dataset

```
qiime feature-table filter-features --i-table table-nonchimeric.qza --p-min-samples 3 --o-filtered-table table-nonchimeric-gt3.qza

qiime feature-table summarize --i-table PAX /split-lib/uchime table-nonchimeric-gt3.qza --o-visualization table-nonchimeric-gt3.qzv --m-sample-metadata-file sample-metadata.tsv

qiime feature-table filter-seqs --i-data rep-seqs-nonchimeric.qza --i-table table-nonchimeric-gt3.qza --o-filtered-data rep-seqs-nonchimeric-gt3.qza
```

7) Assign Taxonomy

Import reference data set into Q2

qiime tools import --input-path sh_taxonomy_qiime_ver7_97_s_01.12.2017 --output-path UNITE_97.qza --type 'FeatureData[Sequence]'

qiime tools import --input-path sh_taxonomy_qiime_ver7_97_s_01.12.2017 --output-path UNITE_97_taxa.qza --type 'FeatureData[Taxonomy]'

qiime feature-classifier classify-consensus-vsearch --i-query split_library_output/uchime-dn-out/rep-seqs-nonchimeric-gt3.qza --i-reference-reads UNITE_database/UNITE_97_ref.qza --i-reference-taxonomy UNITE_database/UNITE_97_taxa.qza --output-dir UNITE_assigned_taxonomy/

#View data

qiime metadata tabulate --input classification.qza --visualzation classification.qzv

8) Export data into format usable in R

qiime taxa collapse --i-table split_library_output/uchime-dn-out/table-nonchimeric-gt3.qza --i-taxonomy UNITE_assigned_taxonomy/classification.qza --p-level 4 --output-dir Level4/

qiime tools collapsed-table.qza --output-dir exported-collapsed-table

biom convert -i feature-table.biom -o Order.txt --to-tsv

APPENDIX 2

16S Analysis in QIIME2

Workflow

1) Import into Q2 as SampleData[PairedEndSequencesWithQuality]

2) Dereplicate in vsearch

3) OTU picking w vsearch

4) Chimera filtering

5) get rid of OTUs with abundance < 3

6) Taxonomy assignment

7) Contingency based filtering

8) Train naive bayes classifier for SILVA 16S 132 release

9) Assign taxonomy using SILVA

10) Export feature tables as txt and biom

Code

1) Import data into Qiime2

##Because this data was already demultilpexed, I can import directly into QIIME2 without first importing into QIIME1##

qiime tools import --type 'SampleData[PairedEndSequencesWithQuality]' --input-path Reads/ --source-format CasavaOneEightSingleLanePerSampleDirFmt --output-path demux-paired-end.qza

2) Join paired ends (should have done in fungal analysis) & summarize

qiime vsearch join-pairs --i-demultiplexed-seqs demux-paired-end.qza --o-joined-sequences demux-joined.qza

qiime demux summarize --i-data demux-joined.qza --o-visualization demux-joined.qzv

3) Filter for quality

qiime quality-filter q-score-joined --i-demux demux-joined.qza --o-filtered-sequences demux-joined-filtered.qza --o-filter-stats demux-joined-filter-stats.qza

4) Dereplicate in vsearch

qiime vsearch dereplicate-sequences --i-sequences demux-joined-filtered.qza --o-dereplicated-table table.qza --o-dereplicated-sequences rep-seqs.qza

5) de novo OTU clustering in vsearch to 97%

qiime vsearch cluster-features-de-novo --i-table table.qza --i-sequences rep-seqs.qza --o-clustered-table table-dn-97.qza --o-clustered-sequences rep-seqs-dn-97.qza --p-perc-identity 0.97

6) Chimera filtering & summarize

qiime vsearch uchime-denovo --i-table table-dn-97.qza --i-sequences rep-seqs-dn-97.qza --output-dir uchime-dn-out

qiime metadata tabulate --m-input-file uchime-dn-out/stats.qza --o-visualization uchime-dn-out/stats.qzv

qiime feature-table filter-features --m-metadata-file uchime-dn-out/nonchimeras.qza --i-table table.qza --o-filtered-table uchime-dn-out/table-nonchimeric.qza

qiime feature-table filter-seqs --i-data rep-seqs.qza --m-metadata-file uchime-dn-out/nonchimeras.qza --o-filtered-data uchime-dn-out/rep-seqs-nonchimeric.qza

qiime feature-table summarize --i-table uchime-dn-out/table-nonchimeric.qza --o-visualization uchime-dn-out/table-nonchimeric.qzv

Saved FeatureData[Sequence] to: uchime-dn-out/rep-seqs-nonchimeric.qza
Saved FeatureTable[Frequency] to: uchime-dn-out/table-nonchimeric.qza

7) Contingency based filtering to remove features that have a frequency < 3
qiime feature-table filter-features --i-table table-nonchimeric.qza --p-min-samples 3 --o-filtered-table table-nonchimeric-gt3.qza

qiime feature-table filter-seqs --i-data rep-seqs-nonchimeric.qza --i-table table-nonchimeric-gt3.qza --o-filtered-data rep-seqs-nonchimeric-gt3.qza

qiime feature-table tabulate-seqs --i-data rep-seqs-nonchimeric-gt3.qza --o-visualization rep-seqs-nonchimeric-gt3.qzv

8) Train naive bayes classifier for SILVA 16S 132 release

qiime tools import --input-path silva_132_97_16S.fna --output-path silva_132_97_16s.qza --type 'FeatureData[Sequence]'

qiime tools import --input-path Silva_132_release/SILVA_132_QIIME_release/taxonomy/16S_only/97/taxonomy_7_levels.tsv --output-path Silva_132_release/SILVA_132_QIIME_release/taxonomy/16S_only/97/taxonomy_7_levels.qza --type 'FeatureData[Taxonomy]' --source-format HeaderlessTSVTaxonomyFormat

qiime feature-classifier fit-classifier-naive-bayes --i-reference-reads Silva_132_release/SILVA_132_QIIME_release/rep_set/rep_set_16S_only/97/silva_132_97_16s.qza --i-reference-taxonomy Silva_132_release/SILVA_132_QIIME_release/taxonomy/16S_only/97/taxonomy_7_levels.qza --o-classifier Silva_132_release/trained_classifier

9) Assign taxonomy using SILVA

qiime feature-classifier classify-sklearn --i-reads 171006_Blackwood_Deforest_Bacteria/uchime-dn-out/rep-seqs-nonchimeric-gt3.qza --i-classifier Silva_132_release/trained_classifier.qza --o-classification 171006_Blackwood_Deforest_Bacteria/uchime-dn-out/classified_taxonomy

10) Export feature tables as txt and biom

qiime taxa collapse --i-table uchime-dn-out/table-nonchimeric-gt3.qza --i-taxonomy 171006_Blackwood_Deforest_Bacteria/uchime-dn-out/classified_taxonomy --p-level 4 --output-dir classified_exported/

qiime tools export classified_taxonomy.qza --output-dir classified_exported/

qiime tools export table-nonchimeric-gt3.qza --output-dir OTUs/

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