Plant Invasions and Microbes: The Interactive Effects of Plant-Associated Microbes on Invasiveness of *Phragmites australis*

by

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Dedication

To Eli and Arlo. Always be curious about the world around you.

P.S. I promise to never make you read this

.

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Abstract

The mechanisms driving biological invasions are important for predicting range expansion and developing effective invasive species management strategies but are often difficult to disentangle. One driver of plant invasions may be through differential interactions with belowground microbes, whereby invasive plants gain a disproportionate advantage over natives either through a relatively stronger interaction with mutualists or a weaker interaction with pathogens. I aimed to examine whether invasive *Phragmites australis*, a clonal wetland plant, gains a performance advantage over a related native lineage through interactions with belowground microbial communities.

I explored bacterial, fungal, and oomycete communities associated with native and non-native *Phragmites* in the Great Lakes region and the impacts of those microbial communities on invasiveness. I used a combination of field surveys of natural populations and controlled environment experimental manipulations combined with next-generation sequencing to thoroughly examine whether invasiveness in *Phragmites* is facilitated by interactions wih belowground microbes and which microbial players are most influential. My results were very consistent among all chapters in this dissertation, finding no strong link between invasiveness and belowground microbial communities, and therefore suggesting that belowground microbes are not fostering invasion of *Phragmites australis* in the Great Lakes region. Field surveys provided evidence that belowground microbial communities did not differ between *Phragmites* lineages in roots or rhizospheres of natural populations. Root communities did differ in fungal colonization

and in oomycete richness, but both of those differences were weak and inconsistent among different environmental conditions. In addition, the few differences that were found between lineages were consistently opposite of my expectation that non-native *Phragmites* would be associated with more mutualists and/or fewer pathogens than native. The rhizosphere largely followed the same patterns with one exception: the rhizosphere bacterial communities differed by lineage in large, dense patches of *Phragmites*, but not elsewhere. Given the small magnitude of the observed differences in bacterial communities, and the fact that they only existed in dense, mature patches of *Phragmites*, no differences in functional potential could be attributed to the community differences observed. Taken together, the evidence that I have obtained strongly suggests that any observed differences in soil microbial communities between *Phragmites* lineages may be a consequence rather than a driver of invasiveness.

Consistent with results from natural populations, I also observed that experimentally-conditioned soils differed only slightly between lineages in bacterial community composition and even less so in fungal community composition. The plant response to those slightly different microbial communities was more significant, but again opposite of expectations if microbes were driving invasiveness. Non-native *Phragmites* was overall negatively impacted by the total soil microbiome, whereas native was unaffected by the total soil microbiome, regardless of which lineage conditioned the soil, with bacterial pathogens likely playing a significant role in the negative plant-soil interaction. These findings on lineage-specific plant responses are counter to our expectation that if microbial communities are driving invasiveness, non-native *Phragmites* should derive disproportionate benefits from microbial communities over native.

Given the preponderance of data suggesting that belowground microbes are not drivers of invasiveness in *Phragmites*, it is reasonable to assume that the non-native's invasiveness is

primarily derived from other sources. However, importantly, differential response to similar microbes in native and non-native lineages suggests that microbial manipulation could be a reasonable tool for lineage-specific biocontrol.

Chapter 1 : Introduction

Plant Invasions and Soil Biota

Invasive species are significant agents of global change, having far reaching impacts on biodiversity and ecosystem services worldwide (Elton 1958, Pimentel et al. 2005). However, the mechanisms that underlie invasions are often complex, making it difficult to predict the expansion of invasive species and promote biotic resistance in the invaded biotic community. Invasive plants often derive success by being different from native species, either in niche space, or in fitness (Shea and Chesson 2002, MacDougall et al. 2009). For example, if a resident and invading plant differ in their fitness such that the resident has a consistent performance advantage, the invader will be repelled (biotic resistance) unless their niche difference is large enough to accommodate both species, leading to coexistence. Conversely, if the invader has the fitness advantage, it will exclude the resident, unless their niche difference is large enough to accommodate both species, also promoting coexistence (MacDougall et al. 2009).

Biotic interactions underlie prominent theories on invasion, because they play a significant role in defining the niche and fitness of plants. For instance the enemy release hypothesis posits that native plants are under more serious pathogen pressure than invaders (who are less affected by local pathogens), which leads to expansion of the invasive due to fitness differences (Keane and Crawley 2002, MacDougall et al. 2009). Likewise, the enhanced mutualism hypothesis, whereby invasive plants benefit from mutualisms more strongly than native plants, maximizes fitness differences between invader and residents (Reinhart and Callaway 2006). Thus, interactions

among plants as well as between plants and other organisms have significant implications for invasion.

As an intimate partner in all stages of a plant's life cycle, the plant-associated microbiome considerably influences niche and fitness differences and therefore may play a significant role in plant invasiveness. Soil microbial communities can alter plant performance through pathogenicity, mutualism, or a combination of these factors. The net performance of a plant as mediated by microbes is a result of the **effect** a plant has on its microbiome and the **response** of plant to that microbiome. In general, the balance between a plant's interaction with mutualists and pathogens in its microbiome will have a strong impact on performance (Fig. 1.1). If the strength of interaction with mutualists and pathogens differs between resident and invading species in appropriate ways, the invading species could dominate over the resident (Fig. 1.1; Jiang et al. 2019).

Changes in plant performance resulting from microbial interactions will further impact the ability of a plant to cultivate a microbiome, potentially creating a feedback in the plant-soil system (Ehrenfeld et al. 2005, Revillini et al. 2016, van der Putten et al. 2016). Positive feedbacks occur when association with a particular group of microbes increases plant performance, such that further cultivation of net beneficial microbes can occur, and plant performance is further increased in successive generations. Conversely, in a negative feedback, a plant cultivates microbes with an overall negative impact, thereby tempering performance. As a result, the plant selects for fewer of those antagonistic microbes in successive generations allowing performance to increase back to its original level (Ehrenfeld et al. 2005).

Feedbacks can be measured either in a species-specific (*i.e.,* measuring absolute performance in conspecific *vs.* heterospecific soils) or pair-wise context (*i.e.*, comparing the relative performance of two plant species in soils from each species), depending on the goals.

Species-specific (or direct) feedbacks inform how individual plants are affecting and responding to soil biota in the short term, (independent of how other species are interacting with soils); pair-wise feedbacks incorporate two plants' effects on and responses to soils and inform whether dominance or co-existence between two species is predicted in the long-term (Bever et al. 1997). Positive (or at least less negative) species-specific plant-soil feedbacks (PSFs) are commonly hypothesized as major factors in the expansion of invasive plant species (Klironomos 2002, Mangla et al. 2008, Meisner et al. 2014, Maron et al. 2014). Under the PSF model for invasiveness, the invasive plant experiences a more positive or less negative feedback than native plants (Reinhart and Callaway 2006, Inderjit and Cahill 2015).

Evidence of a widespread role of PSFs in invasions is mixed and has been addressed in three recent meta-analyses (Suding et al. 2013, Meisner et al. 2014, Crawford et al. 2019). Broad patterns indicate that, opposite of expectations, native plants displayed positive species-specific feedbacks, whereas species-specific invasive plant feedbacks were neutral (Suding et al. 2013). Additionally, the strength and direction of PSF differences between native and invasive plants was strongly dictated by the growth form of the plant (Meisner et al. 2014). The pattern of invasive plants experiencing a more positive or less negative species-specific feedback than a native plant was valid for trees, but not for grasses or forbs (Meisner et al. 2014). Thus, although some types of invasive plants may derive benefits from soil biota, it is not a widespread phenomenon, and it may depend on the growth form of the plant. Moreover, native and invasive plants tended to generate slight negative pairwise feedbacks on average, predicting stabilizing coexistence through conspecific negative density dependence (Crawford et al. 2019). This prediction of coexistence is not consistent with the reality of most invasions and thus implies that other factors impact invasive success.

Even if results of PSF studies do agree with patterns in nature, traditionally, studies of plant associated microbes and invasion take a "black box" approach in which soil biota are indirectly assessed via plant responses, leading to difficulty in pinpointing the specific mechanisms of how microbes mediate plant performance (Cortois and de Deyn 2012). Soil microbiomes are complex; as such, some components may be affected more by plants than others, and plants may respond more strongly to some portions than others. For instance, plant responses to arbuscular mycorrhizal fungi (AMF) are generally positive (Klironomos 2002), but the strength of response depends upon the specific plant and AMF combinations (Klironomos 2003), and may not affect native and invasive plants differently (Meisner et al. 2014). Plant responses to pathogen communities are generally negative (Klironomos 2002), but native and invasive plants may (Klironomos 2002) or may not (Suding et al. 2013) respond differently to those pathogen communities. Given this uncertainty in specificity of plant response to mutualists and pathogens, it is important to break open the "black box" and measure the ways that native and invasive plants affect, and respond to, specific microbial taxa. Disentangling the specific taxa that encompass the mutualist and pathogen communities of native and invasive plants will enable management tools to target those taxa that disproportionately affect invasive plants.

Study System

The common reed, *Phragmites australis*, is a clonal wetland plant species that occurs widely throughout North American wetlands and includes both native and invasive haplotypes (Saltonstall 2002). *Phragmites* has been described as a model organism for studying plant invasions (Meyerson et al. 2016). The most common exotic invasive haplotype is of European origin (Haplotype M; from Saltonstall 2002) (hereafter non-native *Phragmites*) whereas the North American native lineage is recognized as *Phragmites australis* subsp. *americanus* (Saltonstall et

al. 2004) (hereafter native *Phragmites*). Non-native *Phragmites* was introduced to the United States from Europe in the early 1800's (Saltonstall 2002) and since has expanded its range to include all lower 48 states and parts of Canada. In the Great Lakes region, non-native *Phragmites* is a serious problem for wetland land managers with over 24000 ha invaded in the coastal zone alone (Bourgeau-Chavez et al. 2013). While native *Phragmites* has been a consistent part of the North American flora for several thousand years (Saltonstall 2002), many populations have been displaced since the expansion of non-native *Phragmites*; presently, the native lineage is considered rare in some regions (Saltonstall 2002, Price et al. 2013, Caplan et al. 2014).

Phragmites reproduces both sexually and asexually, and non-native expansion is driven by both high seed output and rapid vegetative growth. Recent studies indicate that germination and recruitment of non-native seedlings was positively affected by nutrient enrichment and disturbance (Kettenring et al. 2011). In turn, relatively greater germination and recruitment increase the genetic diversity of populations, which then creates a positive feedback by fostering the viability of seedlings (Kettenring et al. 2011, Hazelton et al. 2014). Native *Phragmites* is considered a low-nutrient specialist, is generally slower growing, and native populations are generally less genetically diverse than non-native populations (League et al. 2006, Mozdzer et al. 2010, 2013, Kettenring and Mock 2012).

Native and non-native *Phragmites* also differ in physiology, which could underlie their performance differences. For instance, in brackish marshes of the Mid-Atlantic region of North America, non-native *Phragmites* photosynthesized at a 51 % higher rate than native did and had twice the rate of stomatal conductance (Mozdzer and Zieman 2010). In addition, non-natives produce a much denser photosynthetic canopy with greater stem density and a higher specific leaf area compared to the native type (Mozdzer and Zieman 2010, Mozdzer et al. 2013). These traits

come with a trade-off, wherein the non-native lineage has a much higher demand for nitrogen than its native counterpart (Windham and Meyerson 2003, Mozdzer and Zieman 2010).

Non-native *Phragmites* commonly dominates in eutrophic areas (Windham and Lathrop 1999, Windham and Meyerson 2003, Kettenring et al. 2011, Mazur et al. 2014), although may also dominate in areas not affected by high nutrient inputs. Some have speculated that the increased nitrogen demand in the non-native lineage is nourished by way of a feedback resulting from root zone aeration (Windham and Lathrop 1999, Windham and Meyerson 2003). Both lineages of *Phragmites* distribute air though their stems and rhizomes via convective throughflow, whereby convected gases are transmitted through air spaces in the culm and underground rhizome, and are vented via old broken culms (Armstrong and Armstrong 1990, Brix et al. 1996). This throughflow mechanism results in aeration of the rhizosphere (Armstrong and Armstrong 1990, Colmer 2003). Importantly, the ventilation efficiency is 300% higher in non-native *Phragmites*, relative to the native taxa, due in large part to the differences in stem density between lineages (Tulbure et al. 2012). With fewer old, broken culms, the convective throughflow is slower in the native lineage (Tulbure et al. 2012), leading to probable differences in rhizosphere oxygen concentrations between lineages. Because rates of nutrient cycling are faster in aerated soils, non-native *Phragmites* may benefit from increased rates of nutrient cycling in a more heavily aerated rhizosphere (Windham and Lathrop 1999, Windham and Meyerson 2003).

The trait differences between native and non-native *Phragmites* described above could create conditions under which different communities of microbes may surround each lineage. For example, higher rates of photosynthesis between lineages likely leads to divergence in leaf tissue chemistry between lineages which could act as an environmental filter and select for different groups of microbes in the leaves of each lineage (*sensu* Pellitier et al. 2019). Additionally,

increased productivity could lead to differences in the root exudates that select for particular microbial communities in the rhizosphere (Kuzyakov and Cheng 2001). Therefore, there is reason to believe that these two plant lineages might create conditions that support diverging microbial communities. The focus of this dissertation is to quantify those community differences and explore whether they may facilitate invasiveness in the non-native lineage.

The extent to which the discrepancy in performance between native and invasive *Phragmites* can be explained by interactions with their microbial communities has not been studied. Although many types of microbes associated with *Phragmites* have been surveyed separately, most of these studies have explored how *Phragmites* affects belowground microbes, and very few have measured the response of each lineage to those microbes. For instance, both lineages of *Phragmites* host a diverse community of oomycete pathogens of the genus *Pythium*. Although some of the same *Pythium* taxa appear in populations in North America and Europe populations (Nechwatal et al. 2008, Nelson and Karp 2013, Crocker et al. 2015, Cerri et al. 2017), the communities associating with native and non-native *Phragmites* in North America differed in one study (Nelson and Karp 2013). Bacterial and archaeal rhizosphere communities have also been examined in *Phragmites* lineages in salt marshes of the Chesapeake Bay region of the U.S., with differences found between lineages in archaeal, but not bacterial communities (Yarwood et al. 2016). However, others have found bacterial communities differ strongly between lineages (Bowen et al. 2017).

The relative importance of pathogens and mutualists between *Phragmites* lineages is not well understood. Non-native *Phragmites* hosts a diverse community of fungi both in its native range (Wirsel et al. 2002, Ernst et al. 2003, Wirsel 2004, Angelini et al. 2012) and in its invaded range of North America (Fischer and Rodriguez 2013, Clay et al. 2016, Shearin et al. 2018),

including some that have been found to enhance biomass production (Ernst et al. 2003) and tolerance to stress (Soares et al. 2016b). However, few have explored the putative function of *Phragmites*-associated fungi at a community level (see Allen et al. *in review* for an example in foliar fungi).

Dissertation Synopsis

While previous studies have made significant contributions to our collective understanding of components of the microbial community associated with *Phragmites* (*Phragmites'* effects on microbes), it remains unclear how these individual microbial associations cumulatively influence invasiveness in non-native *Phragmites* (*Phragmites'* response to microbes). This dissertation explores the total microbiome (*i.e.*, bacteria, fungi, and oomycetes) of each lineage and their putative function and influence on invasiveness and test the hypothesis that *microbial communities are driving invasiveness*. If so, I would expect invasive plants to derive disproportionate benefits from microbial communities over natives. To address this hypothesis, I investigated microbes at the interface of plant and soil in the field and experimentally manipulated microbial communities in controlled conditions to characterize microbial community structure and function.

Chapter 2 examines communities of three microbial groups (bacteria, fungi, and oomycetes) inhabiting roots of both lineages in the field. This represents the first comprehensive examination of root endophytes in populations of native and non-native *Phragmites*. I investigated the degree to which plant lineage and/or environmental variables drive the communities of endophytic microbes in *Phragmites*. I also explored the functional potential of those endophytes and whether they are potential drivers of invasiveness. This chapter was published in the journal, *Ecosphere* (Bickford et al. 2018).

In Chapter 3, I ask similar questions but focus on rhizosphere soils, where plant selection of microbes occurs. I quantify community composition of bacteria, fungi, and oomycetes in soils making this study is more comprehensive than others that have examined rhizosphere microbes in *Phragmites*. I explored the extent to which plant lineage explains microbial community variation, whether variation in stand size impacts microbial community composition, and whether the functional potential of those communities is associated with invasiveness.

In Chapter 4, I use an experimental approach to test the extent to which microbially-mediated feedbacks occur and which microbial groups are driving feedbacks. This experiment breaks open the "black box" and examines the specific microbial communities conditioned by each lineage. In addition, we apply selective microbial inhibitors to further track the impact of particular microbial groups on the productivity of native and non-native lineages. Therefore, I quantify the lineage-specific effects on microbial communities as well as lineage-specific responses and put those in context of their impacts on invasiveness.

Lastly, in Chapter 5, I summarize and synthesize the major findings of my dissertation and discuss their implications for plant microbial interactions in invasion biology. I also put my findings in context of *Phragmites* management. I conclude my dissertation by briefly outlining potential future avenues for microbial manipulation in *Phragmites* management.

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Figures

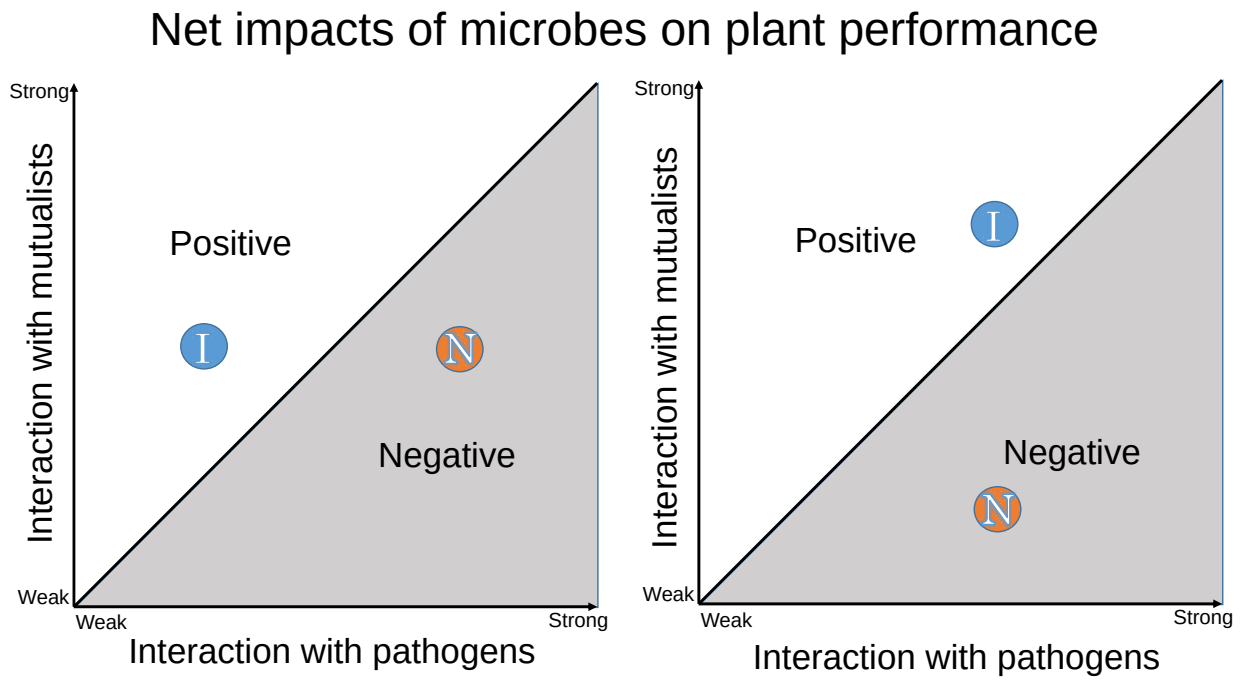

Fig. 1.1 Impacts of microbes on native (circle with "N") and invasive (circle with "I") plant performance. The microbiome may benefit invasive plants over natives if (R) the invasive plant's interaction with pathogens is weaker than natives' (Enemy Release Hypothesis, sensu Keane and Crawley 2002) or (L) the invasive plant's interaction with mutualists is stronger than natives' (Enhanced Mutualism Hypothesis, sensu Reinhart and Callaway 2006).

Chapter 2 : Root Endophytes and Invasiveness: No Difference Between Native and Non-Native *Phragmites* in the Great Lakes Region*

Introduction

The intimate association of plants and their microbiome has significant impacts on plant performance and thereby may be an important driver of invasion success. Recent studies have focused on the plant-associated microbiome of invasive plants, because it could provide a mechanism for their colonization and recruitment, as well as for decreased performance of native species (Thorpe and Callaway 2006, Salles and Mallon 2014, Kowalski et al. 2015). More specifically, if invasive plants cultivate a microbial community with stronger mutualistic effects or less intense pathogenic effects than native plants, then the net effect of their microbiome will be more positive than for native plants (Reinhart and Callaway 2006).

Evidence linking belowground microbial communities to invasive plant success is mixed. For instance, invasive grasses can have stronger positive or less negative plant-soil feedbacks than natives in monoculture (Klironomos 2002) and in mixtures (Kulmatiski 2018). However, a meta-analysis covering many different habitat types from forest to wetland, did not find a consistent trend supporting feedbacks as a mechanism for invasion success (Suding et al. 2013), suggesting that belowground microbial community effects on plant performance may be taxon- or habitat-specific. In addition, plant-soil feedback experiments typically do not identify which microbes are cultivated such that specific microbial mechanisms are unclear. Moreover, microorganisms are phylogenetically diverse and can affect plant performance in many ways, ranging from direct pathogenesis to alteration of site nutrient availability. However, studies that examine plant-

associated microbes often target one specific group of taxa (e.g., bacteria or fungi), thereby ignoring the potential interactions among these diverse organisms that could have important outcomes for plant performance.

One of the most noxious and widespread wetland plant invaders is the common reed, *Phragmites australis* (Cav.) Trin. ex Steud. (Meyerson et al. 2016). This clonal plant occurs widely throughout North American wetlands and includes both native (hereafter native *Phragmites*) and exotic, invasive haplotypes (hereafter non-native *Phragmites;* Saltonstall 2002, Saltonstall et al. 2004). In many Atlantic Coast and Great Lakes wetlands, non-native *Phragmites* is much more productive, and in some cases, displaces native *Phragmites* (Martin and Blossey 2013, Mozdzer et al. 2013, Price et al. 2013). Due to their close phylogenetic relationship, comparison between the non-native and native lineages of *Phragmites* offers a unique opportunity to understand how microbial communities may impact invasive success.

As a species considered invasive in most of North America, *Phragmites* has been well studied, including some exploration of select microbial groups. Previous studies have examined leaf, root, and seed fungal endophytes of non-native *Phragmites* (Clay et al. 2016, Soares et al. 2016a, Shearin et al. 2018), characterized and compared soil oomycete communities (Nelson and Karp 2013, Crocker et al. 2015, Cerri et al. 2017), and compared soil bacterial (Bowen et al. 2017) and archaeal (Yarwood et al. 2016) communities between *Phragmites* lineages. However, the functional significance of *Phragmites*-associated microbiomes remains unknown. To date, no comprehensive survey of root microbial communities and their interactions with native and non-native plant lineages has been performed. A comparison of the microbial communities associated with the two lineages may lend mechanistic insights into the invasiveness of non-native

Phragmites. For example, associating with relatively more mutualists or fewer pathogens, would give the non-native lineage a performance advantage over the native lineage.

Here, we report on an intensive field survey of bacterial, fungal, and oomycete communities associated with the roots of native and non-native *Phragmites* in sites where both lineages co-occur throughout the state of Michigan, USA. We focus on roots as the main interface between the plant and the soil environment and where nutritional mutualisms as well as pathogen attack are common. If performance differences between native and non-native plant lineages are driven by their root microbial communities, then we hypothesize that plant lineage would be a stronger predictor of microbial community differences than environmental variables. Furthermore, mutualist microbes should be more abundant and/or pathogen microbes should be less abundant in the non-native lineage.

Materials and Methods

Study Sites

In August 2015, we sampled roots from native and non-native *Phragmites* individuals at 8 sites distributed across Michigan, USA (Appendix S2: Table S2.1). We sought sites that had at least 3 distinct patches of native and non-native *Phragmites* in close proximity to one another growing under similar environmental conditions (*i.e.*, soil type, hydrology) with no recent history of invasive plant management (*e.g.*, herbicide, burning). Due to the rarity of co-occurring native and non-native *Phragmites* populations that met these criteria (non-native is rare and well-managed in northern Michigan; native is rare in southern Michigan), patch size varied considerably among sites (1 m^2 to 100 m^2) and micro-environmental differences among patches within sites and sample size differences among sites were unavoidable. To account for this variation, we used environmental characteristics as co-variables in our analyses.

At each site, we morphologically identified all *Phragmites* patches as native or non-native. Size of *Phragmites* clones can be quite variable, wherein patches can consist of a single clone or multiple clones. While clones can differ morphologically (Křiváčková-Suchá et al. 2007), such differences are not consistent or distinct enough to reliably use for field identification. To maximize probability that patches represented distinct clones, we considered patches that were separated by at least 10 m to be separate clones. We classified the degree of soil saturation as either unsaturated, saturated, or saturated with surface water, recorded height of water table (if over the surface), and nature of surrounding vegetation. Within each patch, a ramet near the center of the clone was randomly selected. Roots were collected by cutting with a serrated knife in a 10-cm diameter circle around the chosen ramet, exhuming subtending roots, and then placing them in a plastic bag. Leaf samples from the same stem were collected for nutrient analysis. Soil samples (5 cm depth) were collected from the area adjacent to the selected ramet. All samples were kept on ice until returned from the field.

Sample Preparation

Roots were washed with deionized water to remove all soil particles and litter and placed into a sample bag. A subset of the fine roots (< 1 mm dia.) was removed and placed into biopsy cassettes for microscopic analysis, and the remaining roots were stored for molecular analysis. Biopsy cassettes containing fine roots were submerged in 10% KOH at 20 ˚C for 24-48 hours or until roots appeared clear, and then submerged in a mixture of 5% black ink (Sheaffer Pen and Art Supply Co., Providence, Rhode Island) in 5% acetic acid at 95 ˚C for 2 mins (modified from Kosuta et al. 2005). Cassettes were then immediately rinsed with deionized water and a few drops of acetic acid to remove excess stain. Cassettes containing stained roots were stored at 4˚C until microscopic analysis. Stained roots were cut into 1-cm sections, mounted on microscope slides and examined

at 40x magnification for the presence of fungal hyphae following the line-intersection method (McGonigle et al. 1990). While we looked for distinct fungal structures, no vesicles or arbuscules were found. Therefore, counts were restricted to presence/absence of hyphae only.

Fine roots used for molecular analysis were surface sterilized by submerging in 70% ethanol for 1 min, 0.5 % sodium hypochlorite for 3 mins, and 95% ethanol for 30 secs. A subset of sterilized roots was pressed onto petri plates containing Malt Extract Agar for 30 secs and incubated at room temperature for 24 hrs. No growth on the agar was used as an indicator of successful surface sterilization. After surface sterilization, roots were placed into a sterile whirl pack bag and lyophilized in preparation for DNA extraction.

Soils were passed through a 2-mm sieve and oven dried at 60 ˚C for 48 hrs. Dried samples were ground with a mortar and pestle and subsamples from each (0.5 g) were processed in duplicate in a Leco CNS2000 Analyzer (LECO® St. Joseph, MI) to measure carbon and nitrogen. Soil phosphorus was determined colorimetrically following the Bray P1 extraction method (Bray and Kurtz 1945).

Molecular analysis

To prepare for DNA extractions, approximately 50 mg of freeze-dried fine roots was ground in a mortar and pestle with approximately 250 mg autoclaved sand and approximately 1 ml liquid N. The finely ground root sample was then further homogenized with the lysis buffer from Macherey-Nagel Nucleospin Plant II DNA extraction kits (Macherey-Nagel Inc., Bethlehem, PA). All genomic DNA extracts were verified by electrophoresis. Extracts were checked for quality on a NanoDrop UV/Vis spectrophotometer and concentration using a Quant-iT PicoGreen dsDNA kit (Invitrogen, Carlsbad, CA, USA).

All polymerase chain reactions (PCR) for each taxon of interest (fungi, bacteria, oomycetes) were performed using subsamples of the same template genomic DNA. Genomic DNA was diluted to ensure equimolar concentration of template DNA in each PCR reaction. For fungal amplification, ITS1F and ITS4 forward and reverse primers were used (White et al. 1990). For bacterial amplification, the 16s region was amplified using primers 27F and 519R (Lane 1991). Oomycete DNA was amplified using the Oom1F and Oom1R (Arcate et al. 2006). See Appendix S2: Table S2.2 for all PCR conditions, primer sequences, and master mixes. PCR products were purified using the Qiagen MinElute PCR kit and quantified using a Quant-iT PicoGreen dsDNA kit (Invitrogen, Carlsbad, CA, USA).

Sequencing was performed on a PacBio-RS II system utilizing circular consensus technology, which can generate 99.5-99.9% sequence accuracy for DNA fragments ranging from 150 to 500 bp (Travers et al. 2010). Seventeen barcoded samples, pooled in equimolar concentration, were multiplexed per SMRT chip. Three SMRT chips were sequenced per microbial group (9 total SMRT chips) at the University of Michigan Sequencing Core.

Bioinformatics analysis

Raw sequence data were processed using mothur v1.39.5 (Schloss et al. 2009). Operational taxonomic units (OTUs) were clustered at 97% for bacterial sequences. Fungal and oomycete sequences were binned into phylotypes because ITS does not allow for sequence alignment over large datasets and mothur requires a sequence alignment to cluster OTUs. Bacterial taxonomy was determined by comparing representative sequences to the taxa found in the SILVA database (Quast et al. 2018). Fungal taxonomy was assigned based on the UNITE database (Nilsson et al. 2013). For oomycete taxonomy, we created a custom oomycete-specific database from NCBI records. Sequences were compared to this database using the blastn toolkit. Each microbial group was

rarefied according to the sample that yielded the fewest number of sequences to ensure equal sampling across all samples. Fungi were rarified to 200 sequences per sample, bacteria to 459 sequences, and oomycetes to 468 sequences. Sequencing coverage of fungal and bacterial communities was estimated using Good's coverage estimator (Good 1953). Although rarefaction was carried out at a relatively low number of sequences, Good's coverage indicated strong sampling effort at each sample (Appendix S2: Fig. S2.1-S2.3). Sequences were uploaded to the NCBI Sequence Read Archive under SRA accession number SRP160913.

Functional Assignment

To evaluate functional potential of fungal OTUs, we used FUNGuild (Nguyen et al. 2015) which parses fungal communities by trophic mode and functional guilds. We analyzed outputs at the trophic mode level to understand the proportion of the root communities composed of mutualists (symbiotrophs), pathogens (pathotrophs), and likely commensalists (saprotrophs). For bacterial functional potential, we used the BugBase (Ward et al. 2017) tool that groups organisms into functional groups based on KEGG pathways (Ogata et al. 1999) compiled by PICRUSt (Langille et al. 2013). This tool allowed us to view bacterial communities by their oxygen requirements and potential for stress tolerance. Use of this tool required that we reclassify our OTUs using the greengenes taxonomic database (Desantis et al. 2006).

Data analysis

We used Principle Component Analysis (PCA) to visualize environmental gradients at our sample sites and select uncorrelated variables to use in subsequent analysis. We used a two-phased analysis to account for the fact that environmental variables were not balanced with respect to site and lineage. First, we tested differences by site and lineage for root colonization, microbial alpha diversity, and relative sequence abundance using 2-way ANOVA (Type III sum of squares).

Second, we ignored site and assessed the impact of environmental variables (soil nutrients and saturation) on the response variables, including potential interactions with lineage using Analysis of Co-Variance (ANCOVA). Permutational multivariate analysis of variance (PerMANOVA) tested whether plant lineage or site predicted significant microbial community differences among our samples. Homogeneity of dispersions (PermDISP) tested whether microbial community samples differed in their degree of dispersion from their centroid. Additionally, we used Principle Coordinate Analysis (PCoA) with fitting of environmental data to visualize microbial community differences between lineages and potential environmental drivers. All statistical analyses were run in the R environment (R Core Team 2016) with the exception of PerMANOVA and PermDISP, which were conducted in PRIMER-E with PerMANOVA+ (Clarke and Gorley 2006). All R code, notes, and associated data can be accessed on GitHub at https://github.com/wesbick/Root_paper.

Results

Environmental Characteristics

A PCA of our environmental variables revealed that soil saturation, soil nitrogen, and soil phosphorus were uncorrelated. Soil carbon and nitrogen were strongly correlated, so soil carbon is not reported (Appendix S2: Fig. S2.4). Soil nitrogen and phosphorus did not differ significantly by lineage, but patch saturation level was non-random with respect to lineage at our sites (X_2 = 11.99, P = 0.005), wherein patches of non-native *Phragmites* were more likely to be unsaturated and patches of native were more likely to be saturated. Both were equally likely to occur in high water (Appendix S2: Table S2.3), suggesting that differences in growth habitat between lineages at our sites do not represent differential water tolerances, but instead is an artifact of our sampling locations.

Fungal Colonization

Evidence from microscopy revealed that fungal root colonization was significantly greater in non-native *Phragmites* (Fig. 2.1a; ANOVA, $F = 24.57$, $P < 0.001$). Fungal colonization also differed significantly among sites (ANOVA, $F = 6.52$, $P < 0.001$), but site and plant lineage did not interact (Appendix S2: Table S2.4). Of all environmental variables measured, only soil saturation was a significant predictor of fungal root colonization (ANCOVA, $F = 23.47$, $P < 0.001$, Appendix S2: Table S2.5). In fact, the magnitude of the differences between root colonization between native and non-native *Phragmites* depended on the degree of soil saturation (Fig. 2.1b), although the interaction between lineage and saturation was not significant (Appendix S2: Table S2.5, Fig. S2.5). Fungal root colonization was most different between native and non-native lineages in unsaturated conditions and was not different when soil was saturated or submerged. We observed no structures characteristic of arbuscular mycorrhizal fungi.

Alpha Diversity

As one measure of microbial community differences, we analyzed differences in alpha diversity among sites and between lineages. Bacterial and fungal alpha diversity (richness, Shannon-Weiner diversity) differed among sites but not by plant lineage (Appendix S2: Table S2.6) or with any of the measured environmental variable (Appendix S2: Table S2.7, Fig. S2.6). Oomycete community richness differed modestly between the two lineages, with the non-native roots hosting a greater number of species and higher diversity than the native roots (Fig. 2.2; ANOVA, $F = 4.22$, $P = 0.048$, Appendix S2: Table S2.8). Environmental variables did not affect oomycete alpha diversity (Appendix S2: Table S2.9, Fig. S2.7).

Community Composition

Host lineage was not a significant predictor of community composition for any microbial group based on PerMANOVA (Table 2.1). However, site was a significant predictor of variation

for all three microbial groups and significantly interacted with lineage to shape bacterial communities (Table 2.1). PermDISP revealed a marginally significant difference in the dispersion patterns among sites in bacterial (pseudo-F = 5.76, P = 0.072) and fungal (pseudo-F = 4.56, P = 0.064) communities, which indicates that the difference in community detected by PerMANOVA may be due to heterogeneity of dispersion around the centroids of the sites. Given the importance of site for community composition, we explored which environmental variables could account for this spatial variation with PCoA. While soil nitrogen marginally explained community variation in two of the three microbial groups, degree of saturation more consistently explained substantial variation in ordination space for all three groups (Fig. 2.3; Table 2.2).

Taxonomic Analyses

Despite the lack of difference in overall community composition according to the multivariate analyses, relative abundance of some bacterial phyla on roots did differ by plant lineage (Fig. 2.4, Appendix S2: Table S2.6). Relative sequence abundance of the most common bacterial phyla was associated with plant host lineage and not site (Fig. 2.4). While the majority of all bacterial sequences recovered in both lineages were Proteobacteria, non-native *Phragmites* roots host relatively more Proteobacteria, but fewer Firmicutes and Bacteriodetes taxa. The Bacteroidetes were also influenced by site (Appendix S2: Table S2.6). Major bacterial genera are listed in Appendix S2: Table S2.6-S2.7 and Appendix S2: Fig. S2.8)

Relative sequence abundance of fungi did not differ by site or host lineage at the phylum level (ANOVA, Appendix S2: Table S2.4). Over 90% of recovered fungal sequences were from Ascomycota. Minor phyla included Basidiomycota (5.7%), Zygomycota (0.6%), Glomeromycota (0.2%), and unclassified fungi (2.7%; Appendix S2: Fig. S2.9). We expected that Ascomycetes would make up the majority of sequences as most root endophytes are from this phylum. The

extremely low relative abundance of Glomeromycota, the dominant phylum of AMF, was consistent with the lack of AMF structures found via microscopy. Relative abundances of recovered genera differed significantly by site, but not by lineage (Appendix S2: Table S2.4). The dominant genera recovered in roots were *Gibberella* (19.5%), *Tetracladium* (13.4%), *Microdochium* (11.0%) and *Stagonospora* (7.6%; Appendix S2: Fig. S2.10). Environmental variables did not affect relative sequence abundance at the phylum level, but saturation level was a strong determinant of relative abundance in all dominant fungal genera (Appendix S2: Table S2.5). The majority of our recovered oomycete sequences matched most closely to unclassified uncultured oomycete strains (59.4%). The dominant classified genus was *Pythium* which made up 40% of the sequences recovered. *Phytophthora*, *Saprolegnia*, and *Lagena* were recovered in very low abundance. Oomycete genera did not differ in relative abundance by site or lineage (Appendix S2: Table S2.8, Fig. S2.11), or saturation level (Appendix S2: Table S2.9, Fig. S2.7).

Functional classification

Because soil saturation was a strong driver of bacterial community differences, we focused on respiratory mode as a trait that could differentiate groups based on saturation. Additionally, differences in respiratory mode could drive rates of nutrient cycling and has the potential to feedback to plant productivity. We used BugBase to make respiratory mode determinations based on GreenGenes taxonomy of our 16S sequences. While most OTUs were from aerobic bacteria (Fig. 2.5), the relative abundance of anaerobes was marginally determined by plant lineage, with the native lineage hosting more anaerobes than the non-native. Soil saturation was also a predictor of relative abundance of facultative anaerobes (ANCOVA, $F = 3.34$, $P = 0.077$, Appendix S2: Table S2.7). However, plant lineage and soil saturation did not significantly interact in determining relative abundance of bacteria based on respiratory mode, suggesting that plants hosted similar

communities with respect to oxygen requirements in all saturation levels (Appendix S2: Table S2.7, Fig. S6).

Using functional assessment from FUNguild, we compared the relative sequence abundance of the dominant trophic modes of fungi inhabiting *Phragmites* roots (Fig. 2.6). Most taxa were categorized as pathotrophs or saprotrophs, with no difference in the relative abundance of either group between native and non-native lineages (Appendix S2: Table S2.4). Symbiotrophs (mutualists) and pathotroph-symbiotrophs (a hybrid group consisting mostly of Dark Septate Endophytes) also did not differ by site or lineage. Pathotrophs (ANCOVA, $F = 9.13$, $P = 0.005$) and Saprotrophs (ANCOVA, $F = 14.63$, $P < 0.001$) did differ based on soil saturation (Appendix S2: Table S2.5, Fig. S2.5), perhaps reflecting the oxygen demands of these functional groups under various saturation levels. In the fungal communities, there was no evidence for higher mutualist or lower pathogen load on the non-native *Phragmites*.

Discussion

If performance differences between native and non-native plant lineages (e.g., growth rate, productivity, etc.) are primarily driven by their root microbial communities, then we would expect that (1) bacterial, fungal, and oomycete communities will be differentially assembled, comprised of different taxa abundances, OTU abundances, or colonization rates in native and non-native *Phragmites* roots and (2) non-native *Phragmites* roots will associate with relatively fewer microbial taxa capable of pathogenesis and relatively more microbial taxa capable of mutualistic interactions than native *Phragmites* roots. Despite a thorough examination of the root microbial community encompassing three of the major microbial groups (bacteria, fungi, and oomycetes), we could find no evidence to support the hypothesis that performance differences between native and non-native *Phragmites* are driven by their root microbial communities alone. With the

exception of relative abundance of a few bacterial genera and the extent of fungal colonization, plant lineage did not predict root microbial community structure or composition in our study. Instead, site, and specifically soil saturation level, was a stronger predictor of the identity of microorganisms that endophytically colonized roots. Additionally, there was no evidence for functional differences in the root microbial communities that could explain performance advantages observed in the non-native lineage. Consequently, it is unlikely that the invasive capacity of non-native *Phragmites* arises due to a greater abundance of mutualists or a lower abundance of pathogens in roots.

For bacterial root endophytes, soil saturation level was a strong predictor of community composition wherein we observed different bacterial communities under saturated and unsaturated conditions. However, soil saturation only modestly affected the relative abundance of bacterial groups by respiratory requirement or taxonomy. *Pseudomonas*, the most common genus recovered, were slightly less abundant in high water than in unsaturated patches (Appendix S2: Fig. S2.6). Most of the sequences recovered were from aerobic taxa, and the abundance of aerobes did not differ with saturation. Facultative anaerobes were affected by soil saturation level, but they made up a much smaller proportion of bacterial sequences (Appendix S2: Table S2.7, Fig. S2.6). Given that ventilation efficiency differs dramatically between native and non-native *Phragmites* (Tulbure et al. 2012), one might expect to see differences in microbial communities based on respiratory mode. However, the discrepancies in ventilation did not appear to affect endophytic colonization. It may be that, while rhizosphere oxygen concentrations likely depend on ventilation and differ in the two lineages, internal root tissues can remain sufficiently aerobic due to the existence of aerenchyma, despite the differences in surrounding soil. Bacterial endophytes mainly colonize roots from the rhizosphere (Hardoim et al. 2008) or plant litter (Ryan et al. 2008). Thus,

while there may be differences in the rhizosphere bacterial communities between the two lineages (Bowen et al. 2017) and those differences may be in part influenced by ventilation, the broadly habitable, aerobic internal root may select for aerobes among the community of bacteria in the rhizosphere.

For fungal endophytes, soil saturation was also important, influencing colonization rates, community composition, alpha diversity, as well as the relative abundance of many common genera (Appendix S2: Table S2.5). As with bacteria, the soil environment may act as a barrier for colonization. High water levels creating anaerobic soil conditions would make it difficult for germinating spores of AMF or other aerobic filamentous fungi to reach the root surface. While fungal colonization was higher in non-native *Phragmites* roots overall, colonization rate did differ by soil saturation with the largest differences found in unsaturated conditions (Fig. 2.1). Fungal root colonization in native and non-native *Phragmites* lineages has been investigated previously (Holdredge et al. 2010) with no consistent or statistical difference appearing between lineages. That study was conducted in a coastal tidal marsh that likely experiences a high degree of saturation, which may have affected the colonization rates similarly to those in our study. No arbuscules or vesicles were identified in any samples and the relative abundance of Glomeromycota taxa in our samples was extremely low. Although we note the deficiencies of ITS primers in detecting Glomeromycota (Stockinger et al. 2010), given the low levels of AMF sequences recovered in our samples and the results of the FUNGuild analysis, the fungal hyphae observed in our microscopy analysis were likely endophytes or root pathogens rather than AMF mutualists.

The only consistent microbial community difference between native and non-native *Phragmites* was found in oomycete diversity, with the non-native *Phragmites* roots hosting more

diverse oomycete pathogens. While pathogen diversity is not the same as pathogen load, we might expect that the trend would be in the opposite direction to agree with classical enemy release dynamics. Additionally, we did not observe any difference in overall communities of oomycetes between plant lineages that would suggest native and non-native *Phragmites* roots are differentially colonized by oomycete pathogens. Nelson and Karp (2013) compared oomycete communities between native and non-native *Phragmites* and found no overall difference in oomycete richness between plant lineages but discovered higher richness in the native lineage at two of their four sites. Although our results are thus inconsistent with previous findings (Nelson and Karp 2013), they are consistent with the suggestion by Allen et al. (2018) that non-native *Phragmites* attracts more generalist soil pathogens than the native *Phragmites.* Importantly, richness reveals very little about pathogen load or pressure and it is still possible that the two lineages are experiencing different levels of pathogen pressure given differences in pathogen virulence on each plant lineage or surrounding plants (Crocker et al. 2015, Allen et al. 2018).

Functional potential of endophytic bacteria is difficult to determine (Hardoim et al. 2008). While others have found specific endophytic bacteria that provide stress tolerance to invasive *Phragmites* (White et al. 2018), functional determinations often come from culture studies that can measure plant growth response to individual inoculations. Without using an experimental approach, functional potential can be approximated by looking at specific genes (Hardoim et al. 2008), but that requires mapped genomes of OTUs found in the study, which can be difficult to obtain. We instead relied on community and respiratory mode differences between lineages to infer functional differences. We found no such differences between the native and non-native lineages within the roots.

While our study found no differences in root microbial communities of native and non-native *Phragmites,* the complete role of microbes in *Phragmites'* invasiveness is likely much more complex. For example, important microbial community differences driving plant performance differences could exist elsewhere in the phyllosphere or in the rhizosphere. In fact, Bowen et al. (2017) found strong rhizosphere bacteria differences between *Phragmites* lineages, and rhizosphere fungal and oomycete community differences have not yet been studied on a large scale. Additionally, the plant response to microbes could be lineage specific. We know that some oomycetes have lineage-specific virulence in *Phragmites* (Crocker et al. 2015), and that the roles of many endophytic fungi and bacteria are plant and genotype specific (Schulz and Boyle 2005, Hardoim et al. 2008). Therefore, plant responses to microbial communities as well as community differences are important to definitively understand if microbial communities are driving invasiveness in *Phragmites*. Finally, we recognize the limitations of this study in that it relies heavily on publicly available databases of microbial taxonomy (SILVA, NCBI, UNITE) and function (FUNguild, BugBase). While these databases are improving, analyses derived from them are not definitive, but should provide useful trends in functional potential (Nguyen et al. 2015).

Conclusions

We found little evidence of differences in the *Phragmites* root microbiome between lineages at our sites. There was also no evidence that invasive *Phragmites* associated with relatively more mutualist or relatively fewer pathogen microbes than the native *Phragmites*. Thus, it is unlikely that root microbial communities are driving *Phragmites* invasions at these sites. Future studies should explore microbial communities in the rhizosphere or elsewhere in the phyllosphere, as well as differential response to microbes in native and non-native *Phragmites* as potential mechanisms for invasiveness.

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Tables

Table 2.1: Community differences by site and lineage in all microbial groups via PerMANOVA

	Fungi		**Bacteria**		**Oomycetes**	
	Pseudo - F	*P*	Pseudo - F	*P*	Pseudo - F	*P*
Lineage	1.06	0.438	1.24	0.298	1.10	0.389
Site	**1.68**	**<0.001**	**2.43**	**<0.001**	**1.24**	**0.088**
Lineage x Site	0.96	0.616	**1.14**	**0.050**	1.22	0.109

Table 2.2: Environmental fit of Soil Parameters to PCoA ordinations of microbial community compositions

	Fungi		**Bacteria**		**Oomycetes**	
	r^2	*P*	r^2	*P*	r^2	*P*
Soil N	0.096	0.179	**0.309**	**0.004**	**0.135**	**0.097**
Soil P	0.067	0.334	0.049	0.338	0.100	0.193
Saturation	**0.399**	**0.001**	**0.492**	**0.001**	**0.166**	**0.046**

Figures

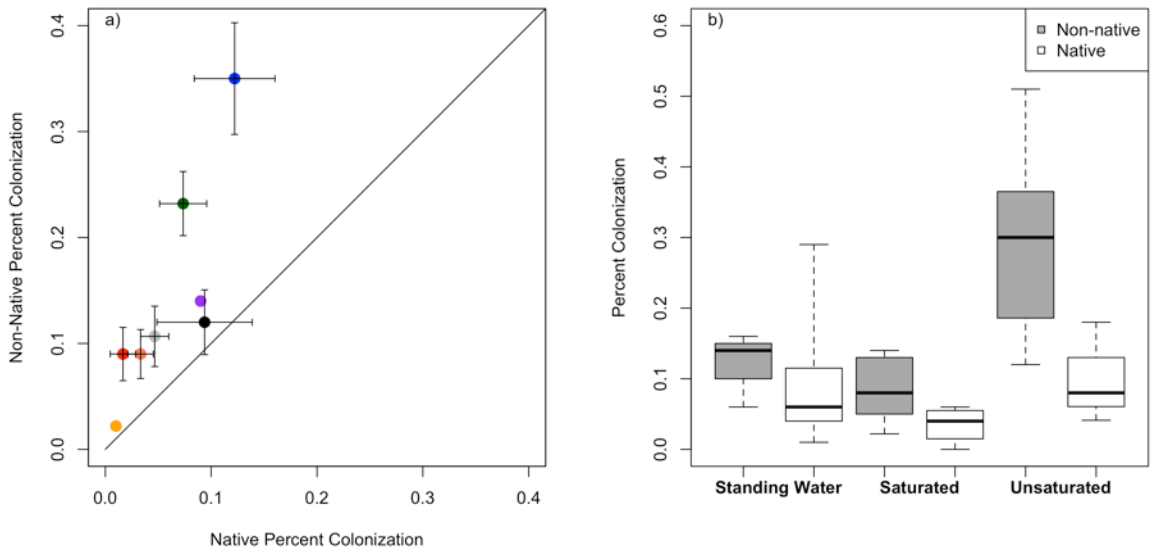

Fig. 2.1: **a)** Percent fungal colonization of roots of Native and Non-native *Phragmites*. Each point represents the average colonization at a site in the non-native patches relative vs. the native patches. Error bars indicate standard error. Diagonal line represents equal colonization among plant lineages. Colors represent sites (blue = BL, red = CB, green = CH, coral = CM, black = CR, brown = PLB, gray = Rt2, purple = SB). **b)** Effects of soil saturation on fungal colonization. Black lines indicate the median; bars indicate the full spread of data.

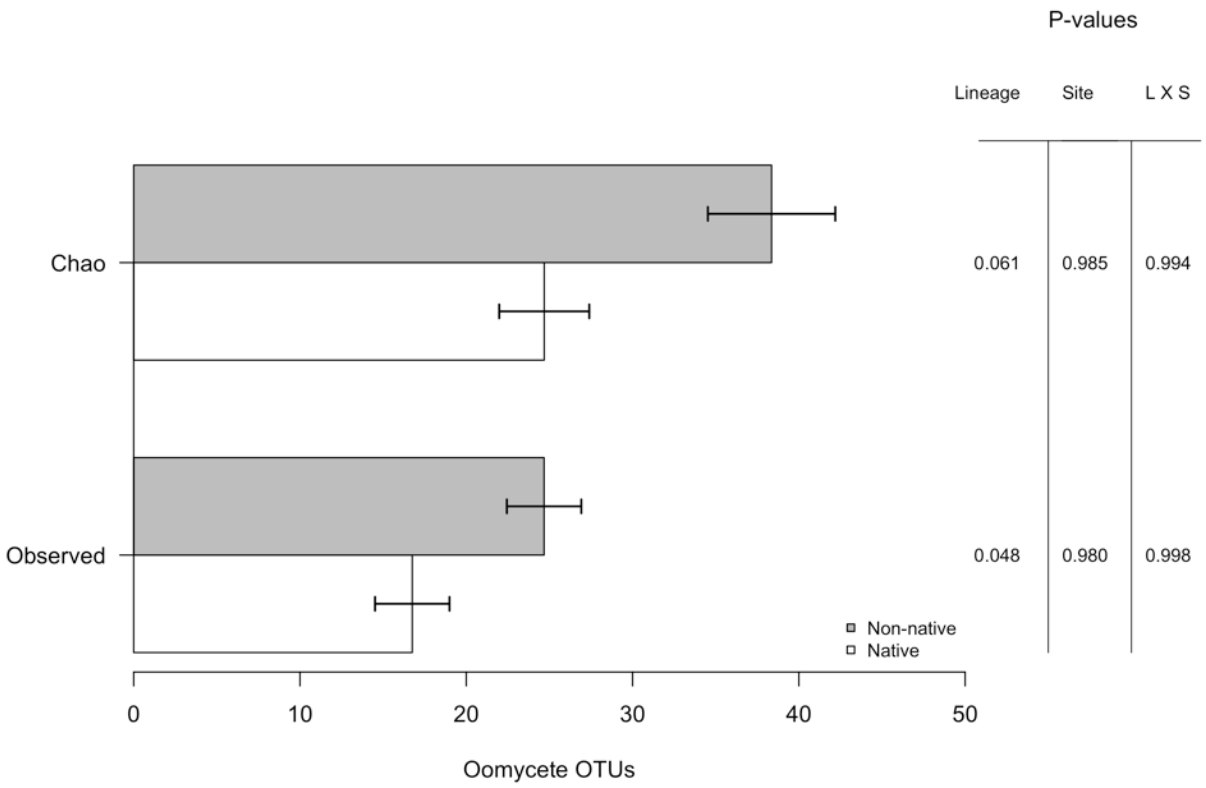

Fig. 2.2: Oomycete Chao Richness and Observed OTUs on Native and Non-Native *Phragmites* Roots. Bars indicate standard error. Significance calculated using ANOVA with Type III Sum of Squares.

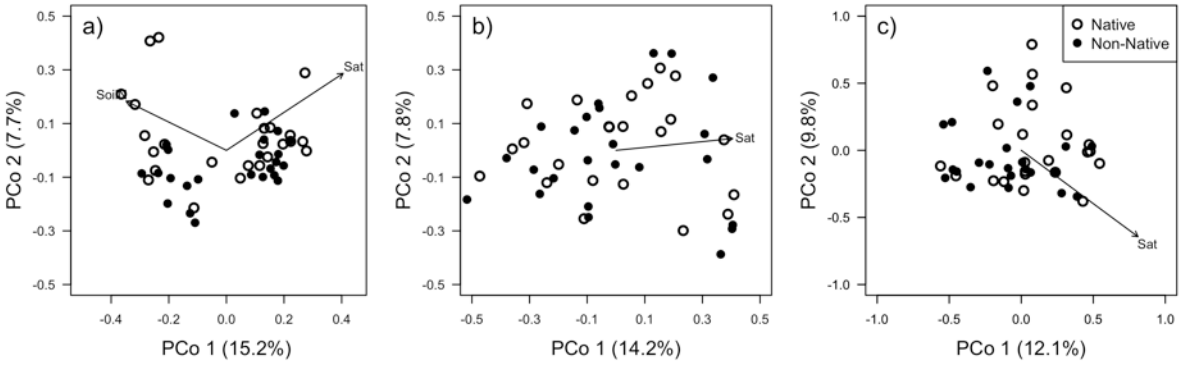

Fig. 2.3: Principle coordinate analyses (PCoA) of **a)** bacterial, **b)** fungal, and **c)** oomycete communities. Points represent the microbial community within a single root sample. Vectors displayed are environmental fit of variables (Soil N, Soil P, and Soil Saturation) to ordination space significant at the a = 0.10 level (See Table 2.2 for *P*-values)

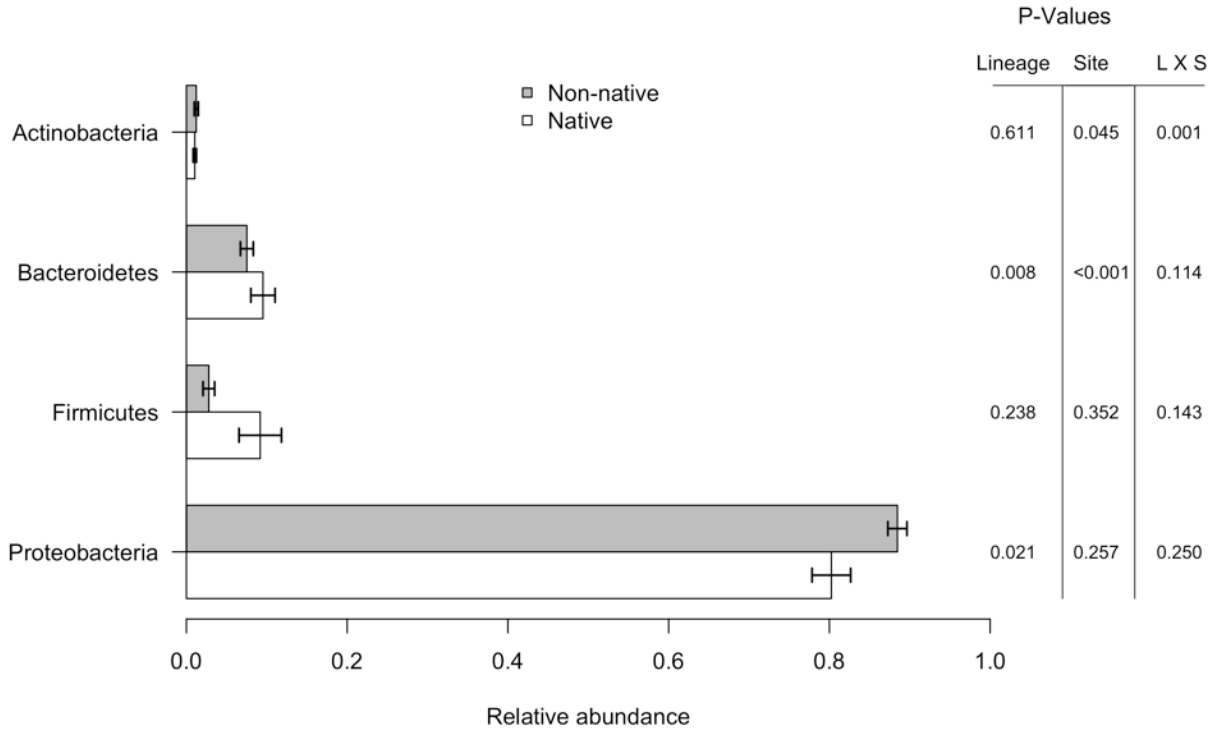

Fig. 2.4: Relative sequence abundance of major bacterial phyla in native and non-native samples. All samples were rarified to a consistent number of sequences (459) making relative abundances comparable. Error bars represent standard error. Significance calculated using ANOVA with Type III Sum of Squares.

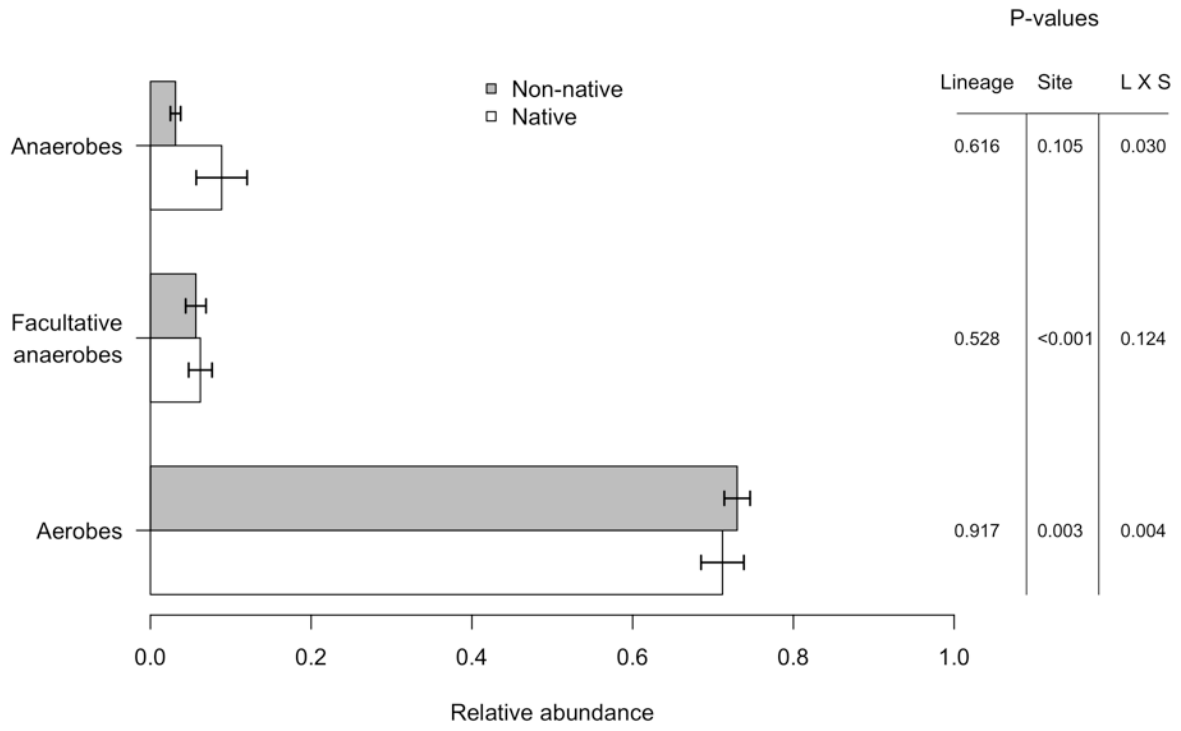

Fig. 2.5: Relative abundance of bacterial classes based on oxygen requirements compared by plant lineage**.** Oxygen-use classes determined using BugBase. Error bars indicate standard error of the mean. Significance calculated using ANOVA with Type III Sum of Squares.

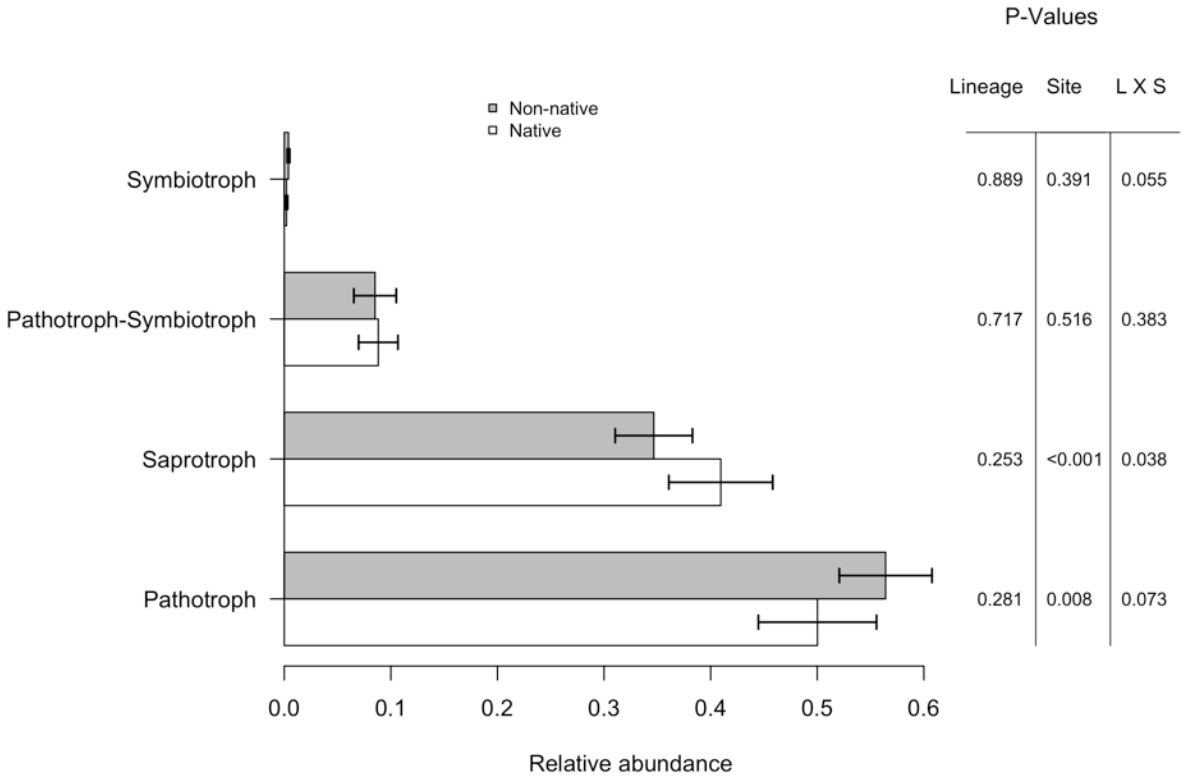

Fig. 2.6: Relative abundance of trophic modes based on FUNguild determinations. Error bars represent standard error of the mean. Significance calculated using ANOVA with Type III Sum of Squares.

Appendix S2

Table S2.1: Site characteristics and environmental variables

Site	Coordinates	Soil Map Unit	Coastal or Inland	No. Samples	Saturation Levels (# of each)	Soil C (%) mean (range)	Soil N (%) mean (range)	Soil P (mg/kg) mean (range)	Tissue C (%) mean (range)	Tissue N (%) mean (range)	Tissue P (%) mean (range)
Bullard Lake Fen (BL)	42˚38'36"N 83˚42'10"W	Houghton Muck	Inland	12	Unsaturated (6)	28.37	1.76	16.84	38.00	2.23	0.15
					Saturated (6)	(18.26 - 38.80)	(1.03 - 2.73)	(0.37 - 86.84)	(12.41 - 43.90)	(0.78 - 3.24)	(0.05 - 0.20)
Chelsea Farm (CH)	42˚18'32"N 84˚03'25"W	Houghton Muck	Inland	12	Unsaturated (9)	10.97	0.60	1.99	41.49	2.32	0.15
					Saturated (3)	(2.87 - 32.70)	(0.08 - 2.13)	(0.34 - 5.64)	(15.35 - 44.91)	(0.88 - 3.54)	(0.03 - 0.24)
Cheboygan Marsh (CM)	45˚39'27"N 84˚28'16"W	Histosols and Aquents	Coastal	6	Saturated (1)	7.17	0.32	3.73	41.78	2.07	0.11
					High Water (5)	(1.37 - 12.61)	(0 - 0.61)	(0.21 - 11.50)	(40.20 - 42.90)	(1.51 - 2.60)	(0.09 - 0.13)
Cecil Bay (CB)	45˚44'52"N 84˚50'57"W	Stony Lake Beach	Coastal	6	High Water (6)	4.97 (0.88 - 16.54)	0.23 (0 - 0.85)	1.60 (0.70 - 4.35)	42.39 (41.03 - 44.00)	2.01 (1.66 - 2.56)	0.11 (0.09 - 0.15)
Sturgeon Bay (SB)	45˚42'30"N 84˚56'46"W	Sandy Lake Beach	Coastal	2	Saturated (2)	1.32 (0.79 - 1.85)	0.05 (0.02 - 0.07)	1.74 (1.47 - 2.01)	42.79 (42.59 - 43.00)	1.37 (1.30 - 1.44)	0.07 (0.06 - 0.07)
Point Le Barb (PLB)	45˚50'51"N 84˚44'28"W	Histosols and Aquents	Coastal	2	Saturated (1)	8.35	0.38	1.29	41.78	2.05	0.12
					High Water (1)	(5.15 - 11.56)	(0.20 - 0.55)	(1.09 - 1.50)	(40.45 - 43.12)	(1.70 - 2.40)	(0.11 - 0.13)
Pointe aux Chenes (Rt2)	45˚54'46"N 84˚52'20"W	Leafriver mucky peat	Coastal	6	High Water (6)	0.86 (0.46 - 1.74)	0.02 (0 - 0.07)	2.99 (1.16 - 6.66)	43.57 (42.95 - 44.14)	1.69 (0.69 - 2.24)	0.08 (0.03 - 0.12)
Castle Rock (CR)	45˚54'39"N 84˚44'18"W	Eastport-Leafriver complex	Inland	6	Saturated (6)	12.58 (3.68 - 23.41)	0.63 (0.16 - 1.29)	12.64 (0.07 - 45.83)	32.57 (9.88 - 43.59)	1.59 (0.51 - 2.46)	0.07 (0.01 - 0.12)

Table S2.2: PCR Conditions and Primer Sequences

Primer Set	Primer	Primer Sequence	Barcode Length	PCR Mastermix	PCR Conditions	Reference
Fungi	ITS1F	5'-CTTGGTCATTTAGAGGAAGTAA-3'	16 bp	2.5 uL 10x Buffer* 0.5 uL BSA 0.5 uL 20uM dNTPs 0.625 uL 20uM ITS1F 0.625 uL 20uM ITS4 0.5 uL Taq* 2 uL Template DNA** 17.75 uL H2O	Initial denaturation: 94℃ for 2 min, 25 cycles, denaturation: 94℃ for 30 s, annealing: 55℃ for 30 s, extension: 72℃ for 45 s (2 min final extension)	White et al. 1990, Gardes and Bruns 1993
	ITS4	5'-TCCTCCGCTTATTGATATGC-3'	16 bp			
Bacteria	27F	5'-AGAGTTTGATCMTGGCTCAG-3'	16 bp	2.5 uL 10x Buffer* 0.5 uL BSA 0.5 uL 20uM dNTPs 0.5 uL 20uM 27F 0.5 uL 20uM 519R 0.5 uL Taq* 2 uL Template DNA** 18 uL H2O	Initial denaturation: 94℃ for 5 min, 25 cycles, denaturation: 94℃ for 30 s, annealing: 55℃ for 60 s, extension: 72℃ for 90 s (10 min final extension)	Lane 1991
	519R	5'-GWATTACCGCGGCKGCTG'3'	16 bp			
Oomycetes (First round)	5.8 SR	5'-TCGATGAAGAACGCAGCG -3'	-	2.5 uL 10x Buffer* 0.5 uL BSA 0.5 uL 20uM dNTPs 0.625 uL 20uM 5.8 SR 0.625 uL 20uM LR7 0.5 uL Taq* 2 uL Template DNA** 17.75 uL H2O	Initial denaturation: 94℃ for 5 min, 30 cycles, denaturation: 94℃ for 30 s, annealing: 47℃ for 90 s, extension: 72℃ for 60 s (10 min final extension)	Vilgalys and Hester 1990
	LR7	5'-TACTACCACCAAGATCT-3'	-			
Oomycetes (Second Round)	Oom1f	5'-GTGCGAGACCGATAGCGAACA-3'	16 bp	2.5 uL 10x Buffer* 0.5 uL 20uM dNTPs 0.625 uL 20uM Oom1f 0.625 uL 20uM Oom1r 0.5 uL Taq* 1 uL Template DNA** 19.25 uL H2O	Initial denaturation: 94℃ for 5 min, 30 cycles, denaturation: 94℃ for 30 s, annealing: 58.4℃ for 30 s, extension: 72℃ for 30 s (10 min final extension)	Arcate et al. 2006
	Oom1r	5'-TCAAAGTCCCGAACAGCAACAA-3'	16 bp			

*Roche Expand High Fidelity PCR System
**DNA was diluted to 5-10 ng per reaction

Table S2.3: Comparisons of environmental characteristics of patches by lineage. Continuous variables compared using t-test; categorical using chi squared test.

	Native mean	Non-native mean	Coefficient	P-value
Soil N	0.52 %	0.63 %	T = 0.492	0.623
Soil P	3.45 mg / kg	8.98 mg / kg	T = 1.089	0.29
Soil Saturation	--	--	χ^2 **= 11.99**	**0.005**

Table S2.4: Results of 2-way ANOVA (Site x Lineage) for selected fungal response variables. Alpha diversity, community composition, phylum relative abundance, and genus relative abundance included. Bold indicates significance at the $\alpha<0.05$ level. Italics indicates significance at the $\alpha<0.1$ level.

		Lineage		Site		Site X Lineage	
	df	F	P	F	P	F	P
Colonization	35	**24.57**	**<0.001**	**6.52**	**<0.001**	1.78	0.122
Shannon	31	0.02	0.894	1.58	0.178	0.80	0.596
Chao	31	0.14	0.708	*2.00*	*0.09*	0.76	0.621
Sobs	31	0.08	0.785	1.49	0.208	1.00	0.450
PCoA Axis 1	31	0.68	0.416	**5.08**	**<0.001**	1.14	0.362
PCoA Axis 2	31	0.18	0.678	**2.56**	**0.033**	0.59	0.761
Asco RA	31	0.34	0.566	*2.16*	*0.066*	1.38	0.250
Basio RA	31	0.19	0.669	1.27	0.295	1.03	0.430
Unclass RA	31	0.00	0.945	*1.95*	*0.09*	0.36	0.919
Zygomycota RA	31	1.13	0.296	0.66	0.704	0.62	0.735
Rozellomycota RA	31	0.00	1.000	0.53	0.801	1.25	0.306
Glomero RA	31	0.84	0.366	1.35	0.262	**2.96**	**0.017**
Gib RA	31	0.58	0.452	**2.64**	**0.029**	0.61	0.746
Micro RA	31	0.03	0.862	0.86	0.548	0.88	0.536
Tetracladium RA	31	0.23	0.638	1.42	0.234	1.25	0.304
Stagonospora RA	31	0.84	0.368	**3.55**	**0.006**	0.38	0.907
Cadophora RA	31	0.45	0.509	0.56	0.781	0.78	0.607
Path RA	31	1.20	0.281	**3.45**	**0.008**	2.10	0.073
Sap RA	31	1.36	0.253	**5.00**	**<0.001**	**2.48**	**0.038**
Path-Sym RA	31	0.13	0.717	0.90	0.516	1.11	0.383
Symbiotroph RA	31	0.02	0.889	1.09	0.391	*2.27*	*0.055*

Table S2.5: Results of ANCOVA for selected fungal response variables. Variables with a significant site effect in Table S2.4 were included for ANCOVA analysis with environmental variables. Bold indicates significance at the $\alpha<0.05$ level. Italics indicates significance at the $\alpha<0.1$ level.

		r2			Lineage		Saturation		Soil P		Soil N		Sat:Lin	
	Residual df	Multiple r2	Adjusted r2	P	F	P	F	P	F	P	F	P	F	P
Colonization	31	**0.7073**	**0.6601**	**<0.001**	**19.97**	**<0.001**	**23.47**	**<0.001**	29.67	<0.001	1.24	0.274	0.58	0.454
Shannon	31	0.1347	-0.0048	0.454	0.16	0.685	**4.26**	**0.047**	0.07	0.790	0.10	0.756	0.22	0.638
Chao	31	0.2068	0.0789	0.185	2.09	0.158	**4.30**	**0.046**	0.80	0.378	0.22	0.646	0.67	0.418
Sobs	31	0.1319	-0.0081	0.468	1.24	0.275	2.52	0.123	0.42	0.524	0.11	0.741	0.43	0.517
PCoA Axis 1	31	**0.4133**	**0.3186**	**0.004**	0.04	0.840	**21.10**	**<0.001**	0.25	0.620	0.44	0.511	0.01	0.940
PCoA Axis 2	31	0.1315	-0.0085	0.470	2.16	0.152	0.01	0.935	0.10	0.753	2.13	0.155	0.29	0.591
Asco RA	31	0.1477	0.0103	0.393	0.35	0.558	2.72	0.109	1.72	0.200	0.00	0.965	0.58	0.452
Gib RA	31	0.2101	0.0827	0.177	0.91	0.347	**6.96**	**0.013**	0.31	0.583	0.05	0.823	0.02	0.901
Stag RA	31	0.2119	0.0848	0.172	0.06	0.804	**6.83**	**0.014**	0.04	0.837	0.37	0.547	1.02	0.321
Path RA	31	*0.2053`*	*0.1617*	*0.061*	0.53	0.471	**9.13**	**0.005**	1.76	0.194	0.51	0.480	0.01	0.944
Sap RA	31	**0.3817**	**0.2819**	**0.008**	0.74	0.396	**14.63**	**<0.001**	2.02	0.165	1.56	0.221	0.19	0.668

Table S2.6: Results of 2-way ANOVA (Site x Lineage) for selected bacterial response variables. Alpha diversity, community composition, phylum relative abundance, and genus relative abundance included. Bold indicates significance at the α<0.05 level. Italics indicates significance at the α<0.1 level.

		Lineage		Site		Site X Lineage	
	df	F	P	F	P	F	P
Shannon	33	1.83	0.185	**12.98**	**<0.001**	**2.32**	**0.046**
Chao	33	1.03	0.318	**2.84**	**0.020**	1.62	0.163
Sobs	33	1.72	0.198	**10.30**	**<0.001**	*2.20*	*0.060*
PCoA Axis 1	33	0.01	0.913	**44.76**	**<0.001**	1.19	0.338
PCoA Axis 2	33	**4.29**	**0.046**	1.55	0.185	1.32	0.274
Proteo RA	33	**5.86**	**0.021**	1.36	0.257	1.37	0.250
Firm RA	33	1.44	0.238	1.16	0.352	1.70	0.143
Bacteroidetes RA	33	**8.05**	**0.008**	**9.40**	**<0.001**	1.83	0.114
Actinobacteria RA	33	0.26	0.611	**2.36**	**0.045**	**4.46**	**0.001**
Pseudomonas RA	33	**7.16**	**0.012**	**5.55**	**<0.001**	**2.84**	**0.020**
Flavobacterium RA	33	0.95	0.337	**5.66**	**<0.001**	1.78	0.125
Janthinobacterium RA	33	0.30	0.590	*2.30*	*0.050*	0.35	0.924
Rhizobium RA	33	**5.83**	**0.021**	**7.68**	**<0.001**	1.60	0.70
Duganella RA	33	*3.18*	*0.084*	1.79	0.123	0.15	0.992
Trichococcus RA	33	0.91	0.346	1,72	0.139	1.69	0.147
Aeromonas RA	33	1.73	0.197	*1.99*	*0.086*	1.38	0.245
Aerobe RA	33	0.01	0.917	**3.88**	**0.003**	**3.85**	**0.004**
Facultative Anaerobe RA	33	0.41	0.528	**4.88**	**<0.001**	1.78	0.124
Anaerobe RA	33	0.226	0.616	1.88	0.105	**2.60**	**0.030**

Table S2.7: Results of ANCOVA for selected bacterial response variables. Variables with a significant site effect in Table S2.6 were included for ANCOVA analysis with environmental variables. Bold indicates significance at the $\alpha<0.05$ level. Italics indicates significance at the $\alpha<0.1$ level.

			r2		Lineage		Saturation		Soil P		Soil N		Sat:Lin	
	Residual df	Multiple r2	Adjusted r2	P	F	P	F	P	F	P	F	P	F	P
Shannon	32	0.189	0.0623	0.220	0.04	0.841	2.57	0.119	1.15	0.290	*3.48*	*0.071*	0.21	0.650
Chao	32	0.1677	0.0376	0.293	2.33	0.137	0.28	0.601	2.19	0.149	1.63	0.211	0.02	0.877
Sobs	32	0.164	0.0334	0.307	0.04	0.840	1.43	0.240	2.03	0.164	2.73	0.108	0.05	0.830
PCoA Axis 1	32	**0.4889**	**0.4091**	**<0.001**	0.01	0.910	**26.37**	**<0.001**	0.53	0.471	*3.22*	*0.082*	0.48	0.494
PCoA Axis 2	32	**0.3694**	**0.2709**	**0.009**	**4.92**	**0.034**	**4.62**	**0.039**	0.32	0.574	**7.47**	**0.010**	1.41	0.244
Proteo RA	32	0.1479	0.0147	0.374	*3.89*	*0.057*	0.36	0.555	0.13	0.722	1.16	0.290	0.02	0.891
Firm RA	32	0.1334	-0.002	0.442	0.85	0.363	0.05	0.823	0.34	0.565	*3.09*	*0.088*	0.60	0.444
Bacteroidetes RA	32	0.1312	-0.005	0.453	*2.99*	*0.094*	1.39	0.247	0.05	0.822	0.26	0.615	0.14	0.706
Actinobacteria RA	32	0.122	-0.015	0.500	0.00	0.987	0.04	0.834	0.03	0.856	0.23	0.631	*4.13*	*0.050*
Pseudomonas RA	32	0.1561	0.0242	0.339	1.74	0.197	*3.48*	*0.071*	0.33	0.570	0.37	0.547	0.00	0.959
Flavobacterium RA	32	0.114	-0.0245	0.542	1.67	0.206	1.90	0.177	0.00	0.957	0.01	0.920	0.53	0.472
Janthinobacterium RA	32	*0.2577*	*0.1418*	*0.076*	0.08	0.773	*3.66*	*0.065*	2.85	0.101	*4.01*	*0.054*	0.50	0.483
Rhizobium RA	32	**0.319**	**0.2126**	**0.025**	2.42	0.130	**9.58**	**0.004**	0.21	0.652	0.46	0.504	2.33	0.137
Duganella RA	32	0.1156	-0.0226	0.534	*3.12*	*0.087*	0.11	0.739	0.05	0.831	0.90	0.350	0.00	0.978
Trichococcus RA	32	0.1866	0.0595	0.228	1.28	0.267	0.58	0.453	0.31	0.584	*3.73*	*0.062*	1.45	0.237
Aeromonas RA	32	0.2406	0.122	0.101	2.06	0.161	1.33	0.257	0.37	0.546	**4.57**	**0.040**	1.80	0.189
Aerobe RA	32	0.1733	0.0413	0.272	0.12	0.729	2.37	0.133	0.37	0.549	*3.83*	*0.059*	0.02	0.884
Facultative Anaerobe RA	32	0.1324	-0.0032	0.447	0.01	0.943	*3.34*	*0.077*	0.08	0.781	0.28	0.603	1.18	0.285
Anaerobe RA	32	0.1558	0.0239	0.340	0.27	0.604	0.58	0.453	0.27	0.608	*3.56*	*0.068*	1.23	0.276

Table S2.8: Results of 2-way ANOVA (Site x Lineage) for selected oomycete response variables. Alpha diversity, community composition, and genus relative abundance included. Bold indicates significance at the α<0.05 level. Italics indicates significance at the α<0.1 level.

		Lineage		Site		Site X Lineage	
	df	F	P	F	P	F	P
Shannon	32	1.97	0.170	0.09	0.997	0.14	0.990
Chao	32	*3.76*	*0.061*	0.18	0.980	0.27	0.944
Sobs	32	**4.22**	**0.048**	0.16	0.985	0.08	0.998
PCoA Axis 1	32	1.44	0.239	1.47	0.219	**2.65**	**0.033**
PCoA Axis 2	32	0.48	0.492	1.03	0.422	0.72	0.637
Pithium RA	32	0.10	0.750	1.79	0.132	0.82	0.563
Uncultured RA	32	0.09	0.764	1.79	0.133	0.82	0.560
Phytopthora RA	32	0.78	0.384	0.69	0.656	1.11	0.376
Lagena RA	32	0.05	0.820	0.51	0.797	0.70	0.650
Saprolegnia RA	32	0.22	0.646	1.38	0.252	0.80	0.580
Albugo RA	32	0.49	0.491	0.80	0.580	0.80	0.580

Table S2.9: Results of ANCOVA for selected oomycete response variables. Variables with a significant site effect in Table S2.8 were included for ANCOVA analysis with environmental variables. Bold indicates significance at the $\alpha<0.05$ level. Italics indicates significance at the $\alpha<0.1$ level.

		r2		Lineage		Saturation		Soil P		Soil N		Sat:Lin	
	Residual df	Multiple r2	Adjusted r2	P	F	P	F	P	F	P	F	P	F	P
Shannon	29	0.1662	0.0224	0.354	1.84	0.185	0.86	0.363	2.14	0.154	0.41	0.525	0.53	0.474
Chao	29	0.2521	0.1232	0.116	**4.64**	**0.040**	0.16	0.691	**4.57**	**0.041**	0.40	0.532	0.00	0.949
Sobs	29	0.2545	0.126	0.126	**6.24**	**0.018**	0.25	0.621	2.73	0.109	0.68	0.416	0.00	0.946
PCoA Axis 1	29	*0.2902*	*0.1678*	*0.064*	**4.89**	**0.035**	*4.11*	*0.052*	1.05	0.314	0.93	0.343	0.87	0.358
PCoA Axis 2	29	0.1495	0.0028	0.424	0.01	0.938	1.93	0.176	0.20	0.656	2.62	0.116	0.34	0.565

Supplemental Figures

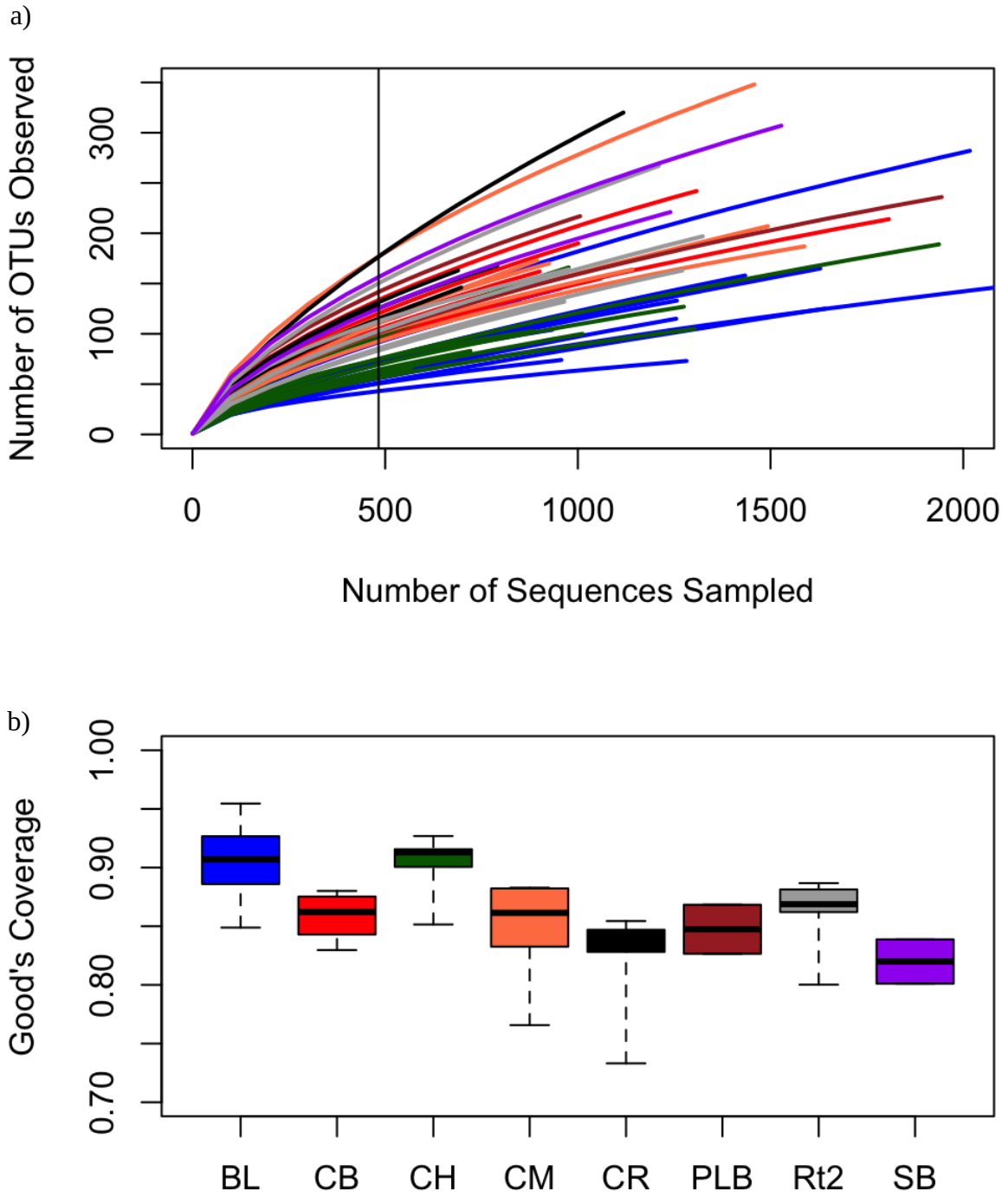

Fig. S2.1: a) Rarefaction curve and b) Good's Coverage by site for bacteria. Colors represent sites (blue = BL, red = CB, green = CH, coral = CM, black = CR, brown = PLB, gray = Rt2, purple = SB).

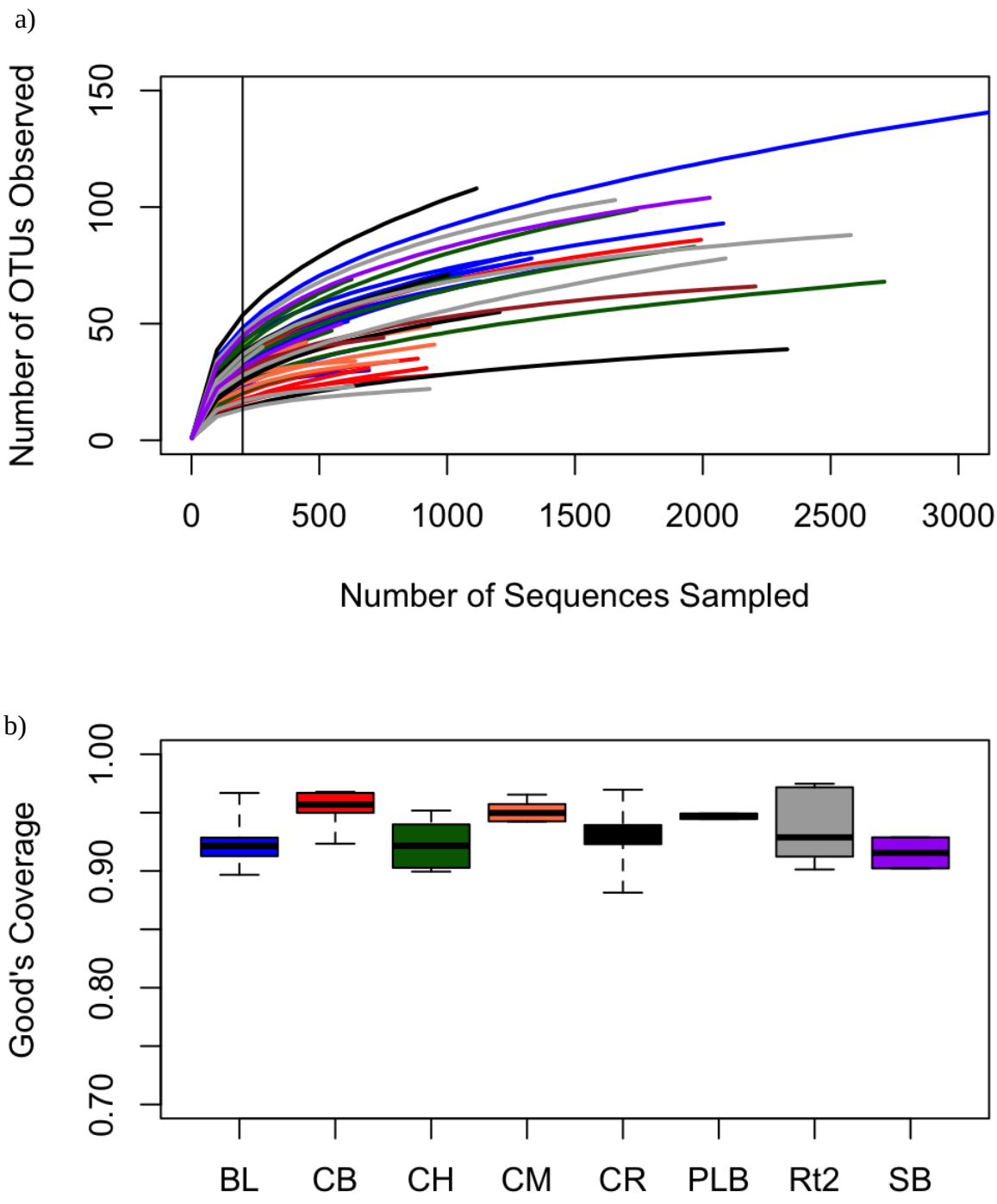

Fig. S2.2: a) Rarefaction curve and b) Good's Coverage by site for fungi. Colors represent sites (blue = BL, red = CB, green = CH, coral = CM, black = CR, brown = PLB, gray = Rt2, purple = SB).

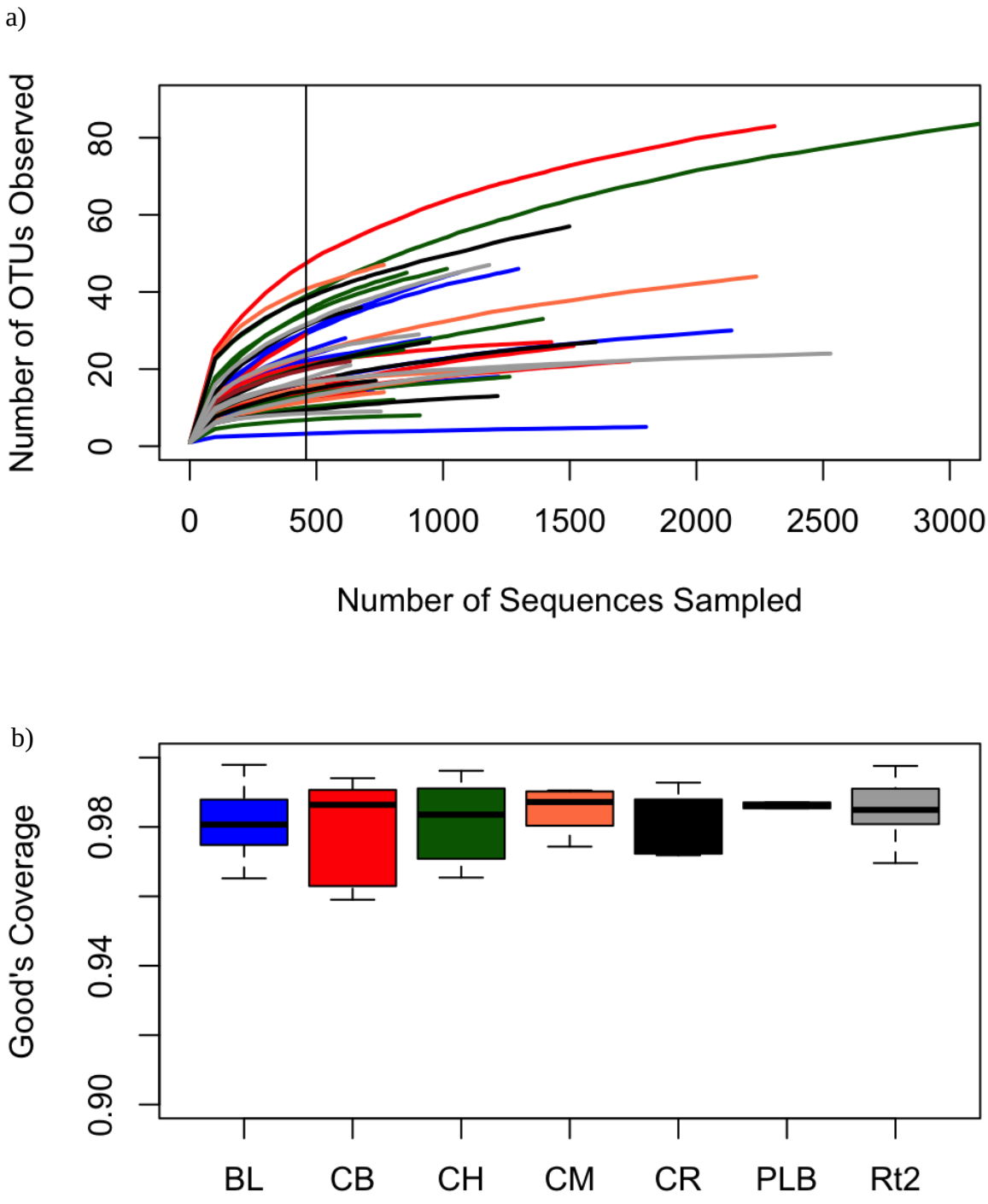

Fig. S2.3: a) Rarefaction curve and b) Good's Coverage by site for oomycetes. Colors represent sites (blue = BL, red = CB, green = CH, coral = CM, black = CR, brown = PLB, gray = Rt2, purple = SB).

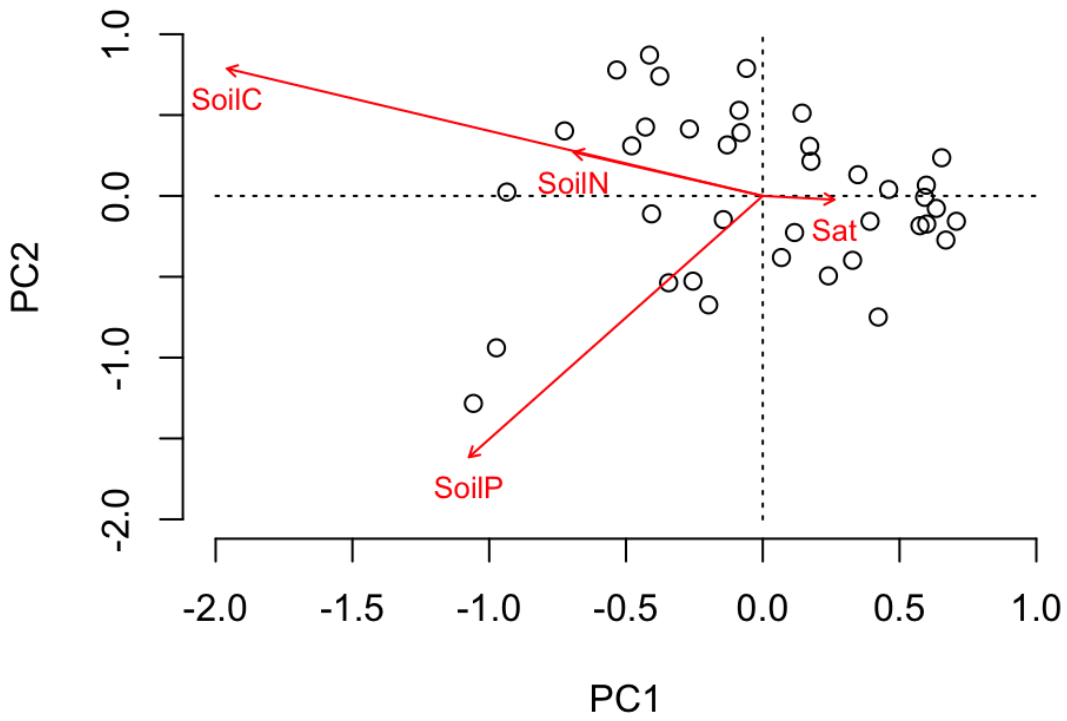

Fig. S2.4: Principle component analysis for environmental variables at all sampling sites Tissue nutrients not shown because there was low variability by sample (Table S2.1) .

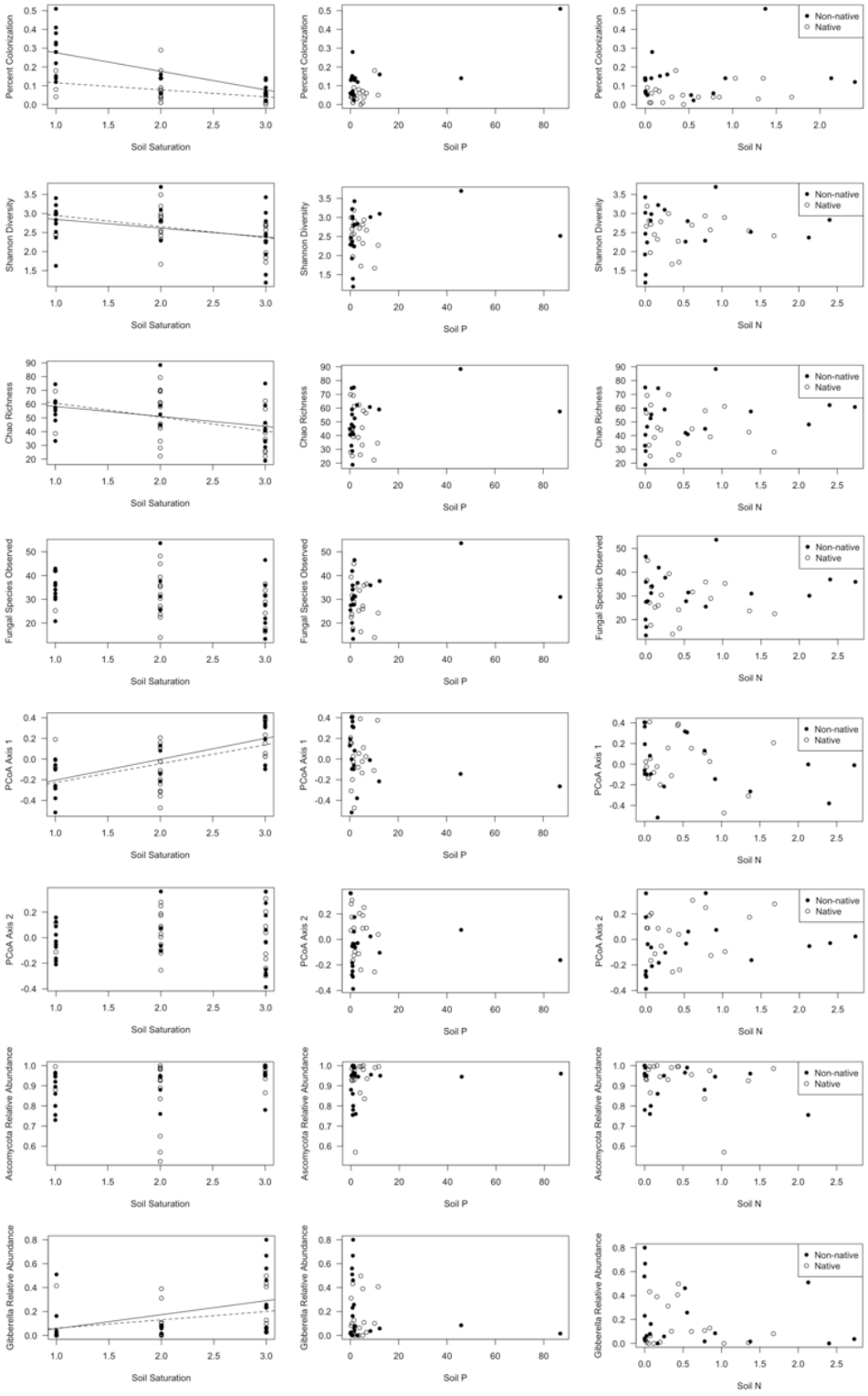

Fig. S2.5: Linear regressions of environmental variable against all Fungal response variables included in the ANCOVA analysis. Regression lines indicate significant relationship at the α<0.1 level.

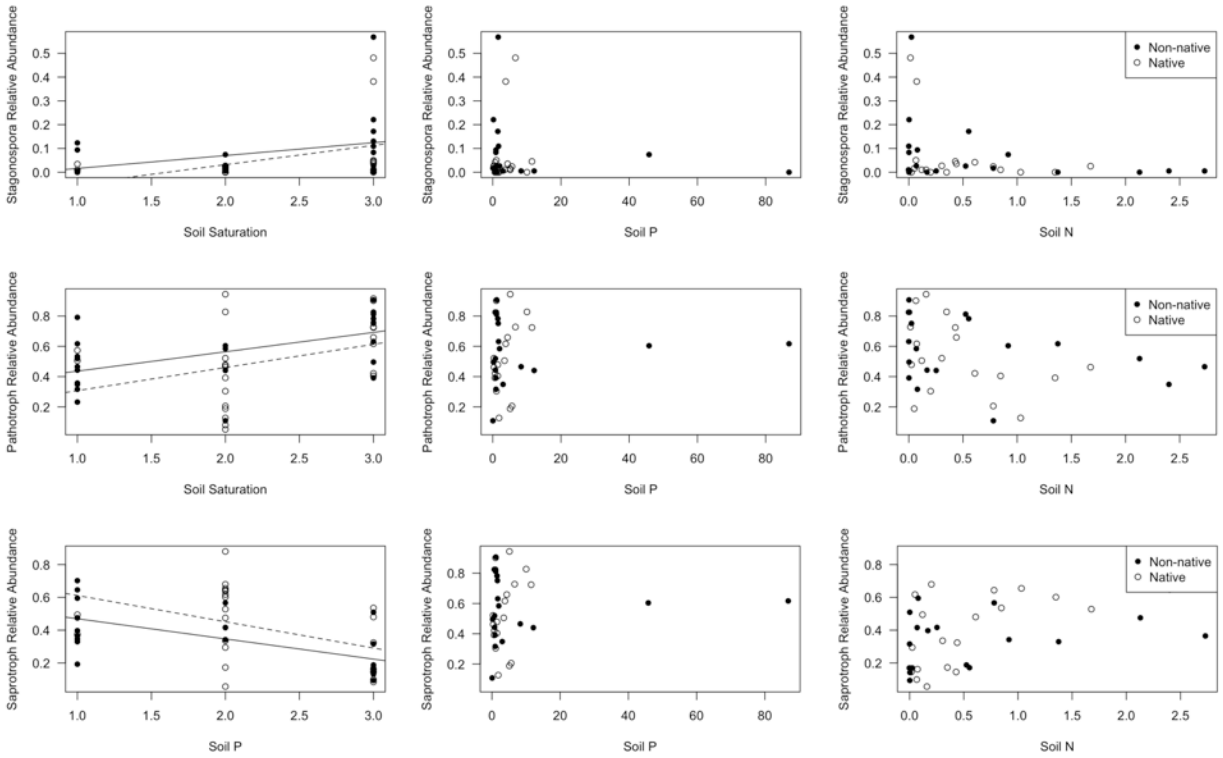

Fig. S2.5 (cont): Linear regressions of environmental variable against all Fungal response variables included in the ANCOVA analysis. Regression lines indicate significant relationship at the α<0.1 level.

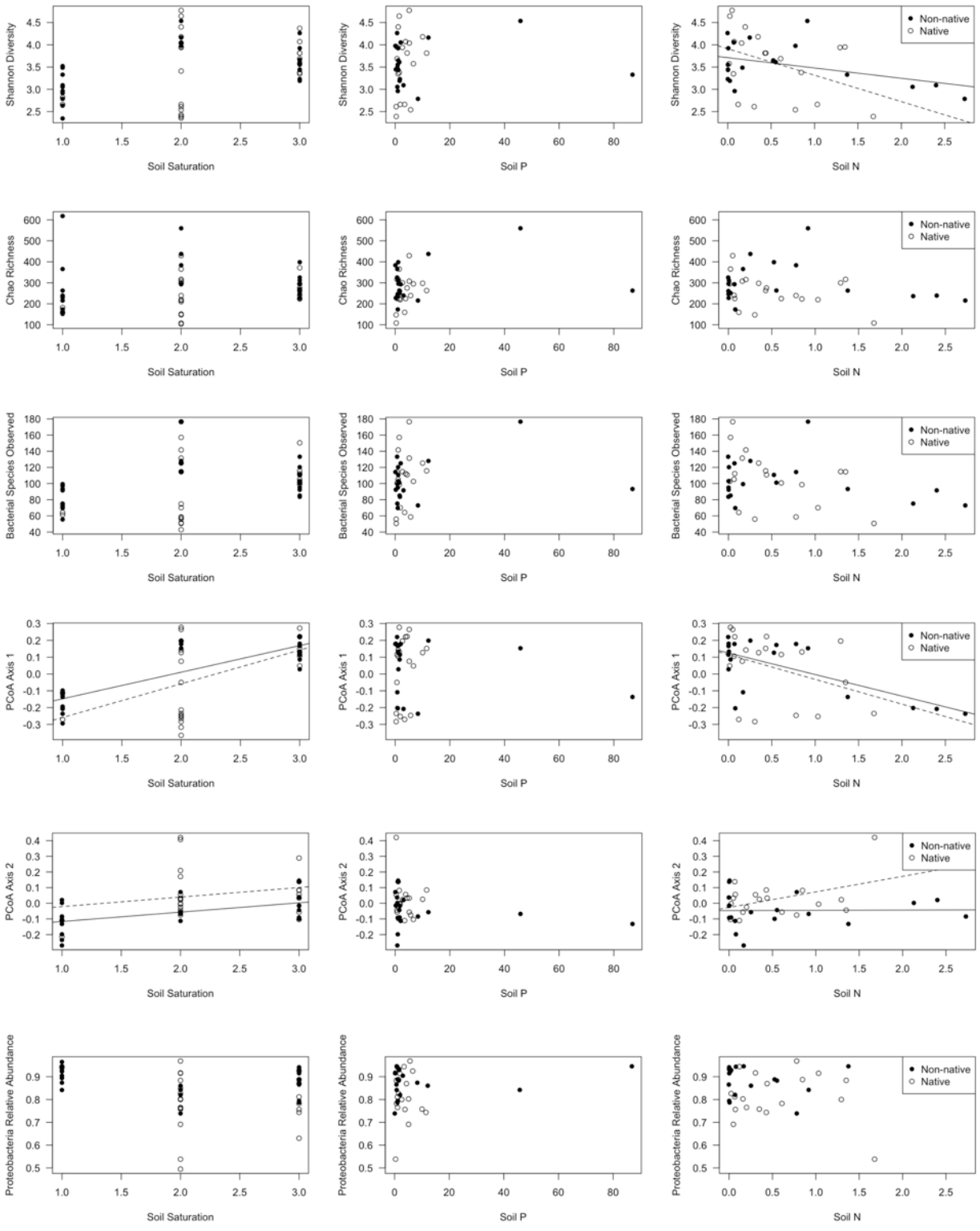

Fig. S2.6: Linear regressions of environmental variable against all bacterial response variables included in the ANCOVA analysis. Regression lines indicate significant relationship at the α<0.1 level.

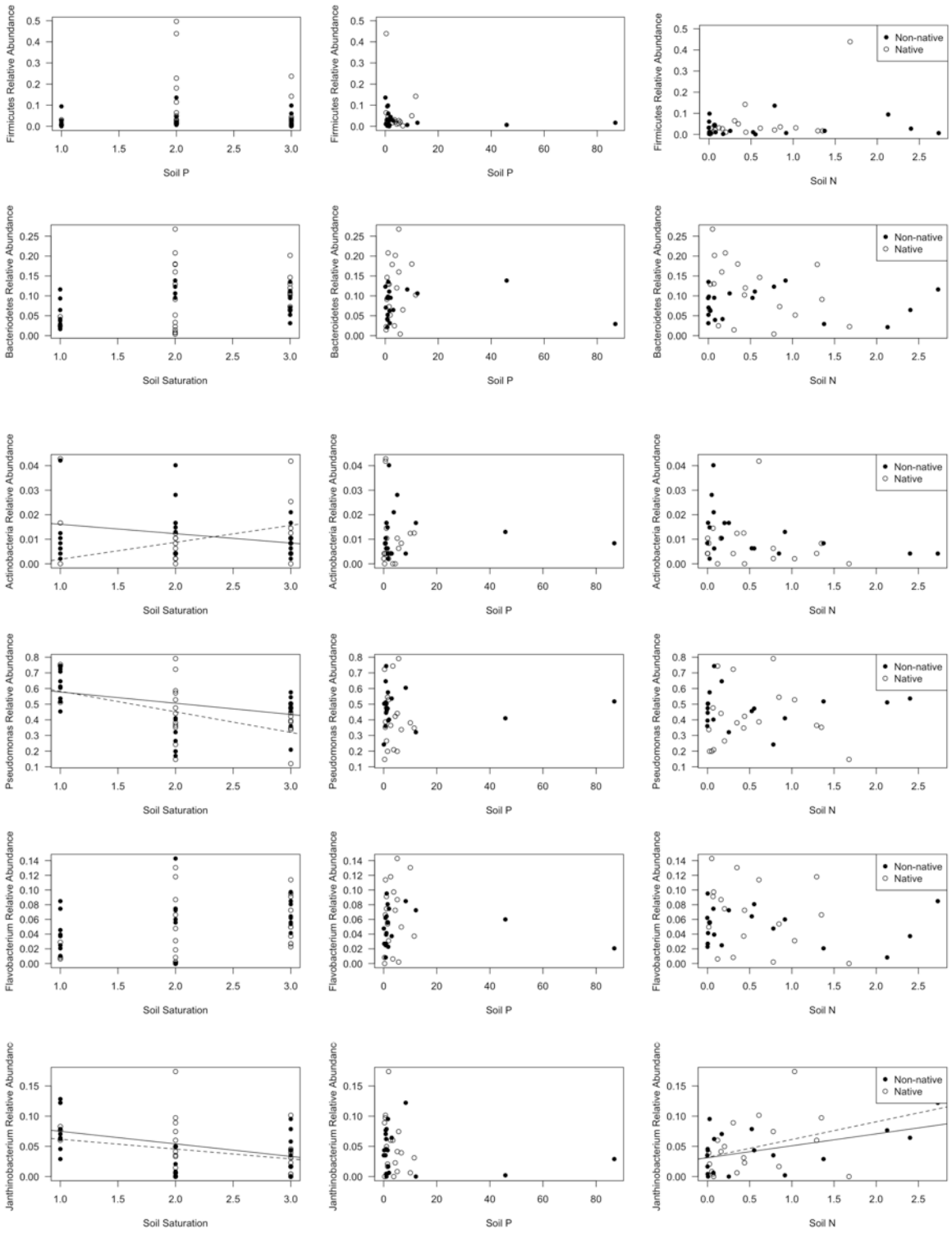

Fig. S2.6 (cont): Linear regressions of environmental variable against all bacterial response variables included in the ANCOVA analysis. Regression lines indicate significant relationship at the α<0.1 level.

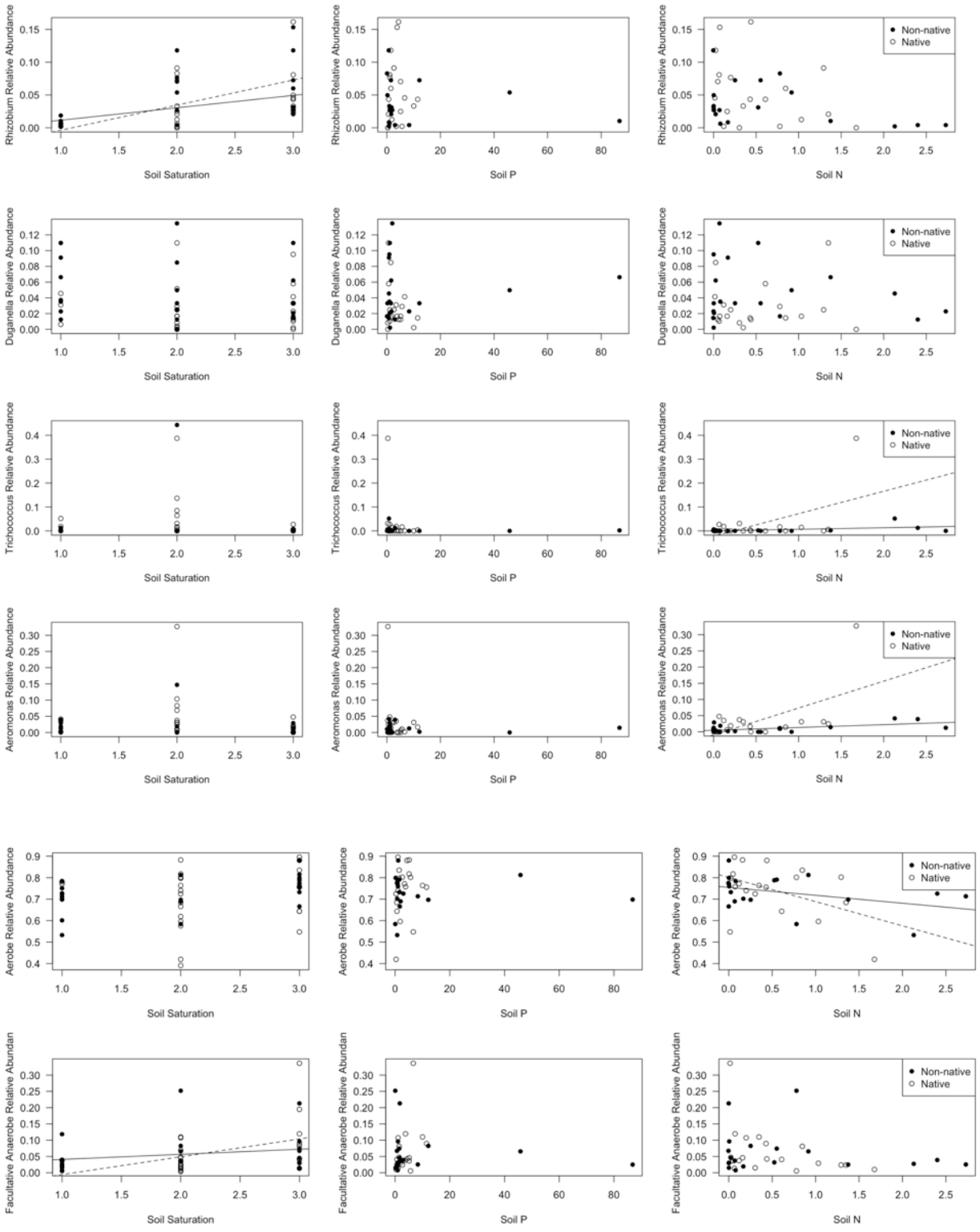

Fig. S2.6 (cont): Linear regressions of environmental variable against all bacterial response variables included in the ANCOVA analysis. Regression lines indicate significant relationship at the α<0.1 level.

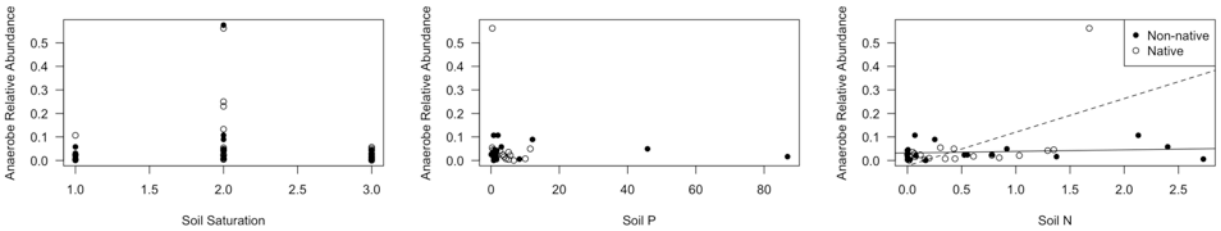

Fig. S2.6 (cont): Linear regressions of environmental variable against all bacterial response variables included in the ANCOVA analysis. Regression lines indicate significant relationship at the α<0.1 level.

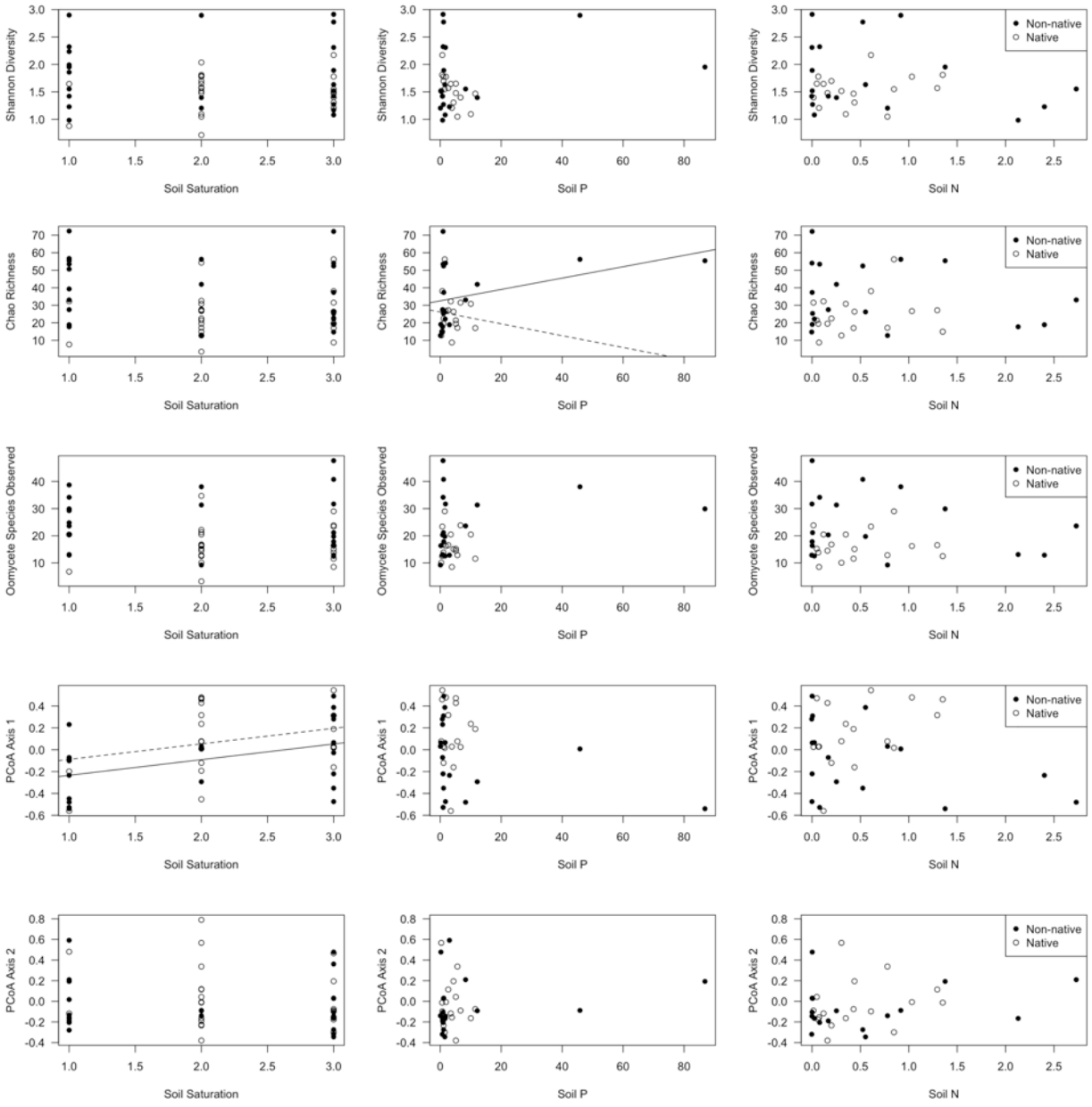

Fig. S2.7: Linear regressions of environmental variable against all oomycete response variables included in the ANCOVA analysis. Regression lines indicate significant relationship at the α<0.1 level.

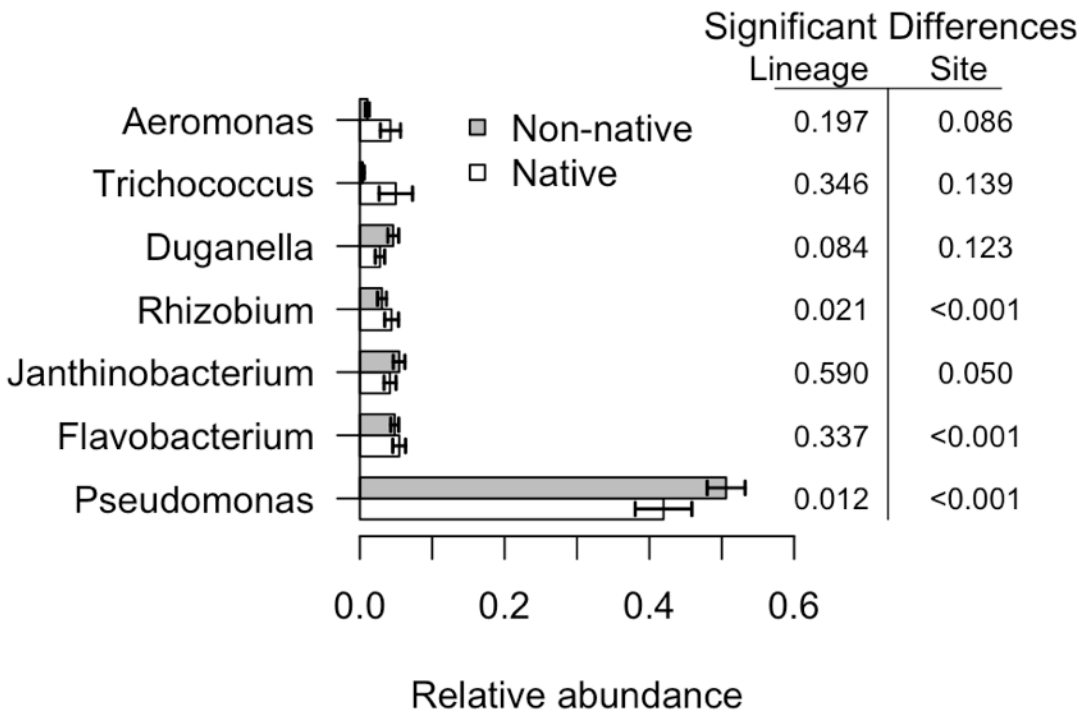

Fig. S2.8: Relative abundance of bacterial genera by lineage. Significance tested by ANOVA

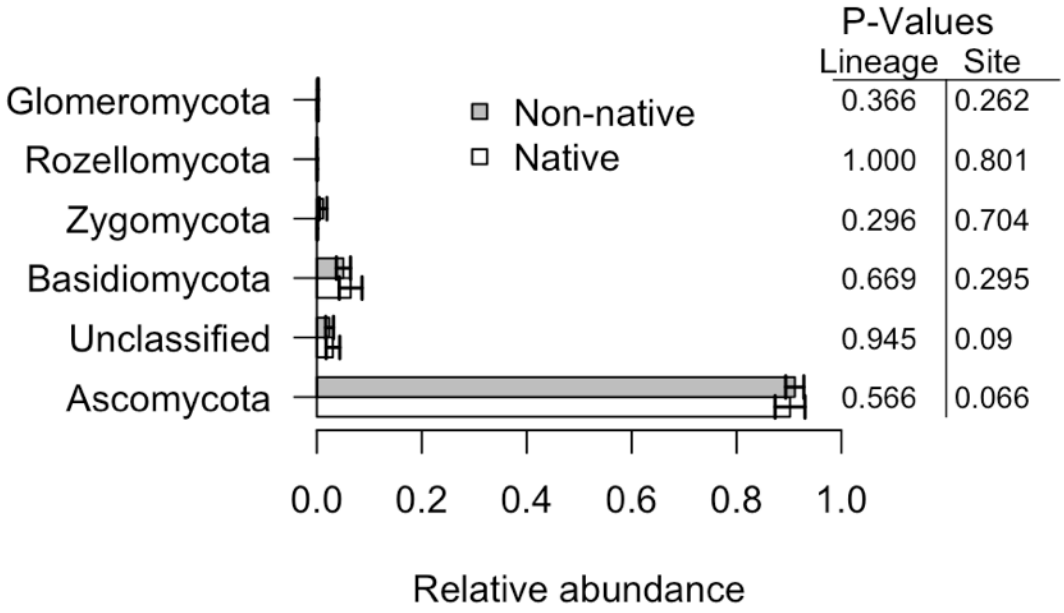

Fig.S2.9: Relative abundance of fungal phyla by plant lineage. Significance determined by ANOVA

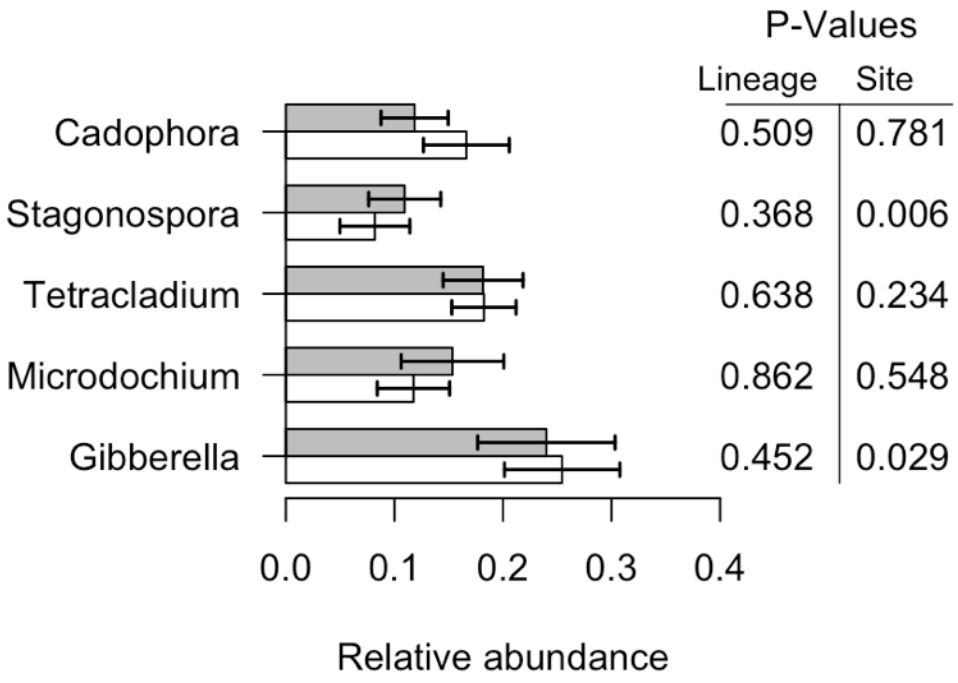

Fig. S2.10: Relative abundance of Fungal genera by plant lineage. Significance determined by ANOVA

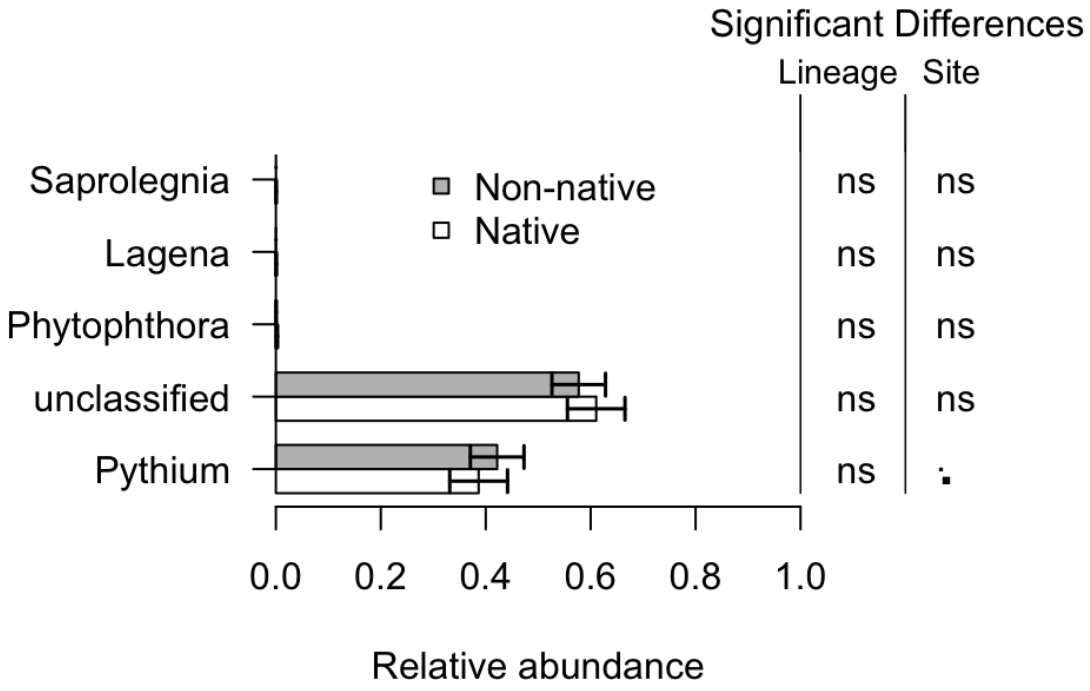

Fig. S2.11: Relative abundance of Oomycete genera by plant lineage. Significance determined by ANOVA

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Chapter 3 : Soil Microbial Communities Do Not Explain the Invasiveness of Non-Native *Phragmites australis*

Introduction

The plant-associated microbiome can dramatically influence plant performance and therefore may play an vital role in driving plant invasions in many ecosystems (Reinhart and Callaway 2006, Kowalski et al. 2015). Plant-microbial interactions span a spectrum from beneficial to antagonistic, and plants may perform better or worse than heterospecifics if their community of microorganisms functionally differs. For example, if invasive plants associate with relatively fewer pathogens than native plants, they will realize performance advantages (Keane and Crawley 2002, Reinhart and Callaway 2006). Similarly, interaction with more mutualists may provide disproportionally stronger benefits to invaders relative to native species (Richardson et al. 2000, Reinhart and Callaway 2006).

Soil dwelling microorganisms may play a prominent role in a plant's invasiveness. For example, a recent meta-analysis found that plant invasions can alter rhizosphere microbial communities, specifically increasing nitrogen mineralization, extracellular enzyme activity, and arbuscular mycorrhizal fungi (AMF) abundance, while decreasing soil pathogen and herbivore abundance (Zhang et al. 2019). Additionally, invasive plants may accumulate pathogens in the soil that are more virulent to native plants than themselves (Mangla et al. 2008, Crocker et al. 2015). Consequently, a better understanding of the microbial interaction between native and invasive plant species will improve our collective understanding of the mechanisms underlying plant invasiveness and may ultimately improve invasive species management outcomes.

Phragmites australis is a cosmopolitan wetland grass with multiple lineages worldwide and is considered a model organism for studying plant invasions (Meyerson et al. 2016). Invasive to North America, the European lineage (*Phragmites australis* haplotype M; hereafter, non-native *Phragmites*) is highly productive, fast growing, and very large, often forming dense monocultures supporting a low overall species diversity. A native lineage in North America (*Phragmites australis* subsp. *americanus*, hereafter native *Phragmites*) is conversely characteristic of low nutrient, high-diversity wetlands and is considered desirable for wildlife habitat (Price et al. 2013). The key to understanding the invasive nature of non-native *Pharagmites* may lie with the community of microorganisms associated with it (Kowalski et al. 2015, Clay et al. 2016, Shearin et al. 2018). For instance, Nelson and Karp (2013) found different rhizosphere pathogen communities (mainly *Pythium* spp.) associated with each lineage, although the total abundance of rhizosphere pathogens did not differ. They speculated that those differences may increase invasiveness of non-native *Phragmites* due to enemy release. Additional evidence indicates that differential virulence of pathogens might favor non-native *Phragmites* over native *Phragmites* and especially over other native species (Crocker et al. 2015).

Importantly, Bowen et al. (2017) showed that bacterial communities in the rhizosphere differed dramatically among the dominant *Phragmites* lineages broadly distributed across the east and west coasts of North America. In fact, geographically distant *Phragmites* populations of the same lineage had more similar bacterial communities than neighboring population of different lineages, suggesting that lineage-specific cultivation drives rhizosphere community composition (Bowen et al. 2017). Despite compelling evidence of lineage-specific bacterial selection in the rhizosphere, the authors failed to find any functional link between bacterial communities and plant performance.

However, further evidence for widespread differences in microbial assemblages between native and non-native *Phragmites* lineages is mixed. For example, in tidal wetlands of the Chesapeake Bay region, *Phragmites* lineages cultivated dissimilar rhizosphere archaeal communities, but contrary to the findings of Bowen et al. (2017), rhizosphere bacterial communities did not differ between lineages (Yarwood et al. 2016). Likewise, a recent study examining root endophytes residing in native and non-native *Phragmites australis* roots in Michigan USA, revealed that root bacterial, fungal, and oomycetes communities did not differ between native and non-native *Phragmites* lineages (Bickford et al. 2018). Instead, root microbial communities were strongly influenced by environmental characteristics, such as soil saturation and nutrient status. Because microbial communities residing in native and non-native *Phragmites* roots did not differ in either composition or function, there was no evidence to suggest that root endophytes contributed to the invasiveness of the non-native lineage.

Here, we expand upon the aforementioned study on *Phragmites* endophytes to examine broad components of the rhizosphere microbiome (*e.g.*, bacteria, fungi, and oomycetes) in native and non-native *Phragmites* populations. We aimed to quantify the rhizosphere microbiome of each lineage and its functional potential to better understand the role of the belowground microbiome in fostering the invasion of non-native *Phragmites*. If performance differences between native and non-native plant lineages are driven by their rhizosphere microbial communities, we would expect (1) that the rhizosphere community of native and non-native *Phragmites* would harbor compositionally dissimilar bacteria, fungi, and oomycete communities and (2) the non-native lineage to associate with more mutualistic and/or fewer pathogenic microbes in rhizosphere soil.

We tested these hypotheses over a range of sites across Michigan, USA that varied in environmental conditions, thereby allowing us to explore additional drivers of microbial

community composition such as soil nutrient content and saturation. We further tested our hypotheses at two sites in Ohio, USA, in which dense and extensive populations of native and non-native *Phragmites* intergrade from nearly pure stands to mixtures of each. Intensive sampling along 20-m transects at these two sites allowed us to explore (1) whether the degree of differentiation differed between dense monoculture stands and mixed plant community zones within the same environment and (2) whether differential rhizosphere cultivation between lineages was detectable at various spatial scales. We included multiple levels of soil proximity to host plant roots in paired samples (rhizoplane, rhizosphere and bulk soil), allowing us to determine if either lineage cultivates a microbial community that is detectably different from the bulk community, and whether the strength or direction of cultivation differs by plant lineage.

Materials and Methods

Site Selection

Our study included 6 sites distributed across Michigan, USA with co-occurring populations of native and non-native *Phragmites* (hereafter Michigan Sites; Appendix S3: Table S3.1) and two sites in Ohio, USA in which native and non-native *Phragmites* stands co-occur and mix (hereafter, Ohio Sites). Sampling protocols differed slightly between the two regions and are described in detail below.

Michigan Sites

In August 2016, we sampled rhizosphere and bulk soils from native and non-native *Phragmites* at 6 sites distributed across Michigan, USA (Appendix S3: Table S3.1). We selected sites that had at least 3 distinct patches of native and non-native *Phragmites* in close proximity to one another, growing under similar environmental conditions (*e.g.*, soil type, hydrology) with no recent history of invasive plant management (*e.g.*, herbicide, burning). Due to the rarity of co-occurring native and non-native *Phragmites* populations that met these criteria (non-native is rare

and well-managed in northern Michigan; native is rare in southern Michigan), patch size and density varied considerably among sites (1 m^2 to 100 m^2). We use the transect sites (described below) to assess whether patch size and density changes the extent of rhizosphere cultivation.

At each site, we morphologically identified all *Phragmites* patches as native or non-native and leaf material from each was collected for later genetic confirmation of lineage (based on chloroplast DNA) using the methods of Saltonstall (2002). We classified the degree of soil saturation as either unsaturated, saturated, or saturated with standing water, and recorded depth of water (if over the surface) and the nature of surrounding vegetation. One ramet near the center of each patch was randomly selected for collection of paired rhizosphere and bulk soils. Using a serrated knife, we cut a 10-cm diameter circle around the chosen ramet, exhuming subtending roots with adhering soil. The root ball was shaken to remove loosely associated soil. To sample rhizosphere soils, we then vigorously shook the root ball in a bag, saving the soil that fell off. Bulk soils were sampled outside of the *Phragmites* patch and paired with rhizosphere soils at the patch level. Leaf samples from the same stem were collected for tissue nutrient analysis. All samples were kept on ice until returned from the field.

Ohio Sites

In September of 2017, we established two 20-m transects within the Cedar Point National Wildlife Refuge, in Ohio USA. The transect sites were established where large, dense native and non-native *Phragmites* patches co-occur and intermix. Each transect contained a zone of non-native *Phragmites* dominance, a mixed zone, and a zone of native *Phragmites* dominance. Each lineage's dominant zone was a near monoculture (i.e. included very few other plant species at low abundance but did not include the opposite lineage); they will hereafter be referred to as monocultures. Samples were collected within 0.5 x 0.5 m plots at 2-m intervals in the monoculture

zones and 1-m intervals in the mixed zones. Within each plot, we assessed plant species composition by counting the stems of each *Phragmites* lineage, identifying other plant species, and estimating total percent plant cover. One *Phragmites* ramet of each lineage was collected within each plot (1 sample in monoculture; 2 in mixed plots). Paired bulk and rhizosphere soils were collected as described above; bulk soils were collected adjacent to the plot in a zone of low stem density to avoid root infuence. The entire root ball with adhering soil particles (rhizoplane soils) from the selected ramet was also collected and returned to the lab on ice.

Sample Preparation

Samples collected from both sampling regions were prepared identically, except for the rhizoplane soils sampled from only the Ohio sites. For soil nutrient analysis, a subset of the bulk soil from each sample was passed through a 2-mm sieve and oven dried at 60 ˚C for 48 hrs. Dried samples were ground with a mortar and pestle and subsamples from each (0.5 g) were processed in duplicate in a Leco CNS2000 Analyzer (LECO® St. Joseph, MI) to measure organic carbon and nitrogen. Soil phosphorus was determined colorimetrically following the Bray P1 extraction method (Bray and Kurtz 1945).

Rhizosphere and remaining bulk soil samples were passed through a sterilized 2-mm sieve and stored at -80°C until DNA extraction. To obtain rhizoplane soils (Ohio sites), we collected ~10 coarse roots randomly from the root ball of each plant using sterile forceps. Sampled roots were placed into a sterile 50 mL centrifuge tube with 30 mL of phosphate buffered saline (PBS). Tubes were vigorously shaken for five minutes, after which the roots were removed. Tubes were centrifuged at 8000 x G for 10 minutes. Supernatant was decanted and the pellet was resuspended in 5-10 mL of supernatant in a 15 mL tube and centrifuged again at 8000 x G for 10 minutes. After

decanting supernatant, each tube containing pelletized rhizoplane soil was stored at -80ºC until DNA extraction.

DNA was extracted from 50 mg (wet weight) of soils using Qiagen PowerSoil PowerLyzer DNA extraction kits. We used manufacturer protocols, with the exception of improvements to reduce ethanol contamination (*e.g.,* extra spins, more frequent transfers to sterile tubes). DNA was eluted with molecular grade water. All genomic DNA extracts were verified by electrophoresis. Extracts were checked for quality on a NanoDrop UV/Vis spectrophotometer and concentration using a Quant-iT PicoGreen dsDNA kit (Invitrogen, Carlsbad, CA, USA).

All polymerase chain reactions (PCR) for each microbial group (*i.e.,* fungi, bacteria, oomycetes) were performed using subsamples of the same template genomic DNA sample. Genomic DNA was diluted to ensure equimolar concentration of template DNA in each PCR reaction. Bacterial amplicons were generated using primers described in Kozich et al. (2013), which target the V4 region of the 16S rRNA gene. Fungal amplicons were produced using primers described by Taylor et al. (2016), which target the ITS2 region of the 5.8S rRNA gene. Oomycete amplicons were generated using primers adapted from Riit et al. (2016) and Taylor et al. (2016) that also target the ITS2 region of the 5.8S rRNA gene. See Appendix for specific primer sequences and PCR conditions. All PCR reactions were performed in triplicate using Phusion High Fidelity DNA Polymerase and master mix (New England BioLabs, MA, USA). Libraries were normalized using SequalPrep Normalization Plate Kit (Life technologies cat # A10510-01) following the manufactures protocol for sequential elution. The concentration of the pooled samples was determined using Kapa Biosystems Library Quantification kit for Illumina platforms (Kapa Biosystems KK4824). The sizes of the amplicons in the library were determined using the Agilent Bioanalyzer High Sensitivity DNA analysis kit (cat# 5067-4626). The final library consisted of

equal molar amounts from each of the plates, normalized to the pooled plate at the lowest concentration. Amplicons were sequenced by the Microbial Systems Molecular Biology Laboratory (MSMBL) at the University of Michigan on the Illumina MiSeq platform, using a MiSeq Reagent Kit V2 500 cycles (Illumina cat# MS102-2003), according to the manufacturer's instructions.

Bioinformatics analysis

Raw bacterial sequence data were processed using mothur v1.39.5 (Schloss et al. 2009). Operational taxonomic units (OTUs) were clustered at 97% for bacterial sequences. Bacterial taxonomy was determined by comparing representative sequences to the taxa found in the SILVA database (Quast et al. 2018). Raw fungal and oomycete sequences were processed using QIIME2 (Bolyen et al. 2019) because QIIME can implement *de novo* sequence clustering of actual sequence variants (ASVs). Fungal sequences were clustered into OTUs at 97% similarity and assigned to taxonomy based on the UNITE database (Nilsson et al. 2013). Oomycete sequences were clustered at 97 % similarity and assigned taxonomy in mothur using a custom oomycete-specific database from the Barcode of Life Database (Ratnasingham and Hebert 2007). Each microbial group was rarefied according to the sample that yielded the fewest number of sequences to ensure equal coverage across all samples. Bacteria were rarified to 13,956 sequences, fungi to 10,608 sequences, and oomycetes to 1000 sequences. Bacterial taxa were analyzed to identify the proportion belonging to common plant pathogen groups using genera found in Wood (1967) and Mansfield et al. (2012). To evaluate functional potential of fungal OTUs, we used FUNGuild (Nguyen et al. 2015), which parses fungal communities by trophic mode and functional guilds. We analyzed outputs at the trophic mode and guild level to group fungal taxa into putative functional groups. All oomycetes were assumed to be pathogens.

We separately analyzed the data collected from the Michigan sites and Ohio sites because the sampling design differed between regions. Microbial alpha diversity and relative sequence abundance was calculated using only the rhizosphere data, and differences with respect to site and plant lineage were assessed using 2-way ANOVA (Type III sum of squares). To explore differences in diversity between paired rhizosphere, rhizoplane, and bulk soil samples, we used paired t-tests. To understand the potential environmental drivers of site differences, we assessed the impact of soil nutrients and saturation on microbial diversity and abundance, including potential interactions with lineage using Analysis of Co-Variance (ANCOVA). Permutational multivariate analysis of variance (Per-MANOVA) tested whether plant lineage or site predicted significant microbial community differences among our samples. Homogeneity of Dispersions (PermDISP) further assessed whether microbial community samples differed in their degree of dispersion from their centroid. Finally, we used Principle Coordinate Analysis (PCoA) with fitting of environmental data to assess and visualize microbial community differences between lineages and potential environmental drivers. All statistical analyses were run in the R environment (R Core Team 2016). All R code, notes, and associated data can be accessed on GitHub at https://github.com/wesbick/rhiz.

Results

Michigan Sites

We found no evidence that native and non-native plant lineages cultivated compositionally different microbial communities; *Phragmites* lineage was not a significant predictor of variation in bacterial, fungal, or oomycete communities (Table 3.1, Fig. 3.1). In contrast, sampling site was a significant predictor of variation in rhizosphere community composition for all three microbial groups (Table 3.1a). However, a significant test for homogeneity of multivariate dispersions (PermDISP) suggested that the site differences in bacterial and oomycete communities may have

been due to differences in dispersion around the centroids, rather than in mean composition (Table 3.1b). Soil saturation, latitude, and soil nutrients were important in structuring bacterial, fungal, and oomycete communities (Fig. 3.1).

Relative abundance of particular microbial phyla found in the rhizosphere also did not strongly differ by plant lineage, providing further evidence that native and non-native plant lineages do not cultivate distinct microbial communities. In bacteria, the most abundant phylum, Proteobacteria, differed marginally (P = 0.098) between native and non-native *Phragmites* lineages; however, the magnitude of difference in relative abundance was small (~2%), making the difference plausibly ecologically unimportant (Fig. 3.2a). Proteobacteria were much more strongly differentiated among sites (Fig. 3.2a), mainly driven by saturation (ANCOVA $P < 0.001$, Appendix S3, Fig. S3.2). Other minor bacterial phyla (*e.g.,* Bacteriodetes, Acidobacteria) also differed among sites, but not plant lineage (Fig. 3.2a). A major factor in these differences among sites seemed to be soil saturation because it significantly impacted abundance of most bacterial phyla (Appendix S3, Fig. S3.2). Proteobacteria ($r^2 = 0.218$, $P = 0.002$) and Chloroflexi ($r^2 = 0.143$, $P = 0.014$) increased with degree of saturation whereas Acidobacteria decreased ($r^2 = 0.525$, $P < 0.001$).

Abundance of fungal phyla did not differ between native and non-native *Phragmites* rhizosphere soil, although many common phyla differed among sites (Fig. 3.2b). Soil saturation was a significant determinant of Glomeromycota (ANCOVA P = 0.016), such that Glomeromycota abundance decreased in saturated soil ($r^2 = 0.189$, $P = 0.005$; Appendix S3, Fig. S3.3). Relative abundance of oomycete families marginally differed between plant lineages. The majority of oomycete sequences recovered belonged to Pythiaceae, and the native lineage hosted relatively more of this family ($P = 0.064$). However, the non-native lineage hosted relatively more

unclassified oomycetes than the native (P = 0.074). Soil saturation and plant host lineage significantly interacted in affecting Pythiaceae (ANCOVA P = 0.015) and unclassified oomycete relative abundance (ANCOVA P = 0.015) such that the non-native lineage hosted slightly less Pythiaceae (r_2 = 0.398, P = 0.016) and more unclassified oomycetes (r_2 = 0.377, P = 0.011) in saturated sites (Appendix S3, Fig. S3.4). The phylogenetic resolution of our recovered sequences did not allow us to compare abundance of Pythiaceae genera or species between *Phragmites* lineages.

We examined the differences in community composition and diversity between rhizosphere and bulk soil samples across the Michigan sites to provide additional context to the lack of community differences seen in rhizosphere communities between the lineages. On average, microbial community composition did not differ between bulk and rhizosphere soils for all three microbial groups (Appendix S3, Fig. S3.6) suggesting that neither *Phragmites* lineage has much influence on soil microbial communities compared to the rest of the wetland plant community. Results were similar when spatial structure was accounted for by pairing at the patch level: communities of bacteria, fungi, and oomycetes still did not differ in composition (PcoA 1 score) between the bulk and rhizosphere soils (Table 3.2). Diversity of bacteria, fungi, and oomycetes also did not differ between rhizosphere and bulk soil samples, when compared between pairs of co-collected samples (Table 3.3; Appendix S3, Fig. S3.5).

Ohio Sites

The intensive sampling arrangement at the Ohio sites allowed us to explore bacterial cultivation at a finer scale than we were able at the Michigan sites and illuminated some subtle, but important microbial community differences between lineages. First, we explored the importance of patch size and density in driving bacterial community differences; we compared the

rhizosphere bacterial communities between lineages in both the monoculture and mixed zones. This analysis illustrated that lineage effects on rhizosphere bacterial communities depend on the relative density of natives and nonnatives (lineage by Stand Type interaction; Per-MANOVA r_2 = 0.023, P = 0.070). Specifically, pairwise comparisons revealed that rhizosphere communities in native monocultures differed from those found in the non-native monocultures (Fig. 3.4; Pairwise Per-MANOVA r_2 = 0.159, P = 0.066, Pairwise PermDISP P = 0.826), while differences between lineages were nonsignificant in mixed zone (Pairwise Per-MANOVA, P = 1.000, Pairwise PermDISP P = 0.852, Fig. 4). This indicates that dominance may factor into the degree of bacterial community differentiation between lineages, wherein larger more dense patches may be more likely to host different bacterial communities.

We also explored the rhizoplane soils for evidence of differential cultivation between lineages. Lineage was a marginally significant predictor of variation across all plots (Per-MANOVA P = 0.075); however, it only accounted for ~2% of the variation in community composition and the differences may be caused by differential dispersion between the groups (PermDISP P = 0.023). Lineages, therefore, show little separation graphically (Appendix S3, Fig. S3.6). Thus, across mixed and monoculture zones, we found no evidence of differentiation in microbial communities between lineages, even at a very narrow proximity to root. Interestingly, differences in rhizoplane soils by lineage did not seem to depend on stand size or density as pairwise comparisons showed no differences in community between lineages in monoculture stands (P = 1.000, Appendix S3, Table S3.7), however this result may have been influenced by the small sample sizes of rhizoplane soils in monocultures (n = 8 in Non-native, n = 3 in Native, Appendix S3, Table S3.1).

Next, we took advantage of the paired soil sampling design and compared the bacterial diversity in the rhizoplane soils to both rhizosphere and bulk soil. Rhizosphere soils were more diverse then the adjacent paired bulk soil and more diverse than paired rhizoplane soils. Rhizoplane soils were not different in diversity from bulk soil (Appendix S3, Fig. S3.7). This suggests that more microbial species are present in the more "biologically active" zone of the rhizosphere, but only a subset of those are present in the narrowly defined zone of the rhizoplane. Additionally, there was evidence of some spatial structure in the narrowly defined rhizoplane soils. The community found in the rhizoplane (as described by PCo Axis 1) was different from its paired bulk soil (Paired t-test, $t = -10.376$, $df = 57$, $P < 0.001$, Fig. 3.5). The rhizoplane and bulk soil bacterial communities associated with the non-native lineage were correlated across sampling sites ($r_2 = 0.267$, $P = 0.002$), but those associated with the native *Phragmites* were not ($P = 0.206$). This may indicate some differences in cultivation between lineages, albeit at a very fine spatial scale.

Functional determination

Putative functional determinations of the microbial taxa in the rhizosphere revealed little to explain mechanisms of invasion. Only 0.5% of the bacterial sequences recovered belonged to known bacterial plant pathogens in the Michigan Sites, and of that small portion, potential bacterial pathogens were not differentially abundant between native and non-native (ANOVA $F = 1.575$, $P = 0.215$). Potential pathogens made up 1% of the bacterial sequences in the Ohio sites and also did not differ in abundance between lineages (ANOVA $F = 0.119$, $P = 0.731$). Fungal functional determinations from FUNguild produced a similar result. The majority of sequences that matched the database were likely soil or litter saprotrophs. While a small portion (~3%) were known plant pathogens, the proportion recovered from native rhizospheres was not different from non-native (Fig. 3.6). Relative abundance of arbuscular mycorrhizal fungi (AMF), the group that makes up

the most common fungal mutualists, differed among sites, but not between plant lineages. The differences among sites were likely related to soil saturation as AMF abundance was significantly negatively related to soil saturation (ANCOVA $P = 0.015$). We assume that all oomycete groups are pathogenic and although the relative abundance of one dominant family of pathogens, Pythiaceae, was marginally greater in the native lineage, the relative abundance of unclassified oomycetes (likely matching uncultured oomycetes) differed in the opposite direction (Fig. 3.2c). Given the lack of consistency in lineage differences between oomycete families, we do not have evidence that native *Phragmites* receives higher oomycete pathogen pressure than non-native.

Discussion

We found very little evidence that native and non-native *Phragmites* cultivated microbial communities that drive patterns of invasiveness. Microbial communities were not different with respect to any microbial group we examined at the Michigan sites, and we could find no meaningful differences in functional potential across all sites. The strongest evidence for differential cultivation comes from the Ohio sites in which native and non-native monocultures, but not mixtures, significantly differed in their rhizosphere bacterial communities. Additionally, at the Ohio sites, rhizoplane soils, but not rhizosphere, differed marginally between plant lineages and differed between lineages in their magnitude of difference from paired bulk soil samples. The monoculture zones of the Ohio sites where much of the differences occurred were much larger and denser than the sampling locations in Michigan. Taken together, these observations provide strong evidence that major microbial groups, as well as the presence of beneficial and pathogenic organisms, do not explain invasiveness of non-native *Phragmites.* We argue below that the small differences that do exist are likely a consequence of invasion rather than a cause.

Our results contrast with one previous study exploring rhizosphere pathogens, wherein oomycete communities differed between native and non-native *Phragmites* populations in New York, USA (Nelson and Karp 2013). Although our study did find marginal lineage differences in relative abundance of Pythiaceae and Unclassified Oomycetes, we did not find differences in community composition. It is possible this disparity arises due to sequencing depth in these respective studies. For instance, Nelson and Karp (2013) used a different sequencing platform that allowed much longer reads than our study (~ 475bp *vs.* ~ 275bp). The shorter reads and lower phylogenetic resolution in our study may have contributed to the smaller breadth of oomycete families we observed, thereby affecting community composition. However, in a study of endophytic root communities in the same Michigan sites as studied here, Bickford et al. (2018) found no difference in oomycete communities between *Phragmites* lineages using the same phylogenetic resolution as the rhizosphere data from Nelson and Karp (2013). Therefore, it is likely that our results accurately reflect the oomycete communities in the Great Lakes region.

Our results also contrast with those found in rhizosphere bacterial communities by Bowen et al. (2017). These investigators reported that *Phragmites* lineages cultivated consistent and distinct bacterial communities in the rhizosphere, regardless of geography, environmental characteristics, or temporal variation. The lack of consistency between our studies is surprising and there are no clear environmental explanations that resolve the differences. For instance, while their dataset includes samples collected from *Phragmites* populations along the east, west, and Gulf coasts of the United States, their sites span a broad range of tidal influence and salinity regimes. Therefore, differences in salinity and/or hydrology between our studies are not likely responsible for the different patterns observed. We argue that stand density and degree of dominance may explain the contrast in results.

Bowen et al. (2017) focused primarily on well-established, large, dense *Phragmites* stands in which density differences between lineages may have been very pronounced. In contrast, our Michigan sites were comprised of smaller stands of each *Phragmites* lineage. One potential consequence of differences in density is soil oxygen. Non-native *Phragmites* has a much higher ventilation efficiency than native *Phragmites*, thereby leading to a more oxygenated rhizosphere; this effect is thought to arise from a higher density of *Phragmites* stems in non-native stands (Tulbure et al. 2012). In anoxic wetland soils, an increase in the soil oxygen concentration could plausibly change the composition of bacterial communities, such that more aerobic microbes are present. We speculate that the lack of differences observed in our sites could arise from the small, less dense patches sampled and correspondingly small differences in ventilation between native and non-native lineages at our sites. The fact that the only place where we found differences between lineages was in the dense monoculture zones of the transect sites is consistent with this potential mechanism. Future work should explicitly explore the effects of stand size, density, and soil oxygen concentrations on differential rhizosphere cultivation.

Data from our Michigan sites suggest the rhizosphere microbiome largely mirrors the root microbiome, where we also found no differences between *Phragmites* lineages across three major microbial groups (Bickford et al. 2018). In exploration of the root microbiome, Bickford et al. (2018) speculated that roots may select similar microbial inhabitants across lineages, despite the differences that may occur in the rhizosphere. However, the data presented here suggest, at least in low density patches, the rhizosphere microbial communities are driven by the environment as much as they are in the roots.

The cumulative evidence from our studies of roots, rhizosphere, and bulk soil suggest that at least at low densities, *Phragmites* does not affect microbial communities, but as high-density

monocultures establish, dissimilarity in bacterial communities emerges. We speculate that the differences at high density are a consequence of a successful invasion, rather than driving differential success at the initial stages of invasion. At low density patches, environmental characteristics such as saturation and soil nutrient content, but not lineage were strong determinants of community composition both in the roots (Bickford et al. 2018) and in the rhizosphere (this study). Our data also suggest that neither *Phragmites* lineage cultivates a community that is substantially different from the surrounding bulk soil. We could not distinguish the communities of any microbial group between bulk and rhizosphere soils at the Michigan sites and when we looked for evidence of cultivation at a fine spatial scale at the Ohio Sites, the evidence of cultivation was quite subtle. Rhizoplane soils at the Ohio sites were less diverse in bacteria than rhizosphere soils, indicating that only a small subset colonize that zone, the bacterial community found in the rhizoplane differed from the paired bulk soil, and the degree of difference between rhizoplane and bulk soil was variable in native but consistent in non-native *Phragmites*. Given the magnitude of the differences, the small spatial scale, and the fact that no differences were identified within the roots (Bickford et al. 2018), the functional impact of this difference is likely minimal.

We have no evidence from microbial composition or functional potential to suggest that the differential cultivation of bulk soil, rhizosphere soil or rhizoplane explain the invasiveness of *Phragmites*. In addition to the similarity in composition between native and non-native lineages, both lineages harbored functionally similar microbial communities, consisting mostly of saprotrophic fungi, few known fungal or bacterial pathogens, and a small subset of mutualists (mainly AMF). AMF abundance in the rhizosphere differed among sites, with drier sites hosting higher AMF abundance in the rhizosphere. A similar trend was observed in root fungal colonization with higher colonization in drier sites, although in that case non-native *Phragmites*

was more heavily colonized (Bickford et al. 2018). Therefore, saturation may drive differences in AMF abundance in soils, but recruitment into roots may differ between lineages. However, given the low abundance of AMF in both studies, especially in wet sites in which the non-native lineage is often highly successful, it is not likely a major driver of invasiveness in *Phragmites*. Lacking evidence to support the role of root-associated microorganisms in *Phragmites* invasiveness, we suspect differences in plant performance arise due to other aspects of plant growth.

Although we saw no evidence that *Phragmites* lineages cultivate different communities of soil microbes, except for perhaps at high densities, it is possible that the response to soil microbes differs between lineages to a similar community of microbes. To investigate whether each lineage has a unique response to soil microbes, we would need to take an experimental approach and keep soil communities constant to see how the growth of each lineage is affected by soil microbes (See Chapter 4).

Cumulatively, the results we report here and elsewhere provide little evidence to support the idea that non-native *Phragmites* gains a competitive advantage over native *Phragmites* by altering the composition and function of root-associated microbial communities in soil. Alteration of the soil microbiome may occur in dense monocultures of native and non-native *Phragmites*. However, those differences do not likely drive invasiveness and may in fact be consequences of alteration of the soil physical environment as non-native *Phragmites* increases dominance and increases surrounding soil oxygen concentration relative to native *Phragmites*, so could potentially be important in later stages of maintaining invasion. Our findings suggest that differences in microbial communities between native and invasive *Phragmites* may not drive invasiveness, and therefore future research should focus on interpretation of functional potential or experiments to establish causality within the microbial drivers of plant invasions.

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Tables

Table 3.1: Results of **a)** Per-MANOVA analysis and **b)** homogeneity of multivariate Dispersions (PermDISP) for all three microbial groups in the rhizosphere.

a)	Site				Lineage				Site x Lineage			
	df	F	R_2	*P*	df	F	R_2	*P*	df	F	R_2	*P*
Bacteria	**5**	**3.605**	**0.379**	**0.001**	1	1.084	0.023	0.292	5	1.098	0.115	0.273
Fungi	**5**	**2.511**	**0.313**	**0.001**	1	0.967	0.024	0.503	5	0.920	0.115	0.655
Oomycetes	**5**	**1.769**	**0.275**	**0.001**	1	1.097	0.034	0.243	5	1.040	0.162	0.314

b)	Site			Lineage	
	df	F	*P*	F	*P*
Bacteria	**5**	**3.553**	**0.014**	0.497	0.520
Fungi	5	2.624	0.053	0.362	0.541
Oomycetes	5	2.710	0.046	0.678	0.431

Table 3.2: Paired t-test statistics comparing community composition of paired bulk and rhizosphere soils. Community composition is represented by the scores along PCo1 for each microbial group.

	Variance Explained By PCo1 Axis	Paired - t	df	*P*
Bacteria	21.1%	-0.792	31	0.434
Fungi	14.4%	-1.039	30	0.307
Oomycetes	9.6%	-0.702	25	0.489

Table 3.3: Paired t-test statistics comparing inverse Simpson diversity of paired bulk and rhizosphere soils. Separate paired t-tests within lineage were also non-significant

	Paired - t	df	*P*
Bacteria	0.414	31	0.682
Fungi	0.376	33	0.709
Oomycetes	0.479	25	0.636

Figures

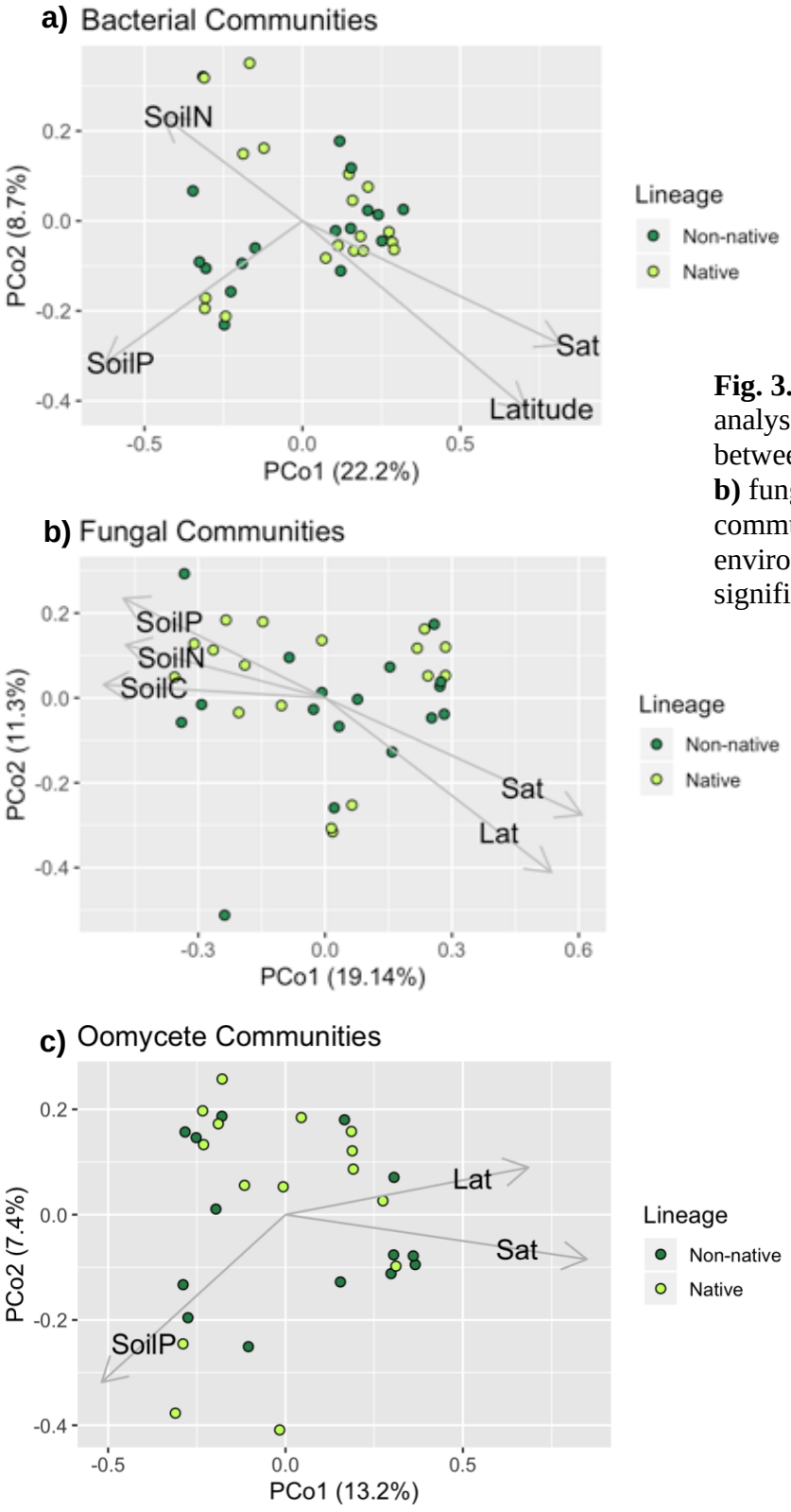


Fig. 3.1: Principle coordinate analysis of Bray-Curtis distances between rhizosphere **a)** bacterial, **b)** fungal, and **c)** oomycete communities. Plotted vectors of environmental variables are significant at the a < 0.05 level.

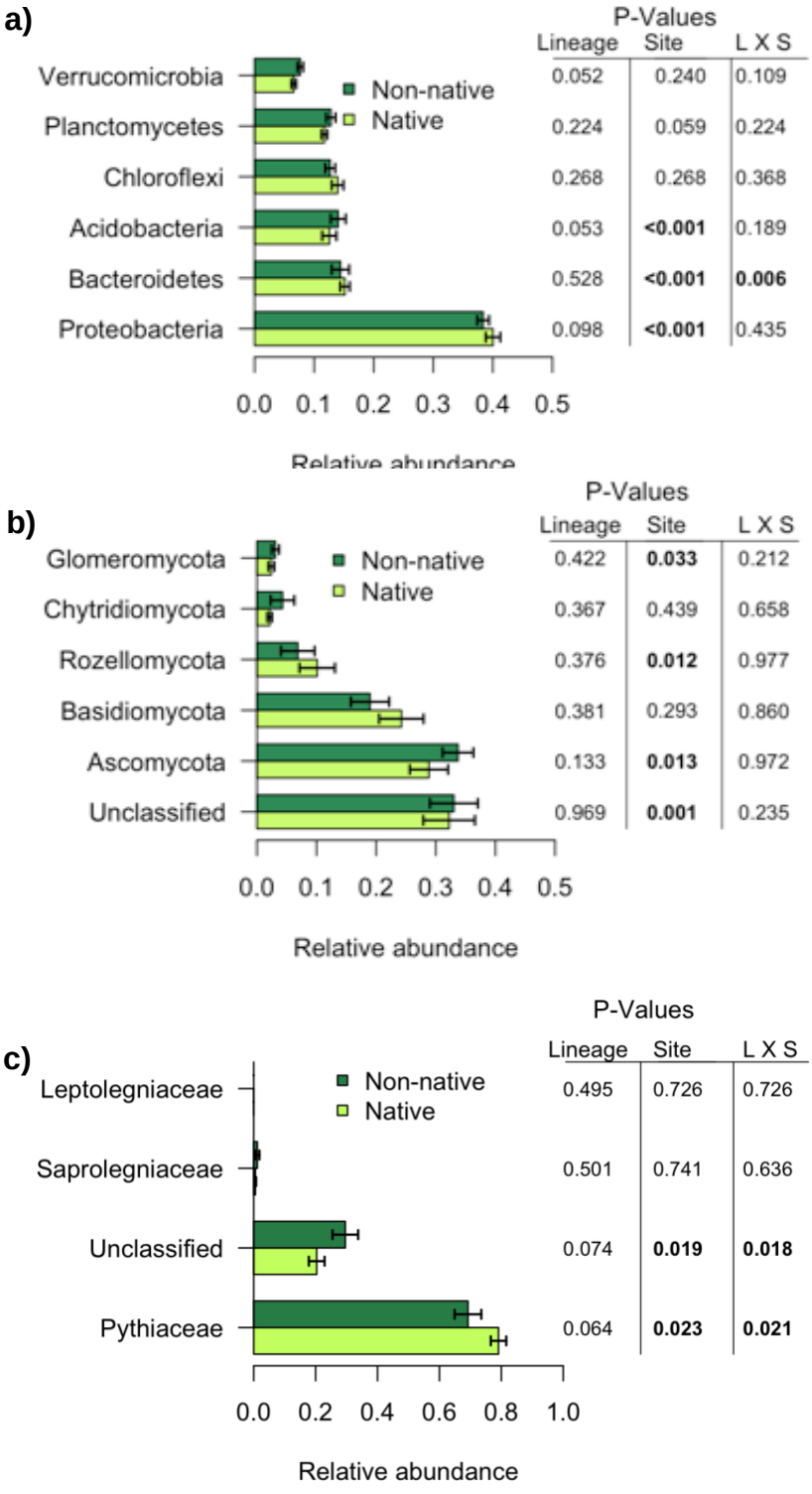


Fig. 3.2: Relative abundance of dominant phyla of **a)** bacterial, **b)** fungal, and **c)** oomycete families found in the rhizosphere. P-Values from a 2-way ANOVA with a Type III sum of squares. Significant P-values in bold.

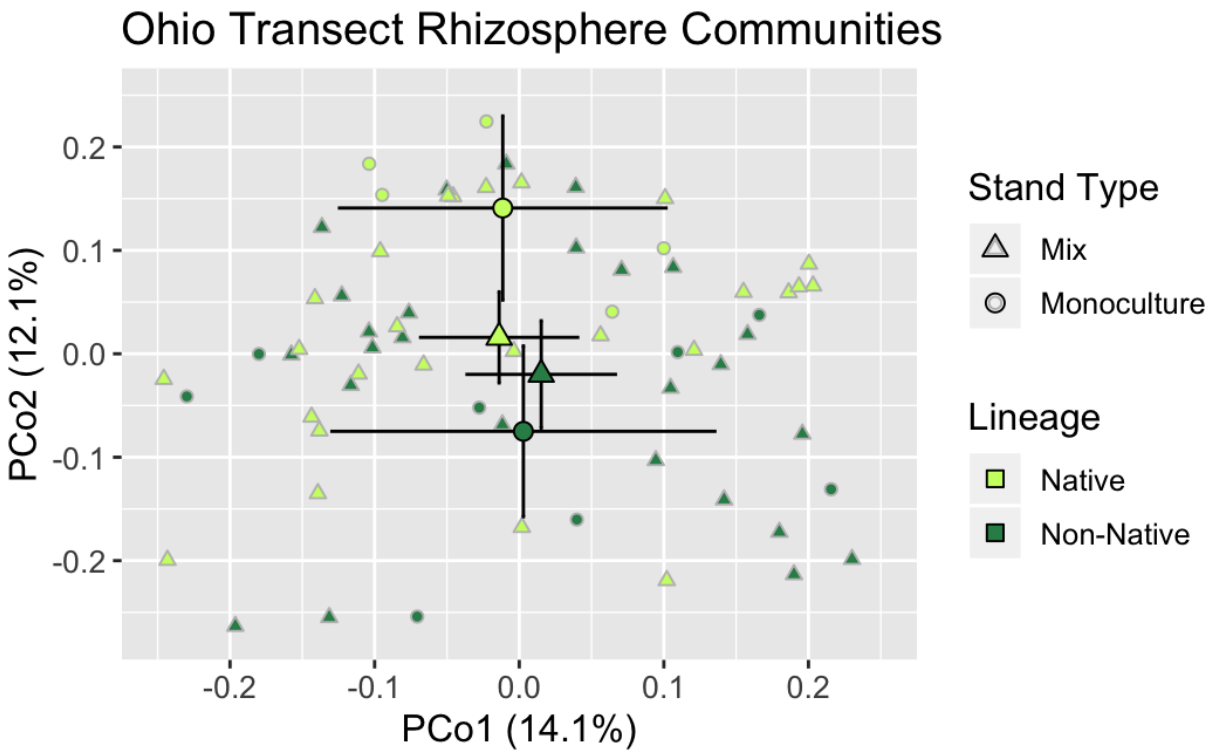


Fig. 3.3: Principle Coordinate Analysis of Bray Curtis distances between rhizosphere bacterial communities in the Ohio sites. Large points indicate centroids of each group with error bars denoting 95% confidence intervals.

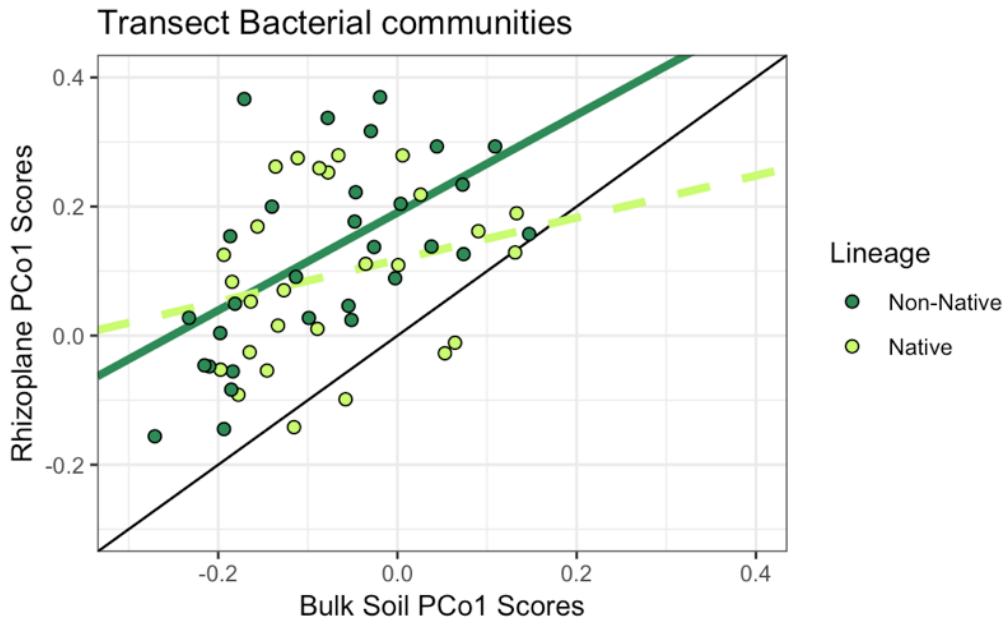


Fig. 3.4: Comparison of bulk and rhizoplane soil communities. Communities are represented by their PCo1 scores based on Bray Curtis distances. PCo1 explained 15.4% of the variance. Black line indicates 1:1 relationship between bulk and rhizoplane community. Colored lines indicate linear regressions. Dashed line not significant at $a<0.05$

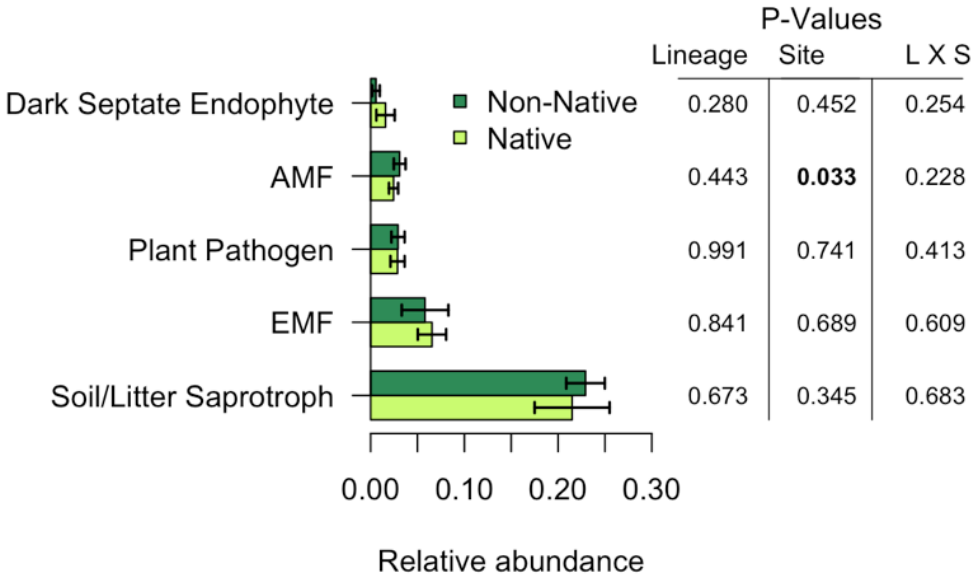


Fig. 3.5: Relative abundance of dominant fungal functional groups found in the rhizosphere. *P*-Values resulted from a 2-way ANOVA with a Type III sum of squares. Significant *P*-values in bold.

Appendix S3

a) **Table S3.1:** Site characteristics and environmental variables for a) all Michigan sites and b) Ohio transect sites.

Site	Coordinates	Soil Map Unit	Coastal or Inland	No. Sampling locations	Saturation Levels (# of each)	Soil C (%) mean (range)	Soil N (%) mean (range)	Soil P (mg/kg) mean (range)	Tissue C (%) mean (range)	Tissue N (%) mean (range)	Tissue P (%) mean (range)
Bay City Rec Area (BC)	43°40' 51"N 83°55' 7"W	Belleville loamy sand	Coastal	6	Saturated (6)	8.42 (3.65 – 21.00)	0.51 (0.21 – 1.34)	5.57 (3.59 – 8.37)	46.07 (45.31 – 46.97)	0.78 (0.41 – 1.60)	0.06 (0.03 - 0.13)
Chelsea Farm (CH)	42˚18'32"N 84˚03'25"W	Houghton Muck	Inland	5	Unsaturated (2) Saturated (3)	15.28 (8.24 - 32.24)	0.78 (0.32 – 1.99)	4.47 (0.30 – 7.00)	44.91 (44.55 – 45.19)	1.14 (0.63 – 1.58)	0.07 (0.04 – 0.15)
Cheboygan Marsh (CM)	45°39'27"N 84°28'16"W	Histosols and Aquents	Coastal	6	Saturated (1) High Water (5)	7.06 (0.88 – 14.53)	0.35 (0 – 0.73)	3.15 (0 – 5.08)	45.63 (43.30 – 47.18)	0.76 (0.40 – 1.40)	0.06 (0.03 – 0.11)
Cecil Bay (CB)	45˚44'52"N 84˚50'57"W	Stony Lake Beach	Coastal	6	High Water (6)	2.15 (1.00 – 6.67)	0.08 (0 - 0.42)	0.50 (0 – 1.19)	46.45 (46.03 – 46.73)	0.72 (0.41 – 0.94)	0.03 (0.02 – 0.05)
Pointe aux Chenes (Rt2)	45˚54'46"N 84˚52'20"W	Leafriver mucky peat	Coastal	6	High Water (6)	0.63 (0.26 – 1.92)	0.04 (0.01 - 0.11)	1.87 (0.23 – 3.00)	45.54 (42.25 – 46.06)	1.00 (0.58 – 1.40)	0.04 (0.02 - 0.08)
Castle Rock (CR)	45˚54'39"N 84˚44'18"W	Eastport-Leafriver complex	Inland	6	Saturated (6)	16.81 (1.59 – 41.64)	0.90 (0.04 – 2.34)	4.29 (0.07 – 10.41)	44.73 (44.19 – 46.06)	0.93 (0.38 – 1.50)	0.05 (0.01 – 0.08)

b)

Site	Coordinates	Soil Map Unit	Coastal or Inland	No. Sampling locations	Stand Types: Bulk Soil Samples (No. Native) (No. Non-native)	Stand Types: Rhizosphere Samples (No. Native) (No. Non-native)	Stand Types: Rhizoplane Samples (No. Native) (No. Non-native)	Saturation Levels (No. of each)	Soil C (%) mean (range)	Soil N (%) mean (range)	Soil P (mg/kg) mean (range)
Ohio Transect 1 (T1)	41°40' 33"N 83°18'18"W	Toledo silt loam	Coastal	44 Total 21 Native 23 Non-native	36 Mix / 8 Mono (18 Mix / 3 Mono) (18 Mix / 5 Mono)	34 Mix / 8 Mono (17 Mix / 3 Mono) (17 Mix / 5 Mono)	32 Mix / 6 Mono (17 Mix / 1 Mono) (15 Mix / 5 Mono)	Saturated (44)	24.16 (18.5 – 32.9)	2.30 (1.9 – 3.2)	7.87 (1.4 – 12.7)
Ohio Transect 2 (T2)	41°40' 33"N 83°17'33"W	Toledo silt loam	Coastal	25 Total 12 Native 13 Non-native	20 Mix / 5 Mono (10 Mix / 2 Mono) (10 Mix / 3 Mono)	19 Mix / 5 Mono (10 Mix / 2 Mono) (9 Mix / 3 Mono)	16 Mix / 5 Mono (8 Mix / 2 Mono) (8 Mix / 3 Mono)	Saturated (25)	17.29 (8.5 – 39.3)	1.41 (0.7 – 2.6)	12.26 (1.0 – 40.5)

Table S3.2: PCR Conditions and Primer Sequences

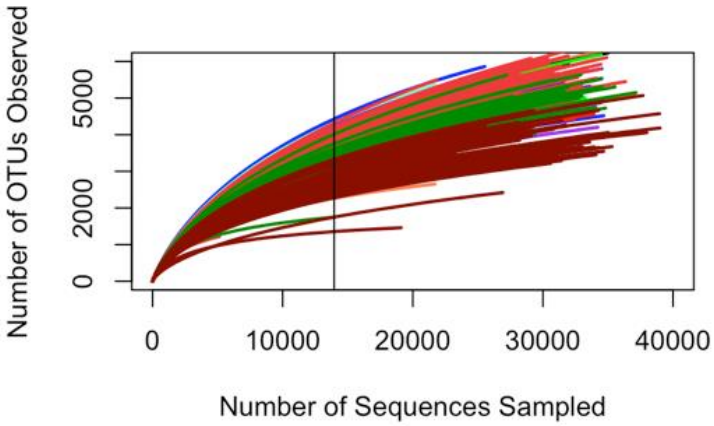
Primer Set	Primer	Primer Sequence	Fused Primer Length†	PCR Mastermix	PCR Conditions	Reference
Fungi	5.8S_Fun	5'-AACTTTYRRCAAYGGATCWCT-3'	65 bp	5.0 uL 5x Buffer* 0.25 uL 20uM dNTPs 0.938 uL 20uM 5.8S 0.938 uL 20uM ITS4 0.25 uL Taq* 4 uL Template DNA** 13.625 uL H2O	Initial denaturation: 94℃ for 3 min, 27 cycles, denaturation: 94℃ for 30 s, annealing: 57℃ for 45 s, extension: 72℃ for 90 s (10 min final extension)	(Taylor et al. 2016)
	ITS4_Fun	5'-AGCCTCCGCTTATTGATATGCTTAART-3'	76 bp			
Bacteria	515F	5'- GTGCCAGCMGCCGCGGTAA-3'	63 bp	5.0 uL 5x Buffer* 0.5 uL 20uM dNTPs 0.5 uL 20uM 515F 0.5 uL 20uM 806R 0.5 uL Taq* 2 uL Template DNA** 18 uL H2O	Initial denaturation: 95℃ for 2 min, 30 cycles, denaturation: 95℃ for 20 s, annealing: 55℃ for 15 s, extension: 72℃ for 5 min (10 min final extension)	(Kozich et al. 2013)
	806R	5'- GGACTACHVGGGTWTCTAAT'3'	69 bp			
Oomycetes (First round)	5.8 SR	5'-TCGATGAAGAACGCAGCG -3'	-	2.5 uL 10x Buffer* 0.5 uL BSA 0.5 uL 20uM dNTPs 0.625 uL 20uM 5.8 SR 0.625 uL 20uM LR7 0.5 uL Taq* 2 uL Template DNA** 17.75 uL H2O	Initial denaturation: 94℃ for 5 min, 30 cycles, denaturation: 94℃ for 30 s, annealing: 47℃ for 90 s, extension: 72℃ for 60 s (10 min final extension)	(Vilgalys and Hester 1990)
	LR7	5'-TACTACCACCAAGATCT-3'	-			
Oomycetes (Second Round)	ITS3oo	5'-AGTATGYYTGTATCAGTGTC-3'	64 bp	5.0 uL 5x Buffer* 0.25 uL 20uM dNTPs 0.125 uL 20uM Oom1f 0.125 uL 20uM Oom1r 0.75 uL DMSO 0.25 uL Taq* 1 uL Template DNA** 17.50 uL H2O	Initial denaturation: 95℃ for 15 min, 30 cycles, denaturation: 95℃ for 30 s, annealing: 55℃ for 30 s, extension: 72℃ for 60 s (10 min final extension)	(Riit et al. 2016)
	ITS4	5'-TCCTCCGCTTATTGATATGC-3'	69 bp			

† Full fused primer included Illumina adapter (29-bp forward; 24-bp reverse), 8-bp unique barcode, 10-bp pad, 2-bp linker followed by the gene specific primer). Reverse adapter was used with forward primer, Forward adapter with reverse.

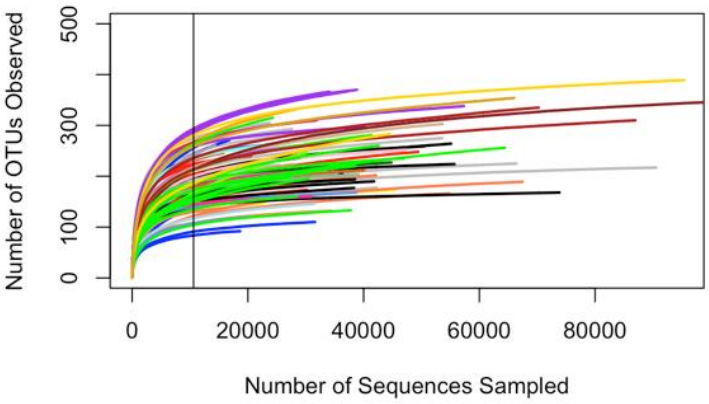
* Phusion High Fidelity PCR Kit (New England BioLabs)

** DNA was diluted to 5-10 ng per reaction

a) Bacteria



b) Fungi



c) Oomycetes

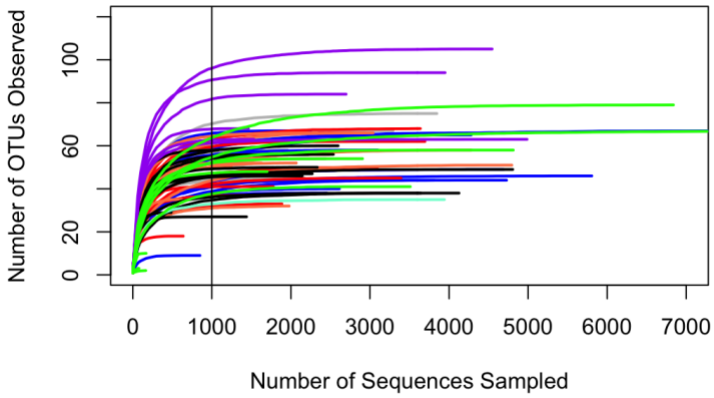


Fig. S3.1: Rarefaction curves for a) bacteria b) fungi and c) oomycete sequences. Colors indicate sampling locations. Vertical lines indicate subsample sequence amount.

Table S3.3: a) ANCOVA table showing the relationship between site, lineage, soil saturation, and nutrients on relative abundance of bacterial phyla. b) ANOVA table showing the relationship between site and plant lineage on the relative abundance of bacterial phyla.

		r^2			Lineage		Saturation		SoilN		SoilP		Sat:Lin	
	Residual df	**Multiple r^2**	**Adjusted r^2**	***P***	**F**	***P***	**F**	***P***	**F**	***P***	**F**	***P***	**F**	***P***
Proteobacteria RA	26	**0.560**	**0.458**	**<0.001**	1.68	0.206	**14.99**	**<0.001**	0.01	0.939	3.17	0.086	1.122	0.280
Bacteriodetes RA	26	0.087	0.000	0.865	0.14	0.708	0.32	0.575	0.10	0.757	0.12	0.732	0.38	0.542
Acidobacteria RA	26	**0.756**	**0.670**	**<0.001**	1.12	0.299	**62.15**	**<0.001**	0.42	0.523	**6.05**	**0.021**	0.02	0.882
Chloroflexi RA	26	0.355	0.206	0.057	0.67	0.420	**12.38**	**0.002**	0.53	0.472	0.42	0.524	0.14	0.710
Planctomycetes RA	26	0.168	0.000	0.528	1.53	0.227	0.02	0.896	0.116	0.737	1.18	0.288	0.00	0.985
Verucomicrobia RA	26	0.271	0.102	0.185	2.25	0.146	**7.29**	**0.012**	0.09	0.758	0.00	0.954	0.00	0.978

	Lineage			Site			Site X Lineage		
	df	**F**	**P**	**df**	**F**	**P**	**df**	**F**	**P**
Proteobacteria RA	1	2.98	0.098	**5**	**6.67**	**<0.001**	5	1.01	0.435
Bacteriodetes RA	1	0.41	0.528	**5**	**9.51**	**<0.001**	**5**	**4.36**	**0.006**
Acidobacteria RA	1	4.17	0.053	**5**	**17.81**	**<0.001**	5	1.64	0.189
Chloroflexi RA	1	1.29	0.268	5	1.38	0.268	5	1.14	0.368
Planctomycetes RA	1	1.56	0.224	5	2.51	0.059	5	1.51	0.224
Verucomicrobia RA	1	4.21	0.052	5	1.46	0.240	5	2.05	0.109

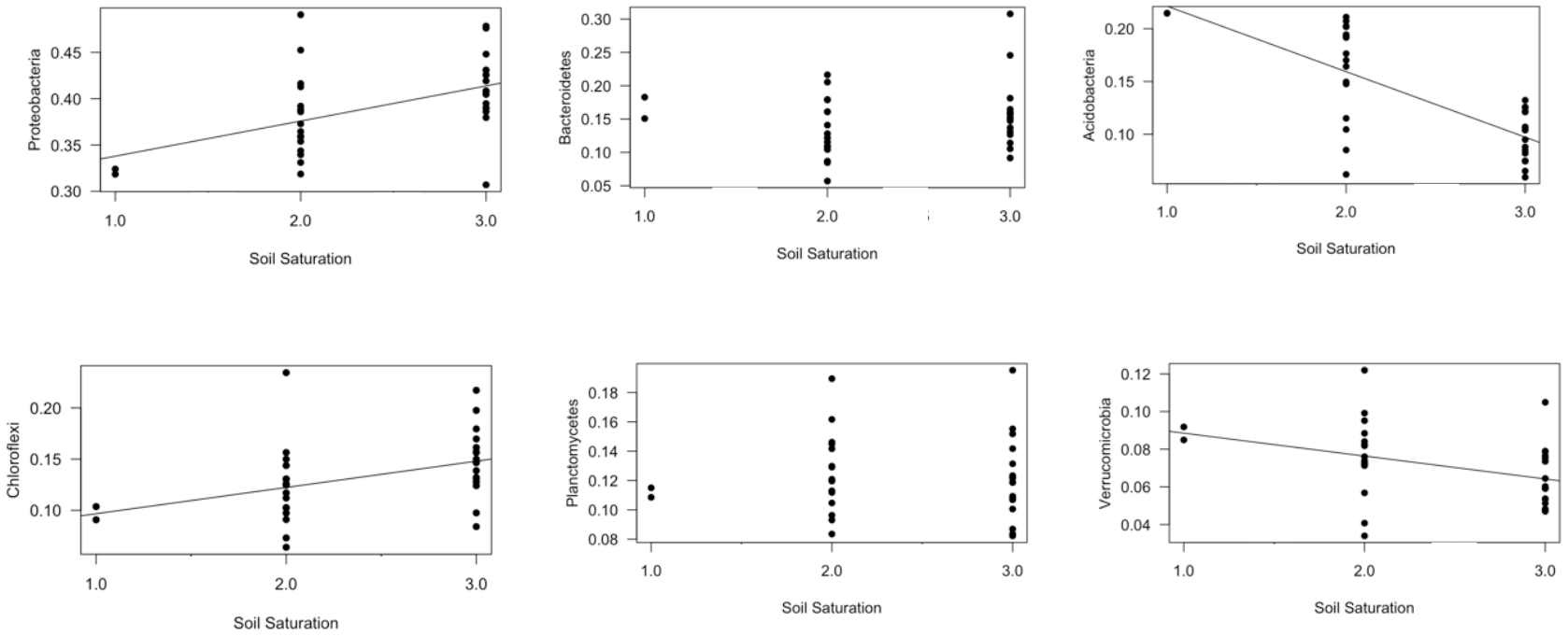


Fig S3.2: Regression of relative abundance of bacterial phyla vs. soil saturation. Trend lines indicate significant correlation ($P < 0.05$) between saturation and the given bacterial phylum.

Table S3.4: a) ANCOVA table showing the relationship between site, lineage, soil saturation, and nutrients on relative abundance of fungal phyla. b) ANOVA table showing the relationship between site and plant lineage on the relative abundance of fungal phyla.

		r^2			Lineage		Saturation		SoilN		SoilP		Sat:Lin	
	Residual df	**Multiple r^2**	**Adjusted r^2**	***P***	**F**	***P***	**F**	***P***	**F**	***P***	**F**	***P***	**F**	***P***
Unclassified RA	25	0.373	0.222	0.051	0.09	0.771	**12.36**	**0.002**	0.60	0.445	0.20	0.657	1.21	0.282
Ascomycota RA	25	0.163	0.000	0.573	1.26	0.272	1.77	0.195	0.45	0.508	1.26	0.273	0.02	0.895
Basidiomycota RA	25	0.203	0.012	0.411	1.11	0.301	3.08	0.091	0.04	0.849	1.28	0.268	0.78	0.384
Rozellomycota RA	25	0.075	0.000	0.911	0.67	0.418	0.51	0.484	0.28	0.600	0.16	0.692	0.02	0.903
Chytridiomycota RA	25	0.060	0.000	0.947	0.80	0.381	0.53	0.471	0.00	0.975	0.07	0.798	0.05	0.830
Glomeromycota RA	25	0.314	0.150	0.119	1.00	0.326	**6.66**	**0.016**	0.32	0.579	0.00	0.997	0.79	0.383

	Lineage			Site			Site X Lineage		
	df	**F**	**P**	**df**	**F**	**P**	**df**	**F**	**P**
Unclassified RA	1	0.00	0.969	**5**	**6.05**	**0.001**	5	1.49	0.235
Ascomycota RA	1	2.44	0.133	**5**	**3.74**	**0.013**	5	0.17	0.972
Basidiomycota RA	1	0.80	0.381	5	1.32	0.293	5	0.38	0.860
Rozellomycota RA	1	0.81	0.376	**5**	**3.80**	**0.012**	5	0.15	0.977
Chytridiomycota RA	1	0.85	0.367	5	1.00	0.439	5	0.66	0.658
Glomeromycota RA	1	0.67	0.422	**5**	**2.99**	**0.033**	5	1.56	0.212

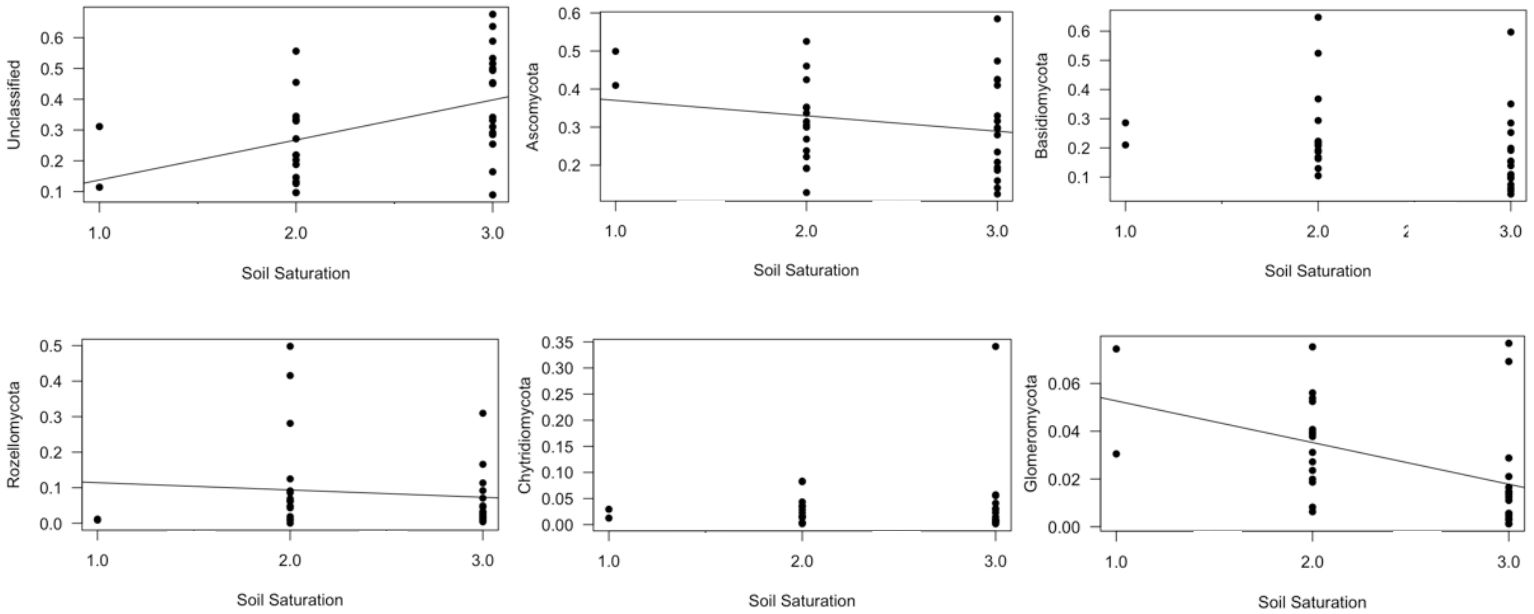


Fig S3.3: Regression of relative abundance of fungal phyla vs. soil saturation. Trend lines indicate significant correlation ($P < 0.05$) between saturation and the given fungal phylum.

Table S3.4: a) ANCOVA table showing the relationship between site, lineage, soil saturation, and nutrients on relative abundance of oomycete families. b) ANOVA table showing the relationship between site and plant lineage on the relative abundance of oomycete families.

a)

		r^2			Lineage		Saturation		SoilN		SoilP		Sat:Lin	
	Residual df	**Multiple r^2**	**Adjusted r^2**	***P***	**F**	***P***	**F**	***P***	**F**	***P***	**F**	***P***	**F**	***P***
Pythiaceae RA	21	**0.463**	**0.309**	**0.028**	**5.40**	**0.030**	3.57	0.073	0.53	0.473	0.59	0.453	**7.11**	**0.014**
Unclassified RA	21	**0.482**	**0.334**	**0.020**	**5.33**	**0.031**	4.16	0.054	0.79	0.383	0.83	0.374	**7.09**	**0.015**
Saprolegniaceae RA	21	0.120	0.000	0.817	0.51	0.485	0.10	0.758	0.47	0.500	0.35	0.559	0.57	0.460
Leptoleniaceae RA	21	0.176	0.000	0.619	1.09	0.309	0.37	0.552	0.03	0.864	0.39	0.540	0.08	0.778

b)

	Lineage			Site			Site X Lineage		
	df	**F**	**P**	**df**	**F**	**P**	**df**	**F**	**P**
Pythiaceae RA	1	3.93	0.064	**5**	**3.51**	**0.023**	**5**	**3.60**	**0.021**
Unclassified RA	1	3.64	0.074	**5**	**3.68**	**0.019**	**5**	**3.76**	**0.018**
Saprolegniaceae RA	1	0.47	0.501	5	0.54	0.741	5	0.69	0.636
Leptoleniaceae RA	1	0.49	0.495	5	0.57	0.726	5	0.57	0.726

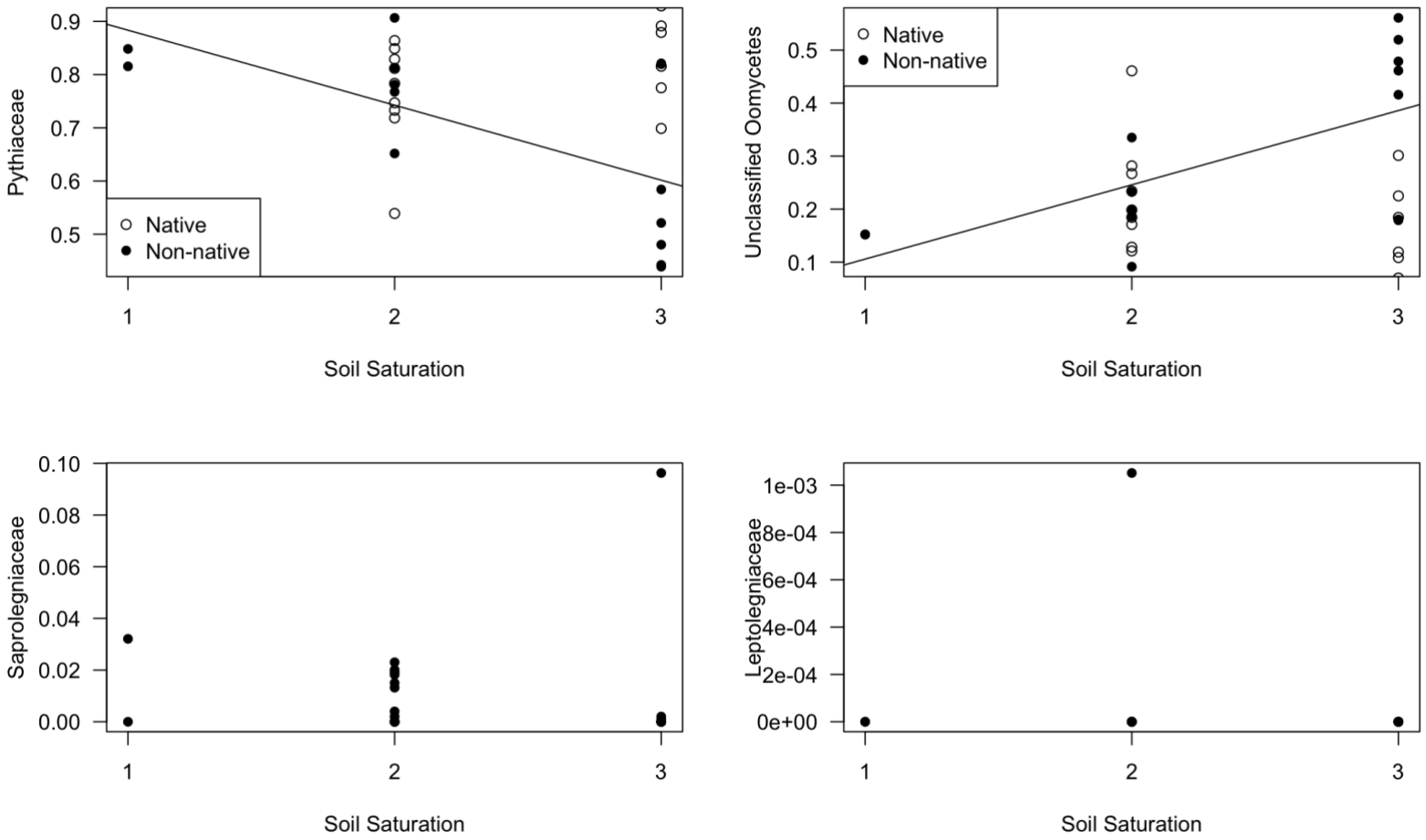


Fig S3.4 Regression of relative abundance of oomycete families vs. soil saturation. Soil saturation levels indicate degree of saturation: 1=Unsaturated, 2=Saturated, 3=Standing water. In a) and b) trendlines indicate significant correlations ($P < 0.05$) between saturation and relative abundance in non-native plants only. Native correlations non-significant (see Table S3.4)

Fig. S3.5

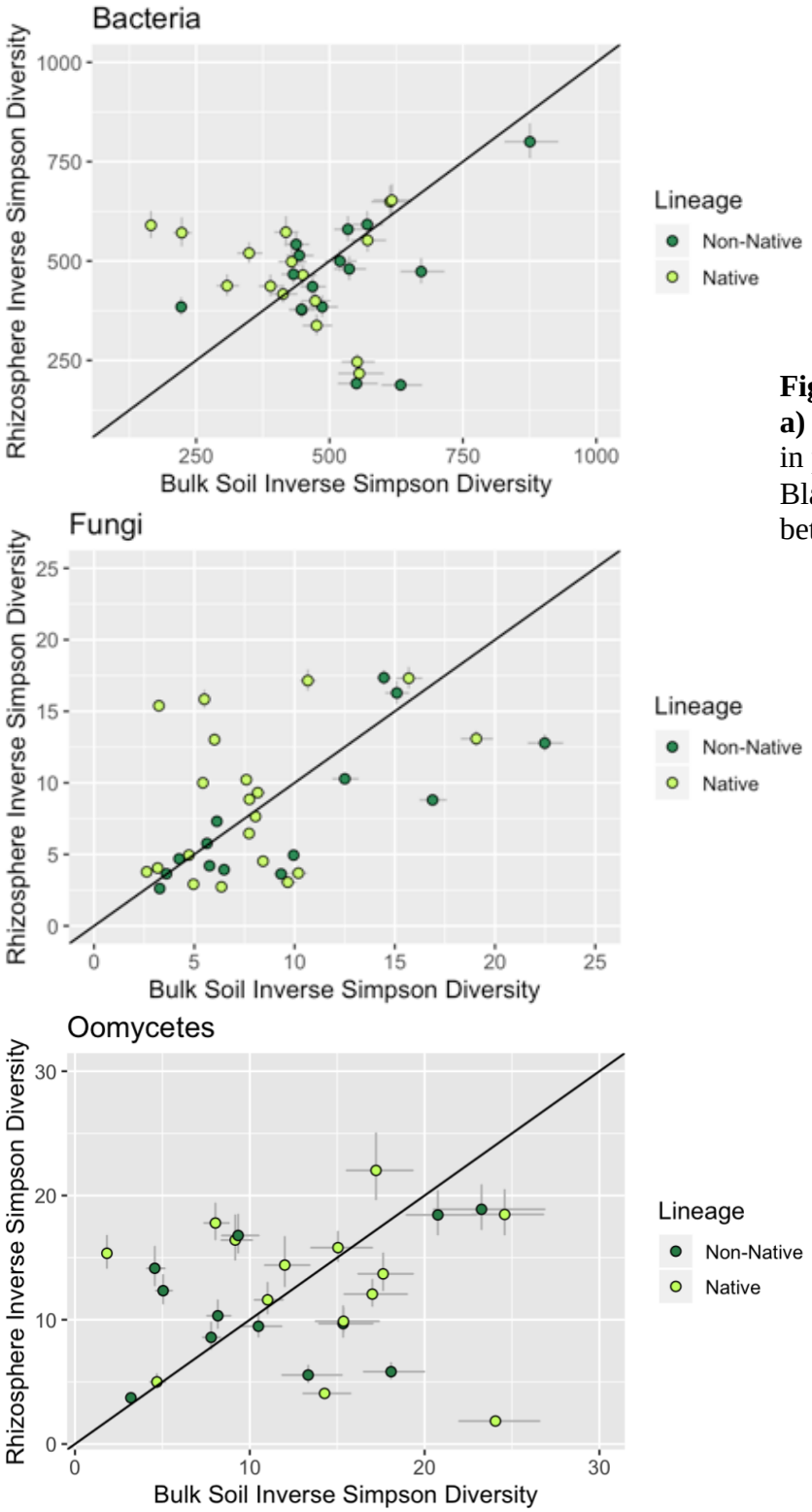


Fig.S3.5: Inverse Simpson diversity of **a)** bacteria, **b)** fungi, and **c)** oomycetes in paired rhizosphere and bulk soils. Black line indicates 1:1 relationship between bulk and rhizosphere diversity.

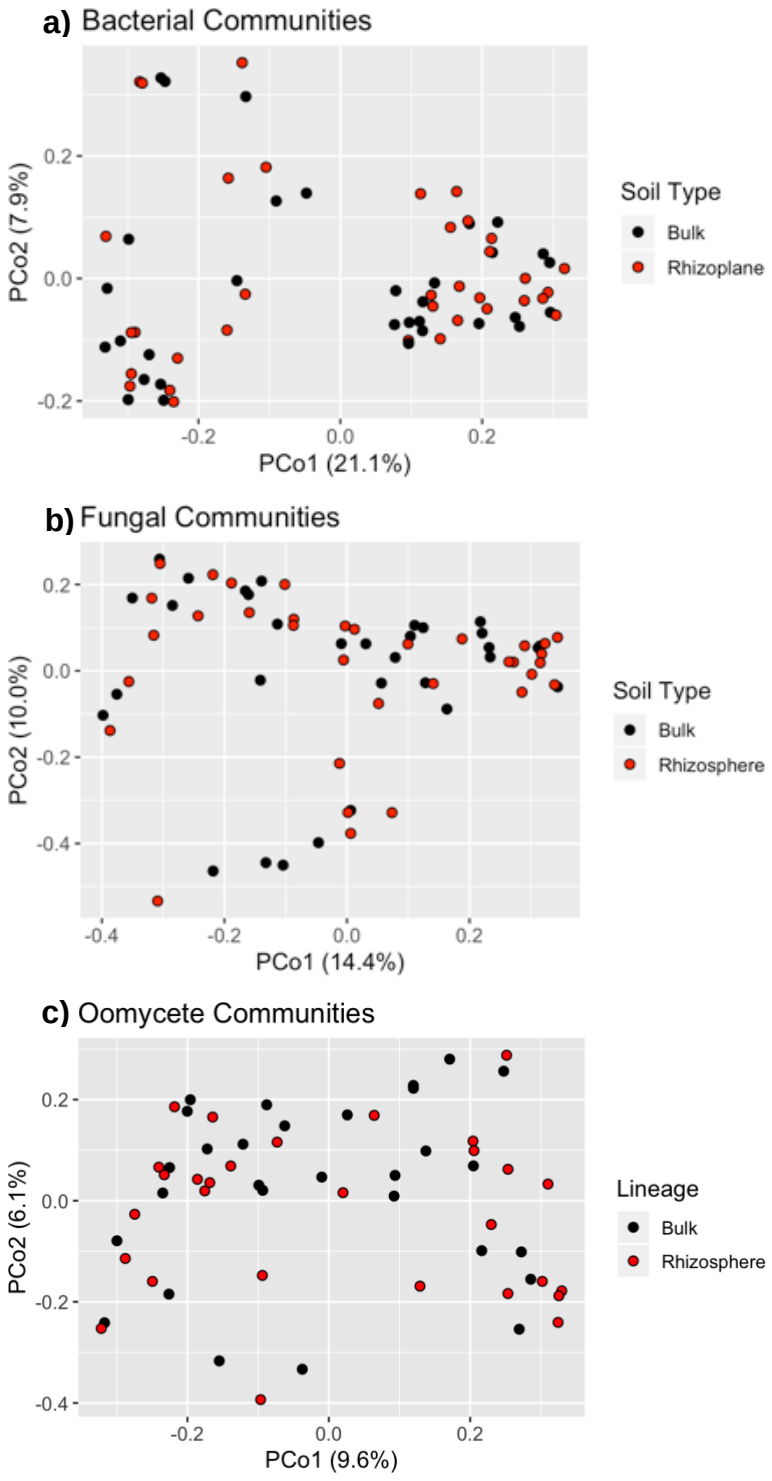


Fig. S3.6: Principle coordinate analysis of Bray-Curtis distances between bulk and rhizosphere **a)** bacterial, **b)** fungal, and **c)** oomycete communities. Bacterial (Per-MANOVA P = 0.969; PermDISP P = 0.958), fungal (Per-MANOVA P = 0.979; PermDISP P = 0.511), and oomycete (Per-MANOVA P = 0.86; PermDISP P = 0.545) communities did not differ between bulk and rhizosphere soils.

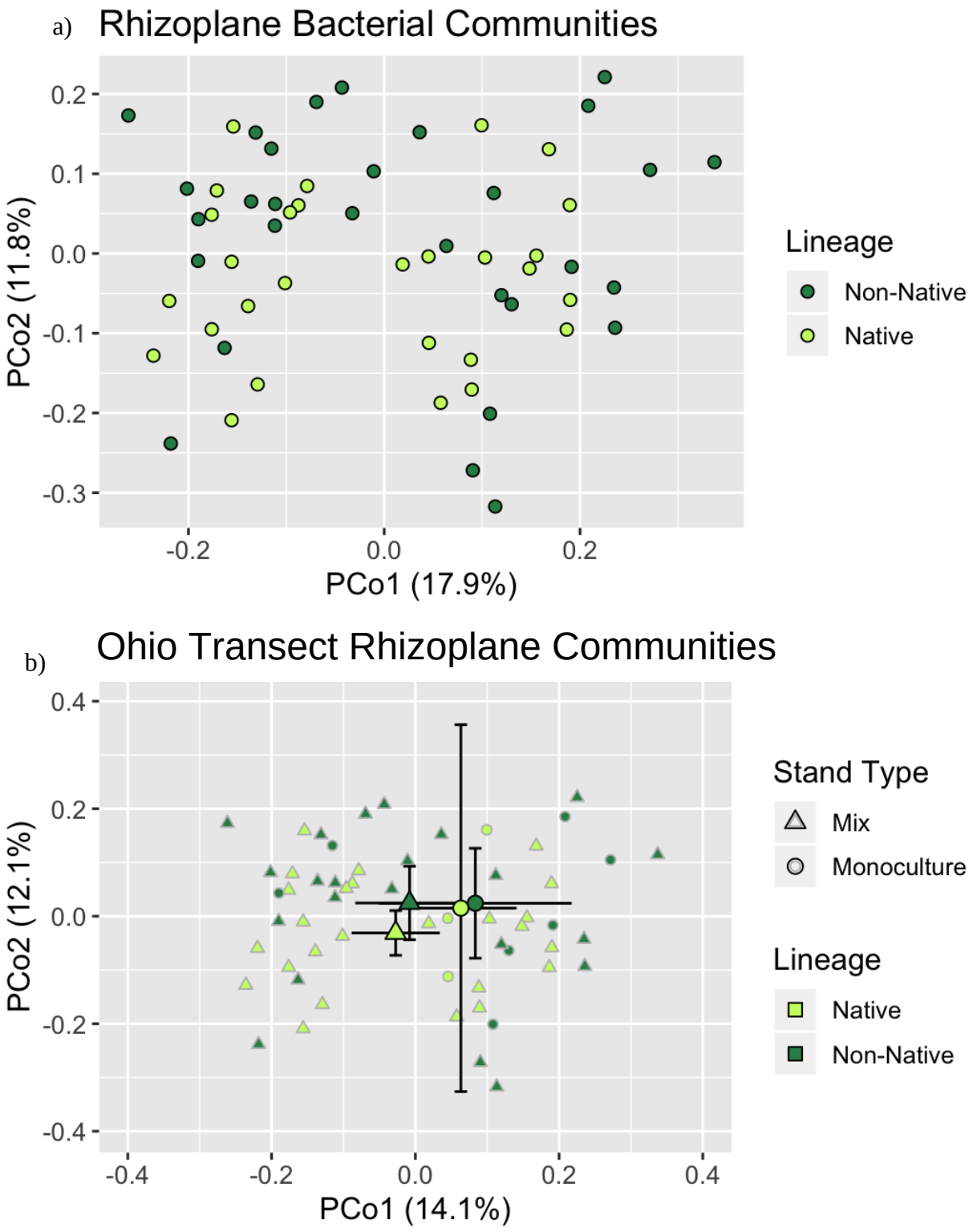


Fig. S3.7: a) Rhizoplane communities by lineage PCoA b) Rhizoplane community differences by stand type (mix and monoculture) and plant lineage (native and non-native)

Table S3.6: Pair-wise Per-MANOVA comparing **rhizosphere** bacterial communities in various zones of *Phragmites*. InvMix: Non-native *Phragmites* focal plant in a mixed zone, NatMix: Native focal plant in a mixed zone, InvMono: Non-native focal plant in a monoculture zone, NatMono: Native focal plant in a monoculture zone. P-values adjusted using a Bonferroni correction.

Pairs	F-value	r^2	*P*-value	*P*-adjusted*
InvMix vs NatMix	0.89	0.017	0.601	1.000
InvMix vs InvMono	1.05	0.032	0.348	1.000
InvMix vs NatMono	1.67	0.054	0.042	0.252
NatMix vs InvMono	1.21	0.036	0.188	1.000
NatMix vs NatMono	1.30	0.043	0.125	0.750
InvMono vs NatMono	2.08	0.159	0.008	0.048

Table S3.7: Pair-wise Per-MANOVA comparing **rhizoplane** bacterial communities in various zones of *Phragmites*. InvMix: Non-native *Phragmites* focal plant in a mixed zone, NatMix: Native focal plant in a mixed zone, InvMono: Non-native focal plant in a monoculture zone, NatMono: Native focal plant in a monoculture zone. *P-values adjusted using a Bonferroni correction.

Pairs	F-value	r^2	*P*-value	*P*-adjusted*
InvMix vs NatMix	1.05	0.023	0.324	1.000
InvMix vs InvMono	0.96	0.033	0.442	1.000
InvMix vs NatMono	0.95	0.040	0.451	1.000
NatMix vs InvMono	1.60	0.049	0.042	0.252
NatMix vs NatMono	1.08	0.040	0.322	1.000
InvMono vs NatMono	0.91	0.092	0.600	1.000

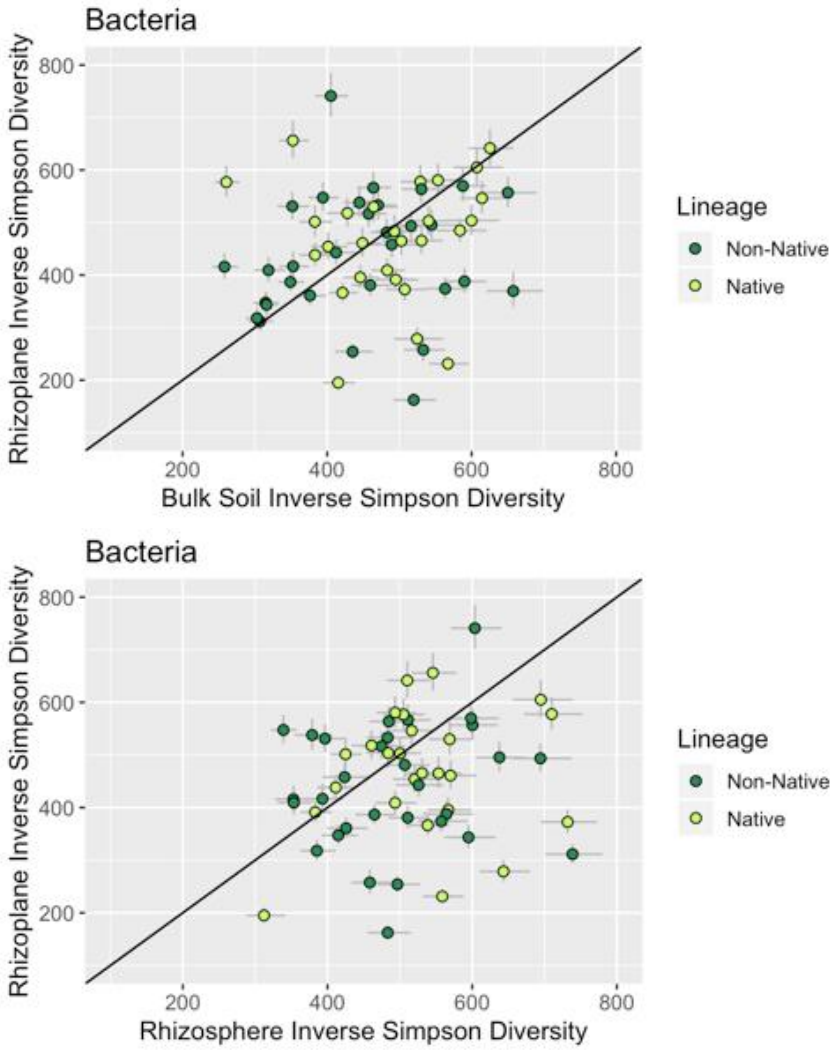


Fig. S3.8: Inverse Simpson diversity in paired **a)** rhizoplane and bulk soils and **b)** rhizoplane and rhizosphere soils from the Ohio transect sites. Black line indicates 1:1 relationship between bulk and rhizosphere diversity. Overall, rhizosphere soils were more diverse then the adjacent paired bulk soil ($t = -2.799$, $df = 64$, $P = 0.007$) and more diverse than paired rhizoplane soils ($t = -3.059$, $df = 54$, $P = 0.003$). Rhizoplane soils were not different in diversity from bulk soil ($t = 0.786$, $df = 57$, $P = 0.435$).

Chapter 4 : How Do Plants and Microbes Interact to Influence Invasiveness in *Phragmites australis*?

Introduction

Soil microorganisms may play a critical role in plant performance during range expansion, and they are thought to facilitate plant invasions in some circumstances (Reinhart and Callaway 2006, Maron et al. 2014, Kowalski et al. 2015). Theory suggests that microbially-mediated invasiveness occurs when invasive plants are disproportionately benefited by their microbial community over native plants (Reinhart and Callaway 2006). The disproportionate benefit for invasive plants may emerge through increased interaction with mutualist microorganisms or decreased accumulation of pathogens relative to native plants.

When a plant alters the soil microbial community in a way that directly affects its own growth, a positive or negative plant-soil feedback (PSF) will be realized over the long term (Bever et al. 1997). Feedbacks can be measured either in a species-specific (measuring absolute performance in conspecific *vs.* heterospecific soils) or pair-wise context (comparing the relative performance of two plant species in soils from each species), depending on the goals. Species-specific (or direct) feedbacks inform how individual plants are affecting and responding to soil biota in the short term, (independent of how other species are interacting with soils); pair-wise feedbacks incorporate two plants' effects on and responses to soils and inform whether dominance or co-existence between two species is predicted in the long-term (Bever et al. 1997). Positive (or at least less negative) species-specific PSFs are commonly cited as a major factor in the expansion of invasive plant species (Klironomos 2002, Mangla et al. 2008, Meisner et al. 2014, Maron et al. 2014). Under the PSF model for invasiveness, native and invasive plants have a differential effect

on soil microbial communities, and, in turn, experience a differential response to the soil microbial community such that the invasive plant experiences a more positive or less negative feedback than native plants (Reinhart and Callaway 2006, Inderjit and Cahill 2015).

Despite compelling theory and several individual validations of these ideas, overall results of PSF studies to date offer limited utility for predicting invasiveness for several reasons. First, the majority of PSF studies take a "black box" approach in which soil biota are indirectly assessed via plant responses, leading to difficulty in pinpointing the specific mechanisms mediating PSFs (Cortois and de Deyn 2012). Second, and perhaps more importantly, recent meta-analyses question the widespread importance of PSFs in driving invasions (Suding et al. 2013, Meisner et al. 2014, Crawford et al. 2019). Opposite of expectations, on average, native plants displayed positive species-specific feedbacks, whereas species-specific invasive plant feedbacks were neutral (Suding et al. 2013). In addition, native and invasive pairs tended to generate slight negative pairwise feedbacks on average, predicting stabilizing coexistence through conspecific negative density dependence (Crawford et al. 2019). This prediction of coexistence is not consistent with the reality of most invasions and thus implies that other factors impact invasive success.

Of course, many factors not related to soil biota could drive invasiveness (Parker et al. 2006), but it is also important to note that soil microbes can still contribute to invasiveness even without PSFs. Complex interactions between plants and microbes can be simplified into (1) effect of plants on microbes and (2) response of plants to microbes (Fig. 4.1). Two co-occurring plants can have the same or different effects and responses and the permutations of differential effects and/or responses determine whether performance differences are mediated through the soil microbial community (Fig. 4.1). Disproportionate benefits for invasive plants may emerge through (1) cultivating a different microbial community with relatively more mutualists or fewer pathogens

than native counterparts (differential effects *and* responses, Fig. 4.1d), or (2) responding more positively or less negatively than natives to a common pool of microorganisms (differential response, Fig. 4.1c). Either mechanism leads to patterns of increased growth and expansion by invasive, relative to native, inhabitants. However, species-specific and pairwise feedbacks are only generated when both effects *and* responses differ between the native and plants (Fig. 4.1d; Bever et al. 1997). Therefore, thorough examination of plant effects on and responses to soil microbes is critical for a fuller understanding of drivers of invasiveness. Additionally, teasing apart the components of the microbial community that are most affected by invasive plants and those that invasive plants respond to the most will improve predictability and management of plant invasions.

Phragmites australis is a cosmopolitan wetland grass with multiple lineages worldwide, and it is often considered a model organism for studying plant invasions (Meyerson et al. 2016). Invasive to North America, the European lineage (*Phragmites australis* haplotype M; hereafter, non-native *Phragmites*) is highly productive, fast growing, and very large, often forming dense monocultures supporting a low overall species diversity. The native lineage in North America (*Phragmites australis* subsp. *americanus*, hereafter native *Phragmites*) is conversely characteristic of low nutrient, high-diversity wetlands and is considered desirable for wildlife habitat (Price et al. 2013). Recent field surveys in North America have largely focused on the effect component of plant-microbe interactions, exploring differential effects of *Phragmites* lineages on rhizosphere communities of soil biota. While some studies have found that native and non-native lineages differed in their communities of bacteria (Bowen et al. 2017), archaea (Yarwood et al. 2016), and oomycetes (Nelson and Karp 2013), the existence and strength of those effects is inconsistent among studies (Bowen et al. 2017, Yarwood et al. 2016, Bickford et al. *in prep*). Further, the differences that exist may be a consequence of soil micro-environmental changes caused by

invasion rather than a driver of invasion (Bickford et al. *in prep*). Most studies of the effect components have studied the soil communities of the rhizosphere, but Bickford et al. (2018) found that the root endophyte communities of bacteria, fungi, and oomycetes did not differ between lineages of *Phragmites* in a study in the Great Lakes. Differential responses have also been documented, albeit to much less significant extent, finding that oomycete pathogen virulence appears to differ between *Phragmites* lineages (Crocker et al. 2015). Only a single study has combined effect and response components and explored species-specific feedbacks, finding that the native and non-native lineage of *Phragmites* both produced weakly negative feedbacks (Allen et al. 2018) suggesting that both lineages may be weakly negatively impacted by their own soil relative to other lineages. When feedbacks are not strongly different from zero, it is difficult to infer whether differential conditioning (effects) and/or responses are occurring (Bever et al. 1997). Thus, it remains unclear whether microbes have any impact on invasiveness of *Phragmites* at all and, if so, which mechanisms are at play in this system. Additionally, it remains unclear which microbial groups are most directly affected by each lineage of *Phragmites*, which microbial groups each lineage responds to strongly, and if differential effects on and/or responses to microbial groups are likely drivers of invasiveness.

Here, we expand upon recent PSF feedback experiments and attempt to isolate effects and responses of native and non-native *Phragmites* lineages to specific microbial groups. If microbial communities surrounding non-native *Phragmites* are driving its invasiveness, we would expect non-native *Phragmites* to be disproportionately benefited by soil microbes by either (1) displaying differential effects on *and* response to soil microbes compared to native *Phragmites* (*i.e.* generating feedbacks, Fig. 4.1d) or (2) responding differently to a similar community of microbes (Fig. 4.1c). First, we look for evidence of species-specific or pairwise feedbacks in our system using a

traditional reciprocal transplant PSF approach. Next, we disentangle the feedback into effects and responses by (a) using advanced molecular methods to peer inside the "black-box" and compare microbial communities cultivated by each lineage and (b) applying targeted microbial inhibitors to soils conditioned by each lineage to compare each lineage's response to broad components of each microbial community. We aim to classify interactions between native and non-native *Phragmites* and their soil microbes into one of the four categories on Figure 4.1 and address whether interactions with their microbial communities likely drive invasiveness.

Methods

We implemented a reciprocal transplant plant-soil feedback approach to address our objectives. The study was designed to assess the growth response of each *Phragmites* lineage to the particular microbial groups cultivated in soils conditioned by each lineage. We also compared growth in conditioned soils to sterilized soils. To identify which microbes most strongly influenced plant responses, we applied microbial inhibitors individually and in combinations to target bacteria, fungi, and oomycetes in pots conditioned by either plant lineage. Each step in the experiment is described in detail below; see Fig. 4.2 for an abbreviated guide.

Plant material collection. Plant belowground material and rhizosphere soils were collected throughout Michigan and Ohio, USA. We sampled from 10 unique genotypes of native and non-native *Phragmites*. At each sampling location, *Phragmites* stands were identified as native or non-native morphologically; plant material was subsequently collected to verify field identification after Saltonstall (2003). Belowground plant material, along with rhizosphere soil, was exhumed from the interior of a patch using a sharpshooter shovel, keeping much of the rhizomes intact and placed into a 5-gal bucket and sealed. All belowground materials were stored in a cold room at 4°C prior to use.

Preparing vegetative clones and soils. Rhizomes from each site were thoroughly washed with deionized water and cut into segments ~50 cm long. Rhizome segments were placed into trays, filled approximately half full of deionized water, and placed under LED growth lights programmed on a 16 hr light/ 8 hr dark cycle within the indoor plant growth facility in the Biological Sciences Building at the University of Michigan. After ~3 days, stem sprouts appeared at the nodes. Trays were drained, rinsed, and refilled every three days to prevent contamination and ensure steady moisture conditions. Stems were allowed to grow for 2 weeks.

Following Kulmatiski and Kardol (2008), we prepared mixes of 70 % sand, 25 % peat, and 5 % field soil inoculum. We triple autoclaved Quikcrete pre-washed and screened play sand and stored it in sterilized 5-gal buckets. We also triple autoclaved Pro-Moss sphagnum peat moss and stored it in sterilized 5-gal buckets. Field soil was gathered from buckets containing rhizomes and homogenized among all locations to create a generalized inoculum (Fig. 4.2a). A subset of that inoculum was triple autoclaved for addition into our "sterile" treatment pots. Appropriate proportions of sand, peat, and field inoculum were homogenized in large batches in a portable cement mixer. Four-hundred pots (15 cm diameter x 11 cm tall) were filled with 1 L of the loosely packed soil mixture.

Rhizomes were cut into 1-cm segments, each containing a node with a single stem and roots. Rhizome cuttings and roots were triple rinsed with deionized water prior to planting. Cuttings were not sterilized because preliminary tests indicated that plants responded negatively to sterilization (submersion in dilute bleach, data not shown). Only stems at least 7 cm tall and with at least 2 leaves were transferred to pots. Half of the pots were planted with native cuttings and half with non-native. All cuttings that did not survive for 2 weeks following initial

transplantation were replaced. Stem height (med = 27 cm, sd = 13.73 cm). root length (med = 13, sd = 9.58), and leaf number (med = 5, sd = 1.28) of the initial cutting was recorded for each pot.

Soil Conditioning Phase. Pots were randomly distributed among benches and subjected to a 16hr light / 8hr dark cycle using ceiling mounted high intensity growth lights. Plants were fertilized initially with 0.5 g/L Fe chelate (Sequestrene 330) and 400 ppm 15-16-17 NPK solution. Pots were irrigated using a dripline irrigation system with individual drippers in each pot for 4 mins twice daily. Plants were fertilized again after 60 days with the same fertilizer mixture as described above. Plants grew under constant temperature conditions of approximately 27 °C for a total of 120 days (Fig. 4.2B).

At the end of the conditioning phase, soil samples were collected in 2 mL cryovials and flash-frozen in liquid nitrogen. Heights of three randomly selected stems in each pot were measured. Plants were harvested from each pot by removing pot-bound below-ground mass from the pot and shaking adhering soil into a sterile 5-gal bucket. Below-ground tissues remained intact and were only broken up to sufficiently remove all soil from roots and rhizomes. Soils were returned to their original pot. Plant materials were separated into roots, rhizomes, and aboveground tissues and dried at 70 °C for 48 hrs. Pots with plants that grew insufficiently or did not survive were not used in the next phase; 320 of the initial 400 pots were used.

Microbial inhibitor treatments. We randomly assigned pots to one of eight microbial inhibitor treatments (1. No inhibitor treatment (control), 2. Antibacterial, 3. Antifungal, 4. Anti-oomycete, 5. Antibacterial and Antifungal, 6. Antibacterial and Anti-oomycete, 7. Antifungal and Anti-oomycete, and 8. Sterile). Pots that received sterilized inoculum prior to conditioning phase were grouped as the Sterile treatment and received no chemical inhibitors. The antibacterial treatment consisted of 7.44 mg/L Streptomycin Sulfate (calculated to equal roughly 5 mg / g dry

soil adapted from Kooijman et al. 2016). The antifungal treatment contained 0.93 mL of 40% Pentachloronitrobenzene (PCNB) per liter of deionized water (following label recommendation for soil drench). The anti-oomycete treatment was comprised of 0.16 mL of 22.5% Mefenoxam (2-[(2,6-dimethylphenyl) methoxyacetylamino]propionic acid methyl ester) per liter of deionized water (following label recommendation for soil drench). Mixed inhibitors were combined at the same rate of undiluted active ingredient as the single inhibitor treatments. The no-inhibitor treatment pots were treated with deionized water. All inhibitors were applied by submerging pots in 2.4 L buckets filled with the corresponding inhibitor solutions. This method minimized air-filled pore space and maximized soil contact with inhibitors. Soils remained submerged for 60 mins then drained to approximately field capacity and returned to their randomized locations on benches.

Seedling Phase. Seeds were collected from field identified and genetically confirmed populations of native and non-native *Phragmites* in Michigan and Ohio. Seeds were cold stratified for 6 weeks, surface sterilized following a 2-day procedure (USGS GLSC, unpublished). On day one, seeds were washed in 97% EtOH for 3 mins, in 1% bleach for 2 mins, triple rinsed in deionized water and stored overnight in deionized water at 4°C. On day 2, seeds were washed in 1% bleach for 5 mins, triple rinsed with deionized water and stored in deionized water at 4°C. Sterilized seeds were plated on 1.5 % Gamborg's media with 0.5 g/L Sequestrine 330 Fe chelate, parafilmed shut, and placed on edge into a growth chamber set at 37°C and 12 hr light / 12 dark cycle. Germinating seedlings were transplanted into sterilized (triple autoclaved) seed-starting medium (SunGro) and placed under LED growth lights programmed on a 16hr light/ 8hr dark cycle. Seedling trays were watered with deionized water containing 0.5 g/L Fe chelate (Sequestrene 330) and 400 ppm 15-16-17 NPK solution. Seedlings were transplanted when they had at least 1 leaf (non-cotyledon).

Seedlings were transplanted into pre-conditioned and treated pots 3 days post soil treatment. All seedlings that did not survive the first week after initial transplantation were replaced. Seedlings grew under constant conditions for 120 days. Stem heights of each plant were recorded at transplanting (med = 5.55 cm, sd = 4.03 cm) and measured weekly. Soil was subsampled 10 days after initial transplantation and at the end of the study period (before plants were harvested). Irrigation was turned off three days prior to harvesting to dry soils. Plants were then removed from pots, shaken and thoroughly manipulated to remove soils from belowground tissues. Plants parts were separated into aboveground tissues, roots, and rhizomes and then dried at 70°C for 48 hrs and weighed.

Soil molecular methods

DNA was extracted from 50 mg (wet weight) of soil (from the end of conditioning phase and the end of the seedling phase) using Qiagen PowerSoil PowerLyzer DNA extraction kits. We used manufacturer protocols, with the exception of improvements to reduce ethanol contamination (*e.g.*, extra spins, more frequent transfers to sterile tubes). DNA was eluted with molecular grade water. All genomic DNA extracts were verified by electrophoresis. Extracts were checked for quality on a NanoDrop UV/Vis spectrophotometer and concentration using a Quant-iT PicoGreen dsDNA kit (Invitrogen, Carlsbad, CA, USA).

Quantitative polymerase chain reactions (qPCR) were performed to quantify relative abundances of fungal and bacterial communities prior to the application of microbial inhibitors (end of conditioning phase) as well as at the end of the seedling phase. All qPCR reactions were performed using subsamples diluted from the same template genomic DNA samples. DNA standards for qPCR were prepared by cloning amplicons generated using primers 338f/518r (bacteria) and ITS1f/5.8s (fungi) from environmental soil DNA with a TOPO TA pCR 2.1 Cloning

Kit (Life Technologies). The inserts were then amplified with M13 primers to create a linear target region (Hou et al. 2010). PCR products were purified using a QIAquick PCR Purification Kit (Qiagen) and quantified using a Quant-iT PicoGreen dsDNA kit. Copy number was then calculated from DNA concentrations and known amplified region length (bacteria or fungi specific insert + M13 region). See Table S4.1 for qPCR reagents, conditions, efficiencies, and R^2. In order to minimize between-run variability, samples collected from the same pot at different time points were analyzed within the same qPCR run. For the same reason, sample order was determined so that the 7 different inhibitor treatments would be evenly dispersed among runs to minimize bias. Data obtained from qPCR was normalized to copies / ng of dry soil; for this conversion, soil moisture content was determined by drying a subsample of approximately 0.5 g, taken from each vial used for DNA extraction, and dried at 105°C for 48 hours. Soils were weighed before and after drying to determine percent moisture content.

All polymerase chain reactions (PCR) for each microbial group (*i.e.*, fungi, bacteria) were performed using subsamples of the same template genomic DNA sample. Genomic DNA was diluted to ensure equimolar concentration of template DNA in each PCR reaction. Bacterial amplicons were generated using primers described in Kozich et al. (2013), which target the V4 region of the 16S rRNA gene. Fungal amplicons were produced using primers described by Taylor et al. (2016), which target the ITS2 region of the 5.8S rRNA gene. See Appendix S4 (Table S4.2) for specific primer sequences and PCR conditions. We did not identify oomycete communities in soils due to of the lack of significant response in the biomass data and the lack of community differences observed in Bickford et al. (in prep). All PCR reactions were performed in triplicate using Phusion High Fidelity DNA Polymerase and master mix (New England BioLabs, MA, USA). Libraries were normalized using SequalPrep Normalization Plate Kit (Life technologies cat

A10510-01) following the manufacturer's protocol for sequential elution. The concentration of the pooled samples was determined using Kapa Biosystems Library Quantification kit for Illumina platforms (Kapa Biosystems KK4824). The sizes of the amplicons in the library were determined using the Agilent Bioanalyzer High Sensitivity DNA analysis kit (cat# 5067-4626). The final library consisted of equal molar amounts from each of the plates, normalized to the pooled plate at the lowest concentration. Amplicons were sequenced by the Microbial Systems Molecular Biology Laboratory (MSMBL) at the University of Michigan on the Illumina MiSeq platform, using a MiSeq Reagent Kit V2 500 cycles (Illumina cat# MS102-2003), according to the manufacturer's instructions.

Bioinformatics analysis

Raw bacterial sequence data were processed using mothur v1.40.1 (Schloss et al. 2009). Operational taxonomic units (OTUs) were clustered at 97% for bacterial sequences and assigned to taxonomy by comparing representative sequences to the taxa found in the SILVA database (Quast et al. 2018). Raw fungal sequences were processed using QIIME2 (Bolyen et al. 2019) which can implement *de novo* sequence clustering that does not require sequence alignment. Fungal sequences were clustered into OTUs at 97% similarity and assigned to taxonomy based on the UNITE database (Nilsson et al. 2013). Each microbial group was rarefied according to the sample that yielded the fewest number of sequences to ensure equal coverage across all samples. Bacteria were rarified to 10,019 sequences and fungi to 993 sequences. Full bioinformatics workflows can be found on GitHub at https://github.com/wesbick/PSF.

Data Analysis

Seedling biomass was square root transformed to conform to a normal distribution. Feedbacks and response to treatments were calculated using Cohen's d.

$$(1)\ Cohen's\ d\ =\ \frac{x_t - x_c}{pooled\ sd}$$

Where $x_t = Heterospecific\ conditioned\ soil$ and $x_c = Conspecific\ conditioned\ soil$ in feedbacks (using no-inhibitor controls) and $x_t = Treated\ Soil$ and $x_c = no\ inhibitor\ Control$ (within same seedling and conditioning lineage) when assessing response to inhibitor treatments. We used t-tests to assess significant differences between conspecific and heterospecific conditioning lineages. We calculated pair-wise feedbacks (I_s) following Bever et al. (1997) and Crawford et al. (2019).

$$(2)\ I_s = \alpha_A - \alpha_B - \beta_A + \beta_B$$

Where α_A is plant A's performance in conspecific soil, α_B is plant B's performance in heterospecific soil, β_A is plant A's performance in heterospecific soil, and β_B is plant B's performance in conspecific soil. To assess response to the total microbiome (sterile vs. live), we subset the biomass data to include only no-inhibitor pots (n = 60) and performed a 3 way ANOVA (Type III Sum of Squares) with conditioning lineage (2 levels), seedling lineage (2 levels), and soil inoculum (2 levels) as main effects. To test response to inhibitor treatments, we standardized biomass of each seedling to the mean biomass of the sterile treatment to enable comparison between native and non-native seedlings that differed in size and analyzed results as a 3 way ANOVA (Type III Sum of Squares with conditioning lineage (2 levels), seedling lineage (2 levels), and inhibitor treatment (7 levels) as main effects.

To explore microbial community composition, we calculated Bray-Curtis distances and used permutational multivariate analysis of variance (Per-MANOVA) to test whether conditioning lineage, seedling lineage, or inhibitor treatment resulted in significant microbial community differences among our samples at the end of the conditioning phase and at the end of the seedling phase. Pairwise Per-MANOVAs (comparing all possible pairs) were also calculated to identify

which inhibitor treatments significantly impacted microbial community composition. *P*-values generated from pair-wise comparisons were adjusted using a Benjamini-Hochberg adjustment (Benjamini and Hochberg 1995). Homogeneity of Dispersions (PermDISP) further assessed whether microbial community samples differed in their degree of dispersion from their centroid. We used Principle Coordinate Analysis (PCoA) to assess and visualize microbial community differences between lineages and inhibitor treatments. Finally, we used a 3-way ANOVA (Type III Sum of Squares Factors: seedling lineage, conditioning lineage, inhibitor) to test responses of particular microbial taxa to each conditioning lineage, seedling lineage, and inhibitor treatment. All statistical analyses were run in the R environment (R Core Team 2016). All microbial community analyses were performed on each timepoint (pretreatment, end of study) separately. All R code, notes, and associated data can be accessed on GitHub at https://github.com/wesbick/PSF.

Results

Feedbacks

Neither native (F = 1.199, *P* = 0.289) nor non-native *Phragmites* (F = 1.882, *P* = 0.187) displayed species-specific feedbacks significantly different from zero (Fig. 4.3a). That is, nether performed significantly differently in the soil conditioned by the other, relative to their own. Interestingly, both lineages trended toward negative feedbacks, indicating both lineages tend to do better in soils of the other lineage. For feedbacks to favor non-native *Phragmites*, we would expect stronger positive or less strong negative species-specific feedbacks in the non-native relative to the native, leading to a positive pairwise feedback. However, we observed similar magnitude of feedbacks in each lineage. Despite the weak strength of species-specific feedbacks, the pair-wise feedback model predicts coexistence (Fig. 4.3b) because both performed better in heterospecific

soil. Below, we will unravel specific plant-microbial interactions related to plant performance and invasiveness using the conceptual framework of plant-microbial effect and response (Fig. 4.1).

Differential effects on the soil microbiome

At the end of the conditioning phase, both bacterial and fungal communities differed significantly between soils conditioned by native and by non-native *Phragmites*, but conditioning lineage accounted for less than 2% of the variance in community composition of both microbial groups (Fig. 4.4). Thus, native and non-native *Phragmites* affected microbial communities differently, but only slightly. In fact, soil fungal communities also differed in their dispersion around the centroid, and thus may not actually differ significantly (Fig. 4.4). Lineages did not significantly differ in relative abundance of any of the most common bacterial phyla (Fig. S4.2) but several fungal phyla did differ: soils conditioned by non-native *Phragmites* were comprised of fewer Chytridiomycota ($P < 0.001$) and (marginally) Glomeromycota ($P = 0.059$), but more Ascomycota ($P = 0.021$) than native-conditioned communities (Table 4.1).

Differential response to total soil microbiome

In response to the total microbial community, non-native *Phragmites* decreased growth relative to sterile conditions, regardless of which lineage conditioned the soil (Fig. 4.5, Table 4.2). In contrast, native *Phragmites* did not respond to the total microbial community as confirmed by the significant interaction between inoculum and seedling lineage (Table 4.2). Thus, microbes had an overall negative effect on non-native biomass, but a neutral effect on native *Phragmites* biomass, indicating differential response to the same total microbial community.

Effectiveness of inhibitor treatments

To evaluate the effectiveness of the microbial inhibitors, we first explored the effect of inhibitors on bacterial and fungal community composition pre-treatment and at the end of the study

and found evidence that the inhibitors did, in fact, modify their intended target communities. Interestingly, soil bacteria and fungi both changed in community structure, but not in abundance. Total bacterial and fungal gene copy numbers were not affected by the treatments (Appendix S4, Fig. S4.3), suggesting the microbial inhibitor treatments acted more like a disturbance to the targeted community than a removal of that group.

Bacterial community composition was significantly altered in response to the treatments that included the antibacterial agent (FUNBAC, BAC, BACOOM). Prior to applying the inhibitors, there were modest differences in bacterial communities among the treatment groups, however the variance explained by the treatment group was quite low in all with the exception of the sterile soil treatment, which were autoclaved at the beginning of the study (Fig. 4.6a; Pair-wise Per-MANOVA $P = 0.0028$, Table S4.3). At the end of the study, overall bacterial community composition was significantly altered in response to the treatments such that all communities that received the antibacterial agent were similar to each other (Fig. 4.6b; Pair-wise Per-MANOVA $P = 0.003$, Table S4.4). Additionally, the bacterial community composition following the treatments containing fungicide significantly differed from the control treatment (Pair-wise Per-MANOVA $P = 0.003$, Table S4.4), indicating a possible interaction between bacteria and fungi in the soil.

Similarly, before inhibitors were applied, only the soil fungi community of the sterile treatment differed from the other treatment groups (Fig. 4.6c; Pair-wise Per-MANOVA $P < 0.001$, Table S4.5). In response to the inhibitor treatments, the fungal communities shifted, wherein fungal communities receiving either antibacterial or antifungal agents significantly differed from sterile and no-inhibitor controls (Fig. 4.6d; Pair-wise Per-MANOVA $P < 0.05$, Table S4.5). Having established that the inhibitor treatments were effective in changing soil microbial communities, we interpret plant response to the treatments as response to microbial communities.

Because of the importance of this group for potential positive feedbacks, we further examined Glomeromycota, the fungal phylum that encompasses all arbuscular mycorrhizal fungi (AMF). Glomeromycota made up just ~2% of all sequences and were marginally more common in the native-conditioned soils ($F = 3.78$, $P = 0.059$, Table 4.1). Despite their low relative abundance, Glomeromycota were affected by the microbial inhibitor treatments (Fig. 4.7), declining in response to all three treatments receiving antifungal inhibitors (FUN, FUNOOM, and FUNBAC $P < 0.05$; Table S4.7) The abundance of AMF remained higher in the native-conditioned soils at the end of the study ($F = 13.94$, $P < 0.001$) and those soils' AMF were more strongly impacted by antifungal treatments (95% confidence intervals, Fig. 4.7; Conditioning lineage x Inhibitor interaction $F = 2.46$ $P = 0.021$, Table S4.7).

Differential response to major microbial groups

We standardized all biomass to the mean biomass of the sterile treatment (from the same seedling lineage) and compared standardized seedling biomass among inhibitor treatments, seedling lineages, and conditioning lineages. Standardized biomass significantly differed among inhibitors and seedling lineages (Table 4.3), but not conditioning lineage. Interestingly, there was no seedling lineage x inhibitor interaction indicating that standardized biomass across all inhibitor treatments was not affected by the seedling lineage. In Fig. 4.8, we further examine the response to the inhibitor treatments and how those differed by seedling lineage and conditioning lineage. In soils conditioned by non-native *Phragmites*, non-native *Phragmites* significantly increased biomass production in two of the treatments in which bacteria were treated (95% confidence intervals, Fig. 4.8a); however, the native lineage did not respond to these treatments. When the soils were conditioned by native *Phragmites*, both lineages responded similarly to treatments including antibacterial agents. This may suggest that the non-native *Phragmites* cultivates

community comprised of bacterial pathogens that are more virulent towards itself than towards the native lineage. However, native *Phragmites* cultivates a bacterial community that is more generally harmful to both lineages. In non-native conditioned soils, both lineages similarly decreased biomass production under the antifungal treatment, but in native soils, only non-native responded significantly to the antifungal treated soil. These results indicate that native and non-native *Phragmites* exhibit differential responses to microbial communities and that the bacterial community is the strongest driver of plant response.

Discussion

In our study, the direction of plant response in each lineage did not appear to be congruent with a role for soil microbes in invasions. The cumulative evidence we have obtained suggests that although both lineages of *Phragmites* very weakly differentially affect microbes, both lineages respond negatively to soil biota, especially bacteria. Moreover, the non-native lineage is more negatively impacted than its native counterpart. Therefore, interaction with soil microbes does not appear to be driving invasiveness in the non-native lineage, at least in the Great Lakes region of North America.

Feedbacks

Consistent with Allen et al. (2018), we detected weak negative feedbacks in both lineages, indicating that lineages demonstrated differential responses generated by differential microbial community cultivation. Additionally, the pairwise feedback indicated that these two lineages should coexist due to conspecific density dependence, which is inconsistent with field observations of strong invasiveness of non-native *Phragmites*. The inconsistency between PSF predictions and experimental evidence may suggest that feedbacks are not the primary drivers of invasion in this system or may reflect challenges inherent in predicting invasions with PSFs.

Recent meta-analyses have questioned the importance of PSFs in driving invasiveness as species-specific and pair-wise feedbacks on average do not conform to invasion theory (Suding et al. 2013, Crawford et al. 2019). In addition, a recent empirical study compared theoretical plant community composition obtained using PSF trained and null models to long-term common garden communities (Kulmatiski 2019). They found that PSFs can predict plant community composition of native plants, but not non-native plants. In common garden, natives grew worse and non-natives grew better than predicted by PSFs. The author suggests that the experimental conditions in PSF studies where species are grown in monoculture, do not accurately resemble interactions in natural communities (Kulmatiski 2019).

Similarly, Schittko et al. (2016) found PSF experiments performed in controlled, glasshouse conditions did not accurately reflect reality in the field. Although some differences in feedbacks were found in the glass house, they found no evidence that invasive or native species differed in the direction or extent of PSF responses under field conditions. They suggest that controlled, glasshouse conditions that focus exclusively on effects of soil biota ignore other potentially significant interactions (*e.g.,* herbivory). However, fluctuations in environmental conditions may also influence PSFs in the field. A recent study found that over the long term, inconsistent year-to-year environmental conditions may undermine the stabilizing forces of negative PSF leading to more positive feedbacks and favoring non-native grasses (Duell et al. 2019). Thus, the disparity between our experimental results and patterns of dominance in the field could arise from a variety of circumstances. However, given the agreement in data from this study, Bickford et al. (2018), and Bickford et al. (in prep) all suggesting that microbes are not driving invasiveness of non-native *Phragmites*, it is unlikely that soil biota in the field behave differently in field conditions such that they drive patterns of dominance in the non-native lineage.

Differential effects

The evidence of differential lineage effects on soil microbes in our study is consistent with recent field studies that have uncovered bacterial community differences; however, the strength of lineage differences in microbial communities across studies and regions is quite variable. For instance, Bowen et al. (2017) found very strong differences between bacterial communities among lineages of *Phragmites* across distant populations, suggesting that *Phragmites* lineages exhibit strong differential effects on rhizosphere soil microbes. The strength of differences displayed in our study more closely mirror those found in Bickford et al. (in prep) who suggested that slight differences in soil bacterial communities may arise between lineages of *Phragmites* as a consequence of invasion rather than driving the initial invasion. Experimentally cultivated bacterial communities differed very slightly in our study (Fig. 4.4a) and may have only been detectable because of the dense monocultures produced in our pots over the course of the 120-day conditioning phase (mean: 7 stems / pot; equivalent to ~400 stems / m_2).

Fungal communities did not detectably differ between conditioning lineages in our study (Fig. 4.4b). No differences in soil fungal communities have been found in the field (Bickford et al. in prep; but note that Yarwood et al. 2016 found differences in fungal biomass between *Phragmites* lineages), consistent with our results. However, the abundance of important fungal phyla (*e.g.* Glomeromycota) did differ in our study, such that native conditioned soils contained slightly more. In contrast, past studies exploring AMF colonization in *Phragmites* lineages have found that non-native tends to host a higher abundance of AMF in roots but abundance depends on site variation (Holdredge et al. 2010, Bickford et al. 2018).

Differential Responses

We also showed that lineages are capable of distinct responses to the same microbial community. When antibacterial treatments were applied to soils cultivated by non-native conditioned soil, non-native *Phragmites* responded positively, but the native lineage did not respond, implying that non-native is more strongly negatively affected by self-cultivated bacteria. If this differential response was driving performance differences in natural populations, all else being equal, we would expect native to out-perform non-native in soils cultivated by non-native. In other words, natives should be capable of invasion into existing non-native populations. Obviously, that scenario does not reflect reality, so it is likely that plant-microbial interactions are not the primary driver of performance differences between lineages in our study.

Microbial drivers of invasiveness

Although native and non-native *Phragmites* are capable of differential effect on microbes and differential response to microbes, we have no evidence these interactions drive invasiveness. In fact, the interactions we observed impact plant performance in the opposite direction than we would expect from invasion theory. For instance, (1) native and non-native *Phragmites* generated weak feedbacks of the same magnitude, (2) the total microbiome had a negative impact on non-native productivity and did not affect native, (3) bacteria negatively impacted non-native *Phragmites*, especially those that were self-cultivated, (4) the pairwise feedback for native and non-native *Phragmites* was negative. These data suggest that the non-native lineage is more vulnerable to belowground pathogen pressure, specifically that it is more consistently hampered by bacterial pathogens.

Evidence for the importance of mutualists for either lineage in this study is minimal. Neither lineage responded significantly to soil communities treated with antifungal agents, although both trended negative, possibly suggesting the loss of mutualist fungi. Indeed,

Glomeromycota relative abundance was negatively impacted by the antifungal treatments (Fig. 4.7), and this drop in mutualistic fungi may have contributed to the decline in biomass in response to the antifungal treatment (Fig. 4.8). However, the majority of the Glomeromycota sequences were found in native-cultivated soils (Table 4.1), and the AMF abundance of those soils was most impacted by antifungal treatments (Fig. 4.8). Despite the stronger impact on native soils, the non-native seedlings were more strongly impacted by antifungal treated, native-cultivated soil (Fig. 4.6b), suggesting the negative response to anti-fungal treated soils may not be caused by inhibition of mutualistic fungi. Interestingly, the bacterial community composition also shifts in response to the antifungal treatment (Fig. 4.5a), indicating that the negative seedling response to antifungal treated soils may be a result of multiple interactions between bacteria and fungi whereby negative bacterial interactions increase in the absence of fungi. Therefore, we do not see strong evidence that enhanced mutualisms is a dominant mechanism our study.

Our results suggest that factors other than interactions with soil biota are likely the main drivers of invasion of non-native *Phragmites*. Importantly, non-native *Phragmites* exhibits greater rates of photosynthesis, stomatal conductance, a larger photosynthetic canopy, higher specific leaf area and greater nitrogen content than the native lineage (Mozdzer and Zieman 2010, Mozdzer et al. 2013), which may underlie its invasiveness more than feedback on soil microbial communities. These disparities show up in glasshouse-grown plants and naturally occurring populations verifying the heritability of these differences (Mozdzer and Zieman 2010). Therefore, given inherent performance advantages in the non-native variety, soil biota may play an insignificant role in invasiveness. However, our results importantly show that native and non-native *Phragmites* are capable of differential response to similar soil biota which may be important for management efforts. As interest grows in microbially mediated biocontrol of non-native *Phragmites* (Kowalski

et al. 2015), differential responses to microbes by each lineage could allow biocontrol efforts to target non-native *Phragmites* and leave the native lineage unaffected. Based on our results, bacterial pathogens may be a good candidate driving a differential negative response.

Conclusions

Although we have strong evidence that native and non-native *Phragmites* exhibit a differential effect on, and response to soil microbes, there is little evidence that those differences provide advantages to non-native over native. Disentangling plant-microbial interactions into effects and responses enabled us to obtain a fuller picture of which specific microbes matter to each lineage of *Phragmites* and how each lineage responds to them. Based on the results of our previous work (Bickford et al. 2018, Bickford et al. in prep), the data reported here likely reflect conditions in the field. It is therefore likely that interactions with soil microbes play a minor role in invasion and other physiological factors are the primary drivers of invasiveness.

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Tables

Table 4.1: Mean relative abundance of the most common fungal phyla found in soils cultivated by each *Phragmites* lineage. *P*-values result from one-way ANOVA (Type III sum of squares).

	Native Mean Rel. Abundance	Non-native mean Rel. Abundance	P-value
Unidentified Fungi	0.596	0.668	**0.004**
Chytridiomycota	0.213	0.131	< **0.001**
Ascomycota	0.090	0.127	**0.021**
Basidiomycota	0.055	0.039	0.196
Glomeromycota	0.025	0.018	0.059
Rozellomycota	0.021	0.018	0.596

Table 4.2: ANOVA table (Type III sum of squares) comparing biomass between inoculum types (live vs. sterile), soil conditioning lineages, and seedling lineages.

	Sum Sq	**Df**	**F-value**	***P*-value**
CondLin	0.05	1	0.81	0.375
SeedLin	**30.60**	**1**	**49.32**	**<0.001**
Inoculum	0.60	1	0.97	0.332
CondLin:SeedLin	2.00	1	3.22	0.082
CondLin: Inoculum	0.13	1	0.21	0.649
SeedLin: Inoculum	**3.68**	**1**	**5.93**	**0.021**
CondLin:SeedLin: Inoculum	0.00	1	0.00	0.949

Table 4.3: ANOVA table (Type III sum of squares) comparing standardized biomass (total biomass / mean biomass of each lineage in sterile pots) across inhibitor treatments, soil conditioning lineages, and seedling lineages.

	Sum Sq	Df	F-value	P-value
CondLin	0.13	1	1.12	0.291
SeedLin	**2.36**	**1**	**21.21**	**<0.001**
Inhibitor	**5.25**	**6**	**7.86**	**<0.001**
CondLin:SeedLin	0.02	1	0.14	0.711
CondLin:Inhibitor	0.49	6	0.73	0.625
SeedLin:Inhibitor	0.30	6	0.44	0.848
CondLin:SeedLin:Inhibitor	0.38	6	0.57	0.754

Figures

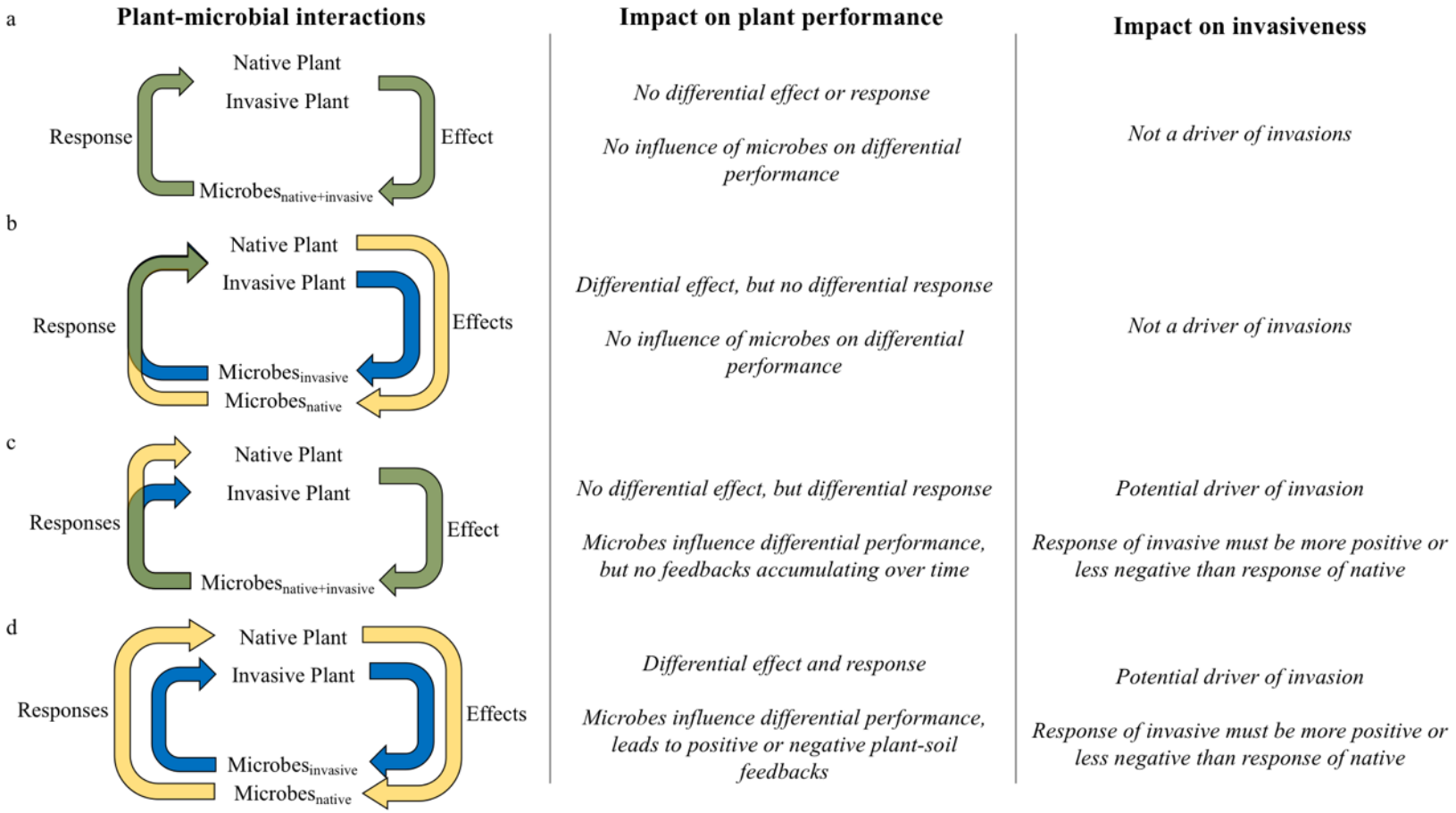


Fig. 4.1: Conceptual model of plant effects on and responses to soil microbes, the impacts on plant performance, and invasions. Yellow and blue arrows indicate individual effect or response; green arrows indicate shared effect or response.

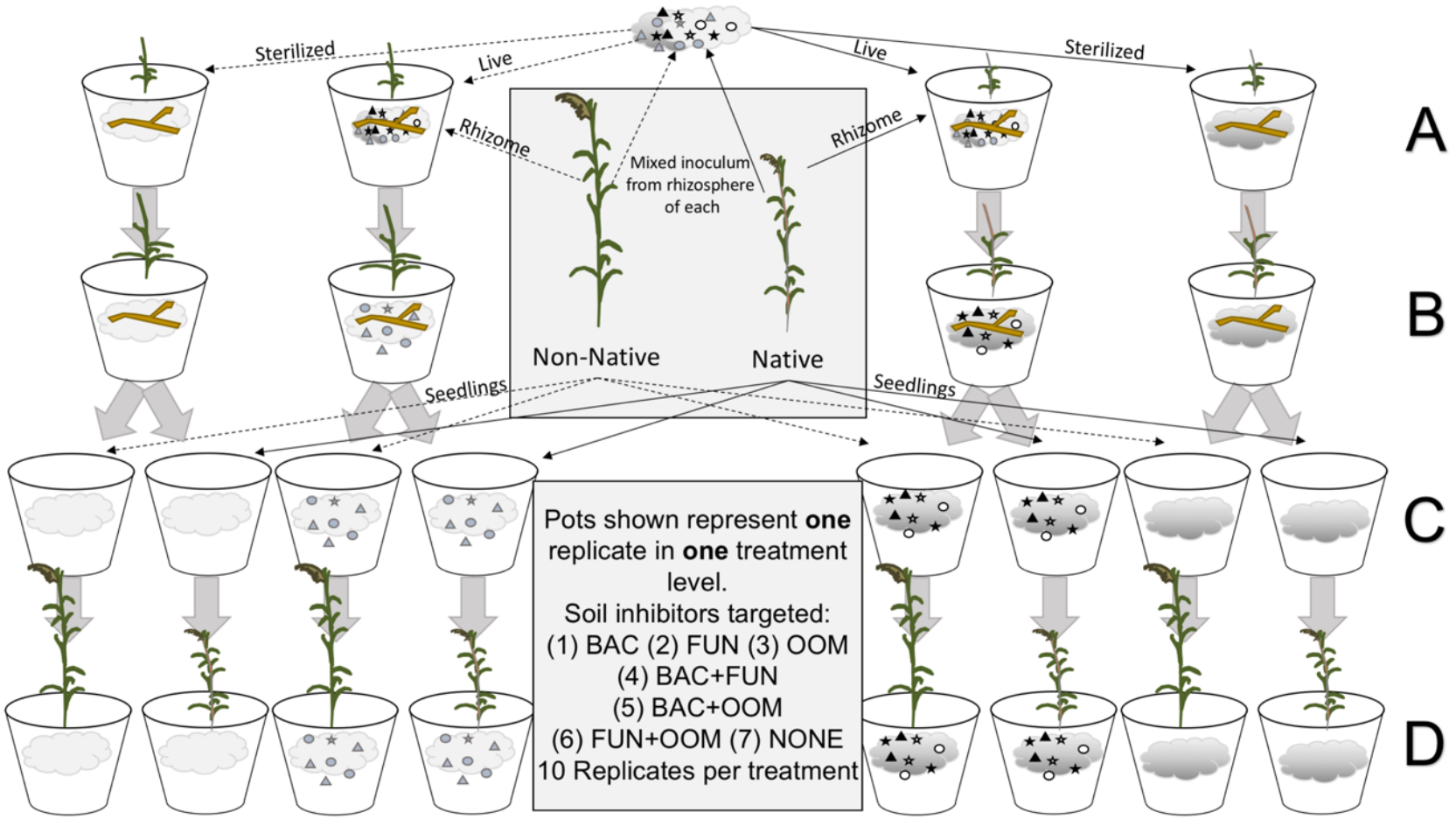


Fig. 4.2: Conceptual model of experimental design. **A:** Pre-sprouted rhizomes of native and non-native *Phragmites* were transplanted in pots with sterile soil plus live or sterile inoculum mixed from rhizosphere of each lineage. **B: Conditioning Phase:** rhizome cuttings grew for 120 days to condition soil microbiota. **C:** Plants were harvested and soil samples were taken to assess conditioned microbial community. Microbial inhibitors were applied to all "live" soils. BAC = Antibacterial, FUN = Antifungal, OOM = Anti-oomycete. Plants and soils were reciprocally crossed so that each lineage was grown in each soil type. **D: Seedling Phase:** seedlings grew for 120 days. Plant biomass was harvested and measured in each pot. Microbial communities were also analyzed in soils at the end of the study.

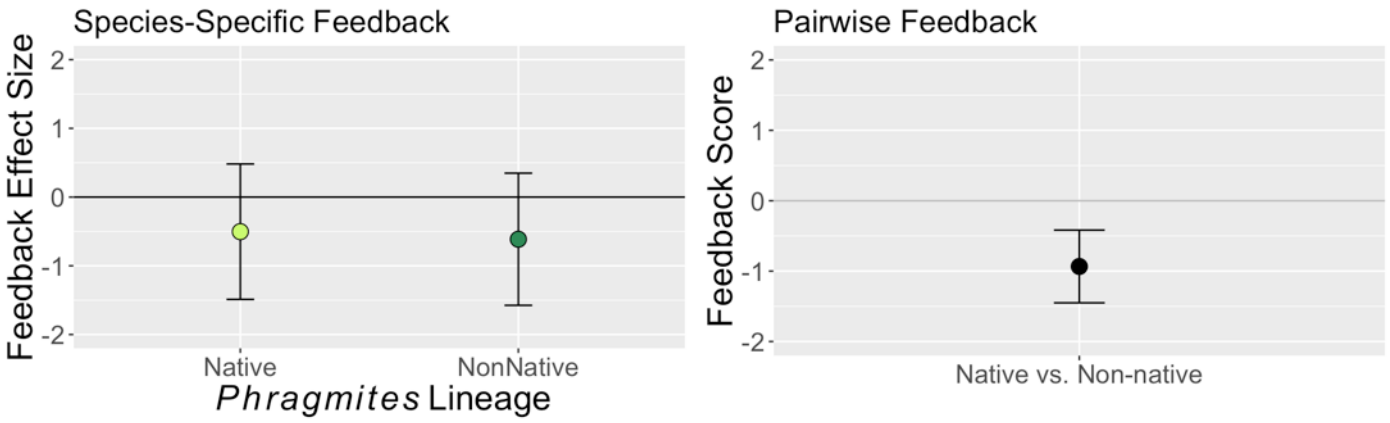


Figure 4.3: a) Species specific feedbacks indicating growth in conspecific relative to heterospecific soil. Feedback calculated using Cohen's d. **b)** Pairwise feedback indicating relative performance of two plant species in soils from each species. Negative value indicates that coexistence is predicted. Error bars represent 95% confidence intervals. All feedbacks calculated using only untreated soils.

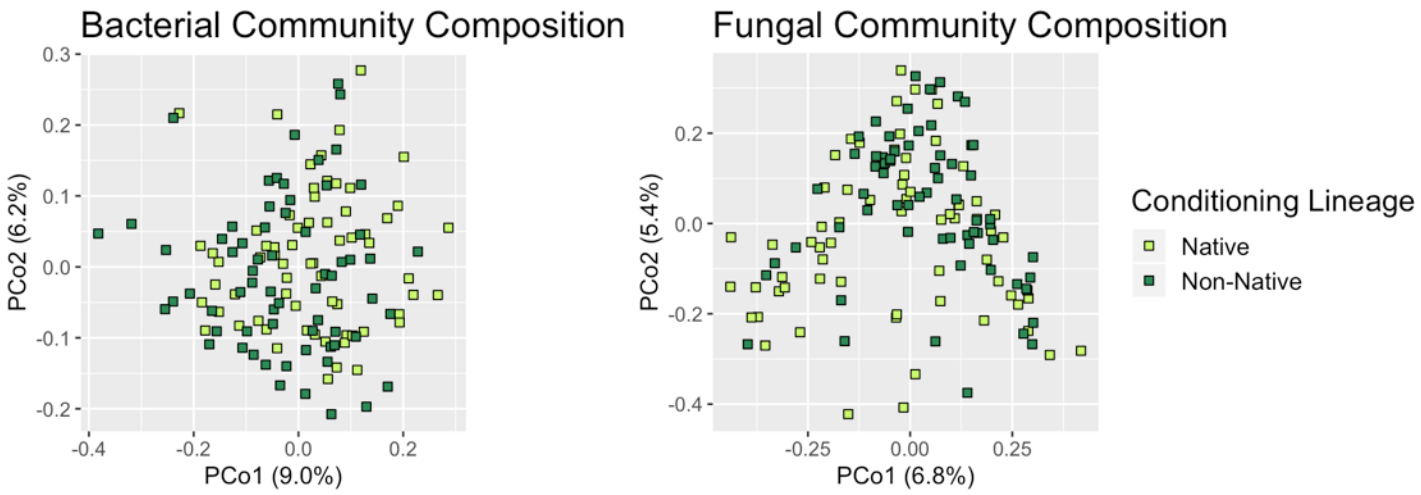


Fig. 4.4: Principle coordinate analysis of Bray-Curtis distances showing composition of **(a)** bacterial and **(b)** fungal communities of pots following soil conditioning phase (pre-treatment). Bacterial communities were slightly, but significantly different by conditioning lineage (Per-MANOVA R^2 = 0.017, P = 0.001, Perm-DISP P = 0.054) Fungal communities were dispersed differently, likely driving slight differences in centroid locations (Per-MANOVA R^2 = 0.019, P = 0.001, Perm-DISP P = 0.004).

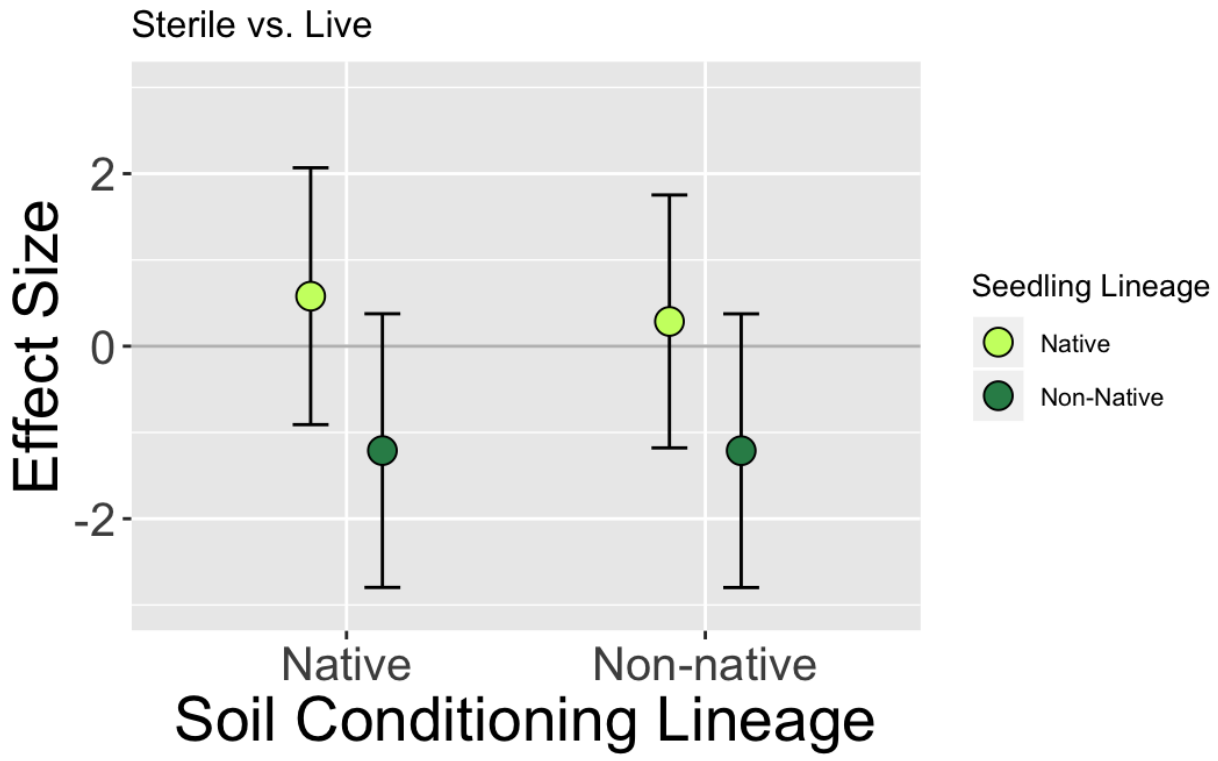
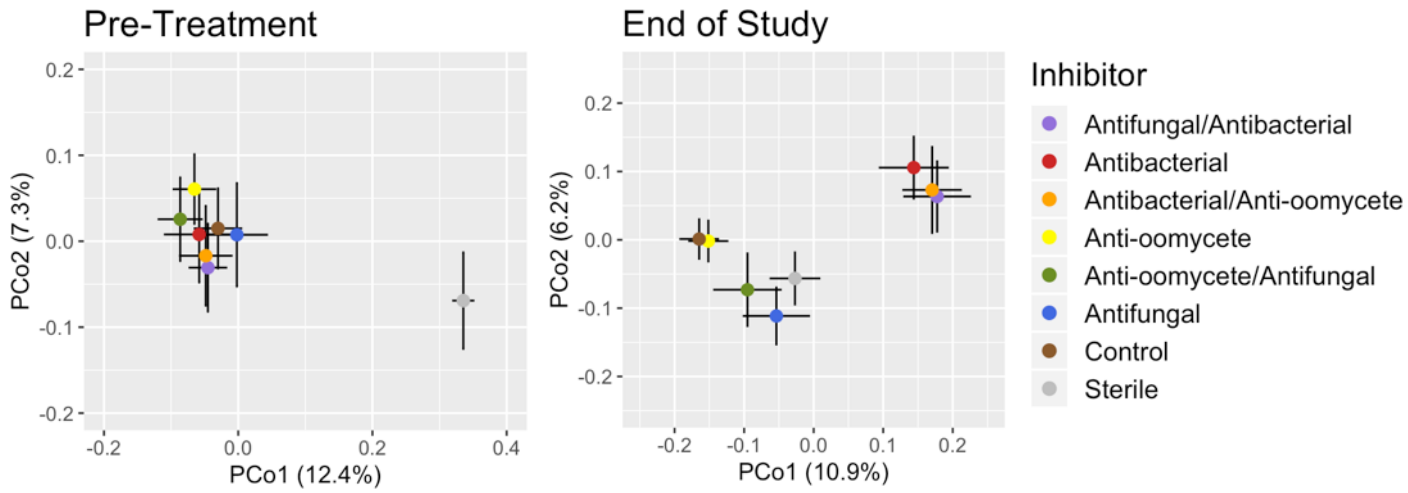


Figure 4.5: Response to sterile and live soils. Negative feedbacks indicate growth was worse in live soils relative to sterile. Feedbacks calculated using Cohen's d.

Bacteria



Fungi

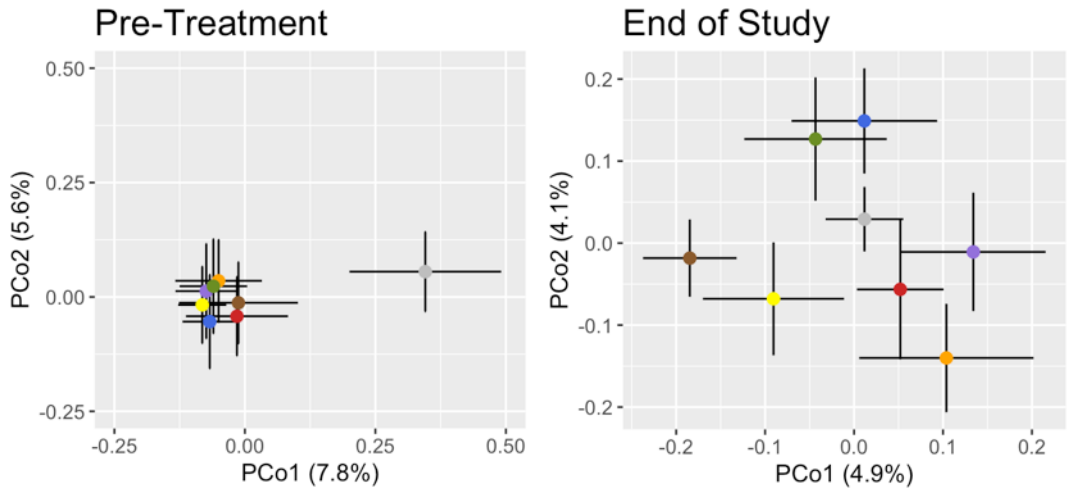


Figure 4.6: Principle coordinate analysis (PCoA) of Bray-Curtis distances representing (a) bacterial and (b) fungal community composition pre-inhibitor treatment and at the end of the study. Both communities shift in response to inhibitor treatments. Plots show centroids of points grouped by inhibitor treatment. PermDISP indicated that dispersion from the centroid did not differ among treatments for either bacteria or fungi.

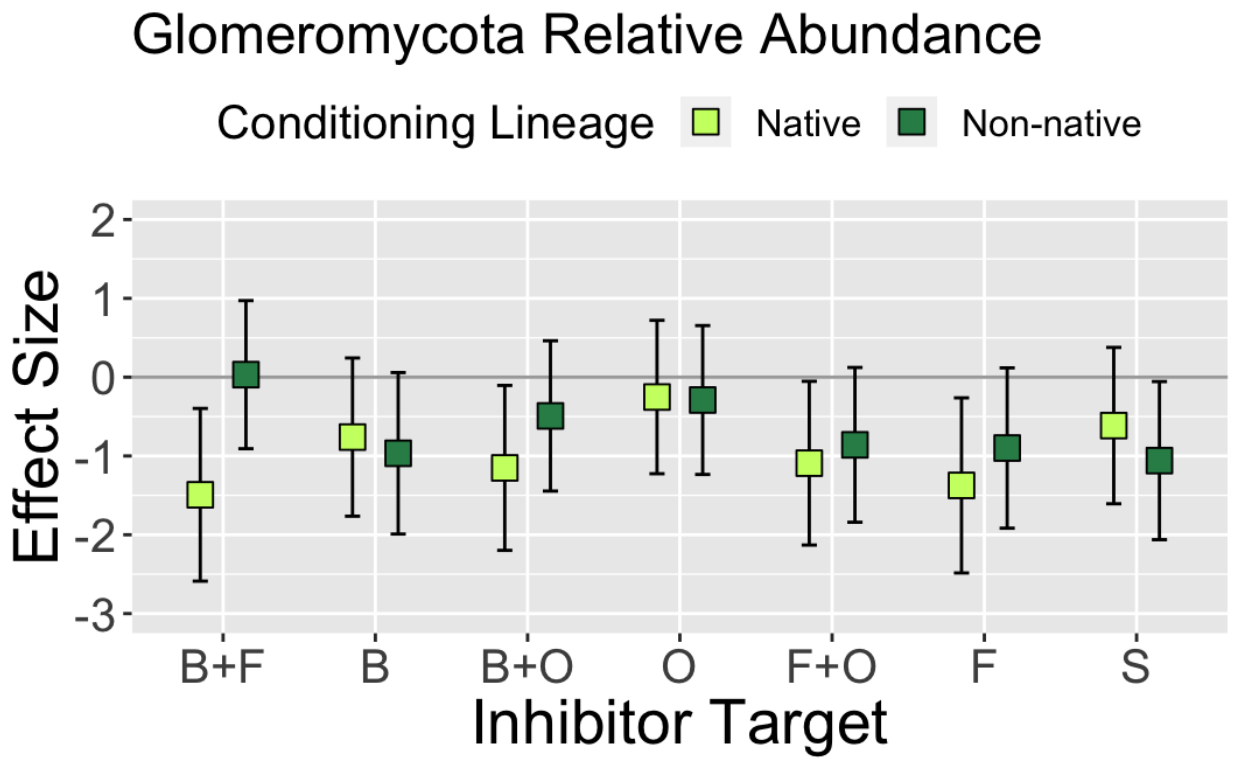


Fig. 4.7: Effect of inhibitor treatments on Glomeromycota relative abundance in fungal communities of soils conditioned by each lineage. All values are relative to control and calculated using Cohen's d. Error bars represent 95% confidence intervals. Values with error not overlapping zero are considered statistically significantly different from control. Inhibitor treatments are abbreviated B+F: Antibacterial/ Antifungal, B: Antibacterial, B+O: Antibacterial/ Anti-oomycete, O: Anti-oomycete, F+O: Antifungal/ Anti-oomycete, F: Antifungal, S: Sterile.

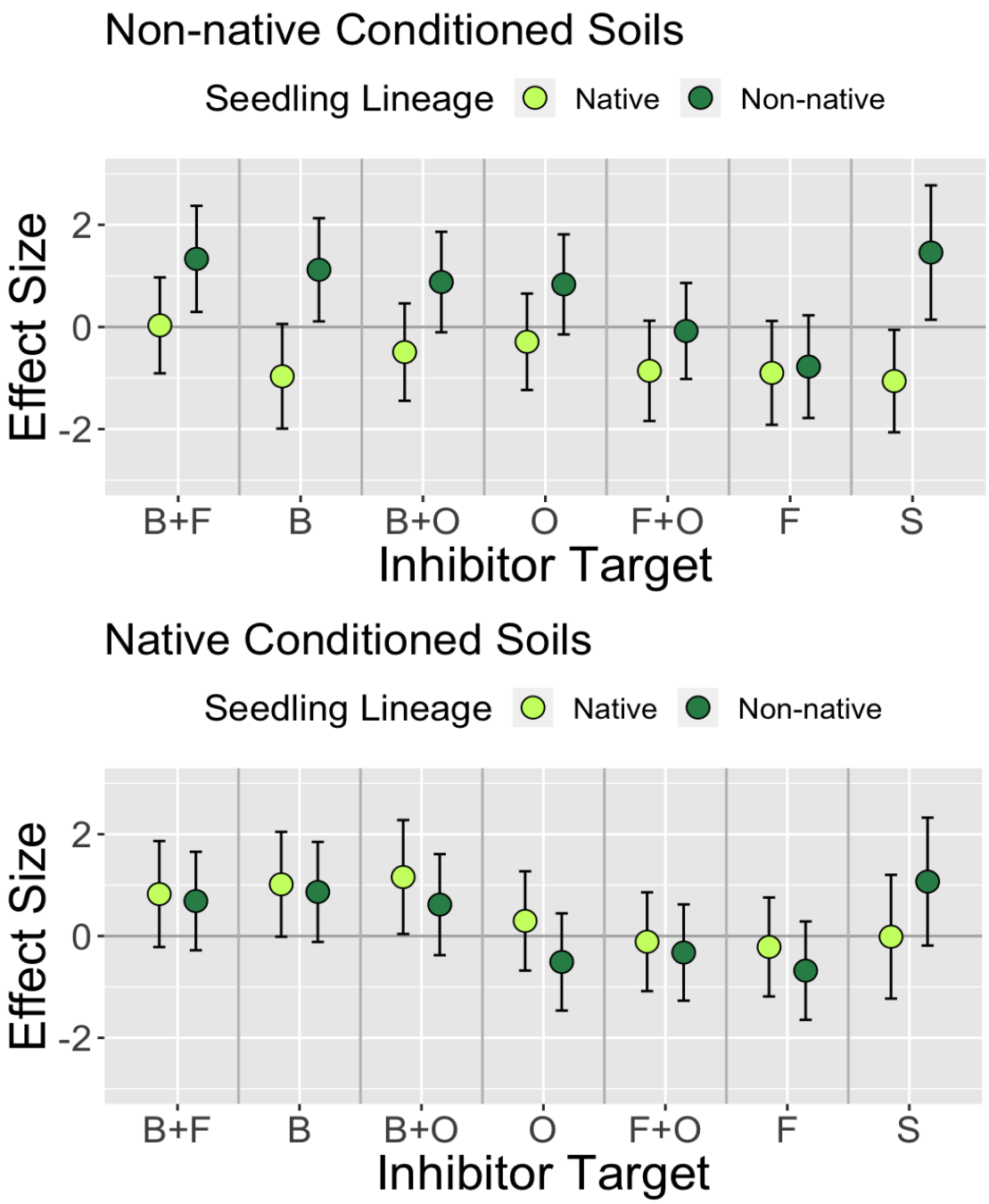


Figure 4.8: Response of seedlings to microbial communities affected by inhibitor treatments in soils conditioned by (a) non-native *Phragmites* and (b) native *Phragmites*. Pairing by soil conditioning lineage illustrates differential response to similar microbial communities. Error bars indicate 95% confidence intervals. Values with error not overlapping zero are considered statistically significantly different from control. Inhibitor treatments are abbreviated B+F: Antibacterial/ Antifungal, B: Antibacterial, B+O: Antibacterial/ Anti-oomycete, O: Anti-oomycete, F+O: Antifungal/ Anti-oomycete, F: Antifungal, S: Sterile.

Appendix S4

Table S4.1: qPCR reagents and conditions used to assess abundance of bacteria and fungi in soils.

	Reagent volumes per reaction (uL)						Reaction conditions (40 cycles)				Quality control	
Focal taxon	**Primers**	**ROX reference dye (500x dilution)**	**Bovine serum albumin (20 mg/mL)**	**Molecular biology grade water**	**Brilliant III Ultra-Fast SYBR Green qPCR Master Mix**	**Target DNA**	**Initial denaturation**	**Denaturation**	**Annealing**	**Extension**	**Average efficiency**	**Average r^2**
Bacteria	0.438 (338f/518r)	0.375	2.5	7.75	12.5	1	95°C 03:00	95°C 00:30	53°C 00:20	72°C 00:20	94.8 ± 3.4%	0.99
Fungi	0.625 (ITS1f /5.8s)	0.375	0.5	9.375	12.5	1	95°C 05:00	95°C 00:45	53°C 00:30	72°C 00:30	97.6 ± 3.0%	0.99

Table S4.2: PCR Conditions and Primer Sequences used for amplicon sequencing of bacterial and fungal communities.

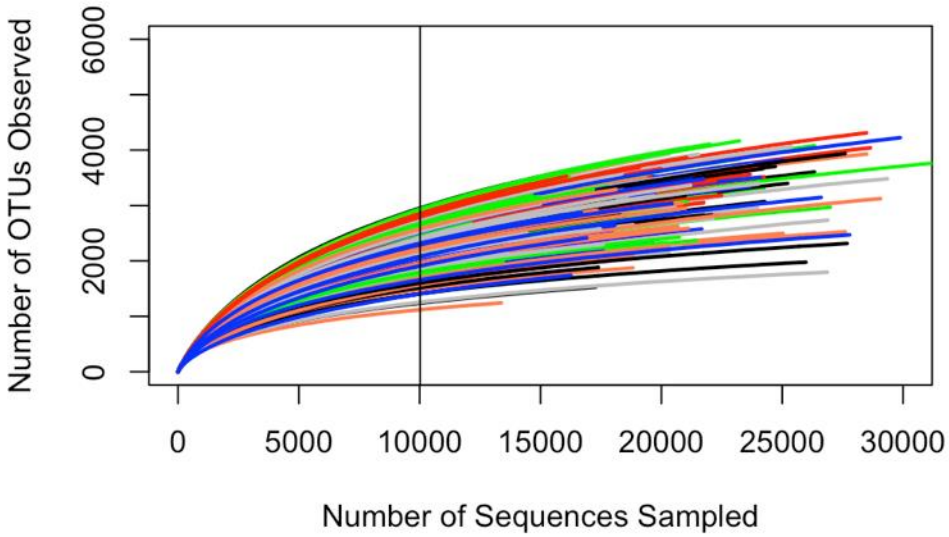
Primer Set	Primer	Primer Sequence	Fused Primer Length‡	PCR Mastermix	PCR Conditions	Reference
Fungi	5.8S_Fun	5'-AACTTTYRRCAAYGGATCWCT-3'	65 bp	5.0 uL 5x Buffer* 0.25 uL 20uM dNTPs 0.938 uL 20uM 5.8S 0.938 uL 20uM ITS4 0.25 uL Taq* 4 uL Template DNA** 13.625 uL H2O	Initial denaturation: 94℃ for 3 min, 27 cycles, denaturation: 94℃ for 30 s, annealing: 57℃ for 45 s, extension: 72℃ for 90 s (10 min final extension)	(Taylor et al. 2016)
	ITS4_Fun	5'-AGCCTCCGCTTATTGATATGCTTAART-3'	76 bp			
Bacteria	515F	5'- GTGCCAGCMGCCGCGGTAA-3'	63 bp	5.0 uL 5x Buffer* 0.5 uL 20uM dNTPs 0.5 uL 20uM 515F 0.5 uL 20uM 806R 0.5 uL Taq* 2 uL Template DNA** 18 uL H2O	Initial denaturation: 95℃ for 2 min, 30 cycles, denaturation: 95℃ for 20 s, annealing: 55℃ for 15 s, extension: 72℃ for 5 min (10 min final extension)	(Kozich et al. 2013)
	806R	5'- GGACTACHVGGGTWTCTAAT'3'	69 bp			

‡ Full fused primer included Illumina adapter (29-bp forward; 24-bp reverse), 8-bp unique barcode, 10-bp pad, 2-bp linker followed by the gene specific primer). Reverse adapter is used with forward primer, Forward adapter with reverse.

* Phusion High Fidelity PCR Kit (New England BioLabs)

** DNA was diluted to 5-10 ng per reaction

a) Bacteria



b) Fungi

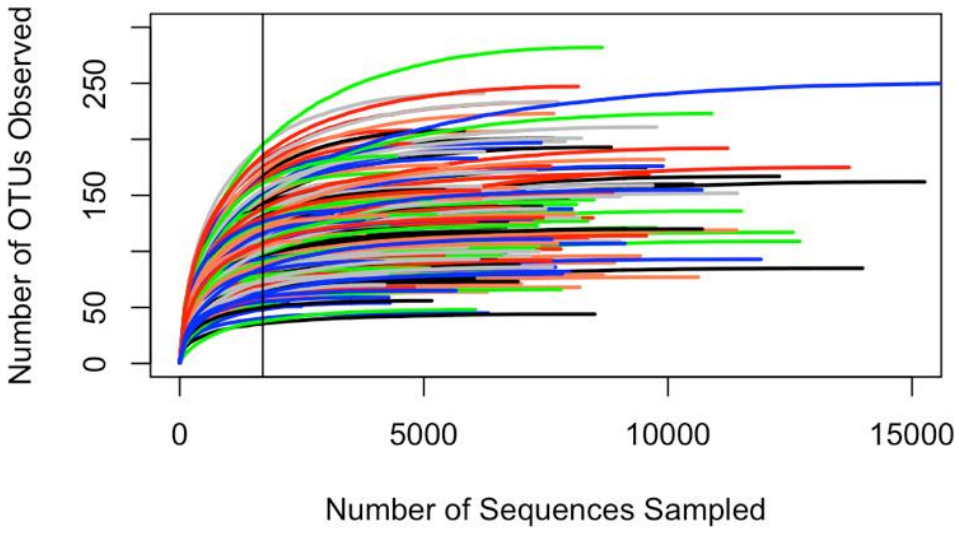


Fig. S4.1: Rarefaction curves for a) bacterial and b) fungal sequences. Colors indicate treatment groups. Vertical lines indicate subsample sequence amount.

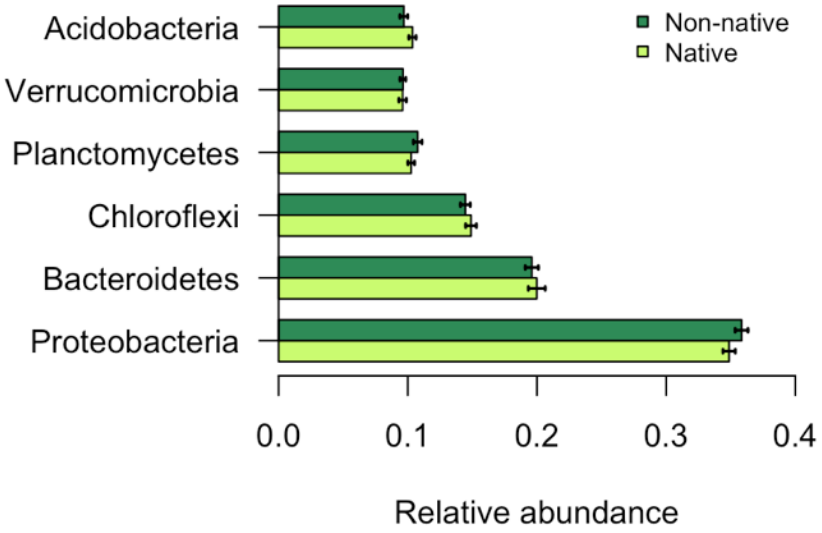
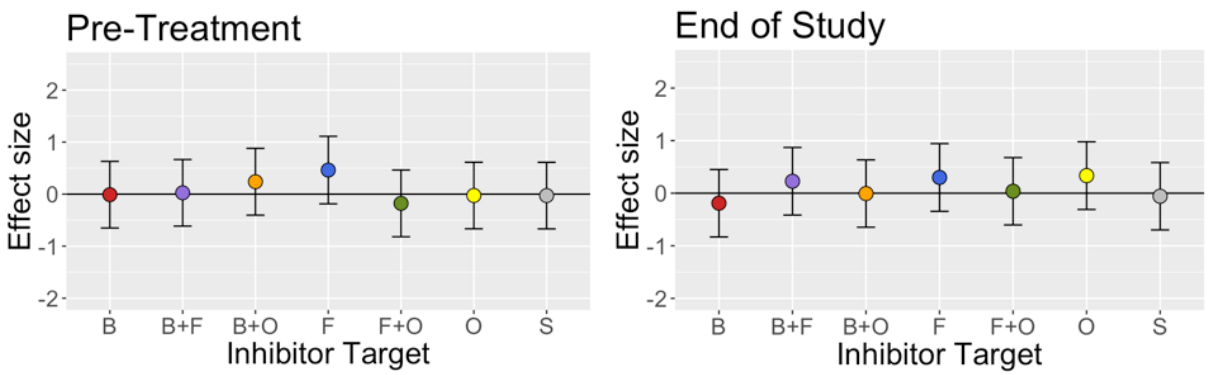


Fig. S4.2: Relative abundance of major bacterial phyla recovered from soils conditioned by each lineage. No lineage comparisons were significant at a <0.05.

a) Bacterial Gene Copy number



b) Fungal Gene Copy Number

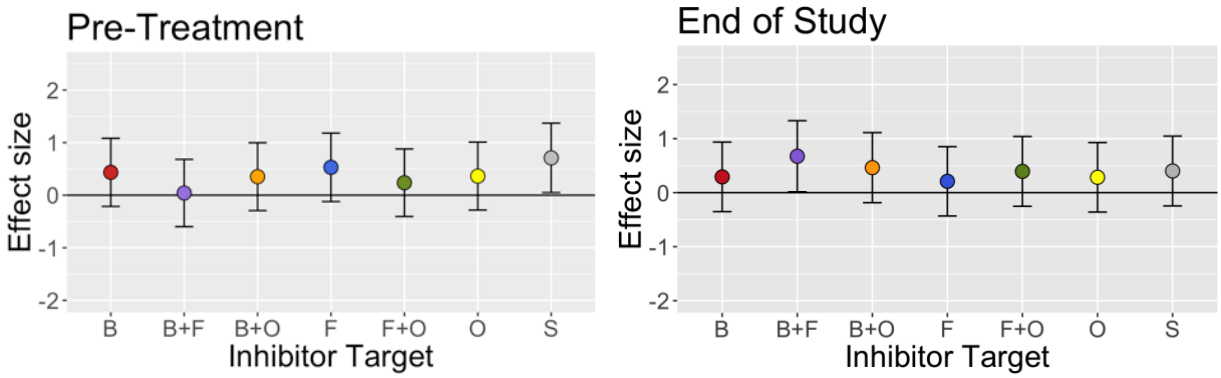


Fig S4.3: a) Bacterial and b) fungal gene copy number changes by inhibitor treatment. Plots show treatments relative to control. Neither microbial changed in abundance in response to the treatments.

Table S4.3: Pairwise Per-MANOVA output comparing **pre-treatment bacterial communities** among all possible inhibitor treatment combinations. P-values were adjusted using a Benjamini-Hochberg correction.

Pairs	F.Model	R_2	p.value	p.adjusted
BAC vs OOM	1.32	0.034	0.0507	0.089
BAC vs FUN	1.11	0.028	0.2240	0.321
BAC vs NONE	1.04	0.027	0.3448	0.402
BAC vs FUNBAC	1.07	0.027	0.3038	0.386
BAC vs FUNOOM	0.89	0.023	0.7241	0.779
BAC vs BACOOM	0.84	0.022	0.8199	0.850
BAC vs STERILE	9.78	0.205	0.0001	**<0.001**
OOM vs FUN	1.67	0.042	0.0046	**0.014**
OOM vs NONE	1.46	0.037	0.0073	**0.019**
OOM vs FUNBAC	1.72	0.043	0.0017	**0.006**
OOM vs FUNOOM	1.37	0.035	0.0289	0.058
OOM vs BACOOM	1.52	0.038	0.0144	**0.034**
OOM vs STERILE	11.33	0.230	0.0001	**<0.001**
FUN vs NONE	1.03	0.026	0.3591	0.402
FUN vs FUNBAC	1.11	0.028	0.2294	0.321
FUN vs FUNOOM	1.47	0.037	0.0214	**0.046**
FUN vs BACOOM	1.08	0.028	0.2615	0.349
FUN vs STERILE	7.59	0.166	0.0001	**<0.001**
NONE vs FUNBAC	1.18	0.030	0.1310	0.204
NONE vs FUNOOM	1.24	0.032	0.0804	0.132
NONE vs BACOOM	1.04	0.027	0.3317	0.402
NONE vs STERILE	9.34	0.197	0.0001	**<0.001**
FUNBAC vs FUNOOM	1.53	0.039	0.0074	**0.019**
FUNBAC vs BACOOM	0.71	0.018	0.9947	0.994
FUNBAC vs STERILE	9.32	0.197	0.0001	**<0.001**
FUNOOM vs BACOOM	1.34	0.034	0.0511	0.089
FUNOOM vs STERILE	11.91	0.239	0.0001	**<0.001**
BACOOM vs STERILE	9.45	0.199	0.0001	**<0.001**

Table S4.4: Pairwise Per-MANOVA output comparing **post-treatment bacterial communities** among all possible inhibitor treatment combinations. P-values were adjusted using a Benjamini-Hochberg correction.

Pairs	F.Model	R_2	p.value	p.adjusted
BAC vs OOM	5.916	0.135	<0.001	**0.003**
BAC vs FUN	5.012	0.117	<0.001	**0.003**
BAC vs NONE	6.180	0.140	<0.001	**0.003**
BAC vs FUNBAC	1.220	0.031	0.093	1.000
BAC vs FUNOOM	5.154	0.119	<0.001	**0.003**
BAC vs BACOOM	0.984	0.025	0.440	1.000
BAC vs STERILE	5.510	0.127	<0.001	**0.003**
OOM vs FUN	2.977	0.073	<0.001	**0.003**
OOM vs NONE	1.172	0.030	0.119	1.000
OOM vs FUNBAC	6.912	0.154	<0.001	**0.003**
OOM vs FUNOOM	2.431	0.060	<0.001	**0.003**
OOM vs BACOOM	6.294	0.142	<0.001	**0.003**
OOM vs STERILE	4.833	0.113	<0.001	**0.003**
FUN vs NONE	2.838	0.069	<0.001	**0.003**
FUN vs FUNBAC	4.793	0.112	<0.001	**0.003**
FUN vs FUNOOM	0.986	0.025	0.432	1.000
FUN vs BACOOM	5.015	0.117	<0.001	**0.003**
FUN vs STERILE	4.024	0.096	<0.001	**0.003**
NONE vs FUNBAC	7.272	0.161	<0.001	**0.003**
NONE vs FUNOOM	2.224	0.055	<0.001	**0.006**
NONE vs BACOOM	6.485	0.146	<0.001	**0.003**
NONE vs STERILE	4.902	0.114	<0.001	**0.003**
FUNBAC vs FUNOOM	5.356	0.124	<0.001	**0.003**
FUNBAC vs BACOOM	1.471	0.037	0.030	0.829
FUNBAC vs STERILE	5.921	0.135	<0.001	**0.003**
FUNOOM vs BACOOM	5.157	0.119	<0.001	**0.003**
FUNOOM vs STERILE	3.964	0.094	<0.001	**0.003**

Table S4.5: Pairwise Per-MANOVA output comparing **pre-treatment fungal communities** among all possible inhibitor treatment combinations. P-values were adjusted using a Benjamini-Hochberg correction.

Pairs	F.Model	R_2	p.value	p.adjusted
BAC vs OOM	0.99	0.026	0.459	0.984
BAC vs FUN	0.88	0.023	0.664	0.984
BAC vs NONE	0.81	0.023	0.790	0.984
BAC vs FUNBAC	0.91	0.024	0.608	0.984
BAC vs FUNOOM	0.84	0.022	0.757	0.984
BAC vs BACOOM	0.90	0.024	0.642	0.984
BAC vs STERILE	**4.19**	**0.099**	**<0.001**	**<0.001**
OOM vs FUN	1.06	0.028	0.343	0.984
OOM vs NONE	1.01	0.030	0.447	0.984
OOM vs FUNBAC	0.74	0.020	0.898	0.984
OOM vs FUNOOM	0.68	0.019	0.957	0.984
OOM vs BACOOM	0.89	0.024	0.662	0.984
OOM vs STERILE	**5.30**	**0.125**	**<0.001**	**<0.001**
FUN vs NONE	1.32	0.037	0.086	0.302
FUN vs FUNBAC	0.87	0.023	0.693	0.984
FUN vs FUNOOM	1.04	0.027	0.365	0.984
FUN vs BACOOM	0.91	0.024	0.610	0.984
FUN vs STERILE	**5.52**	**0.127**	**<0.001**	**<0.001**
NONE vs FUNBAC	0.95	0.028	0.557	0.984
NONE vs FUNOOM	0.86	0.025	0.752	0.984
NONE vs BACOOM	0.70	0.021	0.934	0.984
NONE vs STERILE	**3.65**	**0.097**	**0.001**	**0.002**
FUNBAC vs FUNOOM	0.63	0.017	0.984	0.984
FUNBAC vs BACOOM	0.69	0.019	0.940	0.984
FUNBAC vs STERILE	**5.01**	**0.119**	**0.000**	**<0.001**
FUNOOM vs BACOOM	0.81	0.022	0.813	0.984
FUNOOM vs STERILE	**4.44**	**0.107**	**<0.001**	**<0.001**
BACOOM vs STERILE	**4.55**	**0.109**	**<0.001**	**<0.001**

Table S4.6: Pairwise Per-MANOVA output comparing **post-treatment fungal communities** among all possible inhibitor treatment combinations. P-values were adjusted using a Benjamini-Hochberg correction.

Pairs	F.Model	R_2	p.value	p.adjusted
BAC vs OOM	**1.98**	**0.051**	**<0.001**	**<0.001**
BAC vs FUN	**2.21**	**0.059**	**<0.001**	**<0.001**
BAC vs NONE	**2.71**	**0.070**	**<0.001**	**<0.001**
BAC vs FUNBAC	1.16	0.030	0.212	0.220
BAC vs FUNOOM	**2.96**	**0.074**	**<0.001**	**<0.001**
BAC vs BACOOM	**1.44**	**0.038**	**0.041**	**0.047**
BAC vs STERILE	**1.66**	**0.043**	**0.002**	**0.002**
OOM vs FUN	**2.42**	**0.063**	**<0.001**	**<0.001**
OOM vs NONE	1.15	0.030	0.206	0.220
OOM vs FUNBAC	**2.65**	**0.065**	**<0.001**	**<0.001**
OOM vs FUNOOM	**2.46**	**0.061**	**<0.001**	**<0.001**
OOM vs BACOOM	**2.04**	**0.051**	**0.001**	**0.002**
OOM vs STERILE	**2.26**	**0.056**	**<0.001**	**<0.001**
FUN vs NONE	**2.44**	**0.065**	**<0.001**	**<0.001**
FUN vs FUNBAC	**2.12**	**0.056**	**<0.001**	**<0.001**
FUN vs FUNOOM	1.16	0.031	0.225	0.225
FUN vs BACOOM	**2.53**	**0.066**	**<0.001**	**<0.001**
FUN vs STERILE	**1.88**	**0.050**	**<0.001**	**<0.001**
NONE vs FUNBAC	**3.25**	**0.081**	**<0.001**	**<0.001**
NONE vs FUNOOM	**2.37**	**0.060**	**<0.001**	**<0.001**
NONE vs BACOOM	**2.94**	**0.074**	**<0.001**	**<0.001**
NONE vs STERILE	**2.55**	**0.064**	**<0.001**	**<0.001**
FUNBAC vs FUNOOM	**2.59**	**0.064**	**<0.001**	**<0.001**
FUNBAC vs BACOOM	1.35	0.034	0.089	0.100
FUNBAC vs STERILE	**2.18**	**0.054**	**<0.001**	**<0.001**
FUNOOM vs BACOOM	**3.27**	**0.079**	**<0.001**	**<0.001**
FUNOOM vs STERILE	**2.82**	**0.069**	**<0.001**	**<0.001**
BACOOM vs STERILE	**2.07**	**0.052**	**<0.001**	**<0.001**

Table S4.7: ANOVA output (Type III Sum of Squares) comparing relative abundance of Glomeromycota sequences among treatment groups

Factor	Sum Sq	Df	F-Value	P-Value
INHBTR	0.029	7	3.61	**0.001**
CondLin	0.016	1	13.94	**<0.001**
SeedLin	0.009	1	7.88	**0.006**
INHBTR:CondLin	0.020	7	2.46	**0.021**
INHBTR:SeedLin	0.017	7	2.03	0.056
CondLin:SeedLin	0.000	1	0.00	0.999
INHBTR:CondLin:SeedLin	0.012	7	1.44	0.193

Chapter 5 : Conclusions

In my dissertation, I explored bacterial, fungal, and oomycete communities associated native and non-native *Phragmites* in the Great Lakes region and the impacts of those microbial communities on invasiveness. I used a combination of field surveys of natural populations and experimental manipulations to examine thoroughly whether invasiveness in *Phragmites* is facilitated by plant-soil feedbacks. My results were very consistent among all chapters in this dissertation, finding no strong link between invasiveness and belowground microbial communities, suggesting that belowground microbes alone do not explain invasiveness of *Phragmites australis* in the central Great Lakes region.

In Chapters 2 and 3, I provide evidence that belowground microbial communities did not differ between *Phragmites* lineages in roots or rhizospheres of natural populations. Root communities differed in fungal colonization and in oomycete richness, but both of those differences were weak and inconsistent among different environmental conditions. In addition, the few differences that were found between lineages were consistently opposite of our expectation that non-native *Phragmites* would be associated with more mutualists and/or less pathogens than native. The rhizosphere largely followed the same patterns with one exception: the rhizosphere bacterial communities differed by lineage in large, dense patches of *Phragmites*, but not elsewhere. Given the small magnitude of the observed differences in bacterial communities, and the fact that they only existed in dense, mature patches of *Phragmites*, no differences in functional potential could be attributed to the community differences observed. Taken together, the evidence that I

have obtained strongly suggests that observed differences in soil microbial communities between *Phragmites* lineages may be a consequence rather than a driver of invasiveness.

Consistent with natural populations, the experimentally-conditioned soils in Chapter 4 differed only slightly between lineages in bacterial community composition and even less so in fungal community composition. Despite these small differences in composition, the plant response to those differences was more significant, but again the opposite of that expected if microbes were driving invasiveness. This study showed that non-native *Phragmites* was overall negatively impacted by the total soil microbiome, whereas native was unaffected by the total soil microbiome, regardless of which lineage conditioned the soil. The inhibitor treatments uncovered the substantial role of soil bacteria in driving this response. Both lineages were susceptible to bacterial pathogens; however soil bacteria conditioned by non-native *Phragmites* negatively impacted non-native, but not native *Phragmites*. Both of these findings on lineage-specific plant responses are counter to our expectation that if belowground microbial communities are driving invasiveness, non-native *Phragmites* should derive disproportionate benefits from microbial communities over native.

Given the preponderance of data suggesting that belowground microbes do not explain invasiveness in *Phragmites*, it is reasonable to assume that non-native's invasiveness is derived from other sources. I reviewed the literature on physiological differences between native and non-native *Phragmites* in Chapter 1, and given the observed differences in rates of photosynthesis and stomatal conductance (Mozdzer and Zieman 2010), the non-native lineage may be deriving the majority of its advantage through a higher intrinsic growth rate. However, there are likely many aspects of plant performance in which microbes may be important and at least two scenarios where microbes may be involved in enhancing, although not necessarily inciting, invasiveness.

I observed that rhizosphere bacterial communities differed between lineages of *Phragmites* at high plant densities in Chapter 3 and 4 and speculated that this is likely a consequence of invasion rather than a driver. The mechanism underlying this difference is unknown, but one potential driver could be the difference in soil aeration derived from disparate ventilation efficiency between lineages (Tulbure et al. 2012), thereby creating a more aerobic soil environment in non-native populations than in native. If so, nutrient mineralization rates would be higher in non-native *Phragmites* populations, because microbial decomposition is orders of magnitude more efficient in aerobic conditions. This would lead to increased nitrogen availability and could further augment the performance advantage of non-native *Phragmites* (Windham and Lathrop 1999, Windham and Meyerson 2003). Given its higher nitrogen demand to sustain higher rates of net photosynthesis, this feedback may be a mechanism for non-native *Phragmites* to thrive in more oligotrophic wetlands or may simply improve performance in eutrophic areas. Microbes play an extremely important role in this feedback, but not microbes that would be considered plant mutualists or pathogens. In this system, saprotrophs would be driving the feedback by maintaining high rates of nutrient mineralization and feeding the nutrient demand of non-native *Phragmites*.

Pathogen spillover may be another potential enhancement to invasion provided by belowground microbes associated with non-native *Phragmites*. In chapter 4, we revealed that non-native *Phragmites* responds more negatively to soil microbes than native *Phragmites*. We attributed that phenomenon to increased lineage-specific pathogen pressure in non-native-conditioned soils and concluded that these interactions represent a net negative for non-native's performance. However, just as we found a differential response between the two *Phragmites* lineages, there may be a differential response between non-native *Phragmites* and other native wetland plants. The pathogen build-up could actually be beneficial to non-native *Phragmites* in

the longer term if those pathogens are more virulent to other native plants. Evidence from the literature indicated that many native wetland plants respond negatively to soils conditioned by both *Phragmites* lineages (Crocker et al. 2017) and that there may be a stronger negative legacy in soils cultivated by non-native *Phragmites* (Allen et al. 2018). Thus, although negative response to soils cultivated by non-native *Phragmites* does not explain its invasiveness in the short term, build-up and spillover of those pathogens to other native plants may enhance invasion over longer time scales.

Management Implications

My results indicate that belowground microbes are not likely principle drivers of invasiveness in *Phragmites*. Nevertheless, given the differential response to microbes that we observed in Chapter 4, the possibility of targeting non-native *Phragmites* for control through its microbial community (Kowalski et al. 2015) is still viable. In Chapter 4, I found that growth of self-conditioned non-native *Phragmites* increased following antibacterial treatments, whereas native *Phragmites* did not respond to the same soil. That differential response implies that some bacterial pathogens are more virulent towards the non-native lineage than the native. Therefore, if the specific bacteria causing the response could be identified, these would be candidates for biocontrol. Although developing biocontrol agents is an extremely complicated undertaking, my results indicate that, in theory, belowground bacterial pathogens could negatively impact non-native *Phragmites* in a lineage-specific manner. There is precedent for this type of work in controlling cheatgrass (*Bromus tectorum*) using a strain of *Pseudomonas flourescens* (Tranel et al. 1993a, 1993b) as a bio-control. Its widespread effectiveness is currently being tested in field conditions.

Another potential management target is nutritional mutualisms, given what we know about the difference in nitrogen demand between lineages. Although my data suggest that directly targeting mutualists like AMF may not impact non-native *Phragmites* in a lineage-specific manner, in certain nutrient regimes, non-native *Phragmites* may be more vulnerable to decreases in nutrient uptake given its greater nitrogen demand. For instance, in oligotrophic wetlands, non-native *Phragmites* may rely upon nutritional mutualists more heavily than native to feed its higher nutrient demand (Fig. 2.1 may support this idea).

Non-native *Phragmites* may also have a negative response to disruption of the soil aeration/N-mineralization feedback mentioned in the previous section. If it is benefiting from higher mineralization rates driven by a more aerobic soil environment, a currently used management strategy, mowing or burning the aboveground biomass and subsequently flooding the soils over the cut stems, would disrupt the accelerated convective throughflow and may also slow the rates of N mineralization, further impacting the vitality of the non-native *Phragmites*, especially in oligotrophic wetlands. Cutting *Phragmites* below water has been endorsed as an effective non-chemical management strategy in certain conditions in the Great Lakes region by cutting off the flow of oxygen and essentially drowning the plant (GLPC 2019). An added benefit to this strategy would be the decreased nutrient availability to surviving stems. Mowing and flooding in *Phragmites* stands has been shown to decrease throughflow and decrease oxygen concentrations (Rolletschek et al. 2000), and therefore plausibly decreases N-mineralization rates as well.

The soil legacy of non-native *Phragmites* may be of great importance when considering expansion of existing *Phragmites* populations and the promotion of biodiversity following successful *Phragmites* removal. As conditioning by non-native *Phragmites* may increase the load

of pathogens in the soil (Crocker et al. 2017, Allen et al. 2018, Chapter 4 of this dissertation), other native plants may be negatively impacted during expansion and establishment of native plants following *Phragmites* removal may be complicated. Control of soil pathogens during expansion is not likely viable as it could result in a positive performance increase in the non-native *Phragmites*. However, following removal by other means, control of pathogens would likely benefit recovery of native biodiversity. Additional soil treatments to alleviate the impacts of soil pathogens may be a viable management option.

Importantly, my results indicate that disturbing existing belowground microbial associations in a non-specific manner (*e.g.* antimicrobial application) will not likely curtail non-native *Phragmites'* invasiveness. The lack of strong patterns suggesting that non-native disproportionately benefits from microbes indicates broad spectrum disturbance of belowground microbial communities will not be the "silver bullet" that enables widespread species-specific control of non-native *Phragmites*. Management actions involving belowground microbes will likely need to be targeted towards changing the balance of mutualists and pathogens in the microbiome such that non-native is at a disadvantage relative to native plants. I showed that native and non-native *Phragmites* lineages can have differential effects on and responses to certain microbial groups. Thus, under certain conditions, or in combination with other management actions, targeted management of microbial associations may have a measurable impact. Future studies could explore the (1) possibility of biocontrol using soil bacterial pathogens, (2) disruption of nutritional mutualisms or nutrient feedbacks in oligotrophic wetlands, and (3) reduction of soil legacy effects following *Phragmites* removal.

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