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The Effect of Environmental Perturbations on the Plant Phyllosphere Microbiome

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THE EFFECT OF ENVIRONMENTAL PERTURBATIONS ON THE PLANT PHYLLOSPHERE MICROBIOME

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ABSTRACT

Applying ecological concepts to microbial communities has proved to be challenging, often revolving around the relative importance of stochastic and deterministic processes applied heterogeneously across the habitat in time or space. The phyllosphere is the aboveground surface of plants on which microbial communities, composed primarily of Bacteria, but also including Archaea and microbial eukaryotes, exist and is characterized by stochastic immigration balanced with strong selective forces. Variation in bacterial community composition was characterized across space and time, with special attention given to the exposure of phyllosphere communities to rain as a plausible mechanism of ecological disturbance. Bacterial communities were characterized through targeted Illumina sequencing of the 16S rRNA taxonomic marker gene. Biogeographic patterns of bacteria in the phyllosphere were found to be evident from southern magnolia (*Magnolia grandiflora*) trees 1-452 m apart in a small forest plot. A significant relationship between canopy cover and tree elevation and differences in bacterial abundances but not in bacterial incidence, suggesting that bacterial abundance and incidence in the phyllosphere is shaped by different assembly mechanisms. More broadly, this suggests that environmental parameters and neutral forces may influence spatial patterns in the phyllosphere, even at small spatial scales. Separately, the effects of rain were investigated in both short-term and long-term contexts. First, rain as a short-term disturbance was contrasted against long-term seasonal changes to the phyllosphere bacterial community of broadleaf cattail (*Typha latifolia*) plants collected across an entire year, specifically targeting days before rain events and up to five days

after. Secondly, the effect of rain throughout the tree canopy of *M. grandiflora* was investigated. Across both studies, rain did not appear to have any effect on bacterial community richness, evenness, novel species accumulation, or composition. Instead, longer seasonal trends determined diversity and compositional patterns in *T. latifolia*, while canopy structure had the strongest influence on *M. grandiflora*. These findings suggest that rain does not act as an ecological disturbance towards the phyllosphere bacterial community and that short-term abiotic disruptions may exert minimal influence on its composition and development in comparison to longer trends or spatial heterogeneity which likely influence the plant host as well.

DEDICATION

This work is dedicated to my parents who fostered curiosity and to my wife and daughter who remind me of the importance of sharing moments of discovery.

LIST OF ABBREVIATIONS

rRNA	Ribosomal ribonucleic acid
NGS	Next-generation sequencing
UV	Ultraviolet
EPS	Extracellular polymeric substances
PAR	Photosynthetically active radiation
NOAA	National Oceanic and Atmospheric Administration
DNA	Deoxyribonucleic acid
IR	Infrared
OTU	Operational taxonomic unit
PCA	Principal components analysis
NMDS	Non-metric multidimensional scaling
RDA	Redundancy analysis
AEM	Asymmetric eigenvector maps
UMFS	University of Mississippi field station
AUC	Area under curve
LCBD	Local contributions to beta-diversity

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INTRODUCTION AND BACKGROUND

i. Composition of the Phyllosphere Microbiome

The phyllosphere is the aboveground surface of plants on which microbial communities composed of Bacteria, Archaea, and microbial eukaryotes exist (Vorholt, 2012). Globally, the surface area of the phyllosphere is vast and has been estimated as being several orders of magnitude greater than the land area of the planet (Morris & Kinkel, 2002). As such, it has been an area of interest to microbial ecologists for some time. Studies of the phyllosphere began in the late 1950's with works aimed at exploring the dynamics of individual microorganisms on the surfaces of plant leaves (Last, 1955; Ruinen, 1956). Prior to that time, research on the plant microbiome focused primarily on the relationships of plants with soil microorganisms (Last, 1955), often with an agricultural focus (Sapp, 2004). This applied perspective has carried into phyllosphere research, and until recently, the distribution and population dynamics of single microorganisms, often pathogens, has tended to dominate the phyllosphere literature.

Over the last few decades a variety of culture-independent techniques have been used to identify specific microorganisms and describe microbial assemblages, although analysis of the 16S rRNA gene has become the gold standard (Pace, 1997; Hugenholtz *et al.,* 2002). 16S rRNA-based methods were used to characterize marine and soil microbial communities throughout the 1980's and 1990's, but it was not until 1998 that culture-independent methods were applied to

the phyllosphere in the study of water lily biofilms (Jackson *et al.,* 1998). A more in-depth culture-independent study of the phyllosphere focused on the leaf surfaces of several agricultural plants, which were shown to harbor diverse bacterial communities (Yang *et al.,* 2001). Many culture-independent studies of the phyllosphere in the early 2000's had a similar agricultural focus, often being concerned with preventing plant and human pathogens from colonizing leaf surfaces. While these studies can demonstrate the use of the phyllosphere as a model system for studying pathogen invasion, there exists great potential to use the phyllosphere as a model system for testing broader ecological concepts, and the leaf surface offers a discrete, heterogeneous, and repeatable unit that may be used to investigate ecological mechanisms (Meyer & Leveau, 2012). With the advent of next-generation sequencing (NGS) technologies, microbial communities from the phyllosphere and other environmental samples can now be described with remarkable detail, facilitating the in-depth sampling that may be necessary for ecological studies. As of 2014, at least 37 phyllosphere studies had been published using NGS technologies (Knief, 2014), and the current number is likely to be much greater.

Phyllosphere microbiota represent a diverse array of microorganisms, but are typically dominated by Bacteria. Phyllosphere bacterial assemblages are generally less species rich than those in the rhizosphere or soil (Knief *et al.,* 2012). Alphaproteobacteria are particularly well-represented on the leaf surface and these bacteria play many ecological roles (Ruinen, 1965; Innerebner *et al.,* 2011). Gammaproteobacteria have also commonly been reported in surveys of phyllosphere bacterial community composition (Redford *et al.,* 2010; Vorholt, 2012). Proteobacteria are metabolically diverse, and the phyllosphere bacteria that carry out methyltrophy, nitrification, nitrogen fixation, or anoxygenic photosynthesis are typically representatives of this phylum (Corpe & Rheem, 1989; Fürnkranz *et al.,* 2008; Atamna-Ismaeel

et al., 2012a, b; Watanabe *et al.,* 2016). Bacteroidetes and Actinobacteria are generally the next most dominant bacterial lineages in phyllosphere communities, and both of these phyla are also well-represented in the rhizosphere (Lauber *et al.,* 2009; Philippot *et al.,* 2013). Bacteroidetes in the phyllosphere tend to be from families such as the Cytophagaceae or Chitinophagaceae (Vorholt, 2012; Stone & Jackson, 2016). These organisms are often aerobic and pigmented (Krieg *et al.,* 2011; Yasuyoshi, 2011), suggesting that they are well-adapted to the leaf surface. Phylum Actinobacteria includes members that are plant pathogens, nitrogen-fixing symbionts, and fungal antagonists, as well as decomposers (Qin *et al.,* 2011; Palaniyandi *et al.,* 2013). Many of these roles have not been explored in the phyllosphere environment, but the Actinobacteria *Corynebacterium* has been used as a foliar-applied plant growth promoter (Giri & Pati, 2004). Less is known about the presence and distribution of Archaea in the phyllosphere as they appear to constitute a minor portion of the microbial community (Delmotte *et al.,* 2009; Knief *et al.,* 2012; Vorholt, 2012).

Next generation sequencing of community metagenomes has shown redundant functionality throughout phyllosphere microorganisms, suggesting that environmental conditions (low nutrients, high UV, changing temperature and humidity) select for consistent biological traits, and low functional diversity at the community level (Delmotte *et al.,* 2009; Lambais *et al.,* 2017). Generally, the phyllosphere is dominated by aerobic organoheterotrophs, and metabolic diversity exists primarily in the context of utilizable carbon compounds. Interestingly, proteorhodopsin genes related to anoxygenic photosynthesis were found to be taxonomically widespread in the tamarisk salt cedar (*Tamarix nilotica*) phyllosphere (Atamna-Ismaeel *et al.,* 2012a, b). Proteorhodopsins are light-activated proton pumps that have been implicated as starvation-prevention mechanisms in oligotrophic marine environments (Akram *et al.,* 2013).

Such adaptations seem particularly useful in the phyllosphere, although more studies are needed to address this phenomenon.

Plant species appears to be the predominant factor influencing the composition of the phyllosphere microbial community, although there is substantial variability in microbiome composition even within a single plant species (Hunter *et al.,* 2010; Redford *et al.,* 2010; Laforest-Lapointe *et al.,* 2016). Temporal variation in phyllosphere composition likely represents a combination of leaf age and succession, coupled with environmental variation (Kinkel, 1997) and changes in the microbial composition of the atmosphere (Pedgley, 1991). This variation can be dramatic, and phyllosphere communities differ significantly on seasonal time scales, even on evergreen plants that might be expected to show less influence of seasonality (Jackson & Denney, 2011). Spatial differences in the phyllosphere of individual plant species are less well studied and the extent to which dispersal limitation or environmental gradients drive phyllosphere composition is unclear (Redford *et al.,* 2010; Finkel *et al.,* 2012; Stone & Jackson, 2016).

ii. Environmental Effect on the Phyllosphere Microbiome

Environmental conditions at the leaf surface exert a tremendous influence on microbial populations in the phyllosphere, which in turn determines the interactions that can occur between the plant and its microbiome. Growth on the leaf surface is frequently limited by both water and nutrients, as well as exposure to high levels of ultraviolet (UV) radiation (Vorholt, 2012). Variation in the physical and chemical landscape of the leaf itself (Neinhuis & Barthlott, 1997; Reisberg *et al*., 2013) and patchiness of nutrients on the leaf surface impose additional

constraints for colonizing microorganisms (Remus-Emsermann *et al.*, 2012). Leaf topography changes as the leaf ages, so that plant-microbial interactions are dependent on time as well as the specific characteristics of the host plant (Kinkel, 1997). Most leaf-associated microorganisms occur in areas that are somewhat protected from abiotic stresses and in areas that foster closer interactions with the host plant. Denser populations of bacteria are found in the grooves between plant cells, at the base of trichomes, and near leaf veins and stomatal openings (Kinkel, 1997; Baldotto & Olivares, 2008). Even within a plant species, leaf traits can vary between cultivars (Jenks *et al.*, 1995; Hunter *et al.*, 2010) and even between leaves on the same plant (Beattie, 2002; Cordier *et al.*, 2012), further complicating how the surface interacts with phyllosphere microorganisms.

Moisture availability is a key limitation to microbial growth on the leaf surface (Beattie, 2002). The cuticle prevents moisture from leaving the inside of the leaf and limits how much water remains on the leaf surface (Holloway, 1970; Rentschler, 1971; Neinhuis & Barthlott, 1997). To overcome this challenge, bacteria may form aggregates and biofilms, producing extracellular polymeric substances (EPS) which can resist desiccation (Ophir & Gutnick, 1994; Wilson & Lindow, 1994; Morris *et al.*, 2002). Other bacteria produce surfactants to increase the wettability of the leaf and lessen the ability of the cuticle to limit water accumulation (Knoll & Schreiber, 2000; Schreiber *et al.*, 2005). In addition to these strategies, the leaf surface has a boundary layer with a microclimate that typically has higher humidity than the broader phyllosphere, and this likely mitigates some of the desiccation pressure for microbiota that are in that layer (Burrage, 1971). Microbial populations typically increase following precipitation (Last, 1955; Hirano *et al.*, 1996), when there can be substantial changes in the diversity and composition of the phyllosphere microbiome (Jackson *et al.*, 2006; Copeland *et al.*, 2015).

Although phyllosphere microorganisms have evolved strategies to mitigate potential moisture limitation, the nutrient poor nature of the leaf surface means that growth of these microorganisms is still limited by available nutrients, primarily carbon and nitrogen (Wilson & Lindow, 1994; Mercier & Lindow, 2000). Microorganisms on the leaf surface are generally oligotrophs that can tolerate low nutrient conditions, or are microorganisms that can interact with the host plant to obtain more nutrients (Beattie & Lindow, 1999). Although cuticular waxes are generally resistant to chemical movement, some plant metabolites can move to the leaf surface, supporting microbial growth (Tukey, 1966; Mercier & Lindow, 2000). These compounds may arrive on the leaf surface by excretion from leaf cells, or due to osmotic pressure when the leaf is wet (Tukey, 1970). Plants also release volatile organic compounds that support specific populations of microorganisms; for example, methylotrophic bacteria that metabolize plant-derived single-carbon compounds are abundant constituents of the phyllosphere of many plant species (Omer *et al.,* 2004; Delmotte *et al.,* 2009).

Phyllosphere microorganisms may obtain nitrogen by way of plant-produced amino acids which leach to the leaf surface or by inorganic forms of nitrogen that leach from the apoplast (Tejera *et al.,* 2006). Nitrogen arrives on the leaf surface through atmospheric deposition. Ammonia is typically assimilated by the leaf microbiota, although chemoautotrophic ammonia oxidizers can also be present in the phyllosphere (Bowatte *et al.,* 2015; Watanabe *et al.,* 2016). Oxidized nitrogen species (nitrate, nitrite) are water soluble and their availability changes during rain events (Papen *et al.,* 2002; Guerrieri *et al.,* 2015). Bacterial nitrogen fixation can occur on the leaf surface, likely in pockets of moisture because of the anaerobic constraints of the fixation process (Ruinen, 1965; Jones, 1970; Holland, 2011).

Exposure to UV radiation poses a particular challenge to leaf epiphytes, and bacterial and fungal populations that have been isolated from the phyllosphere are typically more pigmented than those from soil (Stout, 1960; Sundin, 2002). This pigmentation can increase survival in the phyllosphere environment (Sundin & Jacobs, 1999; Jacobs *et al*., 2005). Phyllosphere microorganisms can also express enzymes to handle reactive oxygen species generated from solar radiation, as well as produce DNA protection proteins (Delmotte *et al.,* 2009). Rapid expression of DNA repair mechanisms following UV exposure may be a crucial determinant of phyllosphere survival (Sundin, 2002). However, most of the information on the influence of UV radiation comes from studies at the organismal or population level, and little is known of the effects of UV radiation on the collective microbial community. UV radiation has been shown to increase phyllosphere bacterial diversity (Kadivar & Stapleton, 2003) but also to cause no change (Truchado *et al.,* 2017). Because the influence of solar radiation occurs on a diurnal cycle, day length can also relate to the growth and abundance of certain microorganisms (Sundin & Jacobs, 1999). Thus, growth of microorganisms on the leaf surface may be affected by day lengths that change on a seasonal level; however, no investigations have sufficiently disentangled such effects from other variables that also follow seasonal trends (*i.e.,* temperature, relative humidity). Additionally, because most phyllosphere studies focus on agricultural systems, many studies are concluded at the time of harvest or at the end of the growing season – before seasonal trends may become pronounced.

iii. Ecological Patterns in the Phyllosphere Microbiome

Many theories have been conceived to explain the high species diversity found in

communities with common limiting resources (Hutchinson, 1961; Grubb, 1977; Dybzinski & Tilman, 2007). While microorganisms display a wide variety of metabolic strategies, the phyllosphere has been found to be dominated by heterotrophic taxa, which are likely limited by available carbon (Lindow & Brandl, 2003; Jackson *et al.*, 2006; Vorholt, 2012). However, to what extent competition between phyllosphere bacterial species for carbon or other resources takes place is unknown. Measuring competition and other dynamics of microbial communities *in situ* is difficult, and experiments utilizing only culturable bacterial taxa may have limited application to the highly diverse communities found in nature. Additionally, broadly descriptive NGS studies reveal little about the interactions that shape microbial communities. Ecological diversity theories, borrowed from the community ecology of larger organisms, may therefore frame investigations of the phyllosphere microbial community in such a way as to elicit mechanisms of interaction at work, while still retaining the descriptive power of NGS methods.

Applying ecological concepts borrowed from larger organisms to microbial communities has proved to be challenging. One major point of contention revolves around the relative importance of neutral (stochastic) and niche (governed by predictable, deterministic forces) processes. Studies on both soil and phyllosphere communities have shown that both forces may shape microbial community structure (Fierer & Jackson, 2006; Dumbrell *et al.*, 2010; Maignien *et al.*, 2014). In a recent study of phyllosphere succession, stochastic forces shaped the initial bacterial community, while deterministic forces likely caused these communities to converge over time (Maignien *et al.*, 2014) though this idea was first proposed by Clements (1916). Initial stochasticity in community assembly is likely as the leaf surface is colonized primarily from microorganisms in the atmosphere but is spatially heterogeneous with respect to habitability, so that successful colonization by an organism is determined largely by chance (Kinkel, 1997;

Kinkel *et al.,* 2002; Whipps *et al.,* 2008; Remus-Emsermann *et al.,* 2012). Once on the leaf, microorganisms are exposed to selective forces such as ultraviolet radiation, desiccation, freezing, and nutrient poor conditions (Vorholt, 2012; Stone *et al.* 2018). Such environmental constraints likely select for similarities in community composition, regardless of the initial colonizers. Bacterial phyllosphere assemblages have been shown to group strongly by plant type, indicating that traits specific to the plant host (likely traits related to the leaf surface) also play a large role in determining the composition of these communities (Redford *et al.,* 2010).

While the role of neutral and niche forces in shaping the phyllosphere community has been differentiated (Maignien *et al.,* 2014), the importance of spatial and temporal heterogeneity was not addressed in that study. The phyllosphere is a complex system, heterogeneous at many scales in both time and space. At the smallest spatial scale, available resources distributed unevenly across the leaf affect colonization and growth (Remus-Emsermann *et al.*, 2012). At larger spatial scales, heterogeneity can arise from variation in leaf traits (*e.g.*, composition of cuticular waxes, density of trichomes and stomata) vertically and horizontally throughout the canopy of a single tree (Wildman & Parkinson, 1979; Andrews *et al.,* 1980; Whitman & Schweitzer, 2002). At the scale of a whole forest or woodland, differences between plant species add another layer of heterogeneity (Kim *et al.,* 2012). Finally, at landscape and regional levels, larger environmental gradients may also come into play, and while the importance of these large scale gradients has not been explicitly tested, the issue of forest-scale heterogeneity has been examined by comparing intra- and interspecific differences of plants in microbial community composition (Redford *et al.,* 2010; Kim *et al.,* 2012).

In addition to showing spatial patterns, phyllosphere communities also change temporally, an observation that was noted in the earliest phyllosphere studies (Last, 1955).

Studies on the dynamics of individual microbial populations dominate much of the phyllosphere literature and are predicated on fluxes of individuals over time (see Kinkel (1997) for a well-written synthesis). However, few culture-independent studies have explored the role of temporal heterogeneity in a natural setting. Saprophytic fungal communities have been monitored during leaf senescence and decay, and show both seasonal patterns and rapid successional dynamics during senescence and decomposition (Osono, 2006; Voriskova & Baldrian, 2013). Others have examined changes in phyllosphere bacterial community structure over different seasons (Redford & Fierer, 2009; Jackson & Denney, 2011), but no study has utilized culture-independent methods to specifically examine bacterial community succession on the leaf surface.

While many ecological theories have been developed to explain species diversity and patterns of community structure, few have been applied to phyllosphere research. Theories in community ecology generally rely on two fundamental assumptions about the nature of species interactions, as well as the nature of the habitat in which species occur. The first assumption is that species interactions are either neutral or niche-based, and the second is that either temporal or spatial heterogeneity must be present in the environment in order to maintain diversity. These mechanisms may not necessarily be exclusive (Leibold & McPeek, 2006), and taken collectively, phyllosphere research seems to indicate that all four possibilities (both neutral and niche-based processes, and both temporal and spatial heterogeneity) may be involved in microbial community assembly.

Understanding spatial and temporal heterogeneity is perhaps the most recurrent theme in studies of microbial community assembly. The issue of cosmopolitan dispersal makes this particularly challenging (Baas Becking, 1934; Finlay & Fenchel, 2004). Consequently, most spatial variation in microbial diversity, composition, and function is readily explained by

environmental filtering, and much recent effort has been devoted towards identifying which key factors most strongly influence community structure (Jones & McMahon, 2009; Lindstrom & Langenhelder, 2012; Logares *et al.*, 2013). The phyllosphere is particularly well-suited to studies of spatiotemporal variability because of its strong connection with atmospheric conditions and dispersal, combined with its sensitivity towards environmental variation and understanding microbial patterns in this habitat was the principal objective of this work.

Chapter one explored the influence of spatial heterogeneity at small to intermediate spatial scales through characterization of the phyllosphere community across a small forest. The objective was to identify the relative importance of environmental gradients and dispersal limitation in establishing community patterns. Chapters two and three explore the contribution of rain as agent of temporal heterogeneity, which is particularly understudied with respect to whole community dynamics. While the effect of rain on phyllosphere bacteria is well characterized in the context of pathogen dispersal, the implicit assumption of foliar bacterial removal from the leaf has not been addressed through the perspective of ecological disturbance. The objective of chapter two was to understand the relationship between short-term temporal heterogeneity (*i.e.* rain) and long-term temporal heterogeneity while chapter three was to characterize the ability of the tree canopy (spatial heterogeneity) to mediate the effect of rain. Both chapters represent significant gaps in the current understanding of phyllosphere microbial ecology. Collectively, these projects will provide insight into how phyllosphere communities become established across space and time.

CHAPTER I

SPATIAL VARIATION IN PHYLLOSPHERE COMMUNITIES IN A SMALL FOREST PLOT

i. Abstract

The phyllosphere presents a unique system of discrete and easily replicable surfaces colonized primarily by bacteria. However, the biogeography of bacteria in the phyllosphere is little understood, especially at small to intermediate scales. Bacterial communities on the leaves of 91 Southern Magnolia (*Magnolia grandiflora*) trees 1-452 m apart in a small forest plot were analyzed and fragments of the 16S rRNA gene sequenced using the Illumina platform. Assemblages were dominated by members of the Alphaproteobacteria, Bacteroidetes, and Acidobacteria. Patterns in community composition were measured by both relative-abundance (theta) and presence-absence (Jaccard) dissimilarity metrics. Distance-based Moran's eigenvector map analyses of the distance-decay relationship found a significant, positive relationship between each dissimilarity metric and significant eigenfunctions derived from geographic distance between trees, indicating trees that were closer together had more similar bacterial phyllosphere communities. Indirect gradient analyses revealed that several environmental parameters (canopy cover, tree elevation, and the slope and aspect of the ground beneath trees) were significantly related to multivariate ordination scores based on relative

bacterial sequence abundances; however these relationships were not significant when looking at the incidence of bacterial taxa. This suggests that bacterial growth and abundance in the phyllosphere is shaped by different assembly mechanisms than bacterial presence or absence. More broadly, this study demonstrates that the distance-decay relationship applies to phyllosphere communities at local scales, and that environmental parameters as well as neutral forces may both influence spatial patterns in the phyllosphere.

ii. Introduction and Objectives

The phyllosphere is the aerial portion of plants on which potentially diverse communities composed of Bacteria, Archaea, and microbial eukaryotes exist (Vorholt, 2012). The surface area of the phyllosphere is vast, estimated as being several orders of magnitude greater than the land area of the planet (Morris & Kinkel, 2002). Despite this large area, few studies on the biogeographic distribution of phyllosphere communities have been reported. Rather, the focus has more commonly been on the distribution and population dynamics of single microbial species, typically from a pathological perspective. Most of these spatial studies of the phyllosphere have utilized culture-dependent techniques and focused on the distribution of agricultural pathogens, thus tending to analyze plants that occur in a regular and repeated pattern (*i.e.*, rows of crops; Clayton & Hudelson, 1995). As such, autocorrelation and autoregressive-moving-average (ARMA) models, which are well suited for quantifying disease, are common in the literature (Clayton & Hudelson, 1995). However, ARMA models operate best when applied unidirectionally, while distance methods are probably more appropriate to compare plants that are not regularly distributed. The tendency for communities to become dissimilar at increasing

distances is recognized in broader studies of biogeography, and is known as the distance-decay relationship (Hubbell, 2001).

While there has been increased use of next-generation sequencing (NGS) methods to describe phyllosphere bacterial communities, especially comparing community composition between species (Kim *et al.,* 2012; Kembel *et al.,* 2014), few studies have addressed the issue of biogeographic distribution, despite a call for studies at different spatial scales (Andrews & Harris, 2000). The application of the distance-decay relationship to microbial ecology in general has yielded conflicting results (Green & Bohannan, 2006), and a similar disagreement has emerged from the limited number of studies of phyllosphere assemblages. Compositional differences in the bacterial phyllosphere between different plant species have generally been found to be much greater than intraspecific differences between individuals of the same plant species, even with different geographic origins of the plant host (Redford *et al.*, 2010). Conversely, however, geographic location has been found to be a greater predictor of phyllosphere community composition than plant host species and environmental factors (Finkel *et al.*, 2011, 2012).

The distance-decay relationship is unique in that it may occur by neutral processes if the pattern is caused by dispersal limitation or by niche-based processes if driven by environmental gradients. Previous culture-independent studies of phyllosphere biogeography have utilized spatial scales in the hundreds of kilometers or more, so that little is known of patterns at smaller scales. The goal of this study was to supplement current understanding of spatial patterns of the phyllosphere by investigating whether phenomena such as the distance-decay relationship might apply within a single wooded ecosystem. This study examined the effect of distance on the composition of the bacterial community in the phyllosphere of a single tree species at a small

spatial scale (< 1 km) using NGS methods. We sampled the phyllosphere community of 91 *Magnolia grandiflora* (Southern Magnolia) trees at distances of < 1 m to 450 m apart. By sampling broadleaf evergreens in winter, variation in community composition was tested in a natural setting with limited interference from other tree species. Distances (< 1 km) were hypothesized to affect the bacterial community composition of *M. grandiflora* leaves such that dissimilarity would be higher between communities on trees that were further apart.

iii. Methods

Leaves were initially sampled from 100 *M. grandiflora* trees in Bailey Woods, a 20 ha tract of mature woods adjacent to the University of Mississippi campus in Oxford, Mississippi, USA, in February 2014. The woods are a remnant of old growth forests in northern Mississippi, and are mainly deciduous with *M. grandiflora* accounting for approximately 1% of the total number of trees (Brewer, 2001). The 100 *M. grandiflora* trees sampled account for the majority of *M. grandiflora* trees that grow in this system. GPS coordinates were determined for each tree at the time of sampling. To aid in multivariate plotting, trees were mapped a posteriori to five general clusters based on location within the woods (Figure 1). Unrelated to these five spatial clusters, some smaller trees occurred within close proximity to larger central trees, and may have represented offspring from fallen seeds. These were recorded as separate trees in the same location, and their relationship was noted and used in subsequent analyses.

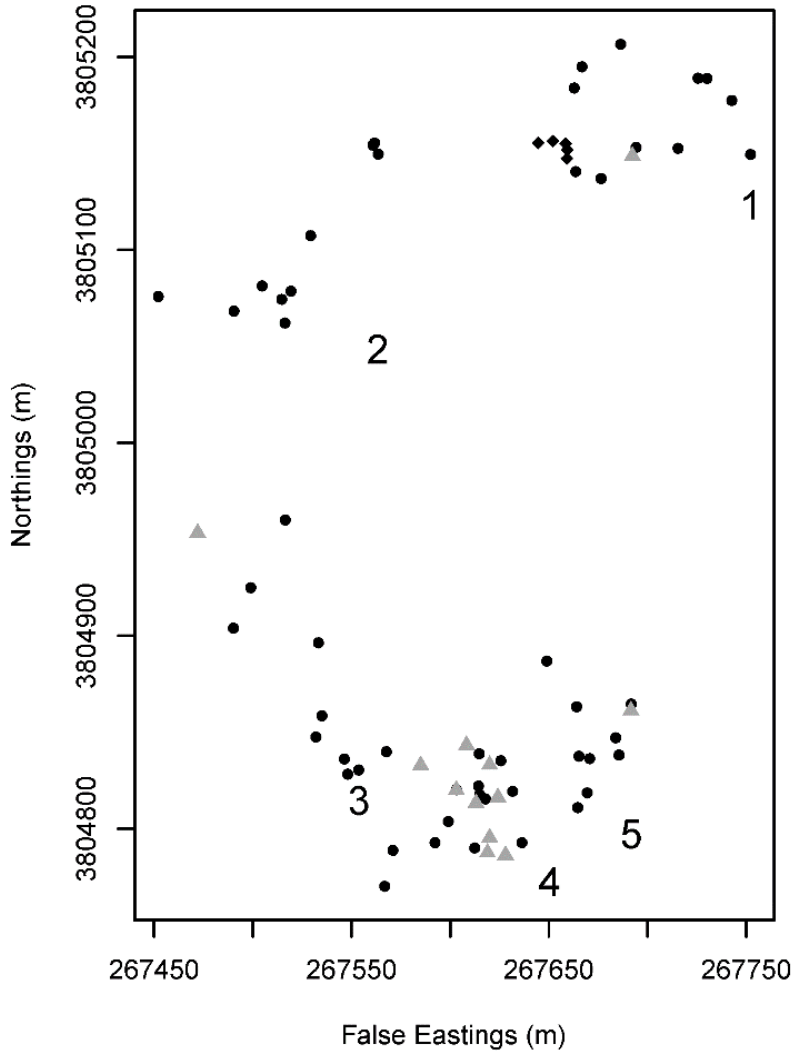

Figure 1. Map of *Magnolia grandiflora* Trees throughout Bailey Woods
Location of 91 *Magnolia grandifolia* trees within a 20 ha woodland in Oxford, MS, USA, that were sampled for phyllosphere communities. Black dots represent single trees while grey triangles represent locations of one parent tree surrounded by one or more smaller offspring. Black diamonds represent a group of trees (14-18) that showed distinct phyllosphere communities from other trees. Numbers indicate hypothetical spatial clusters of trees assigned by geographic or topographic isolation and are placed to the lower right of the indicated area. Location coordinates are expressed using the Universal Transverse Mercator (UTM) coordinate system (zone 6) and with the NAD83 Datum.

Sampling consisted of collecting two leaves (200 leaves total) from each tree, from different branches at approximately 1.5-1.8 m height. Only leaves that displayed no signs of disease or decay (such as browning or spotting) were selected. Leaves were placed in individual sterile sample bags and stored at -20 °C from as soon as possible after sampling (< 2 h) until processed. The circumference of each tree was recorded with measuring tape and then converted to diameter at breast height (DBH). Additionally, at each tree, percent canopy cover was measured using a spherical densiometer. Using the raster package in R (Hijmans, 2015), GPS coordinates were combined with a digital elevation model to obtain the relative elevation, slope, and slope aspect of each tree.

The phyllosphere community was recovered from each leaf by brushing the leaf for two minutes with a sterile toothbrush in sterile TE (pH 8.0) buffer. The resulting suspension was centrifuged (10,000 xg, 2 minutes) and the pellet frozen (-20 °C) for subsequent DNA extraction. DNA was extracted using a PowerSoil extraction kit (MoBio, Carlsbad, CA) following standard procedures, other than that the final volume of DNA eluted was reduced from 100µL to 50µL. DNA samples were analyzed using a dual index barcoding approach targeting the V4 region of the 16S rRNA gene (Kozich *et al.*, 2013). Briefly, DNA (standardized by volume, 1 µL) was combined with 1 µL of each primer (at 10 µM) and 17 µL of AccuPrime Pfx Supermix (Invitrogen, Grand Island, NY). Amplification was conducted through 30 cycles of 95°C (20 s), 55°C (15 s), and 72°C (2 min) after an initial denaturation step of 95°C (2 min) and followed by a final elongation step of 72°C (10 min). Amplification products were standardized with SequalPrep Normalization Plates (Life Technologies, Grand Island, NY) and pooled prior to sequencing. The multiplexed library was sequenced using the Illumina MiSeq platform at the Molecular and Genomics Core Facility at the University of Mississippi Medical Center

(UMMC).

Raw data files (FASTQ) were processed using the Mothur bioinformatics pipeline (Schloss *et al.*, 2009) following the procedures recommended by Schloss *et al.* (2011) and Kozich *et al.* (2013). Rare sequences that were similar to abundant sequences (two base differences), and likely the result of sequencing errors, were merged so that remaining singletons would more legitimately reflect bacterial diversity (see Online Resource 1 for an examination of singleton removal). After removal of other potentially erroneous data, chimeras, and mitochondrial and chloroplast sequences, nine trees (unrelated spatially or by lab procedures) had leaves with < 10,000 valid bacterial sequences and were removed from the dataset, leaving 91 trees in the final analysis, or 182 leaves. All diversity analyses were conducted using operational taxonomic units (OTUs) defined by 97% sequence similarity and by subsampling (1000 iterations) the number of reads to that in the lowest remaining sample (27,719 sequences). After assignment of OTUs, several prominent OTUs classified as "unclassified Cyanobacteria" lineages, which were subsequently (BLAST searches) determined to likely be of chloroplast origin and these were removed prior to further analyses. Beta-diversity was assessed using the abundance-based theta index (Yue & Clayton, 2005) and non-abundance (*i.e.*, presence-absence) derived Jaccard index, which were both used to calculate non-metric multidimensional scaling (NMDS) axes, and specific OTUs correlated to axes scores. Monte Carlo tests were employed to determine if leaves from the same trees were more similar than between different trees. On average, and by both metrics, bacterial communities from leaves of the same tree demonstrated significantly greater similarity than leaves from other trees (Figure 2). Sequences of the remaining leaves were thus merged by tree to give a final dataset of 91 trees, with at least 27,719 sequences per tree. As the data were analyzed, a small cluster of trees (trees 14-18; located in the

western part of cluster 1, Figure 1) appeared to harbor different phyllosphere communities to the other samples. These trees had high richness of distinctly identifiable sequences and were reprocessed separately to ensure that sequences were not incorrectly attributed to them during the processing steps.

Further analyses and figure creation were done using R version 3.0.2 (R Core Team, 2013). To determine the effect of environmental gradients on bacterial alpha-diversity, analysis of covariance (ANCOVA) was performed using DBH, canopy cover, relative elevation, slope, and slope aspect as covariates, and relatedness as a categorical factor. Indirect gradient analyses were performed with those same environmental parameters on multivariate NMDS axes scores using the envfit function in the vegan package (Oksanen *et al.,* 2013). The significance of the distance-decay relationship was assessed with distance-based Moran's eigenvector maps (MEMs). Distance-based MEM analysis was carried out using the PCNM function in the PCNM package (Legendre *et al.,* 2013) and the relationship between all significant eigenfunctions and community dissimilarity matrices was assessed with distance-based redundancy analysis (RDA; using the dbRDA.D function; Legendre *et al.,* 2015). Results of distance-based MEMs are reported along with results from the more commonly used permutation-based Mantel tests using a spatial Euclidean distance matrix between every tree and each community dissimilarity matrix separately. Mothur parameters used for sequence processing and R code used for analyses can be found in Online Resource 2.

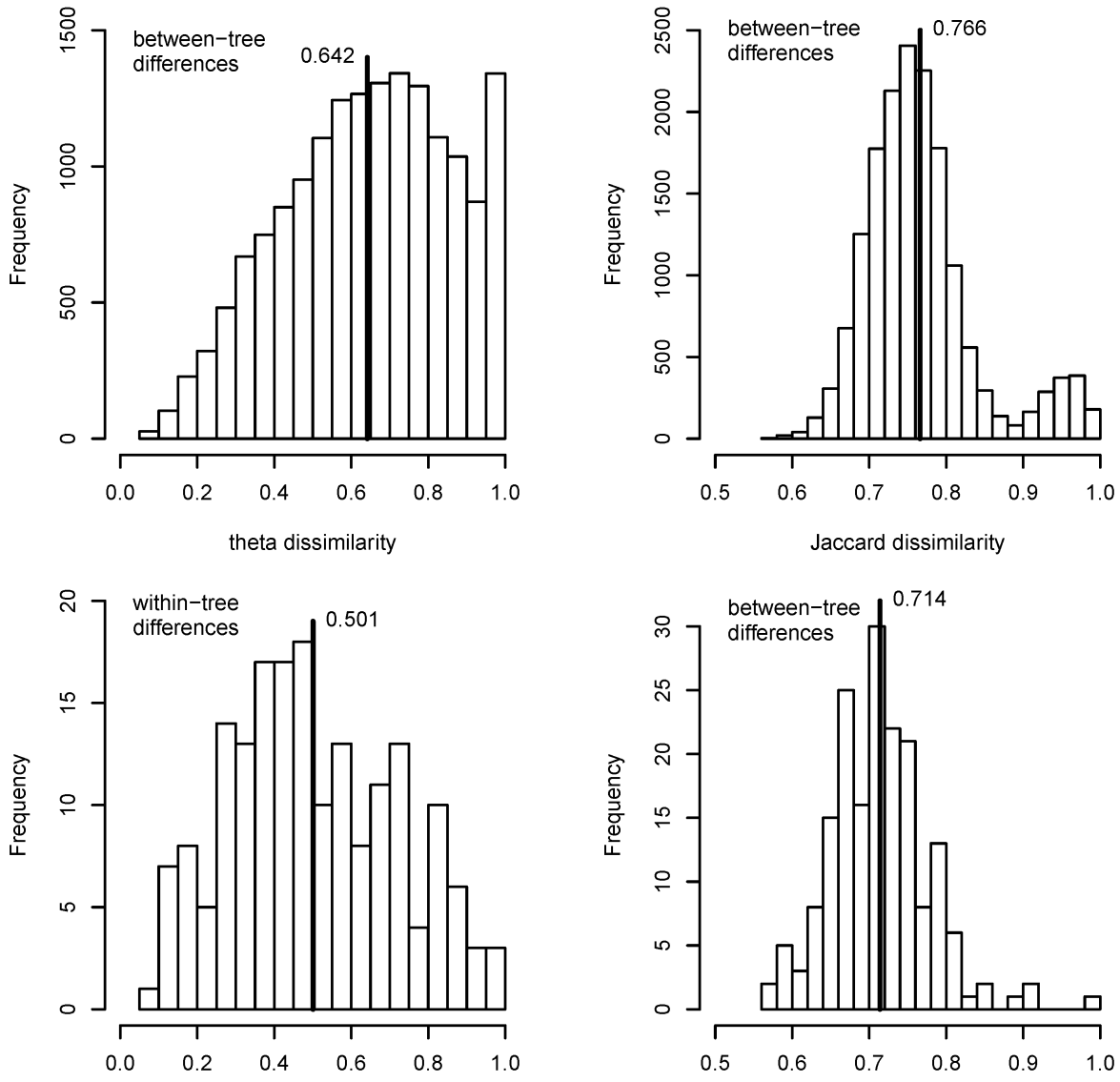

Figure 2. Compositional Similarity of *Magnolia grandiflora* Tree Leaves
Histograms of theta and Jaccard pairwise dissimilarities of bacterial phyllosphere communities grouped as dissimilarities between leaves on different *Magnolia grandiflora* trees (top row) and dissimilarities between leaves on the same tree (bottom row). The bold lines represent group means. Within-tree pairwise dissimilarities were found to be significantly lower using Monte Carlo randomization of the t-statistic ($t_{theta} = 8.67$, $p < 0.001$ and $t_{Jaccard} = 2.56$, $p < 0.001$).

iv. Results

After removal of sequences attributed to Archaea, Eukarya, mitochondria, and chloroplasts, there were 8,704,825 bacterial sequence reads across all samples, consisting of 20,416 unique sequences. Eight bacterial phyla accounted for > 95% of sequence reads: Proteobacteria (49.9% of valid bacterial 16S reads, 82.2% of which were Alphaproteobacteria, Figure 3), Bacteroidetes (12.1%), Acidobacteria (10.1%), Actinobacteria (10.1%), Planctomycetes (6.8%), Verrucomicrobia (4.0%), Armatimonadetes (1.5%), Chloroflexi (1.3%, Figure 3). At a finer taxonomic level, there were 16 OTUs that were represented by > 100,000 reads each, and together accounted for 4,047,874 reads (46.5% of the reads in the dataset). Of these, a member of the Alphaproteobacteria in the order Rhizobiales was the most abundant and accounted for 385,742 sequences. In total, six of these prominent OTUs belonged to the Alphaproteobacteria and included members of the Rhizobiales, Acetobacteraceae, and a Methylobacterium species. Two OTUs represented lineages of Acidobacteria and accounted for 486,194 reads, and could both be attributed to the Acidobacteriaceae. Three OTUs (accounting for 592,344 reads) were lineages of Bacteroidetes. Two OTUs belonging to the Actinomycetales (Actinobacteria) were also abundant (202,250 reads). OTUs representing a lineage in the Planctomycetes and an unidentified member of the Verrucomicrobia were also among the most abundant taxa detected.

The alpha-diversity of phyllosphere bacterial epiphyte communities (measured by the inverse Simpson metric; found in Online Resource 3) was not related to DBH, relatedness, elevation, slope, or slope aspect of the trees. Stepwise model fitting using AIC scores suggested the model that explained the most variation in inverse Simpson scores included only canopy

cover as a significant variable ($F_{1,89} = 4.499$, $p = 0.037$).

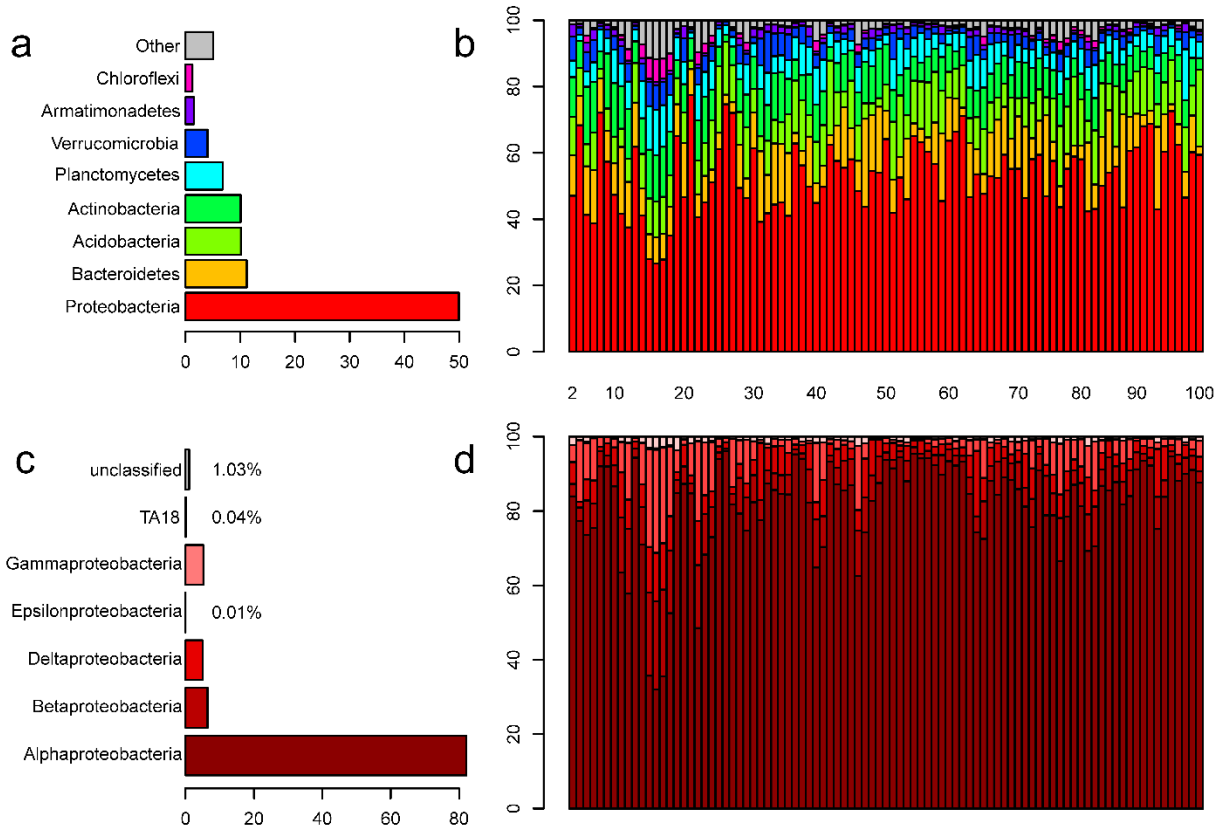

Figure 3. Relative Abundances of Prominent Phyla in the *Magnolia grandiflora* phyllosphere

Bar charts of relative abundances of major bacterial lineages in phyllosphere communities of *Magnolia grandiflora* based off 16S rRNA gene sequence reads. All values are expressed as percentages of 7,025,849 bacterial sequences and 3,572,714 Proteobacteria sequences. Panels show the relative abundances of the eight most dominant bacterial phyla averaged across the whole dataset (a) and for each tree (b), and the relative abundance of Proteobacteria subphyla within that phylum averaged across the whole dataset (c) and for each tree (d).

Indirect gradient analysis indicated that canopy cover, elevation, slope, and slope aspect were significantly related to relative abundance-based NMDS scores; however no additional variables were significantly related to presence-absence-based NMDS scores. AMOVAs of community dissimilarity grouped by the relatedness of the trees showed that theta dissimilarities were not significantly affected by the parent-offspring relationship while Jaccard dissimilarities were ($F_{1,89} = 2.00$, $p = 0.054$ and $F_{1,89} = 1.46$, $p = 0.018$). However, related trees did not group close together, or distinctly away from, non-related trees on either NMDS plots (Figure 4).

By both relative abundance and presence-absence metrics, there was a positive relationship between tree distance and phyllosphere bacterial community dissimilarity (Figure 5a, b). Distance-based MEM (dbMEM) analyses found significant relationships between community dissimilarity and spatial predictor eigenfunctions ($p < 0.001$ in both cases). Because indirect gradient analyses showed significant relationships between several environmental parameters and relative abundance-based community metrics, z-transformed environmental values were included in the predictor matrix of eigenfunctions when assessing the spatial relationship with relative abundance-based dissimilarities. Theta dissimilarities demonstrated higher adjusted R^2 and F-statistic values than Jaccard dissimilarities ($R^2_t = 0.203$, $F_t = 1.500$; $R^2_j = 0.087$, $F_j = 1.199$). Similarly to dbMEM analyses, a partial Mantel test was constructed using dissimilarities of environmental factor scores (created using factor analysis) as the controlling distance matrix, and showed a significant, positive relationship between the relative abundances of bacterial community members and geographic distance between samples ($R_M = 0.244$, $p < 0.001$, Figure 5a). Phyllosphere community dissimilarity based on the presence or absence of bacterial OTUs (Jaccard index) showed a clearer pattern over geographic distance (Figure 5b)

and a slightly higher Mantel correlation coefficient ($R_M = 0.268$, $p < 0.001$). Mantel correlograms indicated that by both dissimilarity metrics, bacterial phyllosphere communities were spatially autocorrelated until 87 m apart (Figure 5c, d). Additionally, correlograms showed that negative spatial autocorrelation (*i.e.*, distance-decay, indicated by negative Mantel correlation coefficients) of bacterial leaf communities was significant between trees that were 330 meters apart.

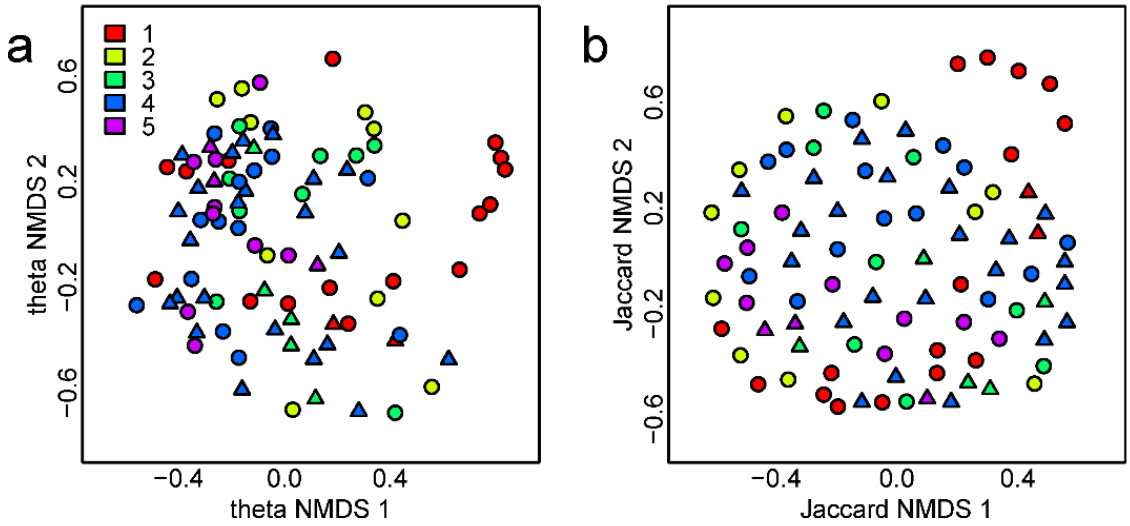

Figure 4. Multivariate Ordinations of *Magnolia grandiflora* Community Composition
Non-metric multidimensional scaling (NMDS) ordinations of community dissimilarity between phyllosphere bacterial communities on leaves of *Magnolia grandiflora* trees as determined from relative-abundance based metrics (theta, a, stress = 0.246) or presence-absence based metrics (Jaccard, b, stress = 0.298). Colors correspond to different spatial clusters of trees (as identified in Figure 1) and triangular points represent parent and offspring trees which occur in close physical proximity while circular points represent non-related trees. Five trees (14-18) in cluster 1 were separated from others by high values of the first theta NMDS axis and high values of both Jaccard NMDS axes.

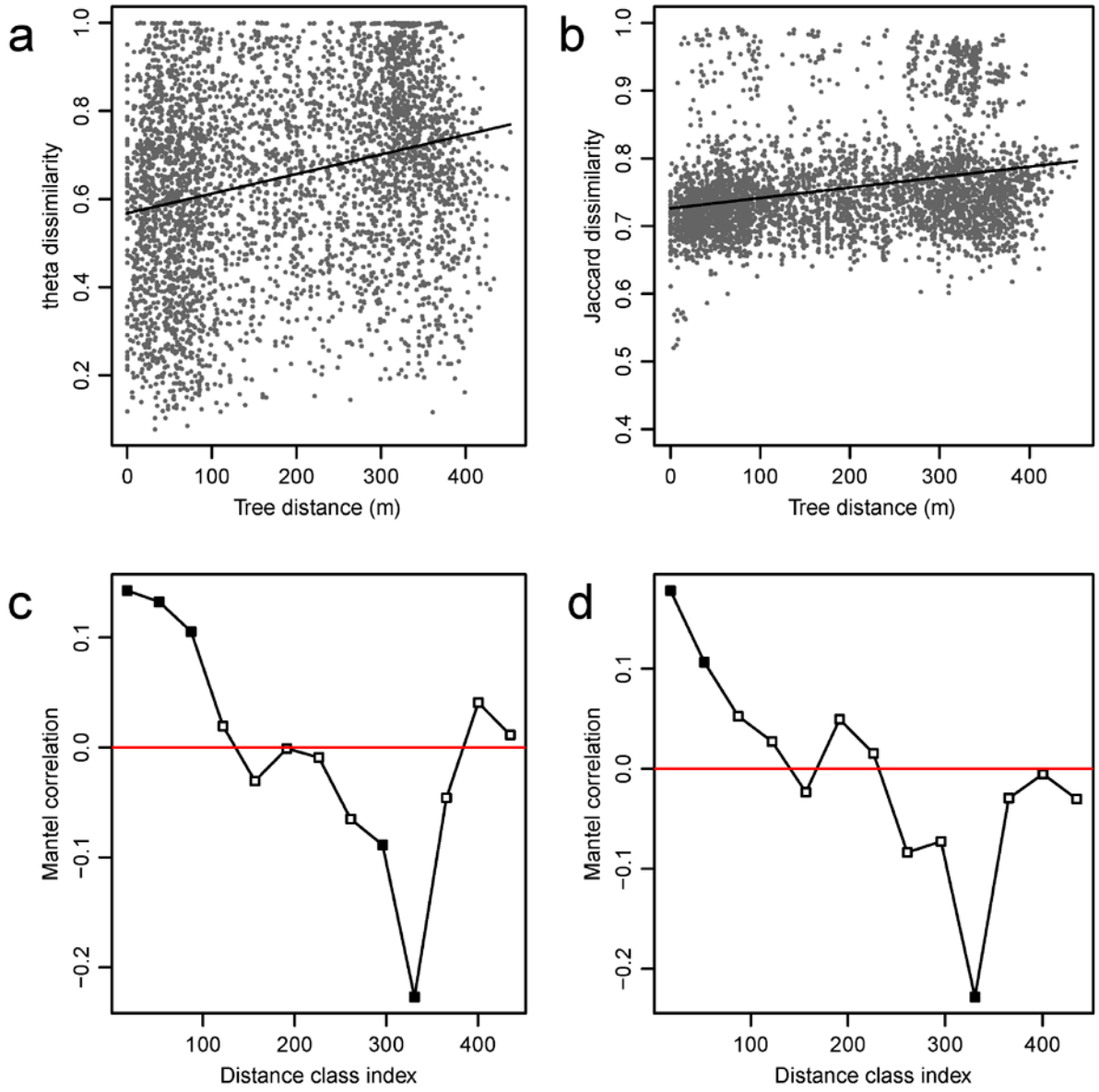

Figure 5. Distance-Decay Relationship in *Magnolia grandiflora* Communities
Distance-decay relationship in bacterial phyllosphere communities as visualized by regression of theta and Jaccard dissimilarities against geographic distances between *Magnolia grandiflora* trees (a, b). Both theta and Jaccard dissimilarities demonstrated positive changes over geographic distance which were confirmed by Mantel tests showing positive coefficients (theta R_M = 0.213, Jaccard R_M = 0.268). Mantel correlograms (c, d) depict Mantel correlation coefficients plotted over distance classes for theta (c) and Jaccard dissimilarities (d). Black dots indicate correlation coefficients significantly different than 0 (α = 0.05 after applying a Holmes correction).

v. Discussion

The predominant bacterial phyla from the trees sampled in this study match closely with those reported by Jackson and Denney who sequenced clone libraries of *M. grandiflora* bacterial leaf communities from a single tree in the same forest, but repeatedly sampled over different seasons from 2007-2009 (Jackson & Denney, 2011). In both cases, Proteobacteria was the most prominent phylum ($\bar{x}$ = 53.1, SE_x = 10.7 from these data, 53-80% from Jackson and Denney), with Alphaproteobacteria being the most prevalent subphylum. The other proportionally abundant phyla detected in this study (Bacteroidetes, Acidobacteria, and Actinobacteria) were also found at similar frequencies by Jackson and Denney (2011), while phyla that were less abundant from this dataset were only transiently present in the clone library data. This suggests that, at least at a broad taxonomic level, there is some consistency to the *M. grandiflora* phyllosphere, and also that comparisons of community composition derived from NGS data to that derived from older cloning-sequencing approaches may be valid.

At a finer taxonomic resolution, specific lineages that were detected by NGS methods in this study were also previously reported in clone libraries. The most prevalent lineages of Alphaproteobacteria reported from *M. grandiflora* clone libraries were the Beijerinckiaceae, Methylobacteriaceae, and the Sphingomonadales (Jackson & Denney, 2011); lineages that were also abundant in the larger Illumina dataset. Some of the Acidobacteria sequences derived from the clone libraries were related to *Terriglobus roseus* and *Edaphobacter* (Jackson & Denney, 2011), and there were numerous sequences identified as a *Terriglobus* species and *Edaphobacter modestum* in the Illumina data. The same patterns were seen for other taxa, and finding such

similarities when comparing samples from multiple trees sampled in 2014 to those taken from a single tree from 2007-2009, analyzed by different approaches, does suggest some consistency to the *M. grandiflora* phyllosphere microbiome.

Both dbMEM and the Mantel test demonstrated a significant relationship between the spatial structure of the trees and the community dissimilarities. However, a recent critique of the Mantel test in this application argues that dbMEM is more powerful for spatial analyses (Legendre *et al.,* 2015). Although Mantel tests found similar results as dbMEM methods in this current study, this may be because of the large sample size of the dataset, and Mantel tests may not be appropriate in this context since spatial data do not meet several numerical assumptions (Legendre *et al.,* 2015). While similar results to Mantel tests may be obtained, future studies on spatial patterns in bacterial communities should utilize the dbMEM framework because of these considerations.

The theta index was used because its values reflect proportional changes in bacterial abundance that are less dependent on the amplification success of any one sample. In contrast, Bray-Curtis dissimilarities are standardized by shared abundance using the denominator in the equation of the metric, but differences in sequencing depth between samples could exaggerate such pairwise differences in community structure. However, despite these contrasting methodologies, when analyses were repeated using Bray-Curtis dissimilarities, the same results were achieved as with the theta metric.

In this study, the diameter of trees (DBH) was used as a rough estimate of overall tree size which can affect the internal and external leaf structures in several ways (*e.g.,* nitrogen content, lignification, and stomatal density; Steppe *et al.,* 2011). However, DBH was not a significant predictor of bacterial community composition on the leaf surface. Other, more direct

and intensive measurements of specific leaf traits may be necessary to elucidate relationships between individual tree physiology and phyllosphere composition. Canopy cover was used as a proxy for radiation and light that could affect the microbial community of the leaf. Canopy cover differences between trees was significantly correlated with community similarity based on abundances of bacterial OTUs (*i.e.,* using the theta index) but not with that based on presence or absence of individual taxa. This suggests that canopy cover may be important in shaping existing bacterial communities, influencing the proportional abundance of certain populations, rather than affecting bacterial immigration and emigration (*i.e.,* dispersal).

Only NMDS axis scores of phyllosphere community dissimilarity expressed as the theta index were significantly associated to environmental variables, suggesting that relative abundances of bacterial OTUs were sensitive to environmental gradients while the presence or absence of specific OTUs was not. This relationship was further supported by the significance of theta dissimilarities with spatial and environmental factors using dbMEM and constrained RDA ordination. This distinction may reflect different mechanisms of microbial assembly. Dispersal and colonization of the phyllosphere from the atmosphere (which in large part determines the bacteria that occur on a leaf) is a purely stochastic process (Kinkel, 1997; Whipps *et al.,* 2008, Remus-Emsermann *et al.,* 2012) that is contrasted by the presence of predictable, selective forces encountered by colonizers after deposition because of conditions on the leaf surface (Kinkel, 1997; Whipps *et al.,* 2008; Vorholt, 2012). Relatedness was significantly associated with Jaccard dissimilarity scores, and likely reflects the very close physical proximity of offspring trees to one another and to their parent tree so that the same OTUs were present.

These findings suggest that environmental gradients may be more important in shaping the relative abundances of existing community members. Stochastic, or neutral, forces may be

drivers of the particular bacterial species on plant leaves. However, leaf age, structure, and morphology were not considered in this study, and these factors may influence bacterial species presence by altering which species may successfully colonize a leaf surface. Further work must be done with the colonization and growth of bacterial communities on leaves, with respect to variation in leaf morphology, in order to determine the true role of neutral forces on phyllosphere community structure.

Previous phyllosphere distance-decay studies have found that dispersal limitations are present along with environmental heterogeneity (Finkel *et al.*, 2011, 2012). The distribution of abundant taxa, in particular, has suggested that environmental heterogeneity is the likely cause of differences in leaf bacterial communities, while distribution patterns in rare bacterial taxa seemed to support dispersal-driven differences (Finkel *et al.*, 2011). At the spatial scales used in this study, dispersal limitation per se may not occur, but small population sizes may prevent rare or uncommon taxa from dispersing evenly throughout a habitat. Cosmopolitan distribution depends, in part, on large population sizes (Green & Bohannan, 2006), which rare taxa necessarily lack.

The issue of dispersal limitation in microbial ecology has largely been explored with communities from continuous habitats (*e.g.,* air, soil, or large water bodies; Lighthart, 1997; Brandão *et al.,* 2002; Horner-Devine *et al.,* 2004; Green & Bohannan, 2006; Galand *et al.,* 2009). The global biogeographic distribution of microbes in isolated hot springs has been examined with both archaeal (*e.g., Sulfolobus*) and bacterial (*e.g., Synechococcus*) lineages and suggests divergent evolutionary histories, which has been attributed to dispersal barriers (Papke *et al.,* 2003; Whitaker *et al.,* 2003; Martiny *et al.,* 2006). At similar scales to those examined in this study, distance-decay pattern of bacterial communities in pools formed by buttressed tree

roots was thought to be caused by (unmeasured) environmental variables, not dispersal limitation (Bell, 2010). However, the method of bacterial description (terminal restriction fragment length polymorphisms) used in that study has biases towards abundant or well-amplified taxa, and analyses were limited to those based on an abundance-based measure of community dissimilarity. If dispersal-based mechanisms are more evident among less common or non-abundant bacterial taxa, the above methods may have prevented these patterns from being observed. In this study we found that the use of both abundance-based and presence-absence dissimilarity metrics demonstrated patterns of community assemblage that would have been difficult to infer otherwise.

The potential influence of both stochastic and deterministic forces make the phyllosphere an intriguing system in which to explore issues of microbial biogeography. The leaf surface offers a discrete, heterogeneous, and repeatable unit that may be used to attain levels of replication that would be prohibitively difficult with communities of larger organisms (Meyer & Leveau, 2012). Using such isolated leaf communities, this study found that while the abundances of bacterial taxa seemed to depend on conditions around the leaf, community membership was not sensitive to the environment and may rather have been due to atmospheric dispersal, deposition, and leaf surface traits. Studies of the phyllosphere can also utilize higher sample sizes, such as the 100 trees (and 200 individual leaves) initially sampled in this study, allowing more confidence than limited sampling of communities of macroorganisms. In addition to spatial patterns such as distance-decay and biogeography, exploration of the phyllosphere bacterial communities in other contexts (such as examining temporal variation) may be applied to ongoing ecological questions relating to community stability and resistance, metapopulations and patch dynamics, and mechanisms contributing to diversity.

CHAPTER II

CONTRIBUTION OF SEASONAL AND CLIMATIC DRIVERS TO PATTERNS IN PHYLLOSPHERE BACTERIAL COMMUNITY STRUCTURE

i. Abstract

Phyllosphere microorganisms are sensitive to fluctuations in wind, temperature, solar radiation, and rain. However, recent explorations of patterns in phyllosphere communities across time often focus on seasonal shifts and leaf senescence without measuring the contribution of environmental drivers and leaf traits. Here we focus on the effects of rain on the phyllosphere bacterial community of the wetland macrophyte broadleaf cattail (*Typha latifolia*) across an entire year, specifically targeting days before and one, three, and five days after rain events. To isolate the contribution of precipitation from other factors, we covered a subset of plants to shield them from rainfall. We used targeted Illumina sequencing of the V4 region of the bacterial 16S rRNA gene to characterize phyllosphere community composition. Contrary to our predictions, rain events did not have a detectable effect on phyllosphere community richness, evenness, or novel species accumulation, regardless of whether the leaves were covered from rain or not. While climatic and leaf-based variables effectively modeled seasonal trends in phyllosphere diversity and composition, they provided minimal explanatory value at shorter time scales. Similarly, we found that bacterial species-area accumulation curves were also contingent on

seasonal patterns. These findings underscore the importance of long-term environmental variation as the main factor influencing the phyllosphere community.

ii. Introduction and Objectives

The unprotected nature of the leaf means that microorganisms of the phyllosphere are subject to movement from wind and rain (Lindemann *et al.*, 1982; Lindemann & Upper, 1985). These fluxes create local patterns in bacterial atmospheric composition (potentially influencing immigration to the leaf surface; Kinkel, 1997; Maignien *et al.*, 2014) and large-scale movement (Morris *et al.*, 2008; Bowers *et al.*, 2011). Wind-induced migration from the leaf surface to the atmosphere is readily apparent from the dominance of phyllosphere-associated lineages in even high-elevation atmosphere samples (Šantl-Temkiv *et al.*, 2013). In contrast, rain is thought to be principally a mechanism of downward immigration wherein organisms travel with precipitation (Constantinidou *et al.*, 1990). Although splash dispersal can remove and transport leaf epiphytes throughout a plant canopy at small scales (Lindemann & Upper, 1985; Cevallos-Cevallos *et al.*, 2012), deposition of bacterial cells on the leaf surface and, especially, population growth following rain are of far more importance (Constantinidou *et al.*, 1990).

Much of the current understanding of the influence of climatic variables on the phyllosphere microbiome come from studies at the organismal or population level. Further, most such studies have been developed around a small group of cultural microorganisms, usually plant pathogens. Little is known about the effects of climate on the collective microbial community. For example, the growth of microorganisms on the leaf surface could be affected by day length that changes at a seasonal level. Temporal variation in phyllosphere community composition

likely represents the combined influence of leaf age and ecological succession, coupled with environmental changes (Kinkel, 1997) and changes in the microbial composition of the atmosphere (Pedgley, 1991). However, few, if any, investigations have disentangled the effects of climatic variables and changes from leaf characteristics that follow seasonal trends. Furthermore, because many phyllosphere studies focus on agricultural systems, these studies are concluded at the time of harvest or at the end of the growing season – before seasonal changes in both leaf characteristics and phyllosphere composition may become pronounced. This is compounded by the lack of recent studies on the species-area and species-time relationships in the phyllosphere. Earlier work on fungal phyllosphere communities has suggested that no such relationship exists between leaf size and species count (Kinkel, 1997) but no work has been conducted using modern sequencing methods.

To address these topics, we characterized the bacterial community of broadleaf cattail (*Typha latifolia*) across an entire year in a natural setting, with specific sampling on days before and after rain events. To understand the effect of rain more completely, we established a treatment group of plants covered by protective 1 m^2 film canopies on rainy days to compare against uncovered plants. We collected climatic data pertaining to precipitation, UV radiation, photosynthetically active radiation (PAR), temperature, relative humidity, and wind speed from NOAA weather station databases, and related them to bacterial alpha and beta-diversity patterns. At a seasonal scale, filamentous fungi dominate the microbiome following lead senescence, with a concomitant loss of bacterial diversity (Voriskova & Baldrian, 2013). We capitalized on the fact that *T. latifolia* leaves remain standing after senescence, allowing us to observe bacterial microbiome development following the shift to senescence. Thus, we explored the relationship between leaf size, senescence, seasonal patterns, and short-term weather impacts. We expected

that reductions in bacterial diversity would occur following leaf senescence. As most bacterial diversity exists at low abundance, and is therefore sensitive to demographic and environmental stochasticity, we predicted that bacterial diversity would be lower initially after rainfall and subsequently increase over five days, but only in plants exposed to rain (*i.e.*, those not under film canopies). We decomposed bacterial beta-diversity into differences driven by either species turnover (loss of one species followed by the gain of another) or by richness differences (asymmetric loss or gain). Lastly, we predicted that bacterial communities would differentiate primarily by leaf senescence and that this difference would be driven by richness differences in bacterial communities rather than by bacterial turnover per se. As environmental variables are often strongly correlated, we utilized machine learning, specifically random forest methodology, to model these variables against bacterial diversity.

iii. Methods

Aerial (*i.e.*, non-submerged) leaves from standing *T. latifolia* plants were collected from the University of Mississippi Field Station (UMFS; 34.4237 N, 89.3859 W) pond 80 using sterile sampling procedures. Leaves selected did not have visible signs of herbivory or disease. Sampling began on April 29th, 2015 and ended on April 25th, 2016. Collection of *T. latifolia* leaves occurred at regular time intervals (semi-monthly) as well as before and after rain events forecast to produce > 2.5 cm of rain. For forecasted rain events, samples were collected 1 d before, 1 d after, 3 d after, and 5 d after. The 1628 m^2 pond was mapped into nine subsections (~ 180 m^2 each) and on regularly scheduled sampling days, material was removed from a single plant in four random subsections. On days when rain was forecasted, 1 m^2 greenhouse

polyethylene film rain canopies (152 µm thick) were erected over *T. latifolia* plants in four randomly chosen subsections (selection was independent between covered and non-covered samples, allowing sampling to occur twice in one subsection) which were sampled along with non-covered subsections (*i.e.,* eight total samples collected; Figure 6). A total of 236 samples were collected over the yearlong sampling period.

Following collection, leaf samples were immediately taken to the laboratory and the phyllosphere community removed by scrubbing in 6 mL of sterile 1 mM sodium bicarbonate buffer. The buffer suspension was centrifuged (7000 rcf for 2 min) and DNA extracted from the resulting pellet using MoBio PowerSoil DNA extraction kits (MoBio Laboratories, Carlsbad, CA) following standard protocols. The V4 region (253 base pairs) of the bacterial 16S rRNA gene was amplified with barcoded targeted primers (515F forward and 806R reverse) (Kozich *et al.,* 2013). Amplified fragments were purified and concentrations standardized using SequalPrep Normalization Plates (Life Technologies, Grand Island, NY), pooled, and paired-end sequenced on the Illumina MiSeq platform at the University of Mississippi Medical Center Molecular and Genomics Core Facility. After microbial community removal, leaves were frozen (-20°C) until imaged as jpeg files using the digital camera of a Samsung Galaxy S4 at 2048 x 1152 resolution from a fixed height. Total leaf area (cm^2) was quantified using ImageJ 1.51 (Rasband, 1997) while the percent of green pixels, and thus green and brown surface area, was calculated using a custom R script (supplementary information 1).

Figure 6. Demonstration of Experimental Rain Covers *in situ*
Clear greenhouse film placed over *Typha latifolia* plants to cover them from rain.

Raw 16S rRNA gene sequence data (FASTQ) were processed using the mothur bioinformatics pipeline following standard protocols (Schloss *et al.,* 2009; Jackson *et al.,* 2015) to remove erroneous sequences from downstream analyses. Remaining sequences were aligned against the SILVA database (version 128) of bacterial sequences (Pruesse *et al.,* 2007), screened to remove Eukarya and Archaea, and classified to operational taxonomic units (OTUs) defined by 97% sequence similarity using the Greengenes taxonomic database (DeSantis *et al.,* 2006; Schloss *et al.*, 2011). The resulting 9,217,422 sequences were classified to 32,546 OTUs. Of these, 9536 OTUs were represented only once across the dataset and were removed leaving 23,010 bacterial OTUs for subsequent analyses.

To account for uneven sequencing depth between samples, samples were standardized (repeated subsampling of 1000 iterations) to the lowest sequence-count sample. However, coverage estimates showed that this fraction of the dataset was not sufficient for calculation of alpha and beta-diversity measures. As such, 31 samples with low sequence counts (< 3000 sequences) were removed to improve subsampling coverage and alpha-diversity measures in the remaining 205 samples. Averaged across subsampling iterations, alpha-diversity was summarized by the Simpson evenness measure (D_{simp}) and Chao estimated richness (S_{chao}), while beta-diversity was summarized using abundance-weighted Bray-Curtis (β_{bc}) and Jaccard (β_j) dissimilarities to measure changes in bacterial abundance and incidence, respectively.

Precipitation data were accessed from the NOAA land-based station located at the UMFS (Abbeville, MS; Station ID: GHCND:US1MSLY0004) from April 1st 2015 through April 30th 2016. Radiation and atmospheric data, including direct and diffuse solar radiation, UV-B, infrared (IR), photosynthetically active radiation (PAR), temperature, relative humidity, and

wind speed – were obtained from the NOAA surface radiation network station in Goodwin Creek, MS (34.2547° N, 89.8729° W; 48 km from the UMFS) for 2015 and 2016. Radiation data (recorded every minute) were subject to quality filtering (any measurement flagged as poor quality was coded as not available, or NA) and combined from separate daily files into one dataset. Missing climatic data from some variables spanned multiple days (up to 29 d) and were imputed from non-missing data using loess trendlines with the glm function in R version 3.4.1.

To understand the extent that prior climatic conditions play in driving bacterial community patterns, means were calculated for all climate variables at 1, 3, 5, 7, and 14-d intervals leading into each sampling day. Net infrared and total net radiation were removed due to poor data quality, and to retain some information about hourly fluctuations, radiation variables were aggregated with daily maximum and minimum values as well as daily means. Along with total precipitation, both rain incidence (rain or no rain) and heavy rain incidence (> 2.5 cm) variables were coded as either 0 or 1 indicating either the absence or presence of rain (or heavy rain) on a given day. From these, the number of days since the last rain event (or heavy rain event) was calculated for each date. Both sums and means were then generated for precipitation variables across the window intervals as above. Samples that were experimentally covered from the rain had all precipitation values set to 0 before these summaries were calculated, and whether or not a sample was covered was included as a factor in statistical analyses.

Non-bioinformatic statistical analyses were conducted in R. To visualize patterns in novel species accumulation across the data set over time, as well as with respect to leaf size, species accumulation curves were constructed. Patterns in these curves may provide insight into how seasonal transitions and leaf size contribute to regional species richness as well as to the conditions that promote novel species accumulation in the phyllosphere. To understand the

extent that leaf size was a factor in the rate of novel species accumulation in the regional species pool, observed curves were created by sorting samples by leaf area and plotted against a simulated null curve representing average per leaf species accumulation across all samples by resampled shuffling of area and diversity measures (9,999 iterations). To test the effect of leaf size (habitat area) on S_{obs}, the area under the curve (AUC) of observed accumulation curves was compared to the AUC of each simulated null curve. Leaf sizes were standardized after sorting so that differences in AUC would be only due to changes in species accumulation.

To test the effect of rain on alpha-diversity in our experimental setting, we fit quadratic regression models of bacterial richness and evenness (both log-transformed) against the number of days since the last rain event (from -1 to 5 d) as well as the interaction between rain and covering treatment (covered or uncovered). To test the effect of rain on the (subsample standardized) abundance of individual OTUs, the same quadratic model was repeatedly run using OTU abundances as the response, and significance assigned to all OTUs whose *p*-values fell within the 0.05 threshold when cumulatively added (ranked by lowest *p*-value). The effect of covering on species accumulation following rain events was tested by setting species richness to zero on the day preceding rain, calculating the number of novel species gained until day five, and generating a regression model with an interaction term between time and covering. Relating alpha-diversity patterns to all climatic variables was accomplished through random forests of S_{chao} and D_{simp} each against climatic data (precipitation, radiation, temperature, and wind), leaf data (total leaf area, green leaf area, brown leaf area, and percent green leaf tissue), and covering. Briefly, random forest are built from multiple individual regression trees which recursively partition samples based on logical assignments of predictor variables and whose performance is measured by the mean squared error between observed values of the response with the means of

groups created by those partitions (Breiman, 1984). To limit the influence of correlations between variables and to attain better predictive capacity, many regression tree models are created, each containing a small subset of the predictor variables available, and individual functions are averaged to generate a single ensemble model (Breiman, 2001). To aid in interpretability and reduce noise, four random forest models were computed, two of richness and evenness against the full 162 variable dataset and two against a reduced dimension dataset of eight variables. To perform dimensionality reduction, permutations of precipitation, radiation, temperature, and wind were each subjected to principal component analysis (PCA). From all PCAs, single eigenvectors were enough to explain most variation in all groups of variables. Model fitting was implemented using the randomForest package for R and train/test validation through caret (Liaw & Wiener, 2002; Kuhn *et al.*, 2017). To minimize correlations between variables, individual trees (500 per test) were limited to a subset of 13 parameters at a time. Random forests tested on the reduced dataset of eight variables utilized only three parameters per tree. Train test validation was performed on an initial, but unfixed (*i.e.*, increasing each test) window of 16 training samples and progressing chronologically by four samples yielding 114 tests total.

Beta-diversity patterns were visualized using non-metric multidimensional scaling (NMDS) on abundance-based Bray-Curtis, Jaccard, and Raup-Crick dissimilarity measures. The Raup-Crick measure explicitly quantifies changes beta-diversity accounting for changes in alpha-diversity (Chase *et al*. 2011). Changes in composition observable by Bray-Curtis and Jaccard ordinations that fail to show in Raup-Crick ordinations are thus likely to be driven by differences in alpha-diversity between samples. The association of individual bacterial OTUs with seasonal patterns was assessed using the indicator function in mothur, based on Dufrêne and Legendre's

metric (Dufrêne & Legendre, 1997). To further understand the contribution of species turnover and differences in alpha-diversity towards community composition, each beta-diversity measure was decomposed into turnover and richness difference fractions using the Podani method (Podani & Schmera, 2011) provided by the beta.div.comp function in adespatial. The local contribution of each sample to beta-diversity (LCBD, including total beta-diversity as well as turnover and richness components) was mapped across time using the LCBD.comp function and the relationship between climate and LCBD explored using the same random forest methodology as employed for alpha-diversity measures.

iv. Results

Contrary to our predictions, we did not observe a significant initial loss and regain of bacterial diversity in the phyllosphere following rain. This was true for both richness and evenness measures (p_{chao} = 0.94, p_{simp} = 0.18), and there was no a significant interaction between leaf covering and quadratic or linear trends in bacterial diversity (p_{chao} = 0.47, p_{simp} = 0.46; Figure 7). Similarly, we observed no difference in novel species accumulation following rain events between covered and uncovered plants (p = 0.585; Figure 7). This pattern was consistent at the level of individual bacterial taxa where we observed no effect of leaf covering on either quadratic or linear responses of OTU frequencies to rain.

Aligning with our initial prediction, we found that phyllosphere bacterial evenness decreased following senescence of *T. latifolia* leaves (as represented by leaf green:brown ratio); however, this pattern was not as clear for bacterial richness (Figure 8). As shown by beta-diversity components over time, community composition was more dynamic in the spring and

summer, and more consistent following senescence (Figure 9). There was a slight increase in species accumulation during the shift from living to senescent leaves (Figure 10), but partitioned beta-diversity components showed that it was driven by differences in species richness between samples (Figure 9). This confirms our original prediction that changes in species diversity would characterize the phyllosphere microbiome shift during leaf senescence. Regional, or gamma, species richness accumulated more slowly than average when samples were ordered by leaf size from small to large, producing a shallower curve and lower AUC value (Figure 10). Species accumulation curves sorted on total leaf area failed to separate above the simulated null curve when sorted high to low ($p = 0.71$) and below the curve when sorted low to high ($p = 0.59$). However, a clearer pattern emerged when considering only green (living) leaf area; in this case, significant separation from the null curve was achieved in both cases (above $p = 0.04$; below $p < 0.001$).

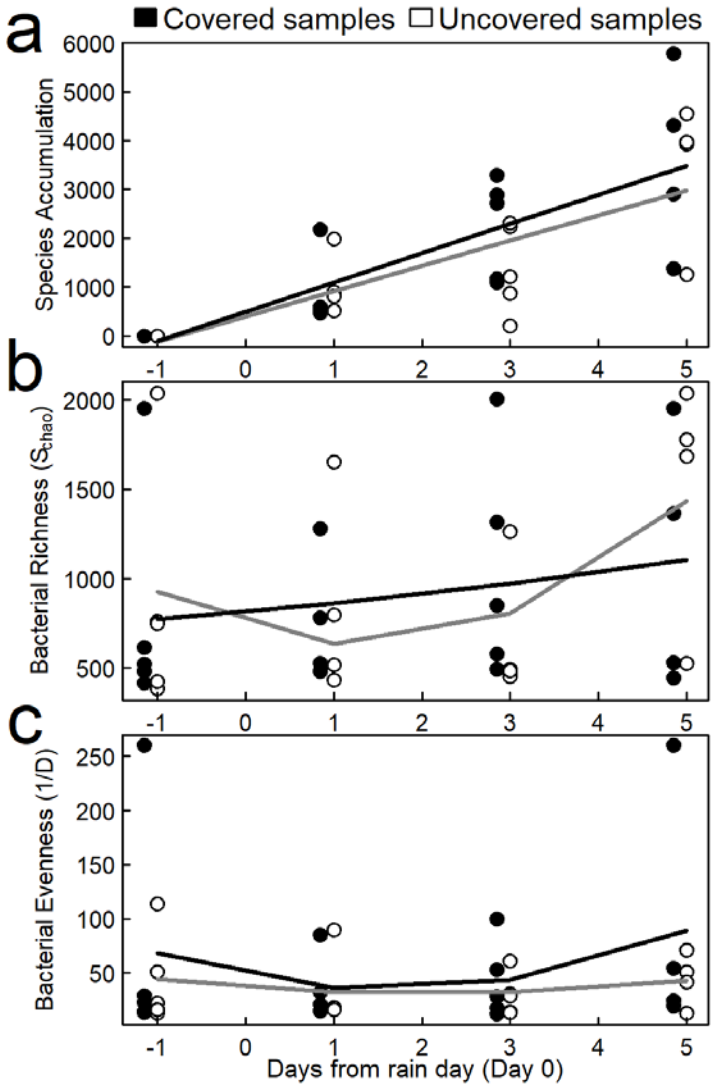

Figure 7. Effect of Rain on *Typha latifolia* Phyllosphere Diversity

Bacterial community diversity in the phyllosphere of *T. latifolia* leaves in response to rain. Points represent means of daily subsamples (four per day for each category) across five separate rain events superimposed against predicted values (lines) based on linear and quadratic models. Black elements represent data from leaves covered from rain, gray/white elements represent data from uncovered samples. (a) Bacterial species accumulation of covered and uncovered leaves. (b) Estimated species richness (S_{chao}) of covered and uncovered leaves. (c) Community evenness (inverse of Simpson's metric) of covered and uncovered leaves.

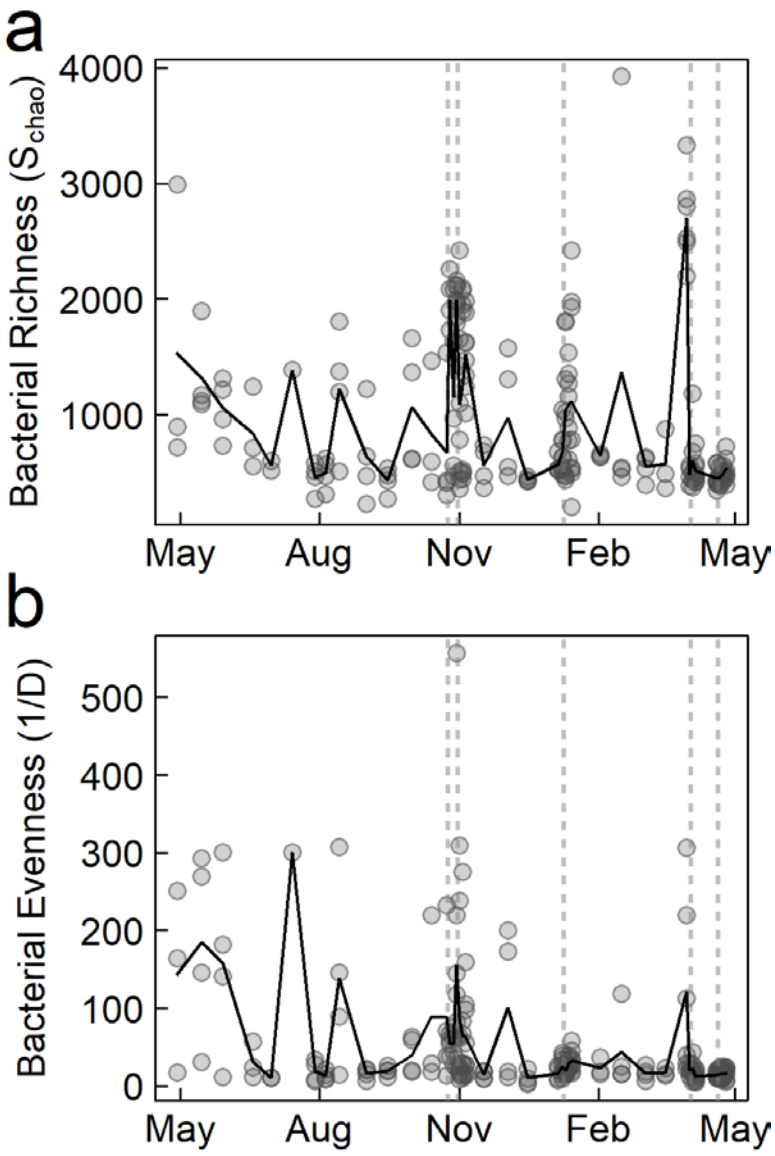

Figure 8. Relationship between Diversity and Rain on the *Typha latifolia* Phyllosphere
Changes in bacterial alpha-diversity in the phyllosphere of *T. latifolia* leaves from April 29, 2015 to April 25, 2016. Points represent diversity measures from individual samples, lines represent average diversity measures at each day of sampling while vertical hashed bars represent measured rain events where sampling frequency was increased to two-day intervals. (a) Estimated species richness (S_{chao}) over time, showing a slight decrease following summer months. (b) Community evenness (inverse of Simpson's metric) over time.

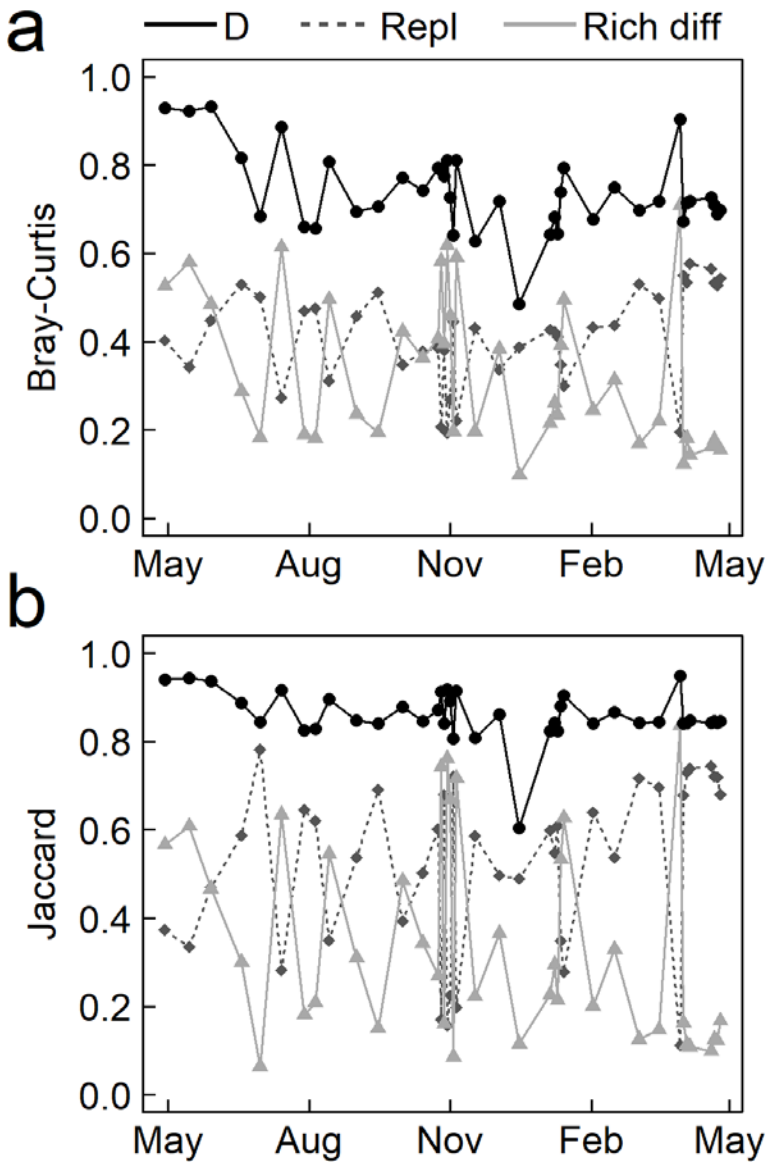

Figure 9. Changes in Beta-diversity over Time in the *Typha latifolia* Phyllosphere
Local contributions of samples to bacterial community beta-diversity, averaged by day, in the phyllosphere of *T. latifolia* leaves from April 29, 2015 to April 25, 2016. Line shades represent contributions to total beta-diversity (D, solid black), replacement-component (Repl, dotted gray), or richness-difference-component (Rich diff, solid gray). Across both measures, the replacement component remained higher while a slight decrease in richness differences over the course of the study was observed. (a) Bray-Curtis beta-diversity with a decrease in overall beta-diversity across time. (b) Jaccard beta-diversity.

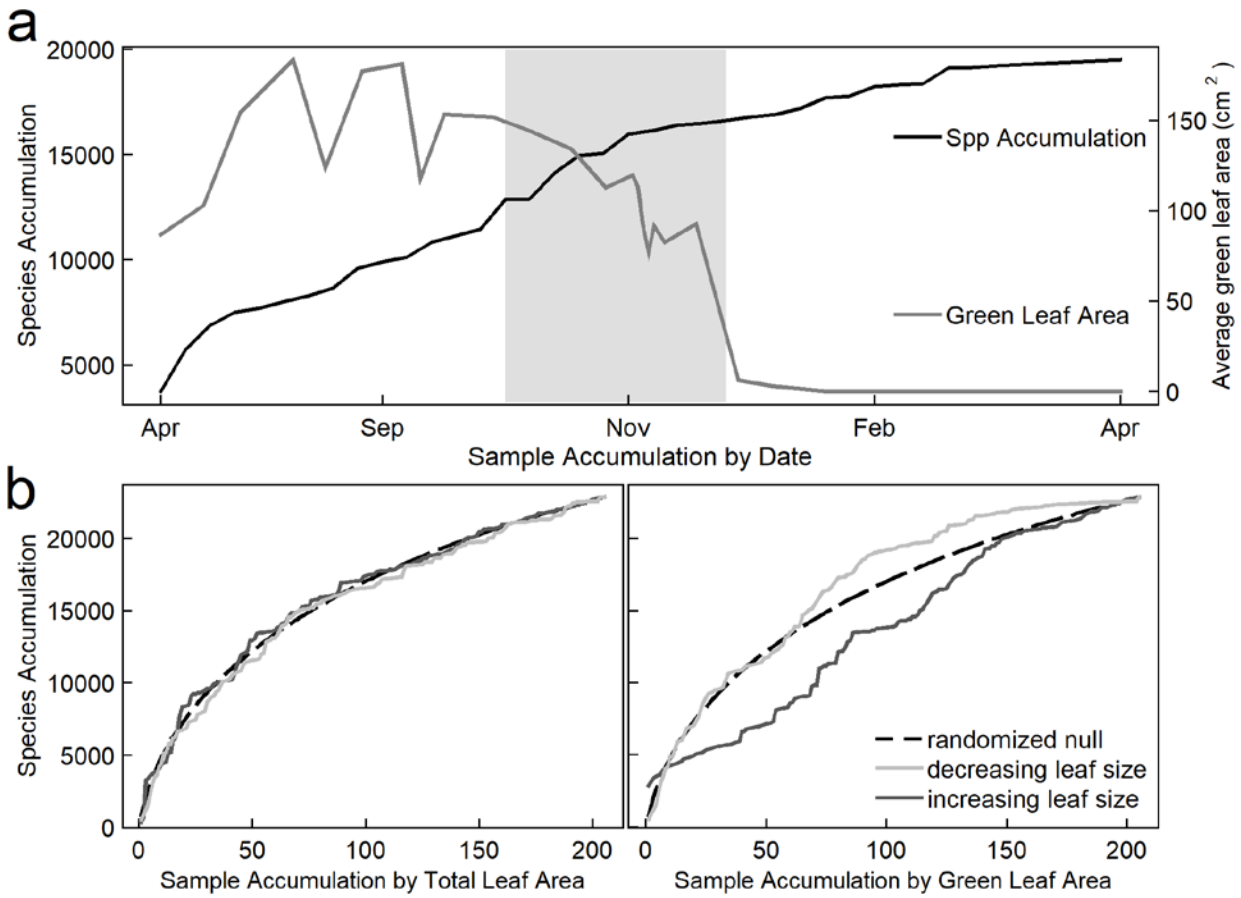

Figure 10. Temporal and Spatial Species Accumulation Curves in the *Typha latifolia* Phyllosphere

Regional species gain with accumulation of individual samples for bacterial communities in the phyllosphere of *T. latifolia* leaves. Curve shape is determined by the order of samples during accumulation. (a) Accumulation of species over time from April 29, 2015 to April 25, 2016 with each date equidistant from its neighbors, superimposed against a period of leaf senescence (highlighted with gray box). (b) Accumulation of species when leaves are sorted from small-to-large (red lines) and from large-to-small (blue lines) both compared to randomized null hypothesis curves. Significant separation from the null curve was not achieved when sorting samples by total leaf area (left) but was when sorting by green leaf area only (right) showing slower species accumulation with small-to-large sorted samples.

Climatic variables were consistently the more informative predictors of phyllosphere bacterial richness while *T. latifolia* leaf traits were more informative predictors of bacterial evenness. Random forest models were able to predict 40.4% of the variation in S_{chao} and 36.8% of the variation in Simpson evenness against the full set of individual variables, and 40.7% and 35.6% on the composite variables created through PCA. There was some disagreement on the most important variables between random forests computed on individual variables and those using composite variables. When computed against individual variables, variation in phyllosphere bacterial richness most consistently related to precipitation and wind speed, while wind was the least explanatory climatic variable in the model built with composite variables (Figure 11). Temperature and radiation were the most consistently important variables in the full model of bacterial evenness, while leaf variables dominated the composite model. In both cases, precipitation was more important to bacterial richness than evenness and whether or not a sample was covered from rain provided minimal explanatory power. Examining the role of rain on bacterial diversity more closely, partial plots returned from random forest models showed that precipitation had a negative relationship with both bacterial diversity and evenness (Figures 12, 13). Following our initial prediction of the importance of senescence, *T. latifolia* green leaf area was consistently and positively associated with higher bacterial evenness, but this relationship was not consistent for bacterial richness.

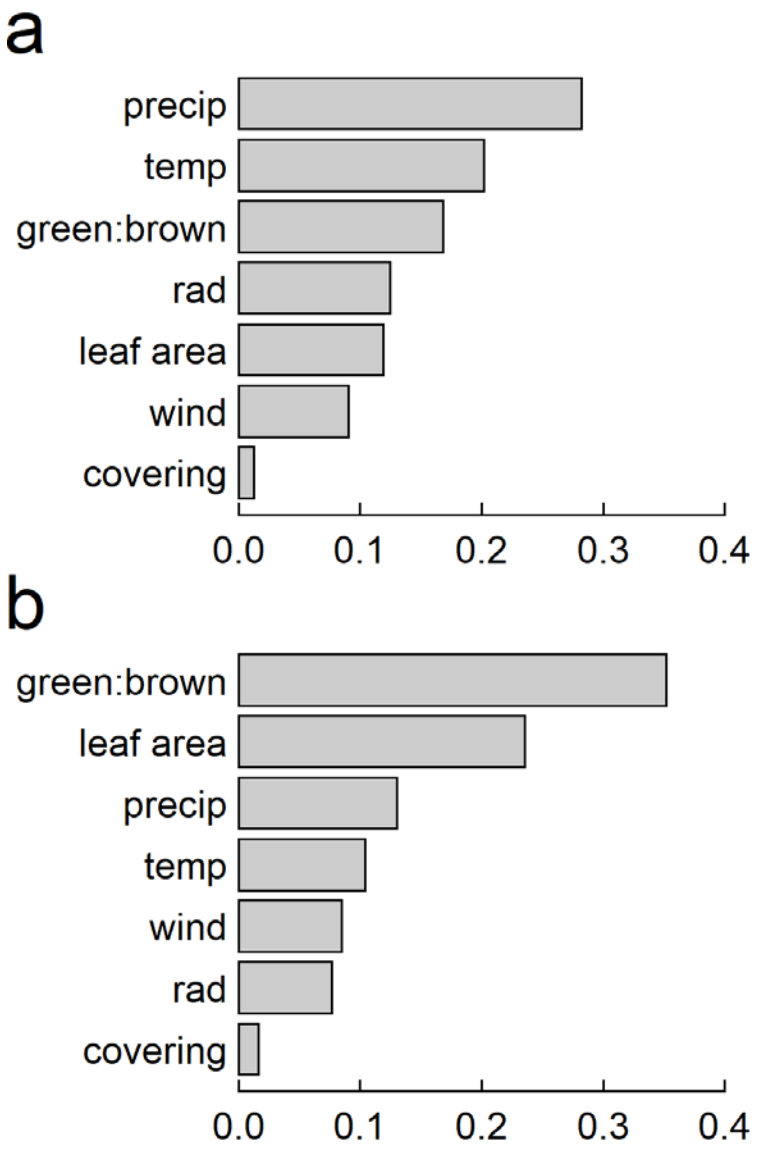

Figure 11. Importance of Climatic Variables in Predicting Phyllosphere Diversity on *Typha latifolia*

Residual sum of squares (RSS) in the response variable explained by each predictor variable, averaged across each random forest regression model and standardized to unit one, indicating their proportional importance in predicting (a) bacterial species richness (S_{chao}) and (b) Simpson evenness in the phyllosphere of *T. latifolia* leaves. Across both measures, leaf traits (the percent of green leaf tissue – green:brown – and total leaf area) are the most important followed by precipitation, temperature, wind, and solar radiation. Experimental covering of leaves during rain events provided minimal predictive power of bacterial diversity.

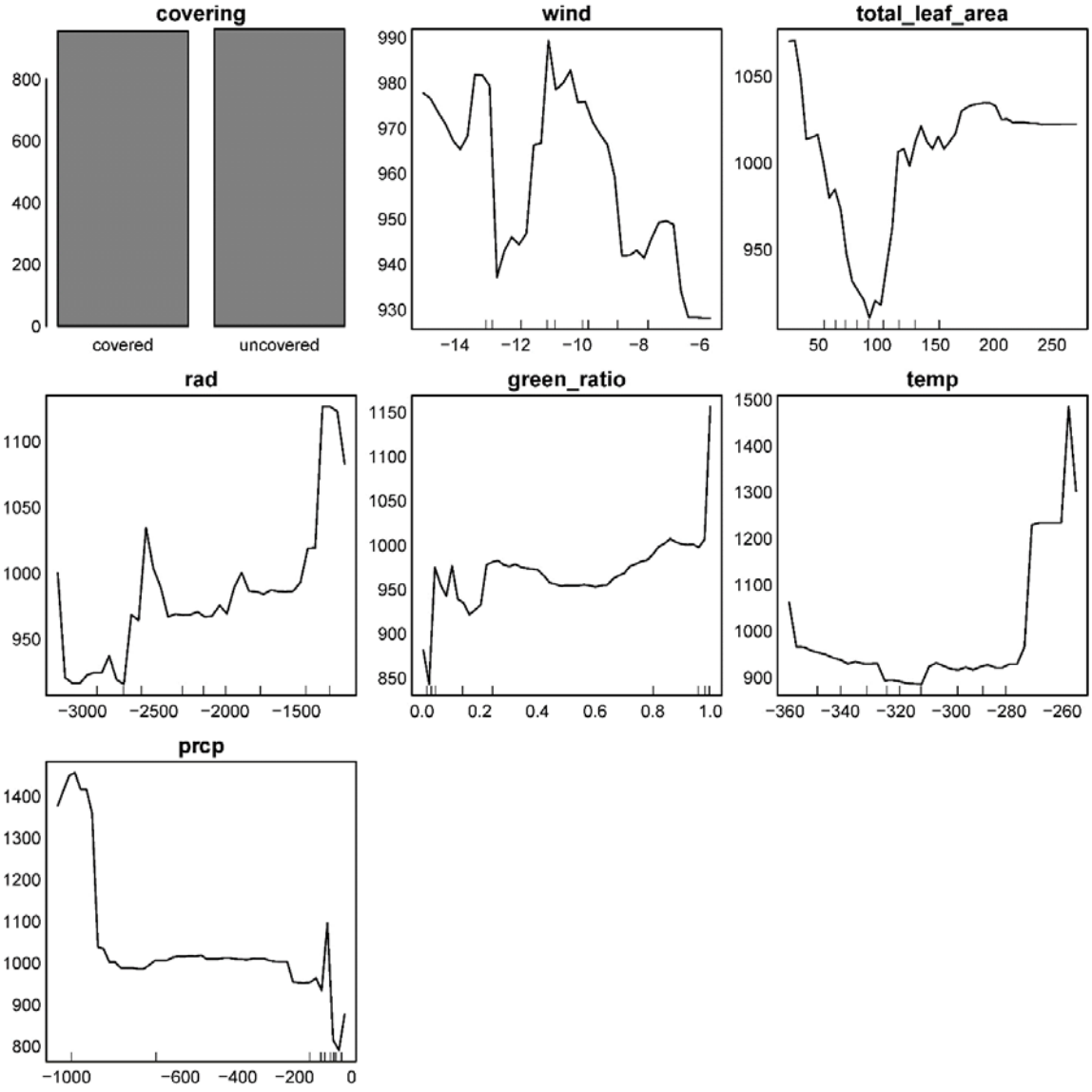

Figure 12. Partial Dependence Plots between Chao Richness and Climatic Variables by Random Forest Modeling

Plots of the marginal effect of each environmental predictor variable along the x-axes (variables created by dimensionality reduction through PCA and are unscaled and un-centered; covering = experimental greenhouse covers; rad = radiation, wind = windspeed, temp = temperature and relative humidity, prcp = precipitation) and raw measures (total_leaf_area = measured leaf area in cm^2, green_ratio = percent of green leaf tissue) on bacterial richness (S_{chao}) of the *Typha latifolia* phyllosphere generated from random forest regression. Lines represent predicted values perceived by the random forest model, indicating trends in S_{chao} (along the y-axes) as a function of each predictor variable, after accounting for the other predictor variables. Wind, and precipitation were generally negatively associated with S_{chao} while radiation, and green:brown leaf color showed positive trends with S_{chao}.

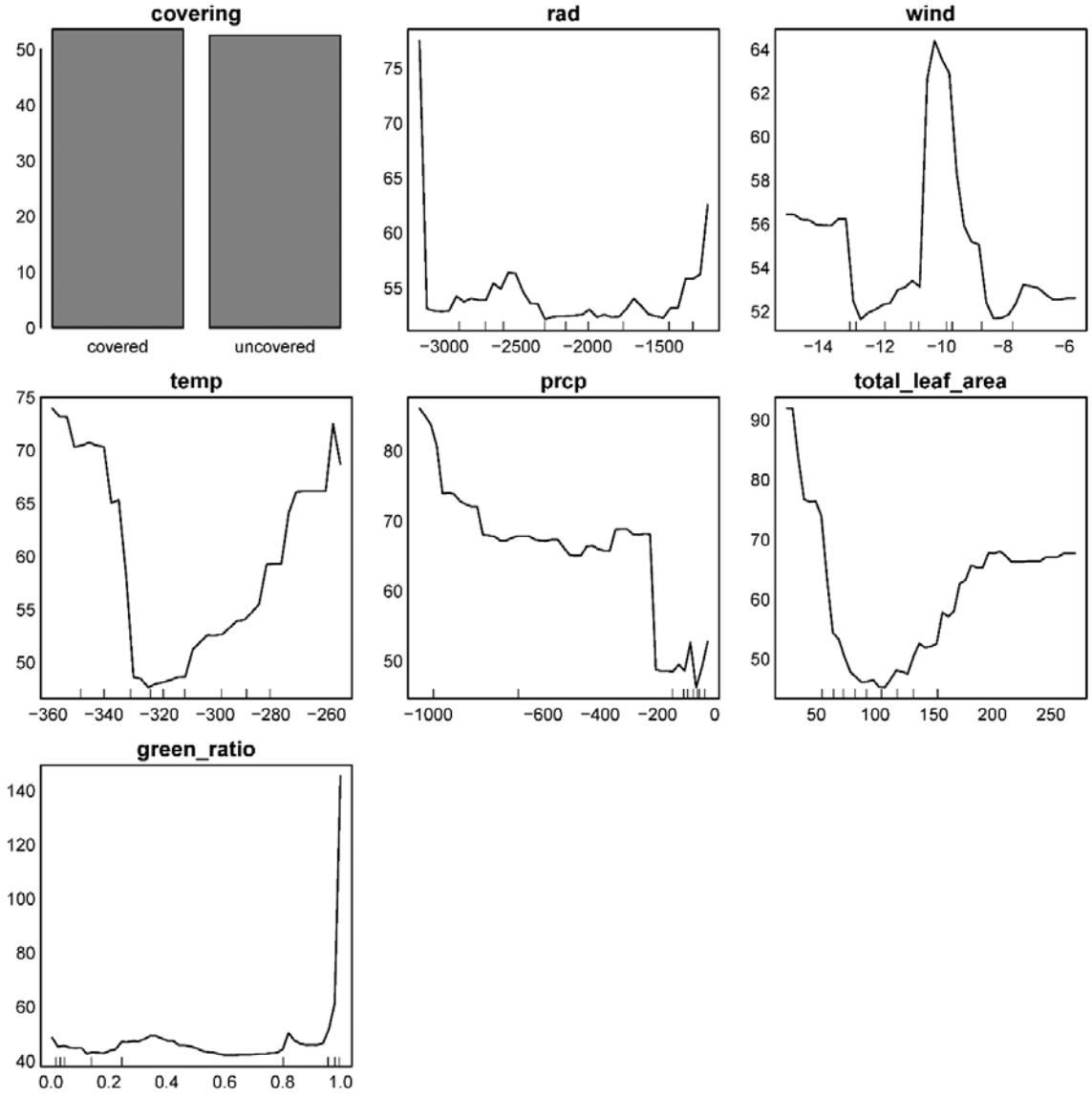

Figure 13. Partial Dependence Plots between Simpson Evenness and Climatic Variables by Random Forest Modeling

Plots of the marginal effects of composite environmental predictor variables (values as in Figure 12; along the x-axes) on bacterial community evenness (D_{simp} along the y-axes) of the *T. latifolia* phyllosphere generated from random forest regression. Precipitation was generally negatively associated with D_{simp} while temperature and total leaf area demonstrate U-shaped curves with low intermediate values of the predictor variable.

Random forests computed on LCBD scores, were generally consistent with those computed on alpha-diversity metrics, though individual beta-diversity components (replacement and richness difference) did not always agree with total beta-diversity. Leaf color (green, brown) was the most important variable across all components of Bray-Curtis diversity as well as total Jaccard (Figure 14). Temperature and precipitation were the next most important variables in all measures, with the exception that precipitation was the most important variable determining the Jaccard measures corresponding to species replacement and richness differences (Figure 14). Wind and solar radiation were less important, relatively, across both measures and their components. Whether or not leaves were covered was minimally important to bacterial composition. Multivariate ordinations demonstrated significant clustering when grouped by senescence using both Bray-Curtis and Jaccard. However, this pattern disappeared when community composition was visualized using Raup-Crick dissimilarities which accounts for differences in alpha-diversity between samples and suggests that compositional changes in beta-diversity were driven alpha-diversity differences.

Bacterial OTUs significantly associated with early-seasonal (*i.e.,* living) and late-seasonal (*i.e.,* senesced) leaves both grouped predominantly into the Proteobacteria (44% and 48% respectively). A larger number of the more frequent sequences obtained from senescent leaves were from the Actinobacteria and Bacteroidetes (8% and 28% from senescent against 0% and 18% from living leaves), while living leaves saw more representation from the Acidobacteria and Verrucomicrobia (12% and 8% from living leaves against 2% for both in senescent leaves). Specific OTUs associated with living leaves more so than senescent leaves included *Agrobacterium vitis*, *Acinetobacter guillouiae*, *Clostridium butyricum*, and an unidentified *Planctomyces* sp ($p < 0.001$ in all cases). In contrast, senesced leaves associated with OTUs that

were generally not classified to the species level but included the genera *Sphingomonas*, *Roseomonas*, *Hymenobacter*, *Rhodoplanes*, and *Spirosima*.

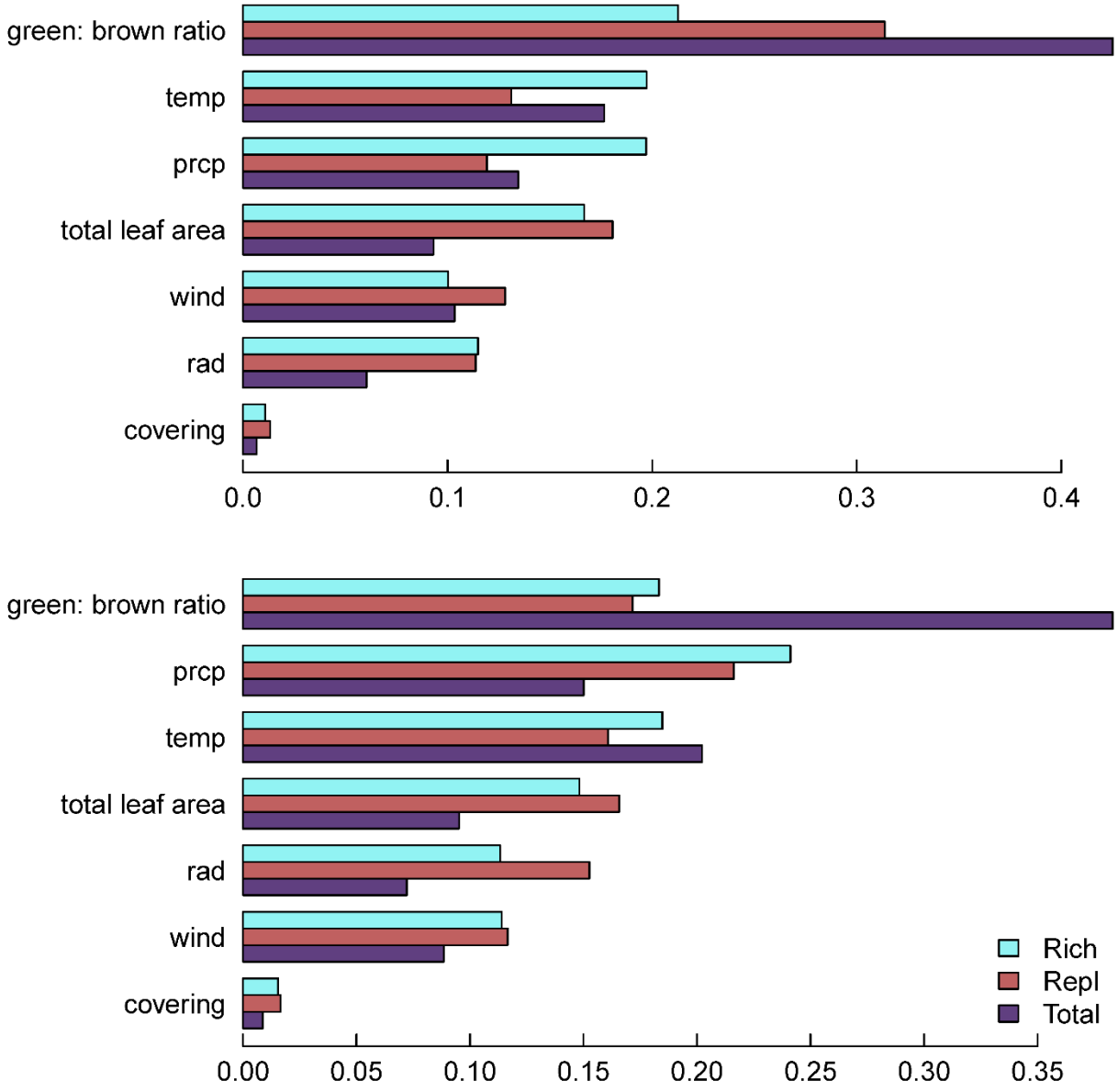

Figure 14. Contribution of Climate to Phyllosphere Community Composition on *Typha latifolia*

Residual sum of squares (RSS) in the response variable explained by each predictor variable in random forest regression of local contributions to beta-diversity (LCBD) using (top) Bray-Curtis and (bottom) Jaccard dissimilarity measures. Dissimilarity measures are further broken down into richness difference (Rich), replacement (Repl; also called species turnover), and total dissimilarity components (Total). Across both measures, the percentage of green leaf tissue (green:brown ratio) is the most important followed by precipitation and temperature. Experimental covering of leaves during rain events provided minimal predictive power of LCBD.

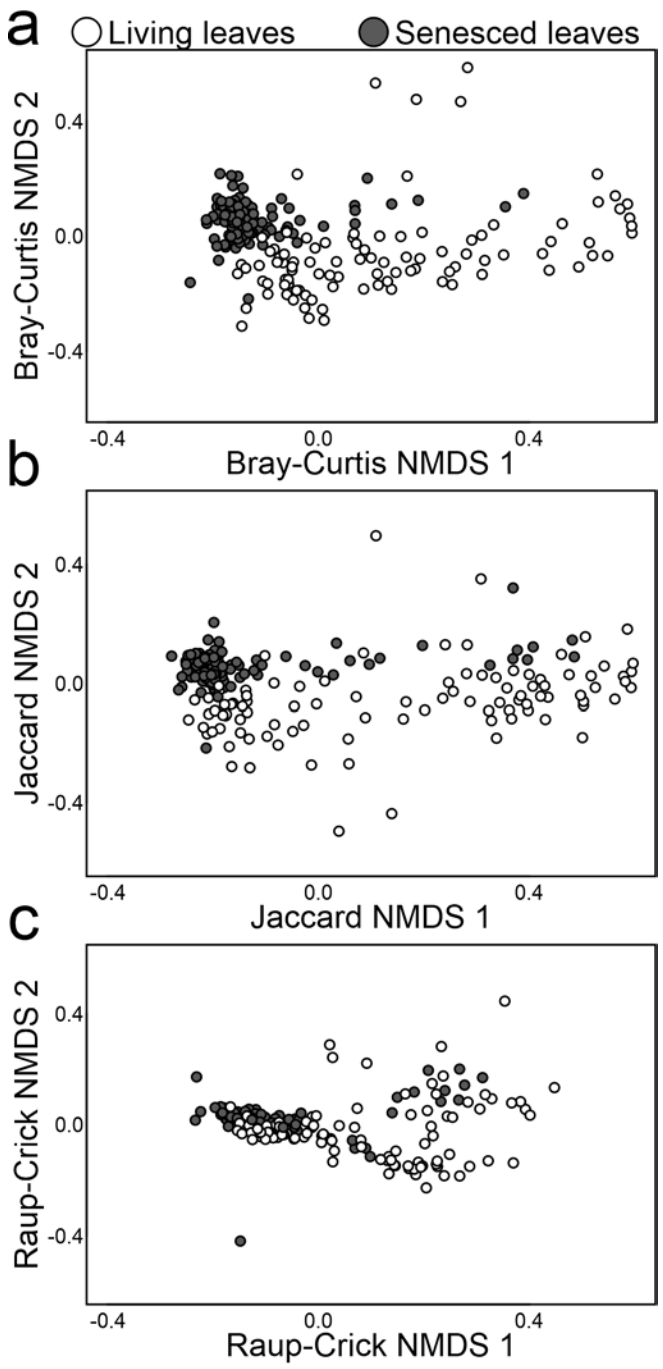

Figure 15. Multivariate Ordinations of *Typha latifolia* Community Composition
Multivariate non-metric multidimensional scaling (NMDS) ordinations of bacterial composition in the phyllosphere of *T. latifolia* leaves from April 29, 2015 to April 25, 2016. Open points represent leaves with at least some green tissue while filled points represent fully senescent leaves (dates after December 15, 2015). (a) Bray-Curtis abundance-based dissimilarities (stress = 0.15). (b) Jaccard presence-absence dissimilarities (stress = 0.11). (c) Raup-Crick presence-absence dissimilarities (stress = 0.06).

v. Discussion

Taken together, our results support previous conclusions of strong seasonal signals in the phyllosphere microbiome (Thompson *et al.,* 1993; Redford & Fierer, 2009; Jackson & Denney, 2011; Williams *et al.,* 2013; Copeland *et al.,* 2015). However, more so than previous studies, we present insight into the ecological mechanics behind the patterns observed. Where our methods differ from the existing body of phyllosphere literature is the implementation of more robust machine learning regression models in combination with the explicit examination of beta-diversity components. Using these approaches we found that bacterial community richness corresponded more to environmental parameters than did community evenness. Further, we supplemented random forest results with an examination of beta-diversity components to show that the period of leaf senescence led to a slight gain in bacterial richness that then drove compositional changes in the phyllosphere community. This gain was short-lived, and afterwards, the component of beta-diversity driven by richness differences remained consistently lower. Consistent with these findings, multivariate ordinations of bacterial community composition demonstrated that after accounting for differences in alpha-diversity using Raup-Crick dissimilarities (Chase *et al.,* 2011), clear grouping based on senescence largely disappeared, indicating that differences in alpha-diversity were important in driving distinction between bacterial communities on living and senesced leaves.

Important taxa associated with living and senescent leaves were generally those found in free-living environments. Prominent OTUs indicative of senescent leaves were often aerobic chemoheterotrophs, broadly categorized as generalist decomposers. Bacterial taxa in the phyllosphere may respond purely to changes in the living vs. senescent leaf surface over time,

but seasonal patterns also appear in the phyllosphere of plants that don't show seasonal senescence (Jackson & Denney, 2011), suggesting that there is at least some climatic influence.

Previous discussions of leaf surface area and microbial diversity have revolved around the importance of competition among organisms on the leaf surface. According to the assumptions of island biogeography, a larger, heterogeneous island is necessary to support more species (MacArthur *et al.*, 1967), and the number of species should increase as area sampled increases (Rosenzweig, 1995; Horner-Devine *et al.*, 2004). Lack of a strong species-area relationship for fungal communities in the phyllosphere has been attributed to the fact that interspecific fungal associations rarely occur across the leaf, and thus diversity is not affected by interspecific competition and not dependent upon leaf size (Kinkel, 1997). Although no similar study has been conducted using bacteria, microscopy has consistently revealed that the majority of phyllosphere bacteria exist in aggregates concentrated in localized areas on the leaf landscape and that aggregates on this landscape may be monospecific or multi-species (Monier, 2006). The nature of bacterial reproduction and species-specific colonization patterns suggest that the majority of bacteria likely interact intraspecifically (Peredo & Simmons, 2018). It is possible that this intraspecific competition introduces strong negative feedback, minimizing interspecific competition, and may be partially responsible for the high diversity of bacteria found in the phyllosphere.

Species-area relationships in the phyllosphere are detectable using modern next-generation sequencing methods but are likely dependent on the types of microorganism studied (bacteria or fungi) and the physical state of the leaf (living or senesced). We tested for these relationships by comparing species-accumulation curves from our data with those generated from a completely stochastic model and found that bacterial species diversity on senescent *T.*

latifolia leaves was invariant to leaf size, although some signal was observable in green leaves. As such, total leaf area was not important in understanding the species-area relationship while green leaf area was. This observation suggests that neutral drivers (*i.e.*, random immigration/emigration) are stronger determinants of bacterial assembly than environmental variation (*e.g.*, leaf condition or climate) after leaf senescence. We did not measure fungal diversity, but given that fungal dynamics have shown strong seasonal trends (Rosenzweig, 1995), we suspect that the existence of a fungal species-area pattern may also be seasonally dependent.

With regard to the effect of rain, we found that experimentally covering plants did not change the accumulation of new bacterial species to the *T. latifolia* phyllosphere or the level of local diversity therein. As rain was an important predictor variable in our random forest models of bacterial diversity, experimental evidence supports our conclusion that seasonal patterns collectively exert a stronger effect on bacterial communities than specific weather events. Previous work has demonstrated a surge in abundance of some microbial taxa following rain (Hirano *et al.*, 1996), but this phenomenon does not appear to lead to any change in diversity at the level of the whole community. Another seasonal phyllosphere study drew similar conclusions to ours with respect to rain (Copeland *et al.,* 2015), with strong seasonal trends and a high initial variability in community composition followed by convergence. While the possibility of rain aiding the succession process was highlighted, there were no other clear consequences of rain on either bacterial community diversity or composition (Copeland *et al.,* 2015). Factors that could limit the impact of rain on the phyllosphere microbial community include the protected nature of bacterial aggregates and biofilms, and structural flexibility in the plant leaf and stalk that minimizes the force exerted from raindrops. As the latter traits may vary among plant species,

the effect of rain on bacterial diversity in the phyllosphere could be plant species specific (Huber *et al.,* 1997), and generalizations of the response of the plant microbiome to rain may be difficult to make.

The failure of the species-area curve to differentiate away from the simulated null curve on senesced *T. latifolia* leaves suggests that phyllosphere bacterial diversity follows stochastic and neutral processes after leaf senescence, while prior to this shift diversity may be influenced by deterministic forces. More importantly, the separation of isolated weather patterns from broad-scale seasonal trends is an important distinction that is often overlooked in surveys of phyllosphere communities. We conclude that bacterial diversity in the phyllosphere is largely unaffected by short-term weather patterns but is particularly sensitive to changes in the leaf surface that occur during seasonal senescence.

CHAPTER III

CONTRIBUTION OF CANOPY STRUCTURE AND RAIN TO PATTERNS IN PHYLLOSPHERE BACTERIAL COMMUNITY STRUCTURE

i. Abstract

The effect of rain on the phyllosphere community has not been extensively explored, especially in the context of spatial variation on the impact of rain throughout the tree canopy. We characterized the response of the phyllosphere bacterial community of the Southern Magnolia (*Magnolia grandiflora*) to rain in the upper, lower, and interior portions of the canopy. We hypothesized that: (1) rain would lead to an initial decrease in phyllosphere bacterial diversity, followed by an increase in diversity on subsequent days, but that this effect would be minimized in the lower and interior portion of the canopy, and that (2) community dispersion of phyllosphere microorganisms would be lower following rain, and similarly contingent on canopy position. We used targeted next-generation sequencing of the V4 region of the bacterial 16S rRNA gene to characterize phyllosphere community composition. In contrast to our predictions, rain did not have any influence on bacterial phyllosphere diversity or on the compositional similarity of phyllosphere communities. Rather, vertical position in the tree canopy and trunk proximity had the strongest influence on phyllosphere diversity and community composition. While rain may be important to the growth and abundance of certain phyllosphere taxa, it does

not appear to constitute a disturbance to the collective phyllosphere community and canopy position exerts a stronger influence on the tree phyllosphere.

ii. Introduction and Objectives

The diversity and composition of the plant microbiome is a critical determinant in plant survival and success in the face of challenging conditions (Stone *et al.*, 2018). In the aerial portion of the plant microbiome, the phyllosphere, a diverse community of leaf-associated microorganisms may protect against plant disease, and thus the ecology of this microhabitat is important to promoting plant health (Balint-Kurti *et al.*, 2010; Vorholt, 2012; Stone *et al.*, 2018). Rainfall can act as a mechanism of microbial movement in the plant phyllosphere. This has been observed for the spread and establishment of plant pathogens, for which rain can cause splash dispersal, where the impact of rain removes epiphytes from the leaf surface and the subsequent splash of water back into the air carries them off the leaf. Splash dispersal has been shown to spread microorganisms throughout a plant canopy at small scales (Lindemann & Upper, 1985; Fitt *et al.*, 1989; Cevallos-Cevallos *et al.*, 2012). Single-species experiments show that rain is important in colonization across the leaf, and models based on individual movement (agent-based models) implicate the disaggregation and spread of bacterial aggregates as the mechanism behind this phenomenon (van der Wal *et al.*, 2013).

Several phyllosphere microorganisms have been found to show large fluctuations in their populations following rain; *Pseudomonas* spp. (Mew & Kennedy, 1982; Hirano *et al.*, 1996), *Xanthomonas* spp. (Weller & Saettler, 1980; Duveiller, 1994; Pietrarelli *et al.*, 2006), and yeasts (Kinkel *et al.*, 1989). However, these studies were accomplished using culture-dependent

approaches and there has been little examination of the effects of rain using culture-independent approaches. The modern studies that have been conducted have yielded inconsistent results. At seasonal timescales, rain has been shown to have either no consequences or only a small effect on phyllosphere community diversity or composition (Copeland *et al.,* 2015; Laforest-Lapointe *et al.,* 2016). In contrast, a strong response in microbial composition and function was observed from phyllosphere communities of the resurrection fern *Polypodium polypodioides* following rewetting by Jackson *et al.* (2006). However it is likely these patterns resulted from physiological changes in the plant host and thus represented an indirect response to rain. No recent studies have examined how canopy structure might interact with the effect of rain on the phyllosphere microbial community. Quantification of bacterial abundance in throughfall and stemflow during rain shows downward movement of bacteria to the soil in rates that differ between tree species, suggesting that canopy structure plays some role in mediating microbial rain response (Bittar *et al.,* 2018). Because most phyllosphere studies have been conducted in agricultural settings where plants exist at equal height, the potential for tree canopies to mitigate the physical and ecological impact of rain on the phyllosphere community is largely unknown.

Because of the importance of the phyllosphere microbial community in plant health (Stone *et al.,* 2018), it is important to clarify the response of these organisms to rain. Further, there is a need to understand the role of spatial and temporal heterogeneity, independently and in concert, in driving microbial assembly patterns. Here, we explore the effect of rain as a disturbance on bacterial community diversity and composition throughout the canopy of the evergreen Southern Magnolia (*Magnolia grandiflora*). Specifically, we examine the differential response to rain events of the phyllosphere on leaves in the upper, lower, and interior portions of the canopy. Our previous research has shown a large proportion of phyllosphere community

membership on *M. grandiflora* occurs infrequently and at low abundance, and organizes neutrally with response towards observable environmental gradients (Stone & Jackson, 2016). With small population sizes, these constituents of the rare biosphere are more likely to be lost if rainfall acts to remove phyllosphere microorganisms from the leaf surface (Pietrarelli *et al.*, 2006). We hypothesized that rainfall would thus lead to an initial decrease in bacterial community diversity, followed by an increase in diversity due to recolonization of the leaf surface from the air and that this effect would be minimized in the lower and interior portion of the canopy where the physical impact of rainfall is lessened by leaves in the upper canopy. With a decrease in the rare and infrequent members of the community, as well as the potential for splash dispersal to move bacteria between leaves and homogenize community composition, rainfall may function to increase compositional similarity between bacterial communities in the phyllosphere. Thus, we hypothesized that community beta-diversity would be lower between leaves following rain, driven by an increase in the proportion of shared species, and that this pattern would be contingent on vertical canopy position.

iii. Methods

Leaves were collected from *M. grandiflora* trees on the University of Mississippi campus in Oxford, MS, USA. All trees were mature (9-12 m high) and located along Magnolia Drive (34.3660 N, 89.5413 W). Collection occurred from May to September 2017 with sampling focused on rain events forecast to produce > 2.5 cm of rain. Sampling occurred 1 d before the predicted rain event, 1 d after, and 3 d after. On each sampling date, ten leaves from each of three *M. grandiflora* trees were removed from the tree with a pole pruner, sterilizing the blades

between each use in 70% ethanol solution (total of 30 leaves per day, or 90 leaves per rain event). Four of the ten leaves from each tree were sampled at 90° intervals around the upper canopy (5-6 m high; facing north, south, east, and west); another four leaves were collected from the same intervals at the bottom of the canopy (2 m). The remaining two leaves were sampled within the interior of the canopy at locations proximal to the trunk (2 m high; facing east and west; 1-2 m from the trunk). Leaves selected did not have visible signs of herbivory or disease. Over the four-month sampling period, a total of 270 samples were collected from three separate rain events.

Following collection, leaf samples were immediately taken to the laboratory and the phyllosphere community removed by scrubbing in 6 mL of sterile 1 mM sodium bicarbonate buffer. The resulting suspension was centrifuged (7000 xg for 2 min) and DNA extracted from the pellet using MoBio PowerSoil DNA extraction kits (MoBio Laboratories, Carlsbad, CA) following standard protocols. The V4 region (253 base pairs) of the bacterial 16S rRNA gene was amplified with barcoded targeted primers (515F forward and 806R reverse; Kozich *et al.*, 2013). Of the 270 samples collected, 15 did not amplify and were removed from further processes. Amplified fragments of the remaining 255 samples were purified and concentrations standardized using SequalPrep Normalization Plates (Life Technologies, Grand Island, NY), pooled, and paired-end sequenced on the Illumina MiSeq platform at the University of Mississippi Medical Center Molecular and Genomics Core Facility. After phyllosphere community removal, leaves were frozen (-20°C) until imaged as jpeg files using a Sony Cybershot digital camera at 2592 x 1944 resolution from a fixed height (20 cm). Total leaf area (cm^2) was calculated using a custom R script and results compared to a validation set of 30 leaves measured by the traditional grid count method of tracing leaf edges on a grid of 1 cm^2

squares and counting squares under which the leaf area occupies (1 for full coverage; estimation of either 0.25, 0.5 or 0.75 for squares with partial coverage). Comparisons of this validation set showed that computed leaf areas accurately approximated grid counts.

16S rRNA gene sequence data (FASTQ files) were processed using the mothur bioinformatics pipeline following standard protocols (Schloss *et al.,* 2009; Kozich *et al.,* 2013; Jackson *et al.,* 2015) to remove erroneous sequences from downstream analyses. Remaining sequences were aligned against the SILVA database (version 128) of bacterial sequences (Pruesse *et al.,* 2007), screened to remove Eukarya and Archaea, and classified to operational taxonomic units (OTUs) defined by 97% sequence similarity using the Greengenes taxonomic database (DeSantis *et al.,* 2006; Schloss *et al.,* 2011). The resulting 3,436,130 sequences were classified to 15,452 OTUs. Of these, 6413 OTUs were represented only once across the dataset and were removed leaving 9039 bacterial OTUs for subsequent analyses.

To account for uneven sequencing depth between samples, samples were standardized (repeated subsampling of 1000 iterations) to the lowest sequence-count sample (1850). However, coverage estimates showed that this fraction of the dataset was not sufficient for calculation of alpha and beta-diversity measures. As such, 39 samples with low sequence counts (< 1850 sequences) were removed which improved coverage estimates for alpha-diversity measures in the remaining 216 samples. Alpha-diversity was summarized by the Simpson evenness measure (D_{simp}) and Chao estimated richness (S_{chao}), while beta-diversity was summarized using the Bray-Curtis (β_{bc}), Jaccard (β_j), and Raup-Crick (β_{rc}) dissimilarities to measure changes in bacterial abundance and incidence, respectively. To confirm that observed significance patterns were not an artifact of subsampling standardization, all tests were re-run on data with the lowest 51 samples removed, bringing subsampling up to 3253 sequences per sample as well as on non-

standardized alpha-diversity measures. All alpha-diversity patterns were robust to changes in subsampling threshold.

Non-bioinformatic statistical analyses were conducted in R version 3.4.1. To test the effect of rain on bacterial community alpha-diversity of *M. grandiflora* leaves, we fitted quadratic regression models of S_{chao} and D_{simp} (both log-transformed) against the number of days since the last rain event (beginning the day before at -1 followed by 1 and 3 d after) as well as the interaction between rain and canopy position variables (lower or upper canopy, interior or exterior) and cardinal direction. To account for the effect of leaf size (habitat area) on alpha-diversity measures, S_{chao} and D_{simp}, linear models were constructed in log-log space. Because linear models identified significant effects of leaf area on alpha-diversity measures, full models of rain and canopy position were calculated using the residuals returned after accounting for the effect of leaf area. To test the association between canopy structure and abundances of individual OTUs, the indicator function was used in mothur, based on Dufrêne and Legendre's metric (Dufrêne & Legendre, 1997), after which data were sorted by indicator value and significance assigned to all OTUs whose *p*-values fell within the 0.05 threshold when cumulatively added.

Beta-diversity patterns were visualized using non-metric multidimensional scaling (NMDS) on Bray-Curtis, Jaccard, and Raup-Crick dissimilarity measures. Bray-Curtis and Jaccard dissimilarities measure changes in bacterial OTU abundance and incidence, respectively, while Raup-Crick dissimilarities indicate differences in bacterial incidence after accounting for pairwise differences in alpha-diversity (Anderson *et al.*, 2011). Raup-Crick dissimilarities were calculated using code adapted from Chase *et al*. to better accommodate large microbial data sets (2011). Across all samples, deviation from "core" composition was defined by calculating homogeneity of dispersion from multivariate centroids through permutation testing using the

betadisper and permutest functions in the vegan package in R (Oksanen *et al.,* 2013). Significant differences in beta-diversity dispersion were further examined using a permutation-based implementation of Tukey's HSD post-hoc test in the vegan package (Oksanen *et al.,* 2013). Differentiation of community composition between canopy and rain groups was assessed using permutational multivariate ANOVA (perMANOVA; Anderson 2001) using the adonis function in the vegan package (Oksanen *et al.,* 2013).

iv. Results

At a broad taxonomic level, seven phyla dominated the community and made up 76.2% of all sequences: the Proteobacteria (41.5% of sequences), Bacteriodetes (13.1%), Actinobacteria (6.8%), Planctomycetes (5.0%), Acidobacteria (4.1%), Armatimonadetes (3.2%), and Verrucomicrobia (2.6%). At a finer taxonomic level, 50.4% of all sequences could be attributed to 20 OTUs, most of which grouped into the Alphaproteobacteria (Table 1), and which included three unclassified *Hymenobacter* spp., three OTUs from two *Sphingomonas* spp. (*S. asaccharolytica* and *S. wittichii*), two *Methylobacterium* OTUs, four OTUs in the Rhizobiales order as well as a Microbacteriaceae sp. Further, 90% of all sequences could be attributed to 295 OTUs (3.9% of all OTUs). The two most abundant taxa in terms of OTUs were *Sphingomonas asaccharolytica* (8.4%), followed by a *Methylobacterum* sp (5.3%).

Table 1. 20 Most Abundant OTUs in the *Magnolia grandiflora* Phyllosphere

Phyla	Class	Order	Family	Genus	Species	Canopy Indicator	p-val	Abund (%)
Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	*Sphingomonas*	*asaccharolytica*	Lower	-	8.43
Proteobacteria	Alphaproteobacteria	Rhizobiales	Methylobacteriaceae	*Methylobacterium*		Upper	0.044	5.34
Actinobacteria	Actinobacteria	Actinomycetales	Microbacteriaceae			Lower	-	4.2
Proteobacteria	Alphaproteobacteria	Rhizobiales				Upper	0.012	4.1
Bacteroidetes	Cytophagia	Cytophagales	Cytophagaceae	*Hymenobacter*		Interior	0.001	4.03
Bacteroidetes	Cytophagia	Cytophagales	Cytophagaceae	*Hymenobacter*		Interior	0.01	3
Proteobacteria	Alphaproteobacteria	Rhizobiales				Upper	0.008	2.29
Proteobacteria						Upper	0.008	2.14
Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	*Sphingomonas*		Upper	-	2.12
Bacteroidetes	Cytophagia	Cytophagales	Cytophagaceae	*Hymenobacter*		Interior	0.001	1.68
Proteobacteria	Alphaproteobacteria	Rhizobiales				Upper	0.001	1.68
Proteobacteria						Interior	-	1.65
Bacteroidetes	Cytophagia	Cytophagales	Cytophagaceae	*Spirosoma*		Interior	0.031	1.58
Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	*Sphingomonas*	*wittichii*	Lower	-	1.43
Proteobacteria	Betaproteobacteria	Burkholderiales	Oxalobacteraceae	*Massilia*		Interior	-	1.38
Acidobacteria	Acidobacteriia	Acidobacteriales	Acidobacteriaceae	*Terriglobus*		Upper	0.046	1.33
Actinobacteria	Actinobacteria	Actinomycetales	Pseudonocardiaceae	*Actinomycetospora*		Interior	-	1.22
Acidobacteria	Acidobacteriia	Acidobacteriales	Acidobacteriaceae			Upper	0.001	0.989
Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	*Sphingomonas*	*wittichii*	Upper	-	0.94
Proteobacteria	Alphaproteobacteria	Rhizobiales	Methylobacteriaceae	*Methylobacterium*		Interior	-	0.93

Table 1. Taxonomic information and canopy preference for the most abundant 20 bacterial OTUs in the *Magnolia grandiflora* canopy ordered by their proportional contribution to the entire dataset (Abund %). These OTUs account for 50.4% of all sequences in the dataset. Blank cells indicate that when no classification was made for a given taxonomic level and OTU. Canopy indication and *p*-value describe whether or not an OTU was significantly associated with samples from any particular canopy location (interior, lower, or upper). Gray entries represent OTUs that have strong associations with a particular canopy position.

Taxonomic composition of phyllosphere bacterial communities in *M. grandiflora* was consistent throughout the canopy both before and after rain at the broad taxonomic level, but several OTUs were linked to certain positions on the canopy. Using a cumulative threshold of $p = 0.05$, 30 OTUs were observed to be significant indicators of certain canopy position, of which 28 were primarily associated with leaves from the interior of the canopy while two were proportionally more abundant in leaves in the upper canopy (Table 2). These two OTUs, an unclassified Proteobacteria and a member of the Acidobacteraceae, were also among the most highly abundant lineages and were 4.8 and 5.6 times more abundant in the upper canopy than the lower canopy. OTUs significantly associated with the interior of the canopy were less abundant, and included two OTUs in the genus *Pseudonocardia* (Actinobacteria) which were 40 and 16 times more abundant in the interior of the canopy, a *Polaromonas* sp. (Betaproteobacteria; 12 times more abundant in the interior), four sequences grouped into family Acetobacteraceae (Alphaproteobacteria; 4-24 times more abundant), two sequences grouped into genus *Deinococcus* (Thermi; 5 and 25 times more abundant), an unclassified *Spirosoma* sp (Bacteroidetes; 15 times more abundant), and an unclassified *Neorickettsia* sp (Alphaproteobacteria; 6 times more abundant; Table 2).

Table 2. Top 30 OTUs Significantly Associated with Canopy Position in *Magnolia grandiflora*

Phyla	Class	Order	Family	Genus	Canopy Indication	Abund (%)	Int:Up
Acidobacteria	Solibacteres	Solibacterales			Interior	0.01	51.55
Bacteroidetes	[Saprospirae]	[Saprospirales]	Chitinophagaceae		Interior	0.03	40.61
Actinobacteria	Actinobacteria	Actinomycetales	Pseudonocardiaceae	*Pseudonocardia*	Interior	0.20	40.45
Cyanobacteria					Interior	0.01	38.02
[Thermi]	Deinococci	Deinococcales	Deinococcaceae	*Deinococcus*	Interior	0.31	24.59
Proteobacteria	Alphaproteobacteria	Rhodospirillales	Acetobacteraceae		Interior	0.05	23.85
Proteobacteria	Alphaproteobacteria	Rhodospirillales	Acetobacteraceae		Interior	0.06	19.70
Proteobacteria	Alphaproteobacteria	Rhizobiales			Interior	0.02	17.33
Actinobacteria	Actinobacteria	Actinomycetales	Pseudonocardiaceae	*Pseudonocardia*	Interior	0.03	16.06
Bacteroidetes	Cytophagia	Cytophagales	Cytophagaceae	*Spirosoma*	Interior	0.01	15.08
Cyanobacteria	Nostocophycideae	Nostocales	Scytonemataceae	*Brasilonema*	Interior	0.02	14.42
Actinobacteria	Actinobacteria	Actinomycetales			Interior	0.03	13.30
Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	*Polaromonas*	Interior	0.45	12.18
Proteobacteria					Interior	0.41	11.01
Proteobacteria	Deltaproteobacteria	Myxococcales	Polyangiaceae		Interior	0.15	10.98
Proteobacteria	Alphaproteobacteria	Rhodospirillales	Acetobacteraceae		Interior	0.04	10.32
Proteobacteria					Interior	0.25	9.96
Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae		Interior	0.19	8.78
Actinobacteria	Actinobacteria	Actinomycetales	Micromonosporaceae		Interior	0.05	6.51
Proteobacteria	Alphaproteobacteria	Rickettsiales	Anaplasmataceae	*Neorickettsia*	Interior	0.03	6.09
Cyanobacteria					Interior	0.05	5.58
Proteobacteria					Interior	0.08	5.22
Acidobacteria	Solibacteres	Solibacterales	MVS-65		Interior	0.01	5.20
[Thermi]	Deinococci	Deinococcales	Deinococcaceae	*Deinococcus*	Interior	0.02	4.60
Proteobacteria	Alphaproteobacteria	Rhodospirillales	Acetobacteraceae		Interior	0.05	4.17
Cyanobacteria					Interior	0.06	3.96
Bacteroidetes	Cytophagia	Cytophagales	Cytophagaceae	*Hymenobacter*	Interior	0.42	3.87
Cyanobacteria					Interior	0.02	3.33
Proteobacteria					Upper	2.14	0.21
Acidobacteria	Acidobacteriia	Acidobacteriales	Acidobacteriaceae		Upper	0.99	0.18

Table 2. Taxonomic information and canopy preference for the 30 bacterial OTUs with the strongest associations towards the interior or upper canopy of *Magnolia grandiflora*. Gray entries indicate OTUs that make are highly abundant and rank within the top 20 most proportionally abundant in the dataset. Int:Up indicates the mean abundance of an OTU in the interior canopy compared to its mean abundance in the upper canopy, expressed as a ratio.

By both richness and evenness measures, alpha-diversity of bacterial communities was predicated on habitat size (Figure 16). Increasing leaf area led to higher S_{chao} values with a slope of 0.33 in log-log space ($F = 24.3$, $p < 0.001$) as well as a corresponding 0.38 unit decrease in D_{simp} ($F = 4.23$, $p = 0.04$). This indicates that communities with higher OTU richness had lower equability between the abundances of different OTUs. After accounting for the effects of leaf area, vertical position and trunk proximity had the most significant influence on bacterial diversity of *M. grandiflora* leaves while the effect of rain was minimal. Canopy structure was the only significant predictor of bacterial richness or evenness ($F_{chao} = 30.8$, $p_{chao} < 0.001$; $F_{simp} = 12.0$, $p_{simp} < 0.001$). Leaves in the lower portion of the canopy supported richer but less even communities than leaves in the upper canopy as evidenced by lower leaves having 21% higher log-S_{chao} values but 51.8% lower log-D_{simp} values (Figure 16). In the interior of the canopy, leaves had a higher richness with 46.6% higher log-S_{chao} values but saw no significant difference in D_{simp} values compared to leaves on the exterior (Figure 17). The cardinal direction of the leaves (north, south, east, or west) did not affect bacterial diversity ($F_{chao} = 1.48$, $p_{chao} = 0.220$; $F_{simp} = 0.896$, $p_{simp} = 0.444$).

Rain did not have any effect on bacterial diversity, and had no immediate influence on the compositional similarity of bacterial communities regardless of rain intensity (Table 3). There was no evidence of any effect of rain on S_{chao} or D_{simp} either directly (by quadratic model terms: $F_{chao} = 0.224$, $p_{chao} = 0.637$; $F_{simp} = 2.04$, $p_{simp} = 0.156$; or linearly: $F_{chao} = 0.151$, $p_{chao} = 0.698$; $F_{simp} = 2.08$, $p_{simp} = 0.155$) or through interactions with canopy location (Figure 18). Samples collected 3 d after rain showed significantly higher dispersion, or compositional variation, between them than samples collected on -1 and 1 d, in opposition to our expectations that rain would make communities more similar ($F = 3.57$, $p = 0.03$). However this pattern was

driven by four outliers (representing 5.6% of samples in the group) that occurred 3 d after rain and increased average community dispersion relative to other days. Removal of these outliers nullified any significant differences in beta-diversity dispersion between the group of samples taken 3 d after rain and other groups ($F = 1.50$, $p = 0.216$).

For all beta-diversity measures, *M. grandiflora* phyllosphere bacterial communities differentiated strongly by canopy position, generally with high differentiation between the interior canopy and the upper canopy and the lower exterior canopy forming an intermediate group between the two (Figure 17; Table 4). Dissimilarities of Bray-Curtis and Jaccard showed strong resemblances when visualized in NMDS ordinations (Figure 17) and PerMANOVA tests of both measures showed that canopy position was the only significant factor associated with changes in community composition (Table 4). Also by both Bray-Curtis and Jaccard dissimilarities, multivariate dispersion was significantly lower in the interior of the canopy than in the upper canopy, indicating that community composition was less variable in the interior canopy than the upper canopy (Table 4). Raup-Crick measures also identified canopy position as the dominant factor influencing community composition (Table 4).

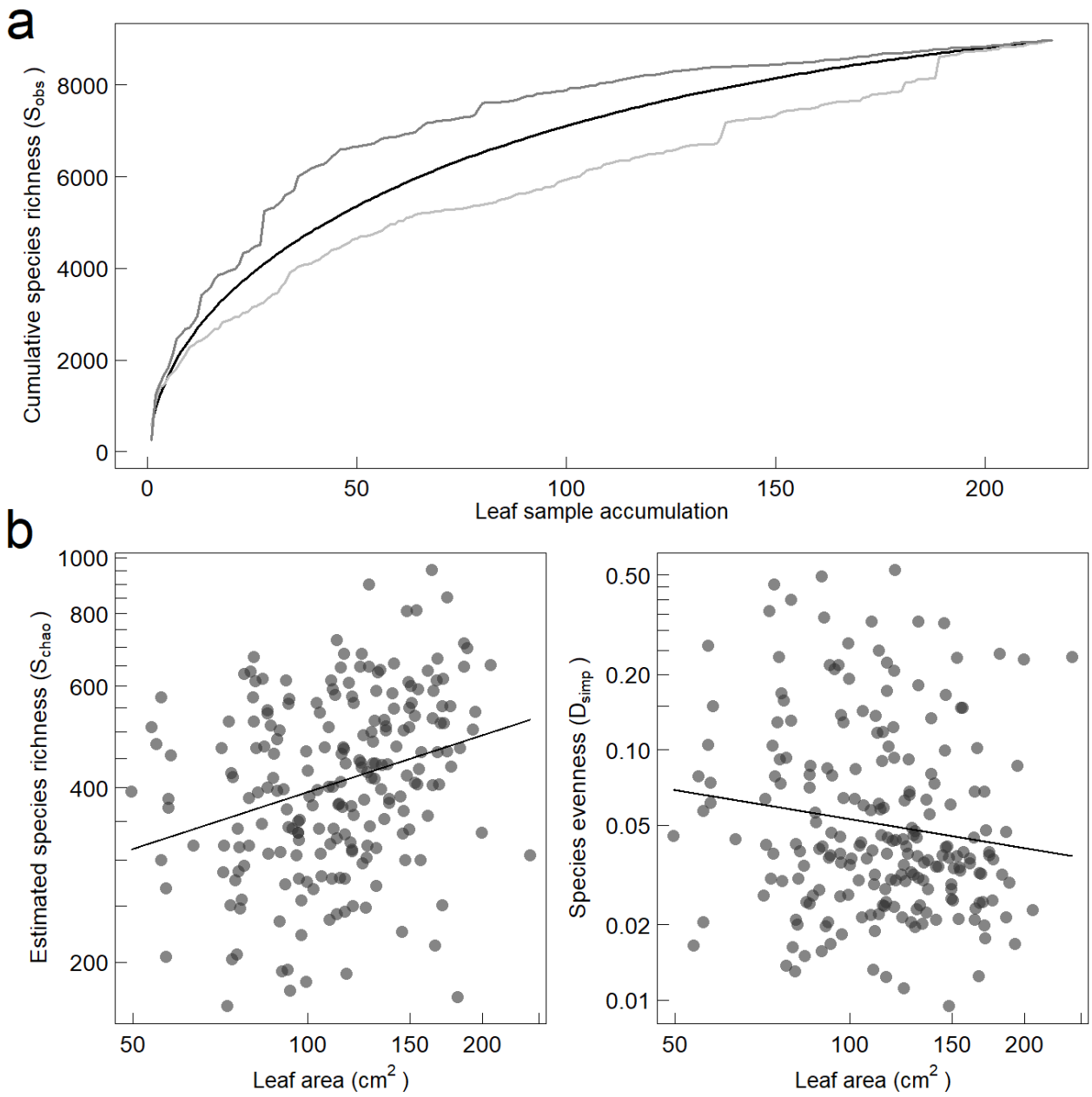

Figure 16. Species-Area Relationship in the *Magnolia grandiflora* Phyllosphere
Bacterial OTU-area relationship for phyllosphere communities on Magnolia grandiflora leaves. (a) Accumulation of bacterial OTUs (S_{obs}) with accumulation of leaf samples (areas standardized to 1) ordered from small to large (light gray) and large to small (dark gray) compared against the randomized null curve (black). Novel species accumulation is significantly slower with smaller leaves and significantly faster with larger leaves (b) Linear trends of OTU richness (S_{chao}) and community evenness (D_{simp}) against individual leaf area (cm^2) in log-log space show significant gains in bacterial richness and decreases in community evenness with increasing leaf size.

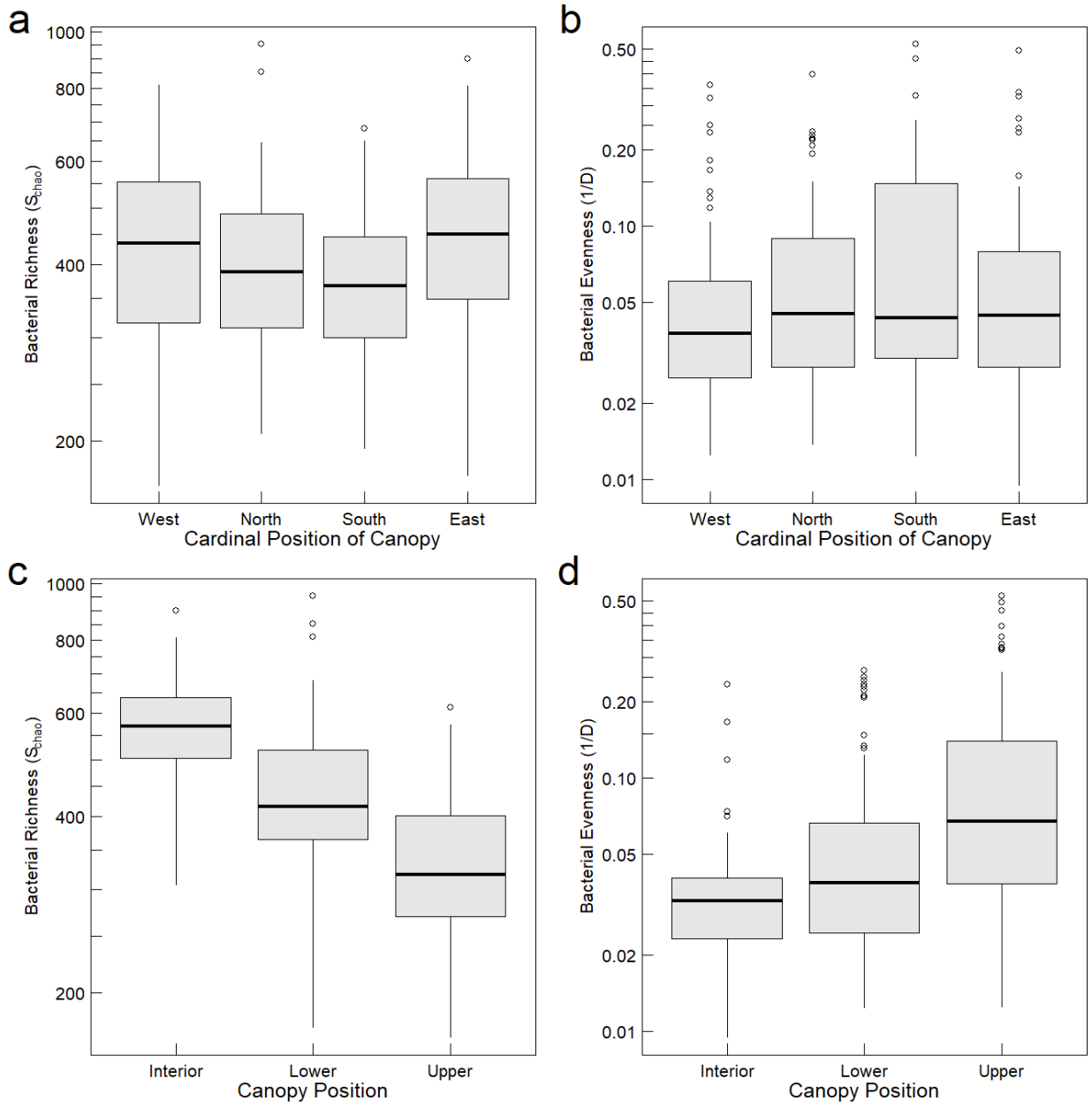

Figure 17. Bacterial Diversity throughout the *Magnolia grandiflora* Canopy
Relationship between bacterial diversity, cardinal position, and canopy structure on the *Magnolia grandiflora* phyllosphere microbiome. Top row: relationship between bacterial diversity and the cardinal direction on the canopy samples were taken from using (a) Chao estimated richness and (b) Simpson's evenness. Bottom row: relationship between bacterial diversity and canopy position (interior, lower exterior, and upper exterior) using (c) Chao estimated richness and (d) Simpson's evenness.

Date	Precipitation (cm)	Average five-day temp (°C)
05/04/2017	6.1	13
06/15/2017	4.3	23.7
09/12/2017	33.3	14.4

Table 3. Precipitation and Temperature of Sampled Rain Events
Recorded precipitation for all rain days sampled from May 2017 to September 2017 as well as five-day average temperatures. Data were obtained from NOAA land-based weather station number GHCND:USC00229079 located in University, MS.

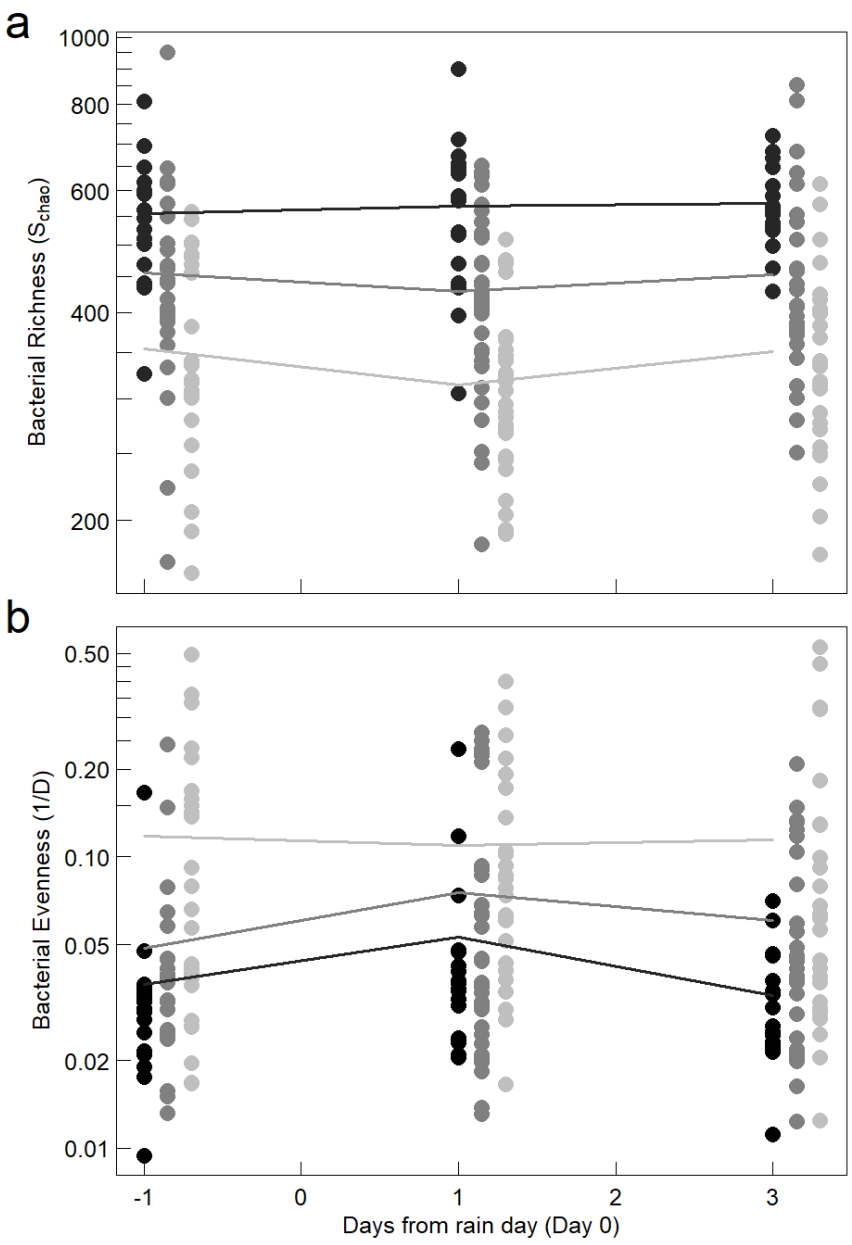

Figure 18. Effect of Rain on Diversity of the *Magnolia grandiflora* Canopy
Relationship between bacterial diversity and rain in the *Magnolia grandiflora* phyllosphere. Dark gray elements represent leaves from the canopy interior, mid gray represents the lower and exterior canopy, light gray represents the upper and exterior canopy. Points indicate individual values while lines indicate predicted values generated from linear models of the relationship between diversity and time. Relationship between bacterial diversity and rain using (a) Chao estimated richness and (b) Simpson's evenness.

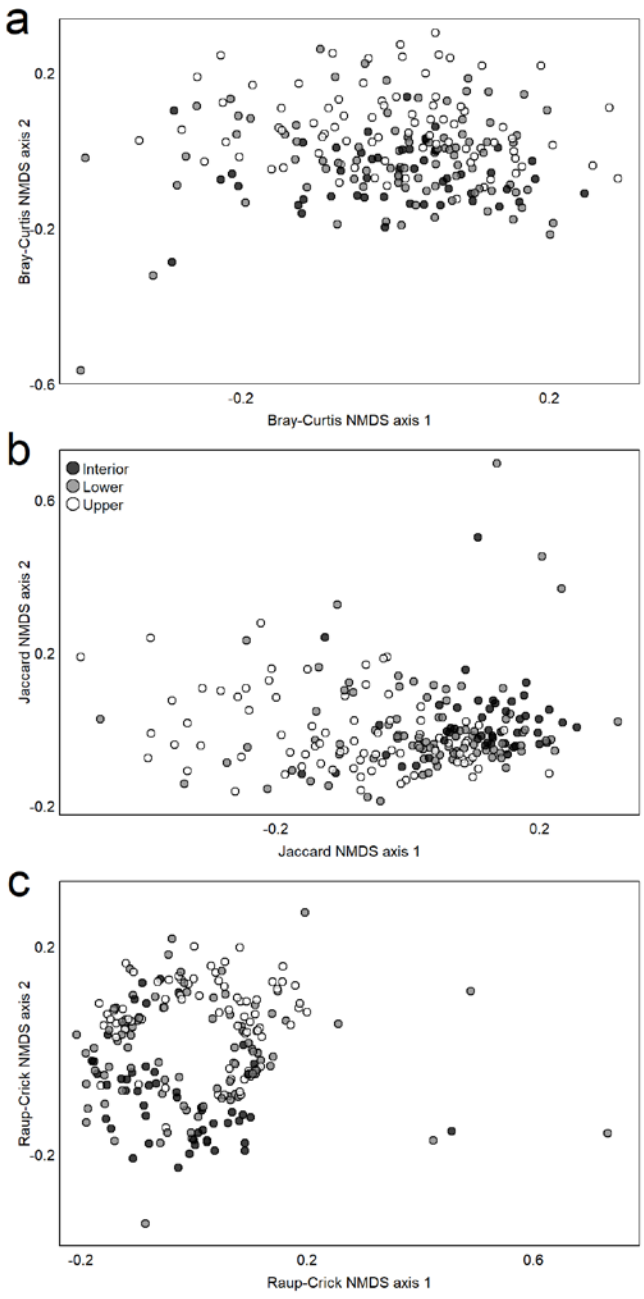

Figure 19. Multivariate Ordinations of *Magnolia grandiflora* Community Composition with Respect to Canopy Position

Non-metric multidimensional scaling (NMDS) ordinations based on (a) Bray-Curtis, (b) Jaccard, and (c) Raup-Crick dissimilarities of bacterial communities in the *Magnolia grandiflora* phyllosphere showing compositional distinction between leaves from different positions of the canopy. Dark gray points represent samples taken from the interior of the canopy. Medium gray points represent samples taken from the lower exterior portion of the canopy while light gray points represent samples taken from the upper and exterior portion of the canopy.

Table 4. Beta-diversity Composition and Dispersion Significance Tests

		Canopy		Rain		Cardinal Position	
		F	*p*	*F*	*p*	*F*	*p*
Composition	Bray-Curtis	6.01	***** 0.001**	1.19	-	1.43	*** 0.041**
	Jaccard	4.42	***** 0.001**	1.00	-	0.01	-
	Raup-Crick	20.48	***** 0.001**	-2.66	-	-0.54	-
Dispersion	Raup-Crick	0.99	-	1.50	-	2.62	*** 0.041**

Table 4. Differences in community composition and dispersion of *Magnolia grandiflora* phyllosphere bacterial communities. Tests were computed for Bray-Curtis, Jaccard, and Raup-Crick dissimilarity measures against canopy position (samples collected from either the interior, lower, or upper of M. grandiflora trees), rain (taken -1, 1 or 3 d after rain), and cardinal position (north, east, south, west). *F*-values were generated by PerMANOVA for compositional distinction, and by permutation tests of Raup-Crick beta-diversity dispersion, each test using 999 permutations.

[+] Removal of four outlier samples nullified significant effect (with outliers: $F = 3.57$, $p = 0.029$).

v. Discussion

Few studies have examined within-canopy variation in phyllosphere communities. Older studies focused on fungal species using culture-based techniques and primarily focused on potential plant pathogens (Carroll, 1979; Wildman & Parkinson, 1979; Andrews *et al.,* 1980). However, recent explorations using next-generation sequencing methods have found similar patterns to those presented here, in that leaves within different levels of the tree canopy can harbor different microbial communities. Fungal composition differentiated between the low, mid, and upper canopy of the coastal redwood (*Sequoia sempervirens*) with higher OTU richness in the mid canopy (23-69 m) and lower in the upper canopy (32-108 m) (Harrison *et al.,* 2016). Similar vertical structure was found for fungal composition in a tropical forest although with a continual decrease in Simpson's diversity from the lower to the upper canopy of 27 plant species (Izuno *et al.,* 2016). Both studies agree with our results of lower OTU richness and higher evenness in the upper canopy. While neither of the above studies examined lateral or horizontal canopy structure, leaves on the exterior of *Ginkgo biloba* tree canopies were shown to support lower richness communities than those more proximal to the trunk which align with our findings (Leff *et al.,* 2015). Species identity has been consistently shown to be one of the strongest factors in shaping community composition in the phyllosphere (Redford *et al.,* 2010; Laforest-Lapointe *et al.,* 2016; Stone *et al.,* 2018) and it is likely that canopy effects may be stronger in some tree species based on variation in leaf physiology throughout the canopy as well as the to the degree that canopy architecture influence distinct microclimates.

Interestingly, Izuno *et al.* (2016) found that fungal community composition had higher multivariate dispersion in the lower canopy levels and were instead more consistent in the upper

canopy, results directly in contrast to our findings. Their results are perhaps more intuitive: presumably harsher environmental conditions exist in the upper canopy (*e.g.*, more exposure to UV and desiccating winds; Brown *et al.*, 1994; Shaw, 2004), and these should consistently select for those microorganisms that are best suited to the habitat. However, after accounting for differences in average alpha-diversity seen in different parts of the canopy, we found no significant difference in multivariate dispersion between the upper canopy, lower, or interior canopy. The lower evenness values observed in the interior canopy suggest that survival does not equate to growth and that abundance in the *M. grandiflora* interior canopy is more concentrated to a few dominant taxa. Extending these conclusions to the upper canopy, these leaves may present a harsher environment for microbial colonization, limiting survival of low-abundance species, thus decreasing species richness and consequently increasing evenness. Habitable sections of the leaf surface exist as a lattice of grooves between cells and other structures that offer protection from harsh conditions (Baldotto & Olivares, 2008). It is likely that bacterial colonizers in the phyllosphere favor different conditions than fungal colonizers, as these two domains seem to be consistently antagonistic in the phyllosphere (Agler *et al.*, 2016) and which would explain the differences in our findings regarding dispersion against Izuno *et al.*'s (2016).

At a high taxonomic level, bacterial OTUs grouped into the same dominant phyla as previous studies conducted in the *M. grandiflora* canopy (Jackson & Denney, 2011; Stone & Jackson, 2016) and these were consistent with respect to canopy position. The dominance by Proteobacterial lineages is also consistent across the broader phyllosphere literature (Ruinen, 1965; Innerebner *et al.*, 2011; Vorholt, 2012). Other important lineages, such the Bacteroidetes, Actinobacteria, Acidobacteria, and Planctomycetes are also well represented in previous studies of the *M. grandiflora* phyllosphere. At a finer taxonomic level, prominent OTUs in the *M.*

grandiflora phyllosphere included those represented by sequences identified as members of genus *Sphingomonas* (Alphaproteobacteria), which were prominent in other *M. grandiflora* studies (Jackson & Denney, 2011; Stone & Jackson, 2016). These organisms are clearly well suited to the phyllosphere habitat and should be considered as a core component of the *M. grandiflora* phyllosphere microbiome. In contrast, *Methylobacterium* spp., another prominent group of Alphaproteobacteria found in this study, have been found to be more variable across seasonal time scales, being less abundant in samples taken from the spring and winter (Jackson & Denney, 2011). A prominent *Methylobacterium* OTU in our dataset was more abundant in the upper portion of the canopy, suggesting that this lineage is more abundant in higher light and higher temperature conditions, or perhaps is more adept in responding to changes in leaf physiology following these same cues. Both *Sphingomonas* and *Methylobacterium* include species that can take advantage of plant-produced carbon sources (Vorholt, 2012). Several lineages of *Hymenobacter* (Bacteroidetes) were observed to be both prominent in this study, and were significantly associated with the interior of the *M. grandiflora* canopy. *Hymenobacter* is an environmentally ubiquitous genus of organoheterotrophs first isolated from Antarctic soil (Hirsch *et al.,* 1998) but seen in the atmosphere (Buczolits *et al.,* 2002), freshwater habitats (Lee *et al.,* 2017) and on irradiated surfaces (Collins *et al.,* 2000). Given its wide distribution and ability to tolerate radiation, desiccation, and oligotrophic conditions (Buczolits & Busse, 2011), it is not surprising that *Hymenobacter* spp. are found in our dataset, but it is interesting that they were consistently associated with the interior canopy, rather than the upper canopy as might be expected. Some *Hymenobacter* are known to degrade chitin, so it is possible that higher abundances in the interior canopy could be tied to increased fungi in that area (Buczolits *et al.,* 2006).

In contrast to the predicted results, rain seemed to have no effect on community composition or diversity regardless of intensity, suggesting that rainfall imposed little physical disturbance on the *M. grandiflora* phyllosphere. Previous studies focusing on single-species dynamics have shown that movement and population growth following rainfall are of far more importance to phyllosphere microorganisms than splash dispersal (Constantinidou *et al.,* 1990; Hirano *et al.,* 1996; van der Wal *et al.,* 2013). However, the species considered in those studies, *Pseudomonas* spp. and *Xanthomonas* spp. (Weller & Saettler, 1980; Duveiller, 1994; Hirano *et al.,* 1996; Pietrarelli *et al.,* 2006; Morris *et al.,* 2008), are both Gammaproteobacteria that can metabolize a diverse array of carbon compounds making them generalists in the phyllosphere, and are typified by fast growth rates (Palleroni, 2005; Saddler & Bradbury, 2005). Thus, these particular species may be well-suited to take advantage of the opportunities afforded by rain. Our results show that at the community level, composed largely of uncultured taxa, the microbiota was intransigent following rain. Besides differences in bacterial growth capacities, the impact of rain on the phyllosphere microbial community could be mitigated by the protected nature of bacterial aggregates and biofilms as well as the structural flexibility of the plant leaf and stalk that minimizes the force exerted from raindrops (Huber *et al.,* 1997). If most phyllosphere organisms are protected in such a way, then rain is likely to cause minimal disruption of existing microbial abundances.

Taken together, our findings demonstrate that canopy position is a stronger determinant of foliar bacterial diversity and structure than rainfall in the *M. grandiflora* phyllosphere. Heterogeneity throughout the canopy, especially with regards to species diversity, is important because higher phyllosphere diversity has been associated with lower disease incidence and intensity (Balint-Kurti *et al.,* 2010), and thus sections of the tree canopy with lower microbial

diversity may thus be more susceptible towards infection, although other factors such as microclimate and disease vectors should also be considered. Leaves in the lower parts of the canopy are more susceptible to disease incidence due to lower radiation while leaves in the upper part may be more susceptible based on increased wetness duration during rain (Calonnec *et al.*, 2013). Few studies have examined how phyllosphere diversity patterns respond to these same cues. We found richer bacterial communities in the lower canopy but a more even distribution of bacteria in the upper canopy and no change in either composition or diversity due to rain. More importantly, we see evidence that variability in phyllosphere bacterial composition is also heterogeneous throughout the canopy in ways that contrast previous work on epiphytic fungi, suggesting that different assembly mechanisms drive these patterns. Future work exploring the relationship between phyllosphere diversity and canopy structure should quantify both bacterial and fungal lineages to understand how these mechanisms manifest on the phyllosphere community.

CONCLUSIONS

There is clearly a great deal of spatial and temporal heterogeneity in communities of the plant phyllosphere. Most significantly, phyllosphere communities did not respond strongly to the effects of rain as was hypothesized in Chapters 2 and 3. Rather, rain and other climatic variables were found to influence phyllosphere diversity and composition of the broadleaf cattail (*Typha latifolia*) at larger seasonal time scales. Although rain was theorized to disrupt the phyllosphere community, similar to environmental disturbances imposed on larger communities (*e.g.*, fire, tornados, floods, excavation), no evidence was found of this being relevant towards leaf epiphytes. Previous research of single-species population dynamics has shown that rain promotes bacterial movement and colonization across the leaf surface; however, these data show that the same response does not extend to the entire bacterial community. It is possible that rain response patterns differ by plant species; however, the alignment between conclusions drawn from studies on cattail and magnolia trees, two plants with very different life histories and growth strategies, suggests that these findings could potentially be generalized to other plant systems.

These studies provide important insights into the natural behavior of the phyllosphere community at a higher resolution than previously encountered and use next-generation sequencing tools that are more inclusive towards uncultivatable lineages. The phyllosphere is a complex system; heterogeneous at many scales. The spatial scales surveyed in recent molecular studies have explored either very large or very small distances (*e.g.*, across hundreds of

kilometers; microscale aggregations on the leaf surface). Future research connecting broad, continental patterns to small-scale and local interactions will require knowledge of dynamics at the forest and canopy level. Chapters 1 and 3 bridge these gaps, and encourage future cross-scale work. Chapter 1 found that phyllosphere community structure could be related to environmental factors (canopy density) when summarized with abundance-based beta-diversity measures (theta) but not when analyzed by incidence-based measures (Jaccard), suggesting that environmental heterogeneity exerts itself more readily on growth and abundance than on incidence of phyllosphere bacteria. In Chapter 3, phyllosphere communities of southern magnolia (*Magnolia grandiflora*) showed variability in both diversity and composition across a small forest, as well as within single canopies. Here, patterns in bacterial richness and evenness suggest that the interior canopy presents an easier habitat for most bacteria to colonize but also allows for fewer species to dominate the community, while the exposed upper canopy is more difficult to colonize and establish, leading to lower richness, higher equitability, and more variable composition. Chapter 2 demonstrated that across time, the most evident factor influencing bacterial community composition was leaf senescence. Green *T. latifolia* leaves harbored higher community evenness and had more compositional variation while senesced leaves harbored more consistent communities dominated by a fewer number of bacterial species.

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VITA

BRAM STONE

EDUCATION

B.S., Portland State University, August 2011

Major: Environmental Science and Management

Minor: Biology

PUBLICATIONS

2018 Stone, B.W.G., E.A. Weingarten, C.R. Jackson. The role of the phyllosphere microbiome in plant health and function. *Annual Plant Reviews* in press.

2017 Caplan, J.S., B.W.G. Stone, C.A. Faillace, J.J. Lafond, J. Baumgarden, T.J. Mozdzer, J. Dighton, S.J. Meiners, J.C. Grabosky, J.G. Ehrenfeld. Nutrient foraging strategies are associated with productivity and population growth in forest shrubs. *Annals of Botany* 119: 977–988.

2016 Stone, B.W.G., C.R. Jackson. Biogeographic patterns between bacterial phyllosphere communities of the Southern Magnolia (*Magnolia grandiflora*) in a small forest.

Microbial Ecology 71: 954–961.

2015 Jackson, C.R., B.W.G. Stone, H.L. Tyler. Emerging perspectives on the natural microbiome of fresh produce vegetables. *Agriculture* 5: 170–187.

GRANTS, FELLOWSHIPS, AND AWARDS

2017 Second place winner of doctoral research, Three Minute Thesis (3MT) Competition. University of Mississippi.

2017 Summer Graduate School Research Fellowship. University of Mississippi.

2016 G. Murray McKinley Research Fund. Pymatuning Laboratory of Ecology, Department of Biological Sciences, University of Pittsburgh.

2015 Graduate Student Council Research Award. University of Mississippi.

2007 Laurels Scholarship. Southern Oregon University.

PRESENTATIONS

2017 Diversity of the plant microbiome in response to environmental disturbance. Invited talk, University of Mississippi Flagship Initiative. Oxford, MS, November.

2017 Co-occurrence of a native and invasive wetland plant species increases rhizosphere bacterial diversity in both species. Ecological Society of America (ESA). Annual Meeting, Portland, OR, August.

2017 Targeted next generation sequencing of microbial communities as a tool for ecological inference. Invited talk, Mississippi Academy of Sciences (MAS) Annual Meeting. INBRE Symposium: Metagenomics to Functional Microbiome. Hattiesburg MS, February.

2015 Biogeographic patterns in phyllosphere microbial communities in a small forest plot provide weak but significant support for the distance-decay relationship. Southeastern Ecology and Evolution Conference (SEEC), Athens, GA, April.

POSTERS

2018 B.W.G. Stone, C.R. Jackson. Temporal Patterns in Enzyme Activity and Bacterial Community Structure of the Phyllosphere of the Wetland Macrophyte Typha latifolia. 12th International Symposium on Biogeochemistry of Wetlands. Coral Springs, FL, April.

2015 Payne, J.T., B.W.G. Stone, J.J. Millar, C.R. Jackson, C.A. Ochs. Microbial enzyme activity in the Lower Mississippi River: Temporal patterns from hourly to monthly time scales. International Society of River Science (ISRS) 4th Biennial Symposium, La Crosse, WI, August.

2015 Stone, B.W.G., C.R. Jackson. Biogeographic patterns in phyllosphere microbial communities in a small forest provide weak but significant support for the distance-decay relationship. American Society of Microbiology (ASM) general meeting, New Orleans, LA, May.

2013 Payne, J.T., J.J. Millar, B.W.G. Stone, C.A. Ochs, C.R. Jackson. Temporal variation of microbial extracellular enzyme activity in the Lower Mississippi River. Poster session at American Society of Microbiology (ASM) meeting, New Orleans, LA, October.

2013 Caplan, J., B.W.G. Stone, J. Grabosky, J. Ehrenfeld. Associations between root traits and shrub productivity in Northeast forests. Poster at Soil Ecology Society (SES) meeting, Camden, NJ, June.

OTHER RESEARCH EXPERIENCE

2014 Research Assistant. Jackson Lab. Department of Biology. University of Mississippi.

2011 Lead Field Technician. Ehrenfeld/Grabosky Lab. Department of Ecology, Evolution and Natural Resources. Rutgers University.

TEACHING

Courses Taught

Biological Sciences (Fall 2013, 2015; Summer 2016), Inquiry into Life (Spring 2015), General Microbiology (Spring 2014, 2015, 2016; Summer intersession 2015; Fall 2016), Principles of Microbiology (Spring 2015, 2016), Microbial Physiology (Fall 2016), General Ecology (Spring 2017, 2018; Fall 2017)

Lectures Given

Biogeochemical cycling, General Microbiology (Spring 2016)

Ecosystem productivity, General Ecology (Fall 2017)

Labs Designed

Invasive species in Mississippi, General Ecology (Spring 2017),

Ecological sampling techniques, General Ecology (Fall 2017)

SERVICE

Departmental

President, Biology Graduate Student Society, 2015–2017

Professional and Societal

Peer review for: *Wetlands* (4), *PLoS One* (1), *Microbial Ecology* (1)

Outreach

Research presentation at university flagship initiative, 2017

University of Mississippi Field Station Science Day, 2017

Judge, Mississippi Region 7 Science and Engineering Fair, 2017