

ABSTRACT

Title of Thesis: UNDERSTANDING THE INFLUENCE OF
MICROBIAL SOURCES AND TIME ON THE
DEVELOPING CREEPING BENTGRASS
MICROBIOME

Joseph Ryan Doherty, Master of Science, 2018

Thesis Directed by: Assistant Professor, Dr. Joseph A. Roberts
Plant Science and Landscape Architecture

Microbial communities are intimately intertwined with many processes affecting the health of plants. There is increasing interest in utilizing microbial communities to increase plant health while reducing management inputs. To that end, bacterial and fungal communities associated with creeping bentgrass were evaluated using next-generation sequencing technologies. Evaluating the impact of resident seed and soil microbial communities revealed introductions of microbes from the seed despite a strong influence from the soil. Observing long-term population dynamics revealed no shifts in fungal diversity over six months, while bacterial diversity increased from emergence to two months post-emergence. Across both studies taxonomic profiling revealed that bacterial and fungal communities were consistently dominated by just a few groups. In both studies, ordination analyses revealed clustering of samples by sampling time. These results show that changes in the microbiome are driven by rare species, and that the turfgrass microbiome is resilient to change over time.

UNDERSTANDING THE INFLUENCE OF MICROBIAL SOURCES AND TIME ON
THE DEVELOPING CREEPING BENTGRASS MICROBIOME

by

Joseph Ryan Doherty

Thesis submitted to the Faculty of the Graduate School of the
University of Maryland, College Park in partial fulfillment
of the requirements for the degree of
Master of Science
2018

Advisory Committee:

Professor Joseph A. Roberts, Chair
Dr. Jo Anne Crouch
Professor Shirley Micallef
Professor Stephanie Yarwood

© Copyright by
Joseph Ryan Doherty
2018

Acknowledgements

First and foremost, I would like to thank my advisor Dr. Joseph Roberts for his unwavering support during the pursuit of this degree. Without his support throughout this process I would undoubtedly not be in the position I am today. To Dr. Jo Anne Crouch for her thorough attention in sharing with me the intricacies of next-generation sequencing platforms, along with troubleshooting sequencing of these platforms. For invaluable assistance in troubleshooting PCR, sequencing and equipment issues many thanks to Dr. Ed Ismaiel and Dr. Catalina Salgado-Salazar. I give my thanks to Dr. Dan Veltri for sharing his knowledge on bioinformatic processing of next-generation sequencing data and the Ruby programming language. Dr. Lisa Beirn I thank you for your help in deciphering the quirks of data analysis for these microbiome data sets. Many thanks to Dr. Shirley Micallef, Dr. Stephanie Yarwood and Dr. Cassandra Swett for serving on my committee providing valuable insight and guidance.

Most importantly I want to thank my family for their continual support through my entire educational journey. All of you have my sincerest thanks! Without your support I never would have made it this far.

Table of Contents

Acknowledgements	ii
Table of Contents	iii
List of Tables	v
List of Figures	vi
Appendix 1	vii
Chapter 1. Review of the Literature: The Turfgrass Microbiome	1
Defining Microbiomes	1
Methods of Investigating Microbiomes	3
Understanding Plant Components and Associated Microbiomes	9
<i>The Phyllosphere</i>	9
<i>The Rhizosphere</i>	11
Microbiomes of Graminaceous Hosts	13
Existing Knowledge on the Turfgrass Microbiome	15
Summary and Thesis Overview	18
Literature Cited	21
Chapter 2. Elucidating the Influence of Resident Seed and Soil Microbiota on the Developing Creeping Bentgrass Microbiome	29
Abstract	29
Introduction	30
Materials & Methods	33
<i>Sterile Growth Conditions</i>	33
<i>Sampling and eDNA Extraction</i>	35
<i>Amplicon Generation and Sequencing Preparation</i>	36
<i>Sequence Manipulation and Analysis</i>	39
Results	40
<i>Amplicon Sequence Variants</i>	40
<i>Composition of Amplicon Sequence Variants</i>	41
<i>Relative Abundances of Bacteria and Fungi</i>	44
<i>Diversity of Rhizosphere Bacterial and Fungal Communities</i>	45
Discussion	53
Literature Cited	61
Chapter 3. Mapping Temporal Shifts in the Creeping Bentgrass Microbiome	69
Abstract	69
Introduction	70
Materials & Methods	73
<i>Growth Conditions</i>	73
<i>Sample Collection and Processing</i>	73
<i>DNA Manipulations</i>	74
<i>Sequence Analyses</i>	76
Results	78
<i>Sequencing Output</i>	78
<i>Diversity of Bacterial and Fungal Communities</i>	79
<i>Taxonomic Composition of Amplicon Sequence Variants</i>	80
<i>Changes in Taxonomy Across Plant Tissue and Time</i>	82
Discussion	89

Literature Cited	95
Appendix 1	101
Literature Cited	103

List of Tables

Table 1. Composition of Bacterial Amplicon Sequence Variants	42
Table 2. Composition of Fungal Amplicon Sequence Variants	43
Table 3. Alpha-Diversity Measures for Microbial Communities Associated with Creeping Bentgrass Grown Under Sterile Conditions	47
Table 4. Centroid and Dispersion Testing of Beta-Diversity Metrics of Microbial Communities Associated with Creeping Bentgrass Grown Under Sterile Conditions	51
Table 5. Centroid and Dispersion Testing of Beta-Diversity Metrics Within Groupings	52
Table 6. Microbial Community Alpha-Diversity Measures for Greenhouse Grown Creeping Bentgrass	87
Table 7. Centroid and Dispersion Testing of Beta-Diversity Metrics for Microbial Communities Associated with Greenhouse Grown Creeping Bentgrass	88

List of Figures

Figure 1.	Taxonomic composition of A) bacterial and B) fungal communities associated with creeping bentgrass grown under sterile conditions	44
Figure 2.	Alpha diversity measures of A) bacterial and B) fungal communities associated with creeping bentgrass grown under sterile conditions	45
Figure 3.	Principal component analyses of Bray-Curtis distance matrices of A) bacterial and B) fungal communities associated with creeping bentgrass grown under sterile conditions	49
Figure 4.	Principal component analyses of Bray-Curtis distance matrices of A) bacterial and B) fungal communities associated with creeping bentgrass grown under sterile conditions	50
Figure 5.	Alpha diversity measures of A) bacterial and B) fungal communities associated with greenhouse grown creeping bentgrass	79
Figure 6.	Principal component analysis of Bray-Curtis distance matrices of A) bacterial and B) fungal communities associated with greenhouse grown creeping bentgrass	80
Figure 7.	Relative abundances of fungal orders associated with greenhouse grown creeping bentgrass	81
Figure 8.	Relative abundances of bacterial phyla associated with greenhouse grown creeping bentgrass	82
Figure 9.	Relative abundances of bacterial families associated with greenhouse grown creeping bentgrass	83
Figure 10.	Relative abundances of bacterial genera associated with greenhouse grown creeping bentgrass	84
Figure 11.	Relative abundances of fungal genera associated with greenhouse grown creeping bentgrass	86

Appendix 1

Supplementary Figure 1.	Relative abundances of bacterial genera associated with creeping bentgrass grown under sterile conditions over time	101
Supplementary Figure 2.	Relative abundances of fungal genera associated with creeping bentgrass grown under sterile conditions over time	102

Chapter 1. Review of the Literature: The Turfgrass Microbiome

DEFINING MICROBIOMES

In recent years there has been a significant increase microbiome research. In 2015, a web-based search using the keyword ‘microbial metagenomics’ returned 31,400 citations (Beirn, 2016). This same search two years later results in nearly double the number of citations at 57,200 (<https://scholar.google.com>; accessed 2017 December 20). The scientific community’s interest in microbiome research is entirely evident based on this 182% increase in “microbial metagenomics” manuscripts published over two years. It is likely that this trend will continue to increase in the years to come.

The microbiome is made up of the resident microorganisms, their genomes, and the surrounding environmental conditions (Marchesi and Ravel, 2015). More simply put, the microbiome is the combination of the metagenome (i.e., all the genetic material present in an environmental sample, consisting of many individual organisms) and the environment. Since 2008, several microbiome projects have been initiated with the goal of mapping and characterizing the microbiomes, varying in terms of the scope and size (American Phytopathological Society, 2016; Gilbert et al., 2010; Turnbaugh et al., 2007). The Human Microbiome Project was launched in 2008 by the National Institutes of Health and was designed to facilitate characterization of the human microbiota to further our understanding of how the microbiome impacts human health and disease (Turnbaugh et al., 2007). The Earth Microbiome Project was launched in 2010 specifically aimed to characterize global microbial taxonomic and functional diversity for the benefit of the planet and humans (Gilbert et al., 2010). The Phytobiomes Initiative was started in 2015

by the American Phytopathological Society with the goal of better understanding the networks of interactions among plants, their environment and the complex communities of microorganisms which inhabit them, including their influence on plant and agroecosystem health and productivity (2016).

Unlike the Human Microbiome Project and the Earth Microbiome Project, whose main foci are the characterization and understanding of microbiome dynamics, the Phytobiomes Initiative covers a wider breadth of research areas, including weather, animals, nutrients, soils, plants, and microbes (Phytobiomes Initiative, 2015). When thinking about the phytobiome, all the aforementioned factors play a role individually, but are also intertwined with one another, forming either a symphony of positive affects leading to enhanced plant health or a cacophony of adverse affects that result in plant decline.

Microbiomes have a wide variety of impacts on plants. Mycorrhizae and rhizobia exchange nutrients with their hosts, helping plants to succeed when nutrient availability is poor (van der Heijden et al., 2008). Endophytes survive within hosts asymptotically and can provide protection from pathogens or harsh environmental conditions (Rodriguez et al., 2009). Nutrient and carbon cycling within the soil is driven by microbial activity, influencing plant health through the availability of nutrients (Arias et al., 2005; van der Heijden et al., 2008). Plant pathogens parasitize their hosts leading to reduced yields, poor playability or reduced aesthetics and economic value, while other microbes can combat the affects of pathogens to reduce or prevent disease (Andrews, 1992; Lo et al., 1997). From a plant pathology perspective, it is thought that understanding plant

microbiomes, will allow for manipulation of microbial communities to combat plant pathogens or enhance plant health.

METHODS OF INVESTIGATING MICROBIOMES

Microbial communities associated with plants are intricate and diverse (Berg and Smalla, 2009). Early methods used to study microbes associated with plants involved culturing those organisms on specialized growth media to view and/or manipulate them in the lab. However, most researchers agree that culture-based methods can only account for a small fraction of the total microbial community present (Saleh-Lakha et al., 2005). For example, Rastogi et al. (2010) estimated that culturing bacteria on tryptic soy agar to account for bacterial abundance in the lettuce phyllosphere only accounted for 0.1-8.4% of the total bacterial abundance observed using real-time PCR. Similarly, other studies have found that only 0.1% to 1% of bacteria present in the soil can be established in pure culture on selective media (Amann et al., 1995; Torsvik and Øvreås, 2002; Torsvik et al., 1990). Advances in culture-based technologies, such as high-throughput microbioreactors, simulated natural environments, or community cultures, have increased the amount of bacterial that can be cultured (Pham and Kim, 2012). Therefore, at least for the present time, reliance on culture-based methods alone to enumerate plant-associated microbes will severely underestimate microbial populations as well as taxonomic diversity.

By utilizing non-culture based investigative techniques in conjunction with culture-based methods, researchers can gain deeper insight into microbial populations. Non-molecular techniques, such as phospholipid fatty acid analysis (PLFA) allow for the

construction of a community profile for the comparison of samples. PLFA is a method of estimating microbial biomass (Frostegard et al., 2010; White, 1983) and provides a general inference as to the types of organisms present in the sample (Frostegard et al., 2010). This is done by comparing the cellular membrane phospholipids of known microorganisms to those of unknown microorganisms in a sample (Frostegard et al., 2010). However, when utilizing PLFA for inferring community composition, caution must be taken, as all organisms within a group may not share a single biomarker and that same biomarker may be shared with organisms from another group, making taxonomic assignment impossible at times (Frostegard et al., 2010). For example, the PLFAs cy17:0 and cy19:0 are typically associated with Gram-negative bacteria, however Schoug et al. (2008) showed these PLFAs are also present in large quantities in a number of Gram-positive bacteria.

Over the past few decades, DNA-based approaches have developed as a more robust tool for studying plant-microbe interactions. Common DNA-based tools include terminal-restriction fragment length polymorphism (T-RFLP), ribosomal intergenic spacer analysis (RISA), PCR-denaturing gradient gel electrophoresis (DGGE), or next-generation sequencing (NGS) technologies and each can provide a more in-depth examination into the microbial communities associated with plants. These DNA based methods all take advantage of the fact that all living organisms possess DNA, therefore allowing analysis of these communities regardless of their capability to be cultured. As such, these methods provide greater resolution of community diversity and may provide a more accurate and comprehensive reflection of the microbial community being investigated relative to culture-based methods or PLFA (Amann et al., 1995). In T-

RFLP, PCR amplified markers are digested with restriction endonucleases and the subsequent size polymorphisms are measured on high resolution sequencing gels (Marsh, 1999). Utilizing PCR amplification of the intergenic spacer between the 16S and 23S subunit rRNA genes in bacteria, RISA generates community profiles on a polyacrylamide gel through electrophoresis of the DNA fragments generated from PCRs (Fisher and Triplett, 1999). Using both T-RFLP and RISA, Micallef and others (2009b) showed that eight different accessions of *Arabidopsis thaliana* L. recruited specific bacterial communities to their respective rhizospheres. Through the use of DGGE, a DNA fingerprinting technique that can be used to separate DNA fragments with the same length but different sequences (Muyzer and Smalla, 1998), Micallef et al. (2009a) investigated the effect of plant age and genotype on the rhizosphere bacterial communities of two different *A. thaliana* accessions. It was found that succession of bacterial communities progressed differently between the two accessions; however, as the plants aged, the rhizosphere and bulk communities converged (Micallef et al., 2009a). This convergence coincided with an expected decrease in root exudate release as the plants aged (Micallef et al., 2009a). Using next-generation sequencing, a high-throughput DNA sequencing technique that allows for rapid sequencing of millions of DNA strands in parallel, biochar applications were evaluated for their effects on soil bacterial and fungal communities (Jenkins et al., 2017). Biochar applications to a short rotation coppice in the UK, a French grassland and a short rotation forestry site in Italy reduced Proteobacteria, Actinobacteria and Acidobacteria compared to pre-treated abundances within the same sites (Jenkins et al., 2017). Biochar applications also

resulted in significant decreases in Gemmataceae and Koribacteraceae within the forestry site in Italy (Jenkins et al., 2017).

While culture-independent methods overcome some of the limitations associated with establishing microorganisms in culture, they are not without their own limitations. Many of the methods previously described require lysing of cells, which is the breaking down of the membrane of cells, to access the DNA for PCR amplification. Lysis of cells and fungal structures differs both within and between microbial groups (Prosser, 2002). Fungal mycelia of different ages lyse differently, potentially introducing biases in molecular based studies of microbial diversity (Prosser, 2002). It has also been observed that various DNA isolation methods can lead to bias due to dominant microbes present. For example, Kozdrój and van Elsas (2000) compared soil bacterial community profiles generated from DNA that was extracted directly (i.e., lysis by bead beating or grinding in liquid N) or indirectly (i.e., cell extraction from soil followed by DNA extraction or combined RNA/DNA extraction). All four methods generated similar profiles, however, cluster analysis showed groupings based on the extraction method utilized (Kozdrój and van Elsas, 2000). These clustering patterns indicated that, even with the same soil sample, different bacterial populations may be detected, solely based on the isolation method used (Kozdrój and van Elsas, 2000; von Wintzingerode et al., 1997). Similarly, Luna et al. (2006) observed that using a single *in situ* SDS-based DNA extraction kit underestimated bacterial ribotypes of marine sediments when compared to a cell extraction protocol. Due to the variability observed through employing different techniques, pooling the results from several different DNA extraction methods may provide a more accurate insight into the microbiome community structure. However,

monetary and time constraints may prohibit the use of combined approaches for large-scale microbiome investigations.

Since the advent of NGS technologies for microbiome analyses, there has been one major issue: how to analyze massive amounts of data in a meaningful manner. Initially the best option was to perform operational taxonomic unit (OTU) clustering from sequences. Using a fixed dissimilarity threshold (commonly 3%), DNA sequences from NGS technologies are clustered into OTUs (Kopylova et al., 2016; Westcott and Schloss, 2015). Subsequently, a sample-by-OTU table is generated, wherein an observation of an OTU in a sample corresponds to an observation of the ‘species’ assigned, and utilized for downstream analyses. While many different methods of OTU clustering have been proposed, two methods are used: 1) closed-reference OTU clustering and 2) *de-novo* OTU clustering. Closed-reference OTU clustering uses a reference database to place reads into an OTU based on their sequence similarity to the database entries (Kopylova et al., 2016) whereas, *de-novo* OTU clustering employs sequence dissimilarities for generating OTUs (Westcott and Schloss, 2015). *De novo* OTU clustering is performed based on a set similarity/dissimilarity threshold without a reference database, which means the clustering of the OTUs themselves depends on the relative abundances of the sampled community (Callahan et al., 2017). Due to this data set dependence, *de novo* OTUs cannot be compared between two different sample sets. Conversely, if utilizing closed-reference OTUs data can be compared across data sets if the same reference database was used for OTU determination. However, biological variation in nucleotide sequences not represented in the reference database will be lost in the final OTU assignment (Callahan et al., 2017). Amplicon sequence variants are inferred *de novo*

under the expectation that biological sequences are more likely to be repeatedly observed than are error-containing sequences.

Increasingly, OTUs are being replaced by amplicon sequence variants (ASVs) which allow for the resolution of sequences down to a single nucleotide difference while controlling errors using improved bioinformatic processing methods (Callahan et al., 2017). Unlike *de novo* OTUs, ASVs are consistently defined by the DNA sequence of the assayed organism (Callahan et al., 2017). This gives ASVs a consistent label that is not dataset dependent and will allow ASVs that are defined from different data sets to be compared (Callahan et al., 2017). There are a myriad of other benefits ASVs hold over more traditional OTU clustering methods. Inference of ASVs are performed on a per sample basis rather than per read basis like OTU clustering (Callahan et al., 2017). As such independent parallelization is easily performed, allowing computation time to scale linearly with increasing sample number, while simultaneously keeping memory requirements flat (Callahan et al., 2017). As with closed-reference OTUs, the consistent labels provided by ASV methods allows for sequence tables (i.e., a sample by sequence variant table providing counts of sequences assigned to an ASV on a per-sample basis) to be merged into a cross-study table. This facilitates analyses across different studies without the need to compile, pool, and reprocess data using *de novo* OTU methods (Callahan et al., 2017). This significantly decreases the time needed to perform meta-analyses for microbiome studies performed on similar environments. One output of microbiome research is using community data to develop diagnostic tools (Baxter et al., 2016). The dataset-dependent labels provided by *de novo* OTU methods exist only in that dataset, however, the consistent labels from closed-reference OTU or ASV methods

can be applied to new datasets to help generate predictive biomarkers through regression or machine learning (Callahan et al., 2017). While both closed-reference OTUs and ASVs utilize consistent labeling, closed-reference OTUs are inhibited by their limitations. Since closed-reference OTUs require the use of a reference database any sequences in the dataset that do not map to the reference database are removed from analysis (Callahan et al., 2017). Considering reference databases are incomplete, this could lead to a significant amount of sequences removed from the dataset, especially in understudied environments (Callahan et al., 2017). Additionally, closed-reference OTUs do not guarantee that the labeled sequence was observed, as these methods still use a similarity threshold to map the input dataset to the reference database, thus masking biological variation in the data (Callahan et al., 2017). Caution should be taken moving forward with this new analysis method, as there are inherent problems using short sequences to represent organisms (Callahan et al., 2017). For example, multiple sequence variants may exist within a single organism if multiple copies of the targeted gene region exist (Callahan et al., 2017). Despite these drawbacks ASVs still present as a more meaningful and applicable method of analyzing microbial community marker gene studies, and as such should become the common place method for these studies.

UNDERSTANDING PLANT COMPONENTS AND ASSOCIATED MICROBIOMES

The Phyllosphere

The phyllosphere is the above-ground component of the plant microbiome, which acts as the interface between the plant and the surrounding environment. Depending on

the plant of interest or the scope of the research, the phyllosphere can be dissected into many different parts, including but not limited to the caluosphere (stems) (Berg et al.; Velmourougane et al., 2006), anthosphere (flowers) (Berg et al., 2015; Rodríguez et al., 2001), carposphere (fruits) (Berg et al., 2015; Mosca et al., 2014), and the endosphere (internal portion of plant) (Berg et al., 2015; Compant et al., 2010). The phyllosphere is highly dynamic and may experience large changes in temperature, humidity, and solar radiation over the course of a single day (Turner et al., 2013). It stands to reason that with these extremes the microbial communities inhabiting the phyllosphere might consist of a small group of highly specialized to surviving harsh conditions (Rastogi et al., 2013).

Inherent variability in microbial populations and communities adds to intricacy of the phyllosphere (Vorholt, 2012). Both the environment and host plant play a major role in the microbial composition of the phyllosphere and variability of each is confounding attempts being made to observe and map the phyllosphere of plants (Vorholt, 2012). The same species of plants inhabiting different places may host vastly different microbial communities (Lindemann et al., 1984; Lindow and Andersen, 1996; Magan and Lacey, 1986). Similarly, plants of different species in the same location may have significantly different microbial populations in the phyllosphere (Kinkel et al., 1996; Lindemann et al., 1984; Lindow et al., 1978). A survey of bacterial communities encompassing the phyllosphere in the Atlantic Forest of Brazil found that the resident microorganisms greatly varied, even in the phyllosphere of trees of the same species, but they could be grouped together by discriminant analysis, a statistical analysis to predict a categorical dependent variable by one or more continuous variables (Lambais et al., 2006). Additionally, DGGE 16S rDNA banding patterns clustered based on plant species,

showing that these plants exhibit selection for their microbiome inhabitants (Yang et al., 2001). Delmotte and others (2009) used DGGE to determine that the phyllosphere bacterial communities of soybean (*Glycine max* (L.) Merr), clover (*Trifolium repens* L.) and *A. thaliana* were all predominantly composed of bacteria belonging to the Alphaproteobacteria, Betaproteobacteria, Gammaproteobacteria, Actinobacteria, and Bacteroides. However, by increasing the taxonomic resolution to the genus levels more nuanced differences appeared. For example, sequencing of excised bands from the DGGE acrylamide gel showed that within the Alphaproteobacteria, reads mapped to the genus *Methylobacterium* were highest in soybean and clover, while reads mapped to the genus *Sphingomonas* were highest in *Arabidopsis* (Delmotte et al. 2009). Additionally, within the Gammaproteobacteria, the genus *Pseudomonas* was consistently detected in clover, soybean and *Arabidopsis* (Delmotte et al., 2009).

The Rhizosphere

The rhizosphere is broadly defined as the interface between the plant and soil, or more precisely, the root and soil. While the phyllosphere is consistently exposed to weather fluctuations, the rhizosphere faces constant changes as a result of root exudation due to sloughing of root cells and mucilage deposition (Turner et al., 2013). These root exudates contain compounds such as amino acids, organic acids, sugars, fatty acids, vitamins, hormones and even antimicrobial compounds (Bais et al., 2006; Bertin et al., 2003). Consequently, root exudates are shown to be driving factors influencing the structure and abundance of the rhizosphere microbiome. Shi et al. (2011) added various root exudate compound solutions to microcosms of pasture soil and measured the response of the microbial communities using DGGE and a PhyloChip, a bacterial 16S

gene microarray for microbial surveys. Relative to sugars the presence of organic acids in the soil exhibited a larger influence on the bacterial communities. For example, 15 proteobacterial taxa were significantly affected by the addition of sugars, whereas 113-349 additional proteobacterial taxa were significantly affected by the addition either maleic acid, quinic acid, or lactic acid (Shi et al., 2011). In addition, soil physical and chemical properties have been shown to be determinants in the microbial community structure (Ranjard and Richaume, 2001). Using selective media to enumerate bacteria, fungi, Gram-negative bacteria, and actinomycetes, Kennedy and Smith (1995) observed higher microbial diversity in cultivated wheat (*Triticum aestivum* L.) fields than a prairie grassland. Adding to the intricacy of the rhizosphere microbiome, Richaume and others (1993) fractionated soil aggregates from an Alfisol, a leached basic or slightly acidic soil with a clay-enriched sub-soil, and enumerated the soil bacteria associated with differing soil particle sizes. With direct counts, staining bacteria with acridine orange, fixing on a 0.2 μ m membrane and enumerating, and indirect counts, plating of diluted soil suspensions onto non-selective nutrient agar, it was observed that the 2-20 μ m soil fraction contained the highest number of bacteria (Richaume et al., 1993). The 50-250 μ m contained the next largest counts of bacteria, with the 20-50 μ m, <2 μ m, and >250 μ m fractions all containing the lowest counts of bacteria (Richaume et al., 1993).

Plant genetics also appear to play a role in the development of the rhizosphere microbiome. Using culture-based methods, Elliot et al. (2004) observed turfgrass species as significant driver in variation for actinomycetes, heat-tolerant bacteria, fluorescent pseudomonads, and Gram-positive bacteria living in the rhizosphere. Similar results were found using both hierarchical clustering analysis and non-metric multi-dimensional

scaling, on T-RFLP and RISA data, showed distinct groups of bacterial communities associated with *A. thaliana* accessions (Micallef et al., 2009b). Moreover, Peiffer et al. (2013) found that even though the effects were small, maize genotype significantly impacted alpha and beta-diversities across different field environments.

In addition to the aforementioned factors that influence the rhizosphere microbiome, plant location may also be a significant factor. A study looking at rhizosphere samples taken from creeping bentgrass (*Agrostis stolonifera* L. cv. “Crenshaw”) putting greens located in North Carolina and Alabama and hybrid bermudagrass (*Cynodon dactylon* (L.) Pers. x *C. transvaalensis* Burt-Davy cv. “Tifdwarf”) putting greens located in Florida and South Carolina found that location was a significant factor explaining the variability in Gram-negative bacteria, aerobic bacteria, actinomycetes, heat-tolerant bacteria, and fluorescent pseudomonads (Elliot et al., 2004). Roots from a single European aspen (*Populus tremula* L.) tree in southern Estonia were observed to contain over 100 species of ectomycorrhizal fungi (Bahram et al., 2010). Overall geography and environmental factors impose strong effects on fungal community structure (Peay et al., 2016).

MICROBIOME OF GRAMINACEOUS HOSTS

There is evidence that members of the Gramineae family exhibit consistent associations with predominant microbes (Donn et al., 2015; Ofek et al., 2014; Johnston-Monje et al., 2016, Knief et al., 2012). In a study of the wheat rhizosphere of two widely grown wheat cultivars, ‘Janz’ and ‘H45’, Proteobacteria, Actinobacteria, and Bacteroidetes were found to be the predominant bacterial phyla (Donn et al., 2015).

Similarly, Johnston-Monje et al. (2016) observed the rhizosphere bacterial fraction of both 'Lenha' and 'BRS 1030' maize varieties to be predominantly comprised of Proteobacteria, Bacteroidetes, and Firmicutes when grown under variable soil types (i.e., iron mine subsoil vs rich, organic anthrosol). Another graminaceous host, rice (*Oryza sativa* L. cv. Angelica, IR-72, and PSB RC80), exhibited this same pattern of Proteobacteria prevalence in both the rhizosphere and phyllosphere, when analyzed through pyrosequencing (Knief et al., 2012). At a deeper taxonomic resolution, within the wheat rhizosphere the Gammaproteobacteria had the highest relative abundance, with Deltaproteobacteria, Alphaproteobacteria and Betaproteobacteria had the next highest relative abundances (Ofek et al., 2014). Based upon these findings it appears that graminaceous plants may consistently associate with Proteobacteria. However, additional research is needed to see if this pattern continues across other graminaceous plants, or even across the plant kingdom.

Management practices, such as organic, e.g., biological based pest management, or conventional, e.g., traditional chemical pest management, and specific management inputs, such as fertilizer and fungicides, have been evaluated for their impacts on the microbiome of graminaceous plants. Utilizing NGS technologies, Proteobacteria, Firmicutes, and Actinobacteria were found to be the predominant bacterial members in the microbiome of wheat grown under conventional and organic management regimes (Gdanetz and Trail, 2017). High rates of nitrogen fertilizer (50 mg N L⁻¹ to 160 mg N L⁻¹) were found to significantly increase bacterial abundance of maize (Zhu et al., 2016). Further analysis showed that increased N rates increased bacterial orders (e.g., Bacillales, Rhodocyclales, and Nitrosomonadales) and genes associated with various parts of the N

cycle (e.g., assimilatory nitrate reduction, nitrification, and denitrification) yet it was undetermined whether increased microbial abundance was due to high N rates, or to increased root exudate production (Zhu et al., 2016).

Looking at the fungal fraction of the microbiome, Gdanetz and Trail (2017) found Dothidiomycetes, Leotiomycetes, and Sordariomycetes to be the predominant classes regardless of whether they were subjected to organic or conventional management practices. Previous work utilizing clone libraries of ITS1/2 rDNA found Sordariomycetes and Leotiomycetes as predominant fungal classes in the wheat root microbiome (Kwaśna et al., 2010). Interestingly, a culture-based study on the wheat root microbiome, rhizosphere, and bulk soil of wheat grown in various production systems found patterns in the predominant members of the fungal fraction of the microbiome similar to the aforementioned studies (Lenc et al., 2015). Applications of various fungicides commonly used to control wheat pathogens decreased fungal community richness and evenness of wheat grown in two different climates in Sweden (Karlsson et al., 2014).

EXISTING KNOWLEDGE ON THE TURFGRASS MICROBIOME

Golf course putting greens, due to their high input management requirements and low organic content root zone, have been thought of as inhospitable for microorganisms (Hodges, 1990; Nunan et al., 2003; Ranjard et al., 2000). However, microbiome research is increasingly showing that turfgrasses host large and diverse microbial communities (Beirn et al., 2016; Crouch et al., 2017; Zhang et al., 2017). Through selective media culturing, it was found that levels of bacteria, fungi, and actinomycetes in the constructed rootzone of a creeping bentgrass putting green were found to be similar to levels found in

native soils (Mancino et al., 1993). In a similar study on creeping bentgrass putting greens, Karp and Nelson (2004) found that bacterial communities of sand-based rootzones showed higher overall diversity and more Gram-positive species. Utilizing several different selective media for enumerating colony forming units (CFUs) of bacteria, Gram-negative bacteria, fluorescent pseudomonads, fungi, actinomycetes, and *Bacillus* spp. in the rhizosphere of newly established putting greens, Bigelow et al. (2002) observed that microbial populations increased rapidly. For example, general bacteria had populations around 10^6 CFUs g^{-1} dry soil at the initial sampling date, and after six months the populations reached higher than 10^8 CFUs g^{-1} dry soil (Bigelow et al., 2002). Similarly, microbial diversity, examined through substrate use pattern and PLFA, was found to be similar in turfgrasses of varying age (e.g., 1, 6, 23, and 95-years old) and in a native pine rootzone (Yao et al., 2006). The only exception being that one- and six-year old stands of turfgrass were less diverse than the native pine and older stands of turfgrass at a depth of 5-15cm; authors attribute this to a lower amount of organic matter in the younger turfgrass stand (Yao et al., 2006). Roberts et al. (2017) isolated 228 bacteria across 32 genera from the foliage of creeping bentgrass over the course of four years. Beirn et al. (2016) used NGS to evaluate the bacterial and archaeal diversity of an annual bluegrass (*Poa annua* L.) putting green and found that even though the turf was subjected to intensive management for several years, the environment still hosted an extensive cohort of microorganisms, with 1.03×10^5 unique bacterial and archaeal OTUs observed (Beirn et al., 2016). Additionally, an Illumina sequencing analysis of the effect of perennial ryegrass establishment on soil bacterial and fungal communities revealed increased bacterial and fungal diversity in turfgrass soil than vacant land soil (Zhang et

al., 2017). While all but four bacterial phyla identified were shared between the turfgrass and vacant land soils, Acidobacteria and Proteobacteria were present at relative abundances 14.9% and 20.7%, respectively, higher in the turfgrass soil (Zhang et al., 2017).

While knowing that turfgrass can host large and diverse microbial communities is valuable, it is even more important to understand how management practices may impact these microbial communities. Although management practices applied to sports turf are aimed at increasing turf aesthetics and playability, it is possible that these practices are also influencing plant-associated microbial communities. Cole and Turgeon (1978) observed that eight-year old Kentucky bluegrass plots treated with the herbicide bandane contained significantly more bacteria than non-treated control plots, whereas no differences in bacterial populations were observed in plots treated with calcium arsenate. In a three-year study by Smiley and Craven (1979), 15 different pesticides were evaluated for their impacts on culturable microbial communities associated with a two-year-old stand of Kentucky bluegrass (*Poa pratensis* L.). Smiley and Craven observed that combination products (i.e., fungicide active ingredient mixtures) reduced fungal populations while increasing bacterial communities, whereas single active ingredient applications had minimal effects on both communities (1979). Similarly, in a culture-based study by Doherty et al. (2017), microbial communities inhabiting a creeping bentgrass putting green phyllosphere were resilient to repeated applications of five different fungicides. While there were some instances where individual microbial groups were affected by some fungicides, the overall impact over the growing season was very minimal (Doherty et al., 2017). Conversely, in a one-year old sand-based putting green, a DGGE analysis

of bacterial and fungal ribosomal RNA showed that chlorothalonil applications positively affected 5 bacterial clones, negatively affected 2 bacterial clones, and a rate dependent effect was seen on two fungal clones (Sigler and Turco, 2002).

Fertilizer applications are frequently made to highly maintained stands of turfgrass, e.g. golf courses and athletic fields, and as such there is interest in what these frequent nutrient applications do to the microbiome of turfgrass. When looking specifically at denitrifying bacteria, Mancino and Torello (1986) found that applications of a nitrate fertilizer did not increase the number of denitrifying bacteria in a five-year-old stand of Kentucky bluegrass. On a creeping bentgrass putting green, applications of both water-insoluble and water-soluble N increased counts of fungi in the soil compared to a non-fertilized control, however, the water-soluble source increased fungal counts more (Mancino et al., 1993). Additional research is needed to fully understand what selections turfgrass management practices are imposing on the microbiome.

SUMMARY AND THESIS OVERVIEW

Microbiomes are an ever-increasing area of interest for scientific research, and the methods used for their investigation are rapidly evolving. Early microbiome research was limited to culture-based methods, which may severely limit the community of microorganisms that researchers are able to detect. This became an acknowledged limitation of culture-based work and researchers began working on new techniques for investigating microbial communities. The currently favored method of investigating the microbiome of a host is next-generation sequencing, which allows for simultaneous high-throughput sequencing of PCR amplicons from DNA regions of interest. This method is

capable of generating millions of reads from multiple samples, from various organismal groups so long as primers are sufficiently developed. New techniques have also provided the ability to simultaneously sequence samples (i.e., multiplexing), which exponentially increases the amount of data collected and provides researchers more statistical power in their analyses.

Turfgrass settings such as golf courses or athletic fields tend to have high management inputs, with fertilizers and pesticides being frequently applied to maximize turfgrass aesthetics and playability. Previous research has provided some insight as to how these management practices may impact the turfgrass microbiome. However, these studies have typically been limited to culture-based methods, which may have provided under-representations of the resident microbial populations. Since much of the microbiome is not culturable using currently available technologies are we missing off-target effects of management inputs on the resident microbiota? Or are these culturable constituents of the microbiome an adequate representation of the effects that management inputs have on the microbiome?

While it is important to thoroughly understand how management practices impact the turfgrass microbiome, baseline knowledge regarding the microbiota that compose the microbiome is needed. By gaining deeper insight into the constituents of the turfgrass microbiome, we may discover novel microbes that can be utilized to promote plant health or combat turfgrass pathogens. The temporal patterns in microbial communities may be important as well. As turfgrass systems age, do they select for a microbiome that is primed for increasing turf vigor? Do pathogens have a threshold they need to pass in

order to cause disease? Can pathogens be mitigated by encouraging other microbiota during seasonal fluctuations?

This thesis sets out to answer two basic questions regarding the turfgrass microbiome. How do resident seed and soil microbiota influence the juvenile creeping bentgrass microbiome? What are the temporal fluctuations in the turfgrass microbiome from seedling emergence through six months? Understanding these basic metrics of the turfgrass microbiome will create a foundation for future work utilizing NGS technologies. Moreover, gaining a better understanding of how the microbiome forms immediately following germination will provide a baseline for the next steps in harvesting the turfgrass microbiome for our own benefit as turfgrass managers.

LITERATURE CITED

- Amann, R.L., W. Ludwig and K.H. Schleifer. 1995. Phylogenetic identification and in situ detection of individual microbial cells without cultivation. *Microbiol. Mol. Biol. Rev.* 59: 143-169.
- American Phytopathological Society. 2016. *Phytobiomes: A roadmap for research and translation*. American Phytopathological Society, St. Paul, MN.
- Andrews, J.H. 1992. Biological control in the phyllosphere. *Annu. Rev. Phytopathol.* 30: 603-635.
- Arias, M.E., J.A. Gonzalez-Perez, F.J. Gonzalez-Vila and A.S. Ball. 2005. Soil health -- a new challenge for microbiologists and chemists. *Int. Microbiol.* 8: 13-21.
- Bais, H.P., T.L. Weir, L.G. Perry, S. Gilroy and J.M. Vivanco. 2006. The role of root exudates in rhizosphere interactions with plants and other organisms. *Annu. Rev. Plant Biol.* 57: 233-266.
- Bahram, M., S. Polme, U. Koljalg and L. Tedersoo. 2010. A single European aspen (*Populus tremula*) tree individual may potentially harbour dozens of *Cenococcum geophilium* ITS genotypes and hundreds of species of ectomycorrhizal fungi. *FEMS Microbiol. Ecol.* 75: 2.
- Baxter, N.T., M.T. Ruffin, M.A. Rogers, and P.D. Schloss. 2016. Microbiota-based model improves the sensitivity of fecal immunochemical test for detecting colonic lesions. *Genome Med.* 8: 37. doi:10.1186/s13073-106-0290-3.
- Beirn, L.A., J.W. Hempfling, C.J. Schmid, J.A. Murphy, B.B. Clarke and J.A. Crouch. 2016. Differences among soil-inhabiting microbial communities in *Poa annua* turf throughout the growing season. *Crop Sci.* doi:10.2135/cropsci2016.06.0463.
- Berg, G. and K. Smalla. 2009. Plant species and soil type cooperatively shape the structure and function of microbial communities in the rhizosphere. *FEMS Microbiol. Ecol.* 68: 1-13. doi:10.1111/j.1574-6941.2009.00654.x.
- Berg, G., A. Erlacher and M. Grube. 2015. The edible plant microbiome: Importance and health issues. In: B. Lugtenberg, editor *Principles of plant-microbe interactions: Microbes for sustainable agriculture*. Springer International Publishing, Cham. p. 419-426.
- Bertin, C., X.H. Yang and L.A. Weston. 2003. The role of root exudates and allelochemicals in the rhizosphere. *Plant Soil* 256: 67-83.
- Bigelow, C.A., D.C. Bowman and A.G. Wollum. 2002. Characterization of soil microbial population dynamics in newly constructed sand-based rootzones. *Crop Sci.* 42: 1611-1614. doi:10.2135/cropsci2002.1611.

- Callahan, B.J., P.J. McMurdie and S.P. Holmes. 2017. Exact sequence variants should replace operational taxonomic units in marker-gene data analysis. *ISME J.* 11: 2639. doi:10.1038/ismej.2017.119.
- Cole, M.A. and A.J. Turgeon. 1978. Microbial activity in soil and litter underlying bandane- and calcium arsenate-treated turfgrass. *Soil Biol. Biochem.* 10: 181-186. doi:https://doi.org/10.1016/0038-0717(78)90094-9.
- Coleman-Derr, D., D. Desgarenes, C. Fonseca-Garcia, S. Gross, S. Clingenpeel, T. Woyke, et al. 2016. Plant compartment and biogeography affect microbiome composition in cultivated and native agave species. *New Phytol.* 209: 798-811. doi:10.1111/nph.13697.
- Compant, S., C. Clément and A. Sessitsch. 2010. Plant growth-promoting bacteria in the rhizo- and endosphere of plants: Their role, colonization, mechanisms involved and prospects for utilization. *Soil Biol. Biochem.* 42: 669-678. doi:http://dx.doi.org/10.1016/j.soilbio.2009.11.024.
- de Souza, R.S.C., V.K. Okura, J.S.L. Armanhi, B. Jorrin, N. Lozano, M.J. da Silva, et al. 2016. Unlocking the bacterial and fungal communities assemblages of sugarcane microbiome. *Sci. Rep.* 6: 28774. doi:10.1038/srep28774.
- Delmotte, N., C. Knief, S. Chaffron, G. Innerebner, B. Roschitzki, R. Schlapbach, et al. 2009. Community proteogenomics reveals insights into the physiology of phyllosphere bacteria. *PNAS* 106: 16428-16433.
- Doherty, J.R., M. Botti-Marino, J.P. Kerns, D.F. Ritchie and J.A. Roberts. 2017. Response of microbial populations on the creeping bentgrass phyllosphere to periodic fungicide applications. *Plant Health Prog.* 18: 44-49. doi:http://dx.doi.org/10.1094/PHP-RS-16-0067.
- Donn, S., J.A. Kirkegaard, G. Perera, A.E. Richardson and M. Watt. 2015. Evolution of bacterial communities in the wheat crop rhizosphere. *Environ. Microbiol.* 17: 610-621. doi:10.1111/1462-2920.12452.
- Elliot, M.L., E.A. Guertal and S. H.D. 2004. Rhizosphere bacterial population flux in golf course putting greens in the southeastern united states. *HortScience* 39: 1754-1758.
- Fisher, M.M., and E.W. Triplett. 1999. Automated approach for ribosomal intergenic spacer analysis of microbial diversity and its application for freshwater bacterial communities. *Appl. Environ. Microbiol.* 65: 4630-4636.
- Frostegard, A., A. Tunlid and E. Baath. 2010. Use and misuse of PLFA measurements in soils. *Soil Biol. Biochem.* doi:10.1016/j.soilbio.2010.11.021.

- Gdanetz, K. and F. Trail. 2017. The wheat microbiome under four management strategies, and potential for endophytes in disease protection. *Phytobiomes* 1: 158-168. doi:10.1094/PBIOMES-05-17-0023-R.
- Gilbert, J.A., F. Meyer, J. Jansson, J. Gordon, N. Pace, J. Tiedje, et al. 2010. The earth microbiome project: Meeting report of the “1st EMP meeting on sample selection and acquisition” at Argonne National Laboratory October 6th 2010. *Stand. Genomic Sci.* 3: 249-253. doi:10.4056/aigs.1443528.
- Hodges, C.F. 1990. The microbiology of non-pathogens and minor root pathogens in high sand content greens. *GCM* 48: 60-75.
- Jenkins, J.R., M. Viger, E.C. Arnold, Z.M. Harris, M. Ventura, F. Miglietta, et al. 2017. Biochar alters the soil microbiome and soil function: Results of next-generation amplicon sequencing across europe. *GCB Bioenergy* 9: 591-612. doi:doi:10.1111/gcbb.12371.
- Johnston-Monje, D., D.S. Lundberg, G. Lazarovits, V.M. Reis and M.N. Raizada. 2016. Bacterial populations in juvenile maize rhizospheres originate from both seed and soil. *Plant Soil* 405: 337-355. doi:10.1007/s11104-016-2826-0.
- Karlsson, I., H. Friberg, C. Steinberg and P. Persson. 2014. Fungicide effects on fungal community composition in the wheat phyllosphere. *PLOS ONE* 9: e111786. doi:10.1371/journal.pone.0111786.
- Karp, M.A. and E.B. Nelson. 2004. Bacterial communities associated with creeping bentgrass in soil and sand rootzones. *USGA Turf Environ. Res. Online* 3: 1-19.
- Kennedy, A.C. and K.L. Smith. 1995. Soil microbial diversity and the sustainability of agricultural soils. *Plant Soil* 170: 75-86. doi:10.1007/bf02183056.
- Kinkel, L.L., M. Wilson and S.E. Lindow. 1996. Utility of microcosm studies for predicting phylloplane bacterium population sizes in the field. *Appl. Environ. Microbiol.* 62: 3413-3423.
- Knief, C., N. Delmotte, S. Chaffron, M. Stark, G. Innerebner, R. Wassmann, et al. 2012. Metaproteogenomic analysis of microbial communities in the phyllosphere and rhizosphere of rice. *ISME J.* 6: 1378-1390. doi:10.1038/ismej.2011.192.
- Koljalg, U., R.H. Nilsson, K. Abarenkov, L. Tedersoo, A.F. Taylor, M. Bahram, et al. 2013. Towards a unified paradigm for sequence-based identification of fungi. *Mol. Ecol.* 22: 5271-5277. doi:10.1111/mec.12481.

- Kopylova, E., J.A. Navas-Molina, C. Mercier, Z.Z. Xu, F. Mahé, Y. He, et al. 2016. Open-source sequence clustering methods improve the state of the art. *mSystems* 1. doi:10.1128/mSystems.00003-15.
- Kozdrój, J. and J.D. van Elsas. 2000. Application of polymerase chain reaction-denaturing gradient gel electrophoresis for comparison of direct and indirect extraction methods of soil DNA used for microbial community fingerprinting. *Biology Fertil. Soils* 31: 372-378. doi:10.1007/s003749900182.
- Kwaśna, H., G.L. Bateman and E. Ward. 2010. Microbiota in wheat roots evaluated by cloning of its 1/2 rDNA and sequencing. *J. Phytopathol.* 158: 278-287. doi:10.1111/j.1439-0434.2009.01595.x.
- Lambais, M.R., D.E. Crowley, J.C. Cury, R.C. Büll and R.R. Rodrigues. 2006. Bacterial diversity in tree canopies of the Atlantic forest. *Science* 312: 1917-1917. doi:10.1126/science.1124696.
- Lenc, L., H. Kwaśna, C. Sadowski and A. Grabowski. 2015. Microbiota in wheat roots, rhizosphere and soil in crops grown in organic and other production systems. *J. Phytopathol.* 163: 245-263. doi:10.1111/jph.12313.
- Lindemann, J., D.C. Arny and C.D. Upper. 1984. Epiphytic populations of *Pseudomonas syringae* pv *syringae* on snap bean and nonhost plants and the incidence of bacterial brown spot disease in relation to cropping patterns. *Phytopathology* 74: 1329-1333.
- Lindow, S.E. and G.L. Andersen. 1996. Influence of immigration on epiphytic bacterial populations on navel orange leaves. *Appl. Environ. Microbiol.* 36: 2978-2987.
- Lindow, S.E., D.C. Arny and C.D. Upper. 1978. Distribution of ice nucleation-active bacteria on plants in nature. *Appl. Environ. Microbiol.* 36: 831-838.
- Lo, C.T., E.B. Nelson and G.E. Harman. 1997. Improved biocontrol efficacy of *Trichoderma harzianum* 1295-22 for foliar phases of turf diseases by use of spray applications. *Plant Dis.* 81: 1132-1138. doi:10.1094/PDIS.1997.81.10.1132.
- Luna, G.M., A. Dell'Anno and R. Danovaro. 2006. DNA extraction procedure: A critical issue for bacterial diversity assessment in marine sediments. *Environ. Microbiol.* 8: 308-320.
- Magan, N. and J. Lacey. 1986. The phyllosphere microflora of ripening wheat and effect of late fungicide applications. *Ann. Appl. Biol.* 109: 117-128.
- Mancino, C.F., M. Barakat and A. Maricic. 1993. Soil and thatch microbial populations in an 80% sand : 20% peat creeping bentgrass putting green. *HortScience* 28: 189-191.

- Marchesi, J.R. and J. Ravel. 2015. The vocabulary of microbiome research: A proposal. *Microbiome* 3: 31. doi:10.1186/s40168-015-0094-5.
- Marsh, T.L. 1999. Terminal restriction fragment length polymorphism (T-RFLP): An emerging method for characterizing diversity among homologous populations of amplification products. *Curr. Opin. Microbiol.* 2: 323-327. doi:https://doi.org/10.1016/S1369-5274(99)80056-3.
- Micallef, S.A., S. Channer, M.P. Shiaris and A. Colon-Carmona. 2009a. Plant age and genotype impact the progression of bacterial community succession in the *Arabidopsis* rhizosphere. *Plant Signaling Behav.* 4: 777-780. doi:10.1093/jxb/erp053.
- Micallef, S.A., M.P. Shiaris and A. Colon-Carmona. 2009b. Influence of *Arabidopsis thaliana* accessions on rhizobacterial communities and natural variation in root exudates. *J. Exp. Bot.* 60: 1729-1742. doi:10.1093/jxb/erp053.
- Mosca, S., M.G. Li Destri Nicosia, S.O. Cacciola and L. Schena. 2014. Molecular analysis of colletotrichum species in the carposphere and phyllosphere of olive. *PLoS ONE* 9: e114031. doi:10.1371/journal.pone.0114031.
- Muyzer, G. and K. Smalla. 1998. Application of denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) in microbial ecology. *Antonie van Leeuwenhoek* 73: 127-141. doi:10.1023/a:1000669317571.
- Nettles, R., J. Watkins, K. Ricks, M. Boyer, M. Licht, L.W. Atwood, et al. 2016. Influence of pesticide seed treatments on rhizosphere fungal and bacterial communities and leaf fungal endophyte communities in maize and soybean. *Applied Soil Ecology* 102: 61-69. doi:https://doi.org/10.1016/j.apsoil.2016.02.008.
- Nunan, N., K. Wu, I.M. Young, J.W. Crawford and K. Ritz. 2003. Spatial distribution of bacterial communities and their relationships with the micro-architecture of soil. *FEMS Microbiol. Ecol.* 44: 203-215. doi:10.1016/S0168-6496(03)00027-8.
- Ofek, M., M. Voronov-Goldman, Y. Hadar and D. Minz. 2014. Host signature effect on plant root-associated microbiomes revealed through analyses of resident vs. active communities. *Environ. Microbiol.* 16: 2157-2167. doi:10.1111/1462-2920.12228.
- Peay, K.G., P.G. Kennedy and J.M. Talbot. 2016. Dimensions of biodiversity in the earth mycobiome. *Nat. Rev. Microbiol.* 14: 434. doi:10.1038/nrmicro.2016.59.
- Peiffer, J.A., A. Spor, O. Koren, Z. Jin, S.G. Tringe, J.L. Dangl, et al. 2013. Diversity and heritability of the maize rhizosphere microbiome under field conditions. *PNAS* 110: 6548-6553. doi:10.1073/pnas.1302837110.

- Phytobiomes Initiative. 2015. What is the phytobiome?
<http://www.phytobiomes.org/about/Pages/What-is-the-Phytobiome.aspx>
- Prosser, J.I. 2002. Molecular and functional diversity in soil micro-organisms. *Plant Soil* 244: 9-17. doi:10.1023/a:1020208100281.
- Ranjard, L., F. Poly, J. Combrisson, A. Richaume, F. Gourbière, J. Thioulouse, et al. 2000. Heterogeneous cell density and genetic structure of bacterial pools associated with various soil microenvironments as determined by enumeration and DNA fingerprinting approach (RISA). *Microb. Ecol.* 39: 263-272. doi:10.1007/s002480000032.
- Ranjard, L. and A. Richaume. 2001. Quantitative and qualitative microscale distribution of bacteria in soil. *Res. Microbiol.* 152: 707-716. doi:[http://dx.doi.org/10.1016/S0923-2508\(01\)01251-7](http://dx.doi.org/10.1016/S0923-2508(01)01251-7).
- Rastogi, G., G.L. Coaker and J.H.J. Leveau. 2013. New insights into the structure and function of phyllosphere microbiota through high- throughput molecular approaches. *FEMS Microbiol. Lett.* 348: 1-10. doi:doi:10.1111/1574-6968.12225.
- Rastogi, G., J.J. Tech, G.L. Coaker and J.H.J. Leveau. 2010. A PCR-based toolbox for the culture-independent quantification of total bacterial abundances in plant environments. *J. Microbiol. Methods* 83: 127-132. doi:<http://dx.doi.org/10.1016/j.mimet.2010.08.006>.
- Richaume, A., C. Steinberg, L. Jocteur-Monrozier and G. Faurie. 1993. Differences between direct and indirect enumeration of soil bacteria: The influence of soil structure and cell location. *Soil Biol. Biochem.* 25: 641-643.
- Roberts, J.A., B. Ma, L.P. Tredway, D.F. Ritchie and J.P. Kerns. 2017. Identification and pathogenicity of bacteria associated with etiolation and decline of creeping bentgrass golf course putting greens. *Phytopathology* 108: 23-30. doi:10.1094/PHYTO-01-17-0015-R.
- Rodríguez, M.A., N. Venedikian and A. Godeas. 2001. Fungal populations on sunflower (*Helianthus annuus*) anthosphere and their relation to susceptibility or tolerance to *Sclerotinia sclerotiorum* attack. *Mycopathologia* 150: 143-150. doi:10.1023/a:1010994317980.
- Rodriguez, R.J., J.F. White Jr, A.E. Arnold and R.S. Redman. 2009. Fungal endophytes: Diversity and functional roles. *New Phytol.* 182: 314-330. doi:10.1111/j.1469-8137.2009.02773.x.

- Saleh-Lakha, S., M. Miller, R.G. Campbell, K. Schneider, P. Elahimanesh, M.M. Hart, et al. 2005. Microbial gene expression in soil: Methods, applications and challenges. *J. Microbiol. Methods* 63: 1-19. doi:<http://doi.org/10.1016/j.mimet.2005.03.007>.
- Schoug, A., J. Fischer, H.J. Heipieper, J. Schnurer and S. Hakansson. 2008. Impact of fermentation pH and temperature on freeze-drying survival and membrane lipid composition of *Lactobacillus coryniformis* si3. *J. Ind. Microbiol. Biotechnol.* 35: 175-181.
- Shi, S.J., A.E. Richardson, M. O'Callaghan, K.M. DeAngelis, E.E. Jones, A. Stewart, et al. 2011. Effects of selected root exudate components on soil bacterial communities. *FEMS Microbiol. Ecol.* 77: 600.
- Sigler, W.V. and R.F. Turco. 2002. The impact of chlorothalonil application on soil bacterial and fungal populations as assessed by denaturing gradient gel electrophoresis. *Applied Soil Ecology* 21: 107-118. doi:[https://doi.org/10.1016/S0929-1393\(02\)00088-4](https://doi.org/10.1016/S0929-1393(02)00088-4).
- Smiley, R.W. and M.M. Craven. 1979. Microflora of turfgrass treated with fungicides. *Soil Biol. Biochem.* 11: 349-353. doi:[https://doi.org/10.1016/0038-0717\(79\)90047-6](https://doi.org/10.1016/0038-0717(79)90047-6).
- Torsvik, V. and L. Øvreås. 2002. Microbial diversity and function in soil: From genes to ecosystems. *Curr. Opin. Microbiol.* 5: 240-245. doi:[https://doi.org/10.1016/S1369-5274\(02\)00324-7](https://doi.org/10.1016/S1369-5274(02)00324-7).
- Torsvik, V., K. Salte, R. Sørheim and J. Goksøyr. 1990. Comparison of phenotypic diversity and DNA heterogeneity in a population of soil bacteria. *Appl. Environ. Microbiol.* 56: 776-781.
- Turnbaugh, P.J., R.E. Ley, M. Hamady, C. Fraser-Liggett, R. Knight and J.I. Gordon. 2007. The human microbiome project: Exploring the microbial part of ourselves in a changing world. *Nature* 449: 804.
- Turner, T.R., E.K. James and P.S. Poole. 2013. The plant microbiome. *Genome Biol.* 14: 209-219.
- van der Heijden, M.G., R.D. Bardgett and N.M. van Straalen. 2008. The unseen majority: Soil microbes as drivers of plant diversity and productivity in terrestrial ecosystems. *Ecology Letters* 11: 296-310. doi:[10.1111/j.1461-0248.2007.01139.x](https://doi.org/10.1111/j.1461-0248.2007.01139.x).
- Velmourougane, K., D.P. Kumari, C.B. Prakasan and Jayarama. 2006. Microbial diversity of popular arabica and robusta selections. *Indian Coffee* 70: 27-31.

- von Wintzingerode, F., U.B. Gobel and E. Stackebrandt. 1997. Determination of microbial diversity in environmental samples: Pitfalls of PCR-based rRNA analysis. *FEMS Microbiol. Rev.* 21: 213-229.
- Vorholt, J.A. 2012. Microbial life in the phyllosphere. *Nat Rev Microbiol* 10: 828-840. doi:10.1038/nrmicro2910.
- Westcott, S.L. and P.D. Schloss. 2015. *De novo* clustering methods outperform reference-based methods for assigning 16s rRNA gene sequences to operational taxonomic units. *PeerJ* 3: e1487. doi:10.7717/peerj.1487.
- White, D.C. 1983. Analysis of microorganisms in terms of quantity and activity in natural environments. In: Slater, J.H., Whittenbury, R., Wimpenny, J.W.T. (eds.), *microbes in their natural environments*. Society of General Microbiology Symposium 34: 37-66.
- Yang, C.-H., D.E. Crowley, J. Borneman and N.T. Keen. 2001. Microbial phyllosphere populations are more complex than previously realized. *PNAS USA* 98: 3889-3894.
- Yao, H., D. Bowman and W. Shi. 2006. Soil microbial community structure and diversity in a turfgrass chronosequence: Land-use change versus turfgrass management. *Applied Soil Ecology* 34: 209-218. doi:http://dx.doi.org/10.1016/j.apsoil.2006.01.009.
- Zhang, W., Z. Zhang, Y. Wang, D. Ai, P. Wen, Y. Zhu, et al. 2017. Effect of turfgrass establishment on soil microbiota using illumina sequencing. *Nat., Environ. Pollut. Technol.* 16: 679-686.
- Zhu, S., J.M. Vivanco and D.K. Manter. 2016. Nitrogen fertilizer rate affects root exudation, the rhizosphere microbiome and nitrogen-use-efficiency of maize. *Applied Soil Ecology* 107: 324-333. doi:https://doi.org/10.1016/j.apsoil.2016.07.009.

Chapter 2. Elucidating the Influence of Resident Seed and Soil Microbiota on the Developing Creeping Bentgrass Microbiome

ABSTRACT

Manipulation of the plant microbiome holds the potential to increase plant health and reduce disease by fostering relationships between plants and beneficial microorganisms. However, we are limited in our knowledge in the members of the microbiome and their population dynamics. This project was developed to gain a fundamental understanding of the influence of resident microflora inhabiting seed and soil exhibit on the juvenile microbiome, and how these communities evolve and mature following seedling emergence. Utilizing a randomized-complete block experimental design creeping bentgrass (*Agrostis stolonifera* L. cv. "007") seeds were planted into sterilized or non-sterilized soil, and grown under sterile conditions in a laminar flow hood for six weeks. Samples of foliage and roots were taken at emergence, and at two, four, and six weeks post-emergence, and soil samples were taken at the end of the experiment. Environmental DNA was extracted, and used to generate PCR amplicons with bacterial 16S and fungal ITS primers. Amplicons were sequenced on the Illumina MiSeq, and sequences were analyzed with the DADA2, phyloseq, and vegan packages in R. Sequencing runs generated 3.32×10^7 reads, which filtered into 2,576 bacterial and 303 fungal amplicon sequence variants (ASVs). Proteobacteria and Eurotiomycetes dominated the bacterial and fungal ASVs, respectively. Bacterial alpha-diversity was lowest at emergence, and no significant changes were observed in fungal alpha-diversity. Taxonomic profiling revealed introduction of two genera, *Sphingomonas* and *Pseudomonas*, from the seed used at planting. These data show consistent microbiome

recruitment despite different soil treatments, highlighting the influence of plant host on the microbiome.

INTRODUCTION

Plant associated microbial communities, herein referred to as the plant microbiome, are complex entities that are intimately involved in many processes that directly impact plants. Some of these processes include pathogenesis, nutrient availability and cycling within the soil, as well as the activation of plant defenses (Arias et al., 2005; Rodriguez et al., 2009; van der Heijden et al., 2008). One area of considerable interest is the manipulation of the microbiome to reduce agronomic inputs and improve plant health (Bakker et al., 2012). To date there have been few successful examples of microbiome manipulations employed to modify plant health. Mendes and others observed that the whole soil microbiome in fields of sugar beets, *Beta vulgaris* L. cv. “Alligator”, is responsible for reducing crown and root rot incidence caused by *Rhizoctonia solani* Kühn and demonstrated that this microbiome-mediated suppression is transferrable to susceptible soils (Mendes et al., 2011). The soil microbiomes selected for by early- or late-flowering genotypes of *Arabidopsis thaliana* L. were used to inoculate soil used to grow three different genotypes of *A. thaliana* (Ler, Be, RLD) and *Brassica rapa* (Panke-Buisse et al., 2014). The Be and RLD genotypes of *A. thaliana* and *B. rapa* exhibited shifts to early- or late-flowering based on the source of the microbiome used as inoculation (Panke-Buisse et al., 2014). Recently a bacterial endosymbiont of *Rhizoctonia solani* AG 2-2IIIB was discovered that enhanced the virulence of the pathogen when inoculated onto creeping bentgrass (Obasa et al., 2017).

Understanding the driving factors behind microbiome recruitment is essential if successful and reproducible microbiome manipulation is ever to be achieved. Plant compartment, host genetics, geographic location, season, and management practices, have all been shown to influence the plant microbiome to varying degrees and have complicated research efforts (Edwards et al., 2015; Gdanetz and Trail, 2017; Lenc et al., 2015; Lundberg et al., 2012; Mendes et al., 2014; Peiffer et al., 2013). For example, Gdanetz and Trail (2017) found that wheat (*Triticum aestivum* L. cv. “25R39”) microbiomes grown under organic or conventional practices were similar. However, Lenc et al. (2015) did find significant differences in the winter wheat (*T. aestivum* L. cv. “Zyta”) root, rhizosphere, and bulk soil microbiomes under organic, integrated, conventional, and monoculture management strategies. However, within each management strategy the crop rotations differed, which may have been an influencing factor on the microbial community composition. Environmental changes are also shown to impact microbial communities. Beirn et al. (2016) documented significant differences in the total archaeal and bacterial communities inhabiting the soil of a *Poa annua* L. putting green over the course of one year (11 June 2014 through 3 June 2015). Seasonal variations were also observed in the microbial biomass of both warm- and cool-season turfgrass soils, with lower biomass observed in September while higher biomass was observed in May and December (Yao et al., 2011). In their analysis, the authors speculated that seasonal differences were driven by competition for nitrogen between microbes and turfgrass (Yao et al., 2011), yet additional research is needed to better define the details of these plant-microbes interactions.

Niches, or the fit of a species living under specific environmental conditions (Pocheville, 2015), may be an additional driving factor in community assemblages. Niche theory suggests that variation in communities allows for partitioning of limited resources between competing organisms, fundamentally based upon the ecological traits differ among species within a community (Leibold and McPeck, 2006), and predict that changes in the composition of a community, microbial or otherwise, are driven by changes in environmental variables (Jongman et al., 1995). When competing for multiple limited resources different organisms have different competitive capabilities for each resource, thus resulting in multiple species coexisting through utilization of these resources (Tilman, 1982; Tilman and Pacala, 1993).

Until recent years, previous research of the turfgrass microbiome has been limited to either culture-based techniques (Bigelow et al., 2002; Buck and Burpee, 2002; Cole and Turgeon, 1978; Mancino et al., 1993) or community fingerprinting techniques (Bartlett et al., 2008; Steer and Harris, 2000). While these studies provide valuable insight into the turfgrass microbiome, culture-based methods may only capture about 0.1-1.0% of the total microbial community (Amann et al., 1995; Torsvik and Øvreås, 2002). With community fingerprinting techniques, such as phospholipid fatty acid analysis (PLFA), taxonomic inference can be problematic, with errors sometimes stemming from biomarkers that may not always be exclusive to an organism (Frostegard et al., 2010). For example, the PLFA markers *cys17:0* and *cy19:0* are typically associated with Gram-negative bacteria, however these markers can be found in large quantities in a number of Gram-positive bacteria (Schoug et al., 2008).

Successful implementation and lowered costs associated with next-generation sequencing (NGS) technologies has increased accessibility to characterize entire microbial communities. To date, published research utilizing NGS approaches to characterize the microbiome of turfgrass is limited to work by Beirn and others (2016), Crouch et al (2017) and Zhang et al. (2017). As a result, our understanding of the developing turfgrass microbiome is still quite limited. While previous research illustrates the significance of environmental factors on microbial composition, it is also likely that species and possible cultivar can have a significant impact as well. One goal of microbiome research is to harness beneficial properties that can be exploited through management. In plants, this may be reducing water, nutrient inputs, or even disease incidence. But to develop management tools that harness the microbiome, it is essential to understand the community as it develops with the plant. Therefore, this project was designed to determine 1) the influence that the microbiota inhabiting seed and soil exhibit on the developing creeping bentgrass (*Agrostis stolonifera* L. cv. “007”) microbiome and 2) how the juvenile creeping bentgrass microbiome develops immediately following emergence from soil. Identifying specific microbial groups that are associated with seed and the developing creeping bentgrass microbiome may provide an opportunity to influence the mature microbiome before final community niches are filled.

MATERIALS & METHODS

Sterile Growth Conditions. A sterile air growth environment was established using modified methods developed from Henry et al. (2006). Briefly, creeping bentgrass plants were established from seed in a sterile air environment through the use of a

laminar flow hood (Purifier™ Clean Bench, Labconco, Kansas City, MO) utilized as a growth chamber for the experiment. Lighting was provided on a 12-hour day-night cycle using fluorescent bulb lighting. The experiment was performed in a central room with minimal airflow to minimize potential contaminants in the experiment. Temperature was maintained at 23° C throughout the trial.

Growth medium was established as a uniform mixture of 85% sand and 15% sphagnum peat moss (sieved) to mimic specifications for putting green rootzone construction (USGA, 2018), hereafter referred to as the soil. Sand was obtained from Egypt Farms White Marsh, MD, and peat was obtained from Premier Horticulture, Inc., Quakertown, PA. For drainage of water, 50 ml polypropylene conical tubes (VWR, Radnor, PA) were modified by drilling a 6 mm hole in the bottom, and then autoclaved. The autoclaved modified conical tubes were filled to the top with soil.

Our trial imposed two treatments, sterilized (i.e., autoclaved) and non-sterilized soil. The sterilized soil treatment was achieved by autoclaving the soil filled conical tubes at 121° C for 30 minutes, three separate times, with 24 hours between each cycle. Sterility was confirmed by collecting water flow through and plating on multiple media in a Labconco Purifier Biosafety Cabinet (Labconco). After autoclaving, 60 ml autoclaved DI H₂O was run through the soil filled conical tubes. Ten µl for the flow through was plated onto four different selective media using a flame sterilized bent glass rod: actinomycete isolation agar (VWR), acidified potato dextrose agar (pH 4.5) (Becton, Dickinson, and Co., Franklin Lakes, NJ), nutrient agar (Becton, Dickinson, and Co.) + 1% sucrose, and King's B medium (Becton, Dickinson, and Co.), to confirm that soil was sterilized. An additional plate of each media was plated with the same autoclaved DI

H₂O used to run through the conical tubes to serve as a control for the sterility confirmation.

Four replicates of each treatment (i.e., autoclaved vs. non-autoclaved soil) were placed into the laminar flow hood in a completely randomized design. Using flame-sterilized forceps, 25 seeds of creeping bentgrass (Seed Research of Oregon, Tangent, OR) were planted into the four replicate autoclaved and non-autoclaved soil treatments. All plants were irrigated daily with 6 ml of autoclaved DI H₂O, and 6 ml of sterilized half-strength Hoagland's solution (Hoagland and Arnon, 1950), made in the lab, was applied twice per week. All irrigation events were delivered with a sterile 25 mL luer-slip syringe (Beckton, Dickinson and Co.). The entire experiment was repeated a second to generate additional data.

Sampling and eDNA Extraction. Using sterilized forceps, foliage and root samples were taken at initial seedling emergence, and at two, four and six weeks post-emergence. Foliage and roots were separated at the chlorophyll line and placed into individual sterile coin envelopes. Sterile 1.5 ml microcentrifuge tubes (Fisher Scientific, Pittsburgh, PA) were filled to the 1.5 ml line with bulk soil from each replicate at the conclusion of each experimental run. Seeds used at planting were also sampled for microbial analysis. Immediately after collections were performed, all samples were maintained at -20° C (-4° F) to preserve microbial populations present at the time of sampling.

Environmental DNA (eDNA), which is DNA extracted from a whole environmental sample as opposed to a single organism, was extracted from all samples using commercially available kits. Foliar and seed samples were extracted with the

DNeasy Plant Mini Kit (Qiagen, Gaithersburg, MD). The DNeasy PowerSoil Kit (Qiagen, Gaithersburg, MD) was used for eDNA extraction from soil and root samples. Plant eDNA extractions consisted of an overnight incubation in 400 μ l solution AP1, solution contained in the kit, at 65° C. Following incubation, 2 microcentrifuge tube capfuls of 450 to 600- μ m glass beads (Acros Organics, Morris Plains, NJ) were added and three runs on a FastPrep-24 (MP Biomedicals, Solon, OH) at 4.5 m/s for 25 seconds were completed, before implementing the guidelines set forth by the manufacturer's protocol in the DNeasy Plant Mini Kit (Qiagen). Initial incubation steps were determined empirically to increase overall yields (data not shown). Soil and rhizosphere eDNA were extracted using a modified protocol provided by the DNeasy PowerSoil kit manufacturer, as initial tests with the manufacturer's standard protocol resulted in poor yields (<10 ng/ μ l), possibly due to low organic content in the soil. An equal volume of 25:24:1 chloroform:phenol:isoamyl alcohol pH 8 (VWR, Radnor, PA) was used to replace 200 μ l of the solution in the PowerSoil Bead tube, followed by adding 0.25 g of soil and 60 μ l of solution C1. Vortexing was replaced with three runs on a FastPrep-24 (MP Biomedicals) at 4.5 m/s for 25 seconds. The manufacturer's protocol was then followed until the elution step where 60 μ l of solution C6 was used for the elution of eDNA.

Amplicon Generation and Sequencing Preparation. Extracted eDNA was quantified using a Nanodrop 1000 (Thermo Fisher Scientific, Waltham, MA) and a Qubit fluorometer (Life Technologies, Grand Island, NY). Based off Qubit quantification, all samples were diluted to 1.5 ng/ μ l for amplicon library generation, which was generated through a two-step PCR process. First, targeted PCR primers were used to amplify a specific gene region-of-interest, i.e., bacterial 16S or fungal ITS. The bacterial 16S

ribosomal DNA V3-V4 hypervariable region was PCR amplified from bacteria using the Ba9F/Ba515Rmod1 primer pair (~500bp; Kittelmann et al., 2013; Weisburg et al., 1991). Fungal communities were characterized through amplification of the internal transcribed spacer region using the ITS3_KYO2-F/ITS4-R primer pair (~350 bp; Toju et al., 2012; White et al., 1990). To the 5' end of each primer, an overhang adaptor sequence, which allows for integration of indices and Illumina sequencing adapters, was added. The forward primer overhang sequence was 5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG-3' while the reverse primer overhang sequence was 5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG-3'. Reverse primers were synthesized in four different versions with 0-3 mixed sequence bases (where N is any nucleotide) and combined into an equimolar mixture for use in subsequent PCR reactions to add diversity to the sequences, as low diversity libraries such as 16S and ITS amplicons do not sequence well on the Illumina MiSeq (Fadrosh et al., 2014). Initial PCR reactions were performed using MangoTaq DNA Polymerase (BioLine, Taunton, MA) in 25 μ L volumes containing 5X PCR buffer (BioLine), 0.2 mM of each dNTP (BioLine), 2 mM MgCl₂ (BioLine), and 10 μ M of each primer. Cycle conditions were: 94°C for 2 min, followed by 30 cycles of 94°C for 1 min, 52°C for 45 s, 72°C for 45 s, followed by a final extension at 72°C for 5 min (Beirn et al., 2016). PCR was confirmed with the QIAxcel Advanced system (QIAGEN, Gaithersburg, MD). After PCR confirmation, 16S and ITS amplicons from the same sample were pooled and purified using the ZR-96 DNA Clean & Concentrator™-5 (Zymo Research, Irvine, CA).

The second PCR step was performed to add unique indices for each sample using the Nextera XT index kit v2 (Illumina, San Diego, CA), to enable multiplexing for

combining samples within a single sequencing run. Indexing reactions were prepared in 40 μ l volumes containing 5X PCR buffer (BioLine), 2 mM MgCl₂ (BioLine), 0.2 mM of each dNTP (BioLine), 5 μ l of each Nextera index primer (Illumina), and 2.5 U MangoTaq DNA polymerase (BioLine). The indices were incorporated with the following cycling conditions: 72°C for 3 min, 95°C for 30 s, followed by 12 cycles of 95°C for 10 s, 55°C for 30 s, 72°C for 30 s, followed with a final extension at 72°C for 5 min (Beirn et al., 2016). Once indices were incorporated, libraries were cleaned using HighPrep PCR (MagBio Genomics, Gaithersburg, MD) and a modified protocol for targeted removal of sequence fragments less than 200 bp. Fragment size and DNA concentration for each sample was calculated from data generated quantifications performed using the QIAxcel Advanced and Qubit fluorometer. Libraries were normalized to 4 nM and combined to form a pooled amplicon library (PAL) for sequencing. A 30% spike-in of PhiX control (Illumina) was added to increase the diversity of the sample. PhiX and 4 nM PAL were denatured with an equal volume of 0.2 N NaOH at room temperature for 5 min. Once denatured PhiX and PAL were diluted further to 10 pM to optimize cluster density in the MiSeq run. Immediately prior to loading the MiSeq the PhiX and PAL mixture was heat denatured at 95°C for 5 min. These were then sequenced as paired-end reads (2x300) on the Illumina MiSeq platform with a 600 cycle MiSeq v.3 Reagent Cartridge (Illumina). For both experimental runs of the entire trial, two additional technical replicates were performed from initial amplicon generation through MiSeq sequencing. All read sequences were output in FASTQ format.

Sequence Manipulation and Analysis. Prior to bioinformatic analysis, non-biological sequences (i.e., primers and Illumina adaptors) were removed from paired-end sequences using cutadapt 1.9.1 (Martin, 2011). Within the cutadapt program, the ‘-p’ option was utilized to parse paired-end files together, failing of one end to pass filter resulted in removal of both ends from the output file to maintain matching reads in downstream analyses. Following removal of non-biological sequences from paired-end files, DADA2 1.6 (Callahan et al., 2016) was used for denoising, joining of reads when appropriate, inference of amplicon sequence variants (ASVs), and taxonomy assignment within the R environment (R Core Team, 2017). Bacterial reads were truncated to 275 bp to remove low quality bases at the end of reads. Fungal reads were joined without trimming or truncation, as these manipulations can impact results due to the length variation of the ITS2 region. Following removal of chimeric sequences from the dataset, taxonomy was assigned for bacteria using a DADA2 formatted release of the GreenGenes database v.13.8 (Callahan, 2016; McDonald et al., 2012) and for fungi using the UNITE general FASTA release v.7.2 (Kõljalg et al., 2013). Any ASVs that were not identified by their respective databases were submitted to a BLAST query and were assigned taxonomy pending successful identification (i.e., 95-100% identity, low E-value, and repeated returns of same taxonomy). Sequences identifications belonging to Plantae, Protista, chloroplast, or mitochondria were removed from the analysis.

All taxonomic and statistical analyses were performed in the R statistical computing environment version 3.4.2, unless stated otherwise (R Core Team, 2017). A *p*-value of ≤ 0.05 was considered significant when calculated, unless explicitly stated otherwise. Graphics were generated using ggplot2 (Wickham, 2009), unless otherwise

stated. Alpha diversity, i.e., species richness and evenness within samples, was assessed through the Shannon diversity index within the R package phyloseq (McMurdie and Holmes, 2013). For alpha diversity metrics, no rarefaction was performed, as estimation of alpha-diversity metrics are not library-size dependent (McMurdie and Holmes, 2014). Differences in alpha diversity between samples was determined using nonparametric Kruskal-Wallis tests with the Benjamini-Hochberg correction applied for multiple pairwise comparisons using the dunn.test package in R (Dinno, 2017).

Beta diversity, i.e., the change in diversity of species between samples, was calculated using Bray-Curtis dissimilarity matrices using the R package phyloseq (McMurdie and Holmes, 2013; Gardener, 2014). Reads were scaled for beta diversity to account for any large differences in sequencing depth (>10x) (Weiss et al., 2017). Distance matrices were subjected to ordination analyses, permutational analysis of variance (PERMANOVA) was used to test community centroids, and homogeneity of variance to test community variance. These were calculated using the ‘adonis’ and ‘betadisp’ functions in the vegan package for R (Oksanen et al., 2017).

RESULTS

Amplicon Sequence Variants. Sequencing of the bacterial 16S and fungal ITS amplicons resulted in 3.32×10^7 reads usable in downstream analyses. Due to poor quality in reverse reads and minimal overlap, only forward reads of bacterial 16S amplicons were utilized in analysis. These reads resulted in 3,126 ASVs, of which 2,705 belonged to bacteria and 421 belonged to fungi, and after quality filtering the final ASV count was 2,576, with 2,273 bacteria and 303 fungi. Foliar samples are included in

results only for visualization of the taxonomic composition of the microbial communities, as poor sequencing depth (<200) resulted in removal from statistical analyses. Technical replicates were not different for each sample and were pooled. Experimental runs are presented separately, as the analyses showed significance for both bacteria and fungi.

Composition of Amplicon Sequence Variants. Bacterial ASVs were predominantly composed of Proteobacteria, Firmicutes, and Actinobacteria at 35%, 17%, and 13%, respectively. Sixteen percent of bacterial ASVs were not identifiable below the kingdom level. The majority of the Proteobacteria were comprised of Alphaproteobacteria (51%), with Gammaproteobacteria and Betaproteobacteria comprising 15% and 14%, respectively (data not shown). On the family level, the family Microbacteriaceae made up 41% of all Actinobacteria in our samples (data not shown). Alicyclobacillaceae was the predominant Firmicute, making up 68% of the family (data not shown).

Fungal ASVs were predominantly of the Eurotiomycete, Sordariomycete, Dothideomycete, and Tremellomycete classes, comprising 42%, 19%, 13%, and 10% of all fungal ASVs, respectively (Table 2). Only six fungal ASVs were not identifiable at the class level (Table 2). The most prevalent class, the Eurotiomycetes, were primarily comprised by the *Penicillium* genus making up 60% of the class (data not shown). The Sordariomycetes were primarily comprised of the genus *Trichoderma* (69%) (data not shown). The Dothideomycetes were comprised of the genera *Mycosphaerella* (31%), *Cladosporium* (17%), and *Alternaria* (14%) (data not shown). Tremellomycetes were predominantly comprised of the genus *Bullera* at 51% (data not shown).

Table 1. Composition of Bacterial Amplicon Sequence Variants

Phylum	ASVs Assigned[†]	Percent of ASVs[‡]
Proteobacteria	818	35.99
Firmicutes	391	17.20
NA [§]	380	16.72
Actinobacteria	302	13.29
Bacteroidetes	199	8.75
Cyanobacteria	80	3.52
Planctomycetes	28	1.23
Acidobacteria	26	1.14
Chloroflexi	10	0.44
TM7	10	0.44
Armatimonadetes	5	0.22
Gemmatimonadetes	4	0.18
Verrucomicrobia	4	0.18
WPS-2	4	0.18
Fibrobacteres	3	0.13
Elusimicrobia	2	0.09
FBP	2	0.09
[Thermi]	1	0.04
Chlorobi	1	0.04
Fusobacteria	1	0.04
Nitrospirae	1	0.04
Spirochaetes	1	0.04

[†]Number of ASVs assigned to the respective phylum.

[‡]Percentage of ASVs assigned to the respective phylum. Not representative of relative abundances

[§]NA indicates ASVs that could not be assigned taxonomy at the class level.

Table 2. Composition of Fungal Amplicon Sequence Variants

Class	ASVs Assigned[†]	Percent of ASVs[‡]
Sordariomycetes	58	19.14
Eurotiomycetes	130	42.90
Dothideomycetes	41	13.53
Tremellomycetes	33	10.89
Mucoromycetes	20	6.60
Microbotryomycetes	5	1.65
Leotiomycetes	10	3.30
NA [§]	6	1.98

[†]Number of ASVs assigned to the respective phylum.

[‡]Percentage of ASVs assigned to the respective phylum. Not representative of relative abundances.

[§]NA indicates ASVs that could not be assigned taxonomy at the class level.

Relative Abundances of Bacteria and Fungi. While individual bacterial ASVs were predominantly identified as Proteobacteria, the Firmicutes had higher relative abundances at ~8% in foliage and ~10-11% in roots (Fig. 1A). Taxonomic composition of bacterial communities was similar between treatments at the phylum level (Fig. 1A). Bacterial communities had minimal shifts in relative abundance over the course of the experiment, as evidenced by taxonomic profiles appearing similar across time points (Supplementary Fig. 1). Relative abundances exhibited the most fluctuations from emergence 4-weeks post emergence, with increases in *Cryocola*, *Curtobacterium*, *Dolichospermum*, *Pseudomonas* and *Sulfobacillus*, and decreases in *Alicyclobacillus* compared to two weeks post emergence (Supplementary Fig. 1).

Non-sterile and sterile soil treatments were not significant, although a higher relative abundance of Tremellomycetes were observed in the sterile treatment (Fig. 1B).

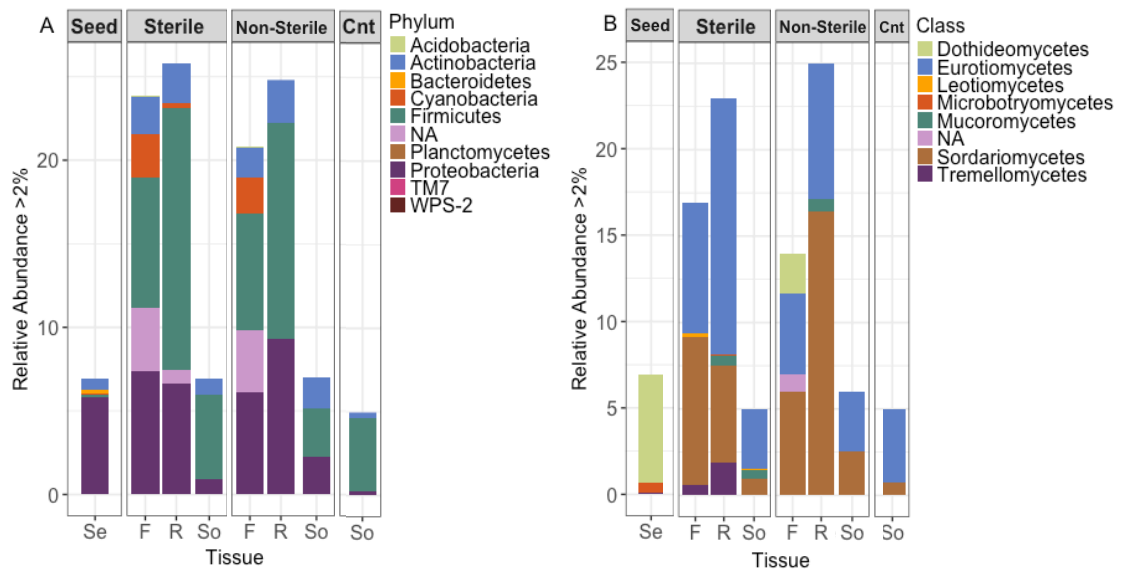


Figure 1. Relative abundances, calculated in phyloseq, of A) bacterial phyla and B) fungal classes associated with creeping bentgrass seed, foliage, roots, and bulk soil under sterile growing conditions. “Se”, “F”, “R” and “So” refer to “Seed”, “Foliage”, “Root” and “Soil”, respectively.

Eurotiomycetes had higher relative abundances (~15%) in root samples compared to foliar samples (~4-6%) (Fig. 1B). Over time there were minimal shifts in the fungal taxa present (Supplemental Fig 2). *Mycosphaerella* was the largest portion of the seed fungal communities at ~4% relative abundance, although, it was not detectable at any other sampling point except at six weeks post-emergence where it was detected only in the foliage at ~0.5% (Supplementary Fig. 2). At two weeks post-emergence there was an increase in the relative abundance of *Trichoderma*, but these levels lowered at subsequent samplings (Supplementary Fig. 2). Root samples from two weeks post-emergence on and soil samples had *Penicillium* at ~5% relative abundance (Supplementary Fig. 2).

Diversity of Rhizosphere Bacterial and Fungal Communities. Significant differences in bacterial alpha diversity were observed in the second experimental run, but not in the first. In the second experimental run, samples taken at the time of leaf emergence had lower diversity than samples taken at 2 and 6 weeks, and diversity at 2 weeks was statistically higher than at 4 weeks (Table 3) (Fig. 2A). These observations

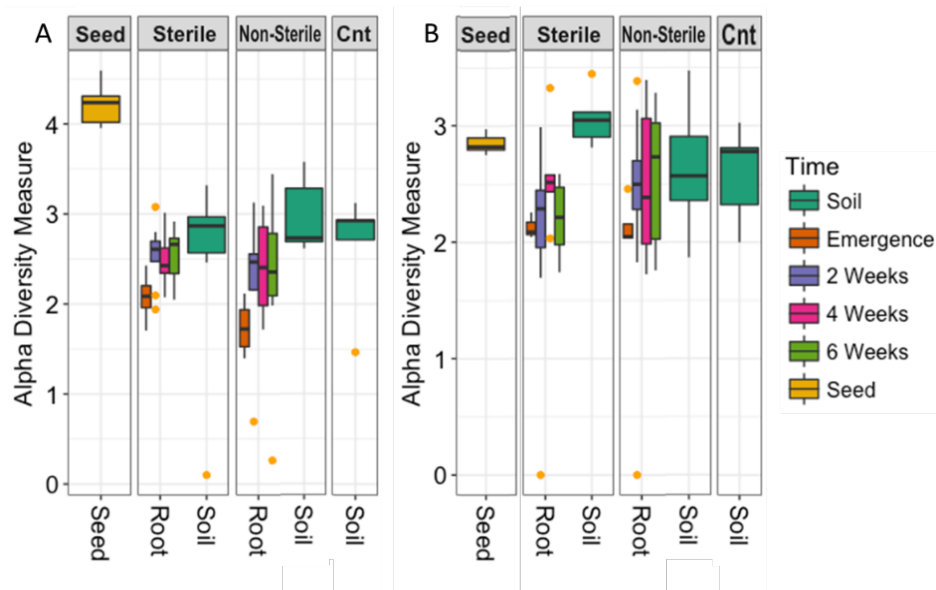


Figure 2. Alpha diversity, as measured through the Shannon diversity index calculated using phyloseq in R, of A) bacterial and B) fungal communities comprising the microbiome of creeping bentgrass grown under sterile conditions. Outliers indicated by orange points.

corresponded with an increase in genera present at low relative abundances from two weeks post emergence forward (Supplementary Fig. 2). In both runs of the experiment, sample types were significantly different, with seed having the highest diversity, followed by soil, then by roots (Table 3) (Fig. 2A). Over the course of the experiment, fungal community alpha diversity was not significantly different in any treatment or at any time point in either experimental run (Table 3) (Fig. 2B).

Table 3. Alpha Diversity[†] Measures for Microbial Communities Associated with Creeping Bentgrass Grown Under Sterile Conditions

Group 1	Group 2	Bacteria				Fungi			
		Run 1		Run 2		Run 1		Run 2	
		Kruskal-Wallis chi ² ‡	p-value§	Kruskal-Wallis chi ²	p-value	Kruskal-Wallis chi ²	p-value	Kruskal-Wallis chi ²	p-value
Treatment		3.372	0.190	3.136	0.210	1.834	0.400	0.496	0.780
Non-Sterile	Sterile	1.736	0.124	-1.317	0.141	1.320	0.280	-0.342	0.366
Non-Sterile	Control	0.241	0.405	1.514	0.195	0.026	0.490	0.686	0.739
Sterile	Control	0.911	0.272	0.753	0.226	0.565	0.429	0.473	0.477
Tissue		9.553	0.010**	20.504	0.001***	3.284	0.190	9.785	0.010**
Roots	Seed	-2.995	0.004**	-3.775	0.001***	-0.783	0.325	-2.542	0.017*
Roots	Soil	-1.324	0.093	-3.119	0.001***	-1.736	0.124	-2.269	0.018*
Seed	Soil	1.848	0.049*	1.639	0.051*	-0.613	0.270	1.084	0.159
Time		3.089	0.210	12.298	0.010**	2.331	0.310	0.591	0.900
Emergence	2 Weeks	-	-	-3.278	0.003**	-	-	-0.762	1.000
Emergence	4 Weeks	-	-	-1.228	0.151	-	-	-0.471	0.957
Emergence	6 Weeks	-	-	-2.478	0.020*	-	-	-0.377	0.706
2 Weeks	4 Weeks	-1.738	0.123	1.999	0.046*	-1.504	0.199	0.276	0.470
2 Weeks	6 Weeks	-0.946	0.258	0.800	0.212	-0.370	0.356	0.373	0.532
4 Weeks	6 Weeks	0.792	0.214	-1.199	0.138	1.086	0.208	0.094	0.463

[†]Alpha diversity, calculated as Shannon's diversity index using phyloseq in R.

[‡]Chi² test statistic calculated through Kruskal-Wallis tests using dunn.test in R.

[§]p-values corrected using Benjamini-Hochberg correction for multiple comparisons.

* Significant at the 0.05 probability level.

** Significant at the 0.01 probability level.

*** Significant at the 0.001 probability level.

Principle component analyses on Bray-Curtis distance matrices showed clustering, to varying degrees, based on soil treatment (Fig 3). Soil and root samples consistently clustered separately from seed samples (Fig. 4). In experimental run 2, within each time point, clustering of bacterial communities became closer as time progressed (Fig 4). PERMANOVA tests of fungal beta diversity revealed that in both runs of the experiment, treatments and tissue type had significantly different centroids, with significant tests of homoscedasticity only for tissue type (Table 4). Across both runs of the experiment, sample type and sampling time had significantly different centroids within sterile and non-sterile soil treatments (Table 4). Dispersion tests were significant for sampling time within the non-sterile treatment of experimental run 1, and for tissue type within both treatments for run 2 (Table 5). Treatments had significantly different centroids within roots and soil in experimental run 2, with a significant dispersion test only for treatment within soils.

PERMANOVA testing of bacterial beta diversity revealed that treatments and sampling times had significantly different centroids for both runs of the experiment (Table 1). Dispersion tests were significant for sampling times in both runs of the experiment, as well as for treatments in run 2. Additionally, bacterial beta diversity was significantly different across tissue types in the second run of the experiment, with a significant homoscedasticity test (Table 1). Within treatments, tissue type and sampling times had significantly different centroids. Tests of homoscedasticity were significant for tissue and time within treatment, except for sample type and time within the sterile treatment of experimental run 1 (Table 2). Within roots, treatments had significantly different centroids across both runs, and sampling times had significantly different

centroids in run 2 (Table 2). Homoscedasticity was only significant for sampling time within roots (Table 2). In run 2 of the experiment treatments had significantly different centroids within soil (Table 2).

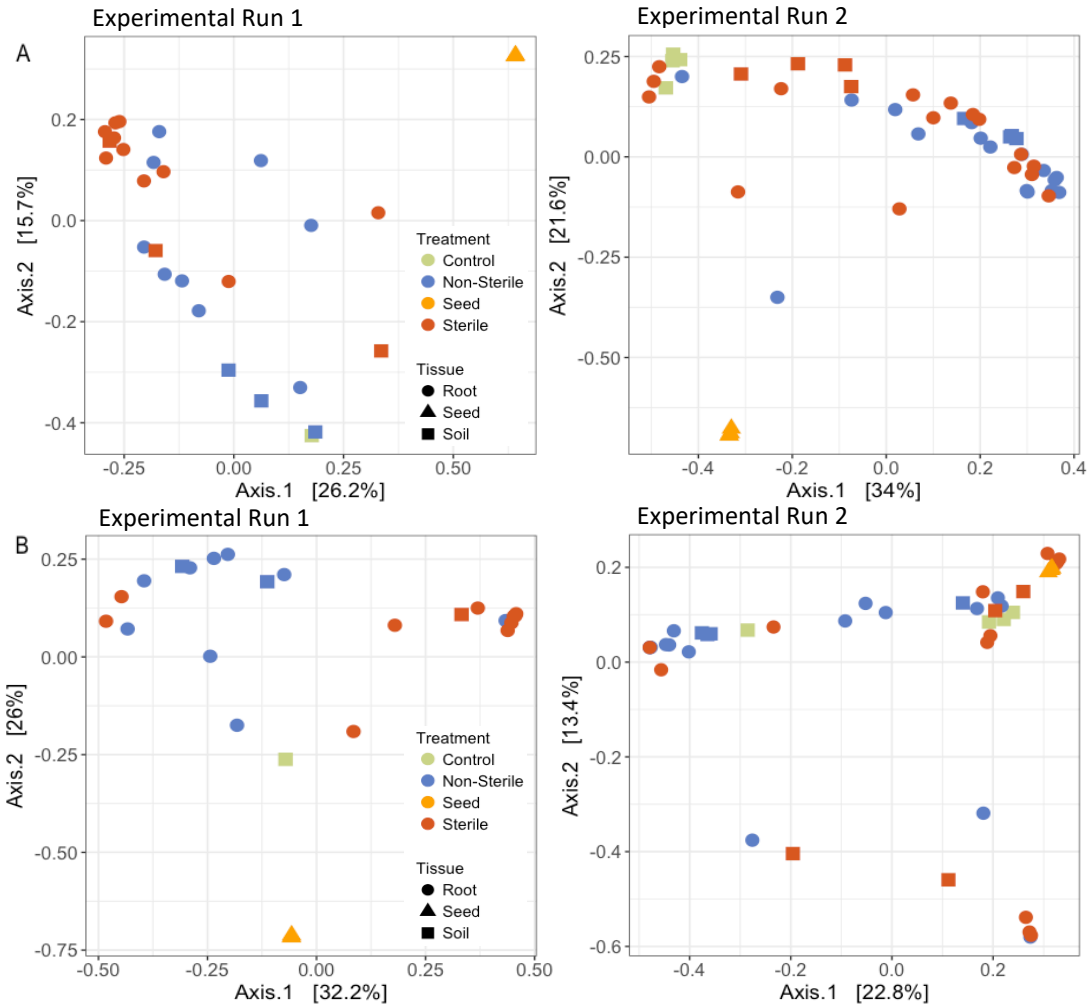


Figure 3. Principal component analysis, completed using phyloseq in R, of Bray-Curtis distance matrices, calculated in phyloseq, of A) bacterial and B) fungal communities of creeping bentgrass grown under sterile conditions. Point shape and color are indicative of tissue sampled and soil treatment, respectively.

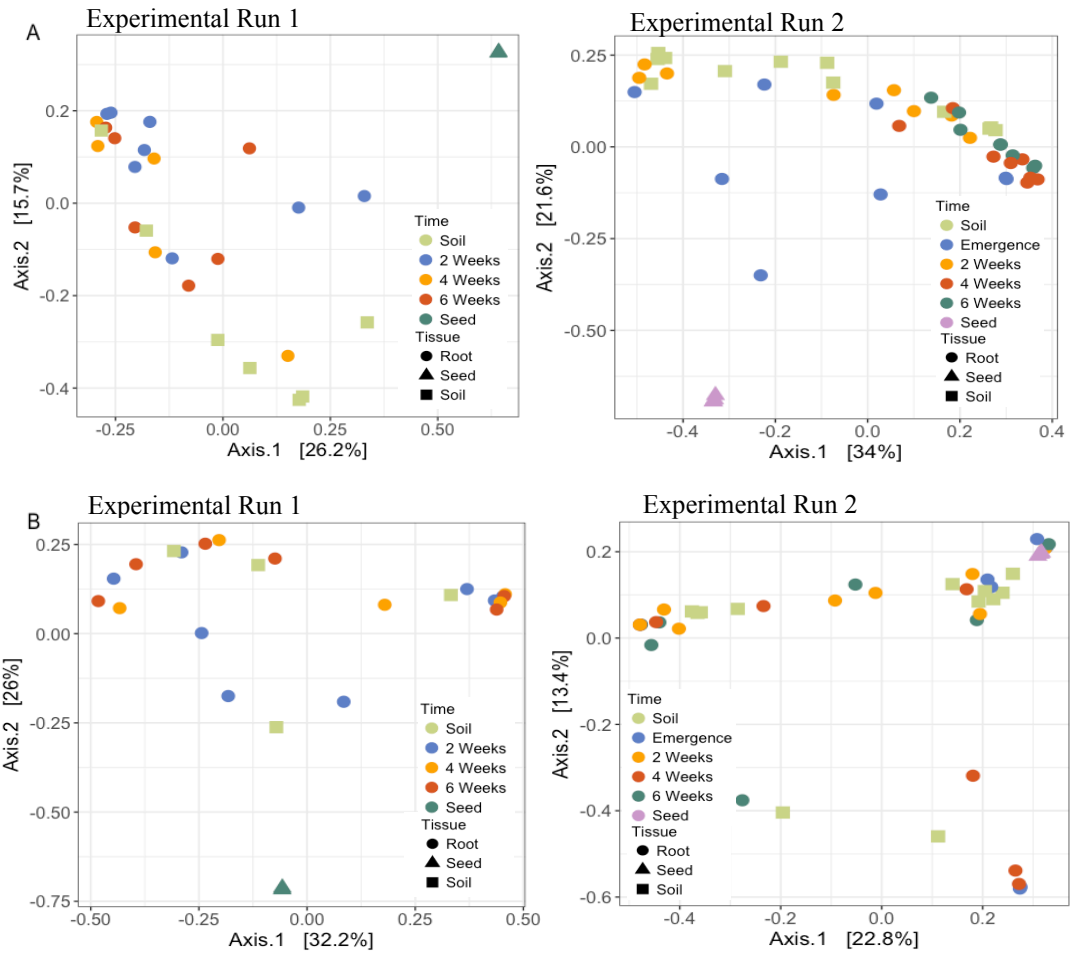


Figure 4. Principal component analysis, completed using phyloseq in R, of Bray-Curtis distance matrices, calculated in phyloseq of A) bacterial and B) fungal communities of creeping bentgrass grown under sterile conditions. Point shape and color are indicative of tissue sampled and time sampled, respectively.

Table 4. Centroid and Dispersion Testing of Beta-Diversity[†] Metrics of Microbial Communities Associated with Creeping Bentgrass Grown Under Sterile Conditions

Beta-Diversity	Bacteria			
	Run 1		Run 2	
	PERMANOVA p-value [‡]	Dispersion p-value [§]	PERMANOVA p-value	Dispersion p-value
Treatment	0.033*	0.090	0.001***	0.019*
Tissue	0.974	0.685	0.001***	0.013*
Time	0.001***	0.019*	0.001***	0.002**
	Fungi			
Treatment	0.002**	0.304	0.001***	0.214
Tissue	0.001***	0.001***	0.001***	0.001***
Time	0.812	0.211	0.189	0.701

[†] Beta diversity metrics calculated as Bray-Curtis dissimilarity matrices using phyloseq in R.

[‡]Permutational analysis of variance p-value calculated using vegan in R.

[§]Dispersion tests used to test community variances, p-values calculated using vegan in R.

* Significant at the 0.05 probability level.

** Significant at the 0.01 probability level.

*** Significant at the 0.001 probability level.

Table 5. Centroid and Dispersion Testing of Beta-Diversity[†] Within Groupings

		Bacteria			
		Run 1		Run 2	
	Group Within	PERMANOVA p-value [‡]	Dispersion p-value [§]	PERMANOVA p-value	Dispersion p-value
Sterile	Tissue	0.002**	0.285	0.001***	0.006**
Sterile	Time	0.001***	0.474	0.001***	0.019*
Non-Sterile	Tissue	0.001***	0.003**	0.001***	0.009**
Non-Sterile	Time	0.001***	0.062	0.001***	0.216
Root	Treatment	0.041*	0.191	0.076	0.505
Root	Time	0.97	0.693	0.001***	0.001***
Soil	Treatment	0.223	0.299	0.001***	0.287
		Fungi			
Sterile	Tissue	0.002**	0.076	0.001***	0.001***
Sterile	Time	0.019*	0.275	0.004**	0.097
Non-Sterile	Tissue	0.003**	0.101	0.001***	0.009**
Non-Sterile	Time	0.008**	0.001***	0.001***	0.462
Root	Treatment	0.023*	0.85	0.024*	0.166
Root	Time	0.826	0.231	0.178	0.712
Soil	Treatment	0.1667	0.208	0.014*	0.098

[†] Beta diversity metrics calculated as Bray-Curtis dissimilarity matrices using phyloseq in R.

[‡]Permutational analysis of variance p-value calculated using vegan in R.

[§]Dispersion tests used to test community variances, p-values calculated using vegan in R.

* Significant at the 0.05 probability level.

** Significant at the 0.01 probability level.

*** Significant at the 0.001 probability level.

DISCUSSION

The goal of this project was to gain a thorough understanding of how the creeping bentgrass microbiome develops following seeding. More specifically, the investigation centered on what influences resident microbial populations play on the developing creeping bentgrass microbiome, and if there is preferential recruitment of certain organisms. To that end, this research project sought to determine what influence resident seed and soil microbiota exhibit on the microbiome assemblage in the developing plant, and how the juvenile microbiome forms over initial growth stages (i.e., 6 weeks) following seedling emergence. Bacterial communities associated with seeds have the potential to increase germination and seedling growth. For example, six plant growth promoting rhizobacteria (*Pseudomonas putida* R-168, *P. fluorescens* R-93, *P. fluorescens* DSM 50090, *P. putida* DSM291, *Azospirillum lipoferum* DSM 1691, and *A. brasilense* DSM 1690) were evaluated for their impacts on maize (*Zea mays* L. cv, “SC 647”) seedling germination and growth following inoculation of seed with a single bacterial strain. The authors found that all tested bacteria, except *A. lipoferum* DSM1691, increased seed germination by up to 18.5% compared to the control and all bacterial inoculants increased seedling fresh weight compared to the control (Gholami et al., 2009). Similarly, volatile organic compounds released by *Bacillus amyloliquefaciens* IN937a and *B. subtilis* GB03 significantly increased total leaf surface area of 2-day old *A. thaliana* seedlings growing in petri dishes, with a center partition separating the bacteria and plants, filled with solid half-strength Murashige and Skoog salt medium (Ryu et al., 2003). Fungi have exhibited benefits to seedling development as well. For example, perennial ryegrass (*Lolium perenne* L.) and tall fescue (*Festuca arundinacea* Schreb.)

seeds from parent plants infected by fungal endophytes exhibited significantly higher germination than seeds from uninfected parent plants (Clay, 1987). Understanding the microbiome as it develops in concert with the plant host could allow for the development of management strategies to harness existing microbiome inhabitants to increase seedling germination and growth.

The bacterial communities in this controlled environment study primarily belonged to the Proteobacteria, Firmicutes, and Actinobacteria. In several field studies of the wheat microbiome, the same dominant bacterial phyla were observed (Donn et al., 2015; Gdanetz and Trail, 2017; Ofek et al., 2014). In the soil of a *Poa annua* putting green the predominant bacterial phyla were identified as Proteobacteria and Acidobacteria (Beirn et al., 2016). Using PCR restriction profile analysis of 16S rDNA extracted from bulk soil, rhizosphere soil, and washed roots of *L. perenne* and *Trifolium repens* L. showed that plant roots have a selective effect towards Gammaproteobacteria and a dominance of *Pseudomonas* (Marilley and Aragno, 1999). Although phylum level identification is an extremely broad classification and covers an immense number of organisms, the consistent dominance of Proteobacteria across many studies may reflect a preferential recruitment of members of this phylum in the plant microbiome. As such there is potential that introduction of beneficial bacteria from the Proteobacteria phylum may prove to be more successful than bacteria from other phyla, though future research would need to confirm this hypothesis.

Within the Proteobacteria there are several organisms of interest when considering enhancing plant health and vigor. The *Burkholderia* genus contains species which are known to produce antibiotic compounds (Pidot et al., 2014), which can

potentially be utilized to combat plant pathogens. Additionally, the bacterial family Sphingomonadaceae contains plant pathogen antagonists, plant growth promoters, and members capable of being utilized in bioremediation (Glaeser and Kampfer, 2014). It is important to note that not all Proteobacteria are beneficial, and some are prominent plant pathogens. Some species within the genus *Bacillus*, which is in the Firmicutes, are known to be plant growth promoters. Two species of note, *B. amyloliquefaciens* and *B. subtilis*, have both been shown to be successful plant growth promoters (Ryu et al., 2003). Actinobacteria are also known for their disease suppression, through antibiotics, lytic enzymes, hyperparasitism, competition, or host defense induction, and plant growth promotion, through increasing nutrient availability or secretion of plant growth regulators (El-Tarabily and Sivasithamparam, 2006; Palaniyandi et al., 2013). Actinobacteria can act as plant growth promoters, this promotion occurs through production of plant growth regulators, siderophores, nitrogen fixation, promotion of symbiosis with nitrogen fixing bacteria or mycorrhiza, phosphate solubilization, and stress alleviation (Palaniyandi et al., 2013). Although it is important to note that while there are beneficial organisms in these groups, there are increasing reports of bacterial problems in turf, such as the emergence of several species such as *Acidovorax avenae*, *Xanthomonas translucens* and *Pantoea annanatis* causing etiolation in turfgrass (Roberts et al., 2017). However, based upon the broad phylum level identification it is possible that the turfgrass microbiome in our study is already primed with beneficial bacteria that can be utilized to increase plant health.

In this study, Ascomycota was the predominant fungal phylum, comprising 65% of all ASVs; however, three phyla—Ascomycota, Basidiomycota and Chytridiomycota—and two subphyla—Kickxellomycota and Mucoromycota—were covered by all ASVs.

The abundance of Ascomycota is consistent with several previous studies (Schadt et al., 2003; O'Brien et al., 2005; Hur et al., 2012). For example, in a phylogenetic analysis of fungi living in the roots of tall oat-grass (*Arrhenatherum elatius* L.), 49 phylotypes were identified, with Ascomycota being the most prevalent at 25 of the 49 phylotypes (Vandenkoornhuyse et al., 2002).

At class level, fungal communities were primarily made up of Dothideomycetes, Eurotiomycetes, Sordariomycetes and Tremellomycetes. With the exception of the Tremellomycetes, other studies have found the same fungal classes to be the predominate the fungal compartment of the plant microbiome. For example, the rhizosphere of wheat under conventional or organic management strategies and in different crop rotations showed consistent dominance of Dothideomycetes, Leotiomycetes, and Sordariomycetes (Gdanetz and Trail, 2017; Lenc et al., 2015). Additionally, a survey of the epiphytic phyllosphere fungi across 57 tree species in a Panamanian tropical lowland rainforest showed Dothideomycetes, Eurotiomycetes and Sordariomycetes being the most prevalent fungal classes (Kembel and Mueller, 2014). Considering no other study found Eurotiomycetes, predominantly *Penicillium* in our study, at the prevalence we did, it is possible this is an artifact of the controlled nature of our study providing limited inoculum entering the microbiome.

Not surprisingly the Ascomycota were the most abundant fungal organisms in the study, considering it is the largest phylum of fungi with over 64,000 described species (Kirk et al., 2008). The most prevalent class, the Eurotiomycetes, includes organisms that are used in fermentation processes in food production, xerophiles and psychrophiles, and those that produce toxic or useful secondary metabolites (Geiser et al., 2006).

Species within the genus *Penicillium*, which made up the overwhelming majority of the Eurotiomycetes, can be plant pathogens (Neri et al., 2006), plant growth promoters (Hossain et al., 2007; Whitelaw et al., 1997), or used to combat plant pathogens through antifungal compound production (Yang et al., 2008). When thinking of combating plant pathogens and increasing plant health, *Trichoderma*, a member of the Sordariomycetes, is commonly used as a biocontrol for controlling some diseases (Howell, 2003). In turfgrass systems *Trichoderma harzianum* strain 1295-22 has been shown to reduce disease severity due to pathogens such as *Sclerotinia homoeocarpa*, *Rhizoctonia solani*, and *Pythium graminicola* (Lo et al., 1997). Similar to bacterial communities associated with plants, it is possible that the fungal fraction of the plant microbiome is already primed with beneficial organisms that could be utilized to improve plant health. Future research projects could be aimed at determining methods of activating these native beneficial bacteria and fungi.

Over the 6-week sampling period of this study, significant changes were observed in alpha diversity of bacterial communities at emergence, where it was lower than all other sampling points. This difference may be driven by the shift in dominant members of the community between seeds, roots, and soil (Fig 1). Similar patterns have been observed in the wheat microbiome, where bacterial alpha diversity was observed to be higher in root tissue at flowering and seed development growth stages compared to the vegetative growth stage (Gdanetz and Trail, 2017). Similarly, wheat rhizosphere bacterial diversity was found to increase as the plants went from vegetative to reproductive growth (Donn et al., 2015). A similar shift was documented from the microbiome of apple flowers, where alpha diversity increased from closed bud to bloom,

a 2-day time difference, followed by a stabilization at all other sampling points (Shade et al., 2013). This shift from closed bud to bloom highlights how quickly diversity can change. Interestingly, researchers observed the bacterial alpha diversity of a *P. annua* rhizosphere increases as time progressed (Beirn et al., 2016). However, authors related differences to environmental changes across the season and not plant age, as the experiment was conducted on a 5-yr old stand of *P. annua*. Given the controlled environment of our study, changes over time on the bacterial communities may also be driven by plant exudates, as plant exudates are shown to be a driving factor in rhizosphere composition (Micallef et al., 2009; Paterson et al., 2007; Shi et al., 2011).

Unlike the observed shift in bacterial diversity, fungal alpha diversity did not significantly change over time. Additionally, community centroids were similar over time, indicating that these communities are remaining similar across sampling times. This lack of change in alpha diversity is similar to what was found in the wheat microbiome, where fungal alpha diversity was similar over the 29 days between vegetative and flowering growth stages across leaf, stem, and root tissue (Gdanetz and Trail, 2017). In organically managed wheat, root tissue had significantly higher α -diversity than stem tissue did during the seed production growth stage (Gdanetz and Trail, 2017). Long term studies may be required to determine if the diversity of plant-associated fungal communities are changing, or if diversity remains constant as plant tissue matures.

Comparison of the initial seed-associated bacterial communities to that of the subsequent rhizosphere bacterial communities show an interesting trend. The bacterial and fungal communities inhabiting the seed were predominantly Proteobacteria and

Dothideomycetes, respectively. However, the bacterial communities of the soil were predominantly Firmicutes. Interestingly, the plant tissue bacterial communities showed a shared dominance of Firmicutes and Proteobacteria. Bacterial communities of the rhizosphere of maize grown on sterile sand and non-sterile soils all contained the same dominant OTUs, indicating the seed as a common source of inoculum (Johnston-Monje et al., 2016). Based on these findings, the bacterial compartment of the plant microbiome can be influenced by the bacteria present on the seed. However, further research is needed to confirm this under field conditions and to evaluate whether beneficial bacterial organisms can be successfully and consistently introduced into the plant microbiome through seed treatments.

Data presented here suggest that bacterial communities inhabiting seed play a role in determining which bacteria are present in the juvenile microbiome of creeping bentgrass. However, fungi were less impacted by the resident seed microflora and more dependent upon what was present in the soil. Only bacterial alpha diversity exhibited a significant change over time, with an initial increase following seedling emergence, whereas fungal community alpha diversity was not significantly changed over time. Based solely on these results, it is believed that fungal communities reached a climax community prior to 2 weeks post-emergence, while bacterial communities have not. Although, since various studies found bacterial alpha diversity to increase as plants matured it is possible that our short six-week sampling period may not have been long enough to capture any potential shifts in alpha diversity. Future research implementing a longer sampling duration is necessary to confirm when and if a climax community is reached. While this closed environment was successful in allowing us to determine the

specific influences of resident seed and soil microbiota on the developing creeping bentgrass microbiome, this same system limited incoming inoculum that would impact the microbiome. Previous studies evaluating similar graminaceous hosts under field conditions have observed similar predominant members of bacteria and fungi as identified in the present study, however, more research is needed to elucidate if these communities are functioning in the same manner. The work presented here provides a framework for developing turfgrass microbiome research. A thorough understanding of the turfgrass microbiome establishment and what influence resident microflora exhibit is necessary if turfgrass scientists hope to manipulate the turfgrass microbiome to increase plant health and productivity.

LITERATURE CITED

- Amann, R.I., W. Ludwig and K.H. Schleifer. 1995. Phylogenetic identification and in situ detection of individual microbial cells without cultivation. *Microbiol. Mol. Biol. Rev.* 59: 143-169.
- Arias, M.E., J.A. Gonzalez-Perez, F.J. Gonzalez-Vila and A.S. Ball. 2005. Soil health -- a new challenge for microbiologists and chemists. *Int. Microbiol.* 8: 13-21.
- Bakker, M.G., D.K. Manter, A.M. Sheflin, T.L. Weir and J.M. Vivanco. 2012. Harnessing the rhizosphere microbiome through plant breeding and agricultural management. *Plant and Soil* 360: 1-13. doi:10.1007/s11104-012-1361-x.
- Bartlett, M.D., I.T. James, J.A. Harris and K. Ritz. 2008. Size and phenotypic structure of microbial communities within soil profiles in relation to different playing areas on a uk golf course. *Eur. J. Soil Sci.* 59: 835-841. doi:10.1111/j.1365-2389.2008.01056.x.
- Beirn, L.A., J.W. Hempfling, C.J. Schmid, J.A. Murphy, B.B. Clarke and J.A. Crouch. 2016. Differences among soil-inhabiting microbial communities in *Poa annua* turf throughout the growing season. *Crop Sci.* doi:10.2135/cropsci2016.06.0463.
- Bigelow, C.A., D.C. Bowman and A.G. Wollum. 2002. Characterization of soil microbial population dynamics in newly constructed sand-based rootzones. *Crop Sci.* 42: 1611-1614. doi:10.2135/cropsci2002.1611.
- Buck, J.W. and L.L. Burpee. 2002. The effects of fungicides on the phylloplane yeast populations of creeping bentgrass. *Can. J. Microbiol.* 48: 522-529.
- Callahan, B.J. 2016. The rdp and greengenes taxonomic training sets formatted for dada2 [data set]. Zenodo. 10.5281/zenodo.158955.
- Callahan, B.J., P.J. McMurdie, M.J. Rosen, A.W. Han, A.J.A. Johnson and S.P. Holmes. 2016. DADA2: High-resolution sample inference from Illumina amplicon data. *Nat. Methods* 13: 581. doi:10.1038/neth.3869.
- Clay, K. 1987. Effects of fungal endophytes on the seed and seedling biology of *Lolium perenne* and *Festuca arundinacea*. *Oecologia* 73: 358-362. doi:10.1007/bf00385251.
- Cole, M.A. and A.J. Turgeon. 1978. Microbial activity in soil and litter underlying bandane- and calcium arsenate-treated turfgrass. *Soil Biol. Biochem.* 10: 181-186. doi:https://doi.org/10.1016/0038-0717(78)90094-9.
- Crouch, J.A., Z. Carter, A. Ismaiel and J.A. Roberts. 2017. The US National Mall microbiome: A census of rhizosphere bacteria inhabiting landscape turf. *Crop Sci.* 57: S-341-S-348. doi:10.2135/cropsci2016.10.0849.

- Dinno, A. 2017. *Dunn.Test: Dunn's test of multiple comparisons using rank sums*. <https://CRAN.R-project.org/package=dunn.test>.
- Donn, S., J.A. Kirkegaard, G. Perera, A.E. Richardson and M. Watt. 2015. Evolution of bacterial communities in the wheat crop rhizosphere. *Environ. Microbiol.* 17: 610-621. doi:10.1111/1462-2920.12452.
- Edwards, J., C. Johnson, C. Santos-Medellín, E. Lurie, N.K. Podishetty, S. Bhatnagar, et al. 2015. Structure, variation, and assembly of the root-associated microbiomes of rice. *PNAS* 112: E911-E920. doi:10.1073/pnas.1414592112.
- El-Tarabily, K.A. and K. Sivasithamparam. 2006. Non-streptomycete actinomycetes as biocontrol agents of soil-borne fungal plant pathogens and as plant growth promoters. *Soil Biol. Biochem.* 38: 1505-1520. doi:<https://doi.org/10.1016/j.soilbio.2005.12.017>.
- Fadrosh, D.W., B. Ma, P. Gajer, N. Sengamalay, S. Ott, R.M. Brotman, et al. 2014. An improved dual-indexing approach for multiplexed 16s rRNA gene sequencing on the Illumina MiSeq platform. *Microbiome* 2: 6. doi:10.1186/2049-2618-2-6.
- Frostegard, A., A. Tunlid and E. Baath. 2010. Use and misuse of PLFA measurements in soils. *Soil Biol. Biochem.* doi:10.1016/j.soilbio.2010.11.021.
- Gardener, M. 2014. *Community ecology: Analytical methods using R and Excel*. Pelagic Publishing, Exeter, UK.
- Gdanetz, K. and F. Trail. 2017. The wheat microbiome under four management strategies, and potential for endophytes in disease protection. *Phytobiomes* 1: 158-168. doi:10.1094/PBIOMES-05-17-0023-R.
- Geiser, D.M., C. Gueidan, J. Miadlikowska, F. Lutzoni, F. Kauff, V. Hofstetter, et al. 2006. Eurotiomycetes: Eurotiomycetidae and Chaetothyriomycetidae. *Mycologia* 98: 1053-1064. doi:10.1080/15572536.2006.11832633.
- Gholami, A., S. Shahsavani and S. Nezarat. 2009. The effect of plant growth promoting rhizobacteria (PGPR) on germination, seedling growth and yield of maize. *International Journal of Biological, Biomolecular, Agricultural, Food and Biotechnological Engineering* 3: 8-14.
- Glaeser S.P., Kämpfer P. (2014) The family Sphingomonadaceae. In: Rosenberg E., DeLong E.F., Lory S., Stackebrandt E., Thompson F. (eds) *The Prokaryotes*. Springer, Berlin, Heidelberg.

- Henry, A., W. Doucette, J. Norton, S. Jones, J. Chard and B. Bugbee. 2006. An axenic plant culture system for optimal growth in long-term studies. *J Environ Qual* 35: 590-598. doi:10.2134/jeq2005.0127.
- Hoagland, D.R., and D.I. Arnon. 1950. The water-culture method for growing plants without soil. California Agricultural Experiment Station Circular. 347: 32.
- Hossain, M.M., F. Sultana, M. Kubota, H. Koyama and M. Hyakumachi. 2007. The plant growth-promoting fungus *Penicillium simplicissimum* gp17-2 induces resistance in *Arabidopsis thaliana* by activation of multiple defense signals. *Plant and Cell Physiology* 48: 1724-1736. doi:10.1093/pcp/pcm144.
- Howell, C.R. 2003. Mechanisms employed by trichoderma species in the biological control of plant diseases: The history and evolution of current concepts. *Plant Dis.* 87: 4-10. doi:10.1094/PDIS.2003.87.1.4.
- Hur, M., Y.W. Lim, J.J. Yu, S.U. Cheon, Y.I. Choi, S.-H. Yoon, et al. 2012. Fungal community associated with genetically modified poplar during metal phytoremediation. *Journal of Microbiology* 50: 910-915. doi:10.1007/s12275-012-2491-9.
- Johnston-Monje, D., D.S. Lundberg, G. Lazarovits, V.M. Reis and M.N. Raizada. 2016. Bacterial populations in juvenile maize rhizospheres originate from both seed and soil. *Plant Soil* 405: 337-355. doi:10.1007/s11104-016-2826-0.
- Jongman, R.H.G, C.J.F. Ter Braak and O.F.R. Van Tongeren. 1995. Data analysis in community and landscape ecology. Cambridge University Press: Cambridge, UK.
- Kembel, S.W. and R.C. Mueller. 2014. Plant traits and taxonomy drive host associations in tropical phyllosphere fungal communities. *Botany* 92: 303-311. doi:10.1139/cjb-2013-0194.
- Kirk, P.M., G.C. Ainsworth, G.R. Bisby and C.A.B. International. 2008. Ainsworth & bisby's dictionary of the fungi. CABI.
- Kittelmann, S., H. Seedorf, W.A. Walters, J.C. Clemente, R. Knight, J.I. Gordon, et al. 2013. Simultaneous amplicon sequencing to explore co-occurrence patterns of bacterial, archaeal and eukaryotic microorganisms in rumen microbial communities. *PLoS ONE* 8: e47879. doi:10.1371/journal.pone.0047879.
- Kõljalg, U., R.H. Nilsson, K. Abarenkov, L. Tedersoo, A.F.S. Taylor, M. Bahram, et al. 2013. Towards a unified paradigm for sequence-based identification of fungi. *Mol. Ecol.* 22: 5271-5277. doi:10.1111/mec.12481.

- Leibold, M.A. and M.A. McPeck. 2006. Coexistence of the niche and neutral perspectives in community ecology. *Ecology* 87: 1399-1410. doi:doi:10.1890/0012-9658(2006)87[1399:COTNAN]2.0.CO;2.
- Lenc, L., H. Kwaśna, C. Sadowski and A. Grabowski. 2015. Microbiota in wheat roots, rhizosphere and soil in crops grown in organic and other production systems. *J. Phytopathol.* 163: 245-263. doi:10.1111/jph.12313.
- Lo, C.T., E.B. Nelson and G.E. Harman. 1997. Improved biocontrol efficacy of *Trichoderma harzianum* 1295-22 for foliar phases of turf diseases by use of spray applications. *Plant Dis.* 81: 1132-1138. doi:10.1094/PDIS.1997.81.10.1132.
- Lundberg, D.S., S.L. Lebeis, S.H. Paredes, S. Yourstone, J. Gehring, S. Malfatti, et al. 2012. Defining the core *Arabidopsis thaliana* root microbiome. *Nature* 488: 86. doi:10.1038/nature11237.
- Mancino, C.F., M. Barakat and A. Maricic. 1993. Soil and thatch microbial populations in an 80% sand : 20% peat creeping bentgrass putting green. *HortScience* 28: 189-191.
- Marilley, L. and M. Aragno. 1999. Phylogenetic diversity of bacterial communities differing in degree of proximity of *Lolium perenne* and *Trifolium repens* roots. *Applied Soil Ecology* 13: 127-136. doi:https://doi.org/10.1016/S0929-1393(99)00028-1.
- Martin, M. 2011. Cutadapt removes adapter sequences from high-throughput sequencing reads. *EMBNet.Journal* 17: 10-12.
- McDonald, D., M.N. Price, J. Goodrich, E.P. Nawrocki, T.Z. DeSantis, A. Probst, et al. 2012. An improved greengenes taxonomy with explicit ranks for ecological and evolutionary analyses of bacteria and archaea. *ISME J.* 6: 610-618. doi:10.1038/ismej.2011.139.
- McMurdie, P.J. and S. Holmes. 2013. phyloseq: An R package for reproducible interactive analysis and graphics of microbiome consensus data. *PLoS ONE* 8: e61217.
- McMurdie, P.J. and S. Holmes. 2014. Waste not, want not: Why rarefying microbiome data is inadmissible. *PLoS Comput. Biol.* 10: e1003531. doi:10.1371/journal.pcbi.1003531.
- Mendes, L.W., E.E. Kuramae, A.A. Navarrete, J.A. van Veen and S.M. Tsai. 2014. Taxonomical and functional microbial community selection in soybean rhizosphere. *ISME J.* 8: 1577. doi:10.1038/ismej.2014.17.

- Mendes, R., M. Kruijt, I. de Bruijn, E. Dekkers, M. van der Voort, J.H.M. Schneider, et al. 2011. Deciphering the rhizosphere microbiome for disease-suppressive bacteria. *Science* 332: 1097-1100. doi:10.1126/science.1203980.
- Micallef, S.A., S. Channer, M.P. Shiaris and A. Colon-Carmona. 2009. Plant age and genotype impact the progression of bacterial community succession in the *Arabidopsis* rhizosphere. *Plant Signaling Behav.* 4: 777-780. doi:10.1093/jxb/erp053.
- Neri, F., M. Mari and S. Brigati. 2006. Control of *Penicillium expansum* by plant volatile compounds. *Plant Pathology* 55: 100-105. doi:10.1111/j.1365-3059.2005.01312.x.
- O'Brien, H.E., J.L. Parrent, J.A. Jackson, J.-M. Moncalvo and R. Vigalys. 2005. Fungal community analysis by large-scale sequencing of environmental samples. *Appl. Environ. Microbiol.* 71: 5544-5550. doi:10.1128/AEM.71.9.5544-5550.2005.
- Obasa, K., F.F. White, J. Fellers, M. Kennelly, S. Liu, B. Katz, et al. 2017. A dimorphic and virulence-enhancing endosymbiont bacterium discovered in *Rhizoctonia solani*. *Phytobiomes* 1: 14-23. doi:10.1094/PBIOMES-08-16-0005-R.
- Ofek, M., M. Voronov-Goldman, Y. Hadar and D. Minz. 2014. Host signature effect on plant root-associated microbiomes revealed through analyses of resident vs. active communities. *Environ. Microbiol.* 16: 2157-2167. doi:10.1111/1462-2920.12228.
- Oksanen, J., F.G. Blanchet, M. Friendly, R. Kindt, P. Legendre, D. McGlenn, et al. 2017. Vegan: Community ecology package. R package version 2.4-5, <https://CRAN.R-project.org/package=vegan>.
- Palaniyandi, S.A., S.H. Yang, L. Zhang and J.-W. Suh. 2013. Effects of actinobacteria on plant disease suppression and growth promotion. *Appl. Microbiol. Biotechnol.* 97: 9621-9636. doi:10.1007/s00253-013-5206-1.
- Panke-Buisse, K., A.C. Poole, J.K. Goodrich, R.E. Ley and J. Kao-Kniffin. 2014. Selection on soil microbiomes reveals reproducible impacts on plant function. *ISME J.* 9: 980. doi:10.1038/ismej.2014.196.
- Paterson, E., T. Gebbing, C. Abel, A. Sim and G. Telfer. 2007. Rhizodeposition shapes rhizosphere microbial community structure in organic soil. *New Phytol.* 173: 600-610. doi:10.1111/j.1469-8137.2006.01931.x.
- Peiffer, J.A., A. Spor, O. Koren, Z. Jin, S.G. Tringe, J.L. Dangl, et al. 2013. Diversity and heritability of the maize rhizosphere microbiome under field conditions. *PNAS* 110: 6548-6553. doi:10.1073/pnas.1302837110.

- Pidot, S.J., S. Coyne, F. Kloss and C. Hertweck. 2014. Antibiotics from neglected bacterial sources. *Int. J. Med. Microbiol.* 304: 14-22. doi:<https://doi.org/10.1016/j.ijmm.2013.08.011>.
- Pocheville, A. 2015. The ecological niche: History and recent controversies. In: T. Heams, P. Huneman and G. Lecointre, editors, *Handbook of evolutionary thinking in the sciences*. Springer, Dordrecht. p. 547-586.
- R Core Team. 2017. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria, <https://www.R-project.org/>.
- Roberts, J.A., B. Ma, L.P. Tredway, D.F. Ritchie and J.P. Kerns. 2017. Identification and pathogenicity of bacteria associated with etiolation and decline of creeping bentgrass golf course putting greens. *Phytopathology* 108: 23-30. doi:10.1094/PHYTO-01-17-0015-R.
- Rodriguez, R.J., J.F. White Jr, A.E. Arnold and R.S. Redman. 2009. Fungal endophytes: Diversity and functional roles. *New Phytol.* 182: 314-330. doi:10.1111/j.1469-8137.2009.02773.x.
- Ryu, C.-M., M.A. Farag, C.-H. Hu, M.S. Reddy, H.-X. Wei, P.W. Paré, et al. 2003. Bacterial volatiles promote growth in *Arabidopsis*. *PNAS* 100: 4927-4932. doi:10.1073/pnas.0730845100.
- Schadt, C.W., A.P. Martin, D.A. Lipson and S.K. Schmidt. 2003. Seasonal dynamics of previously unknown fungal lineages in tundra soils. *Science* 301: 1359-1361. doi:10.1126/science.1086940.
- Schoug, A., J. Fischer, H.J. Heipieper, J. Schnurer and S. Hakansson. 2008. Impact of fermentation pH and temperature on freeze-drying survival and membrane lipid composition of *Lactobacillus coryniformis* s3. *J. Ind. Microbiol. Biotechnol.* 35: 175-181.
- Shade, A., P.S. McManus and J. Handelsman. 2013. Unexpected diversity during community succession in the apple flower microbiome. *mBio* 4: e00602-00612.
- Shi, S.J., A.E. Richardson, M. O'Callaghan, K.M. DeAngelis, E.E. Jones, A. Stewart, et al. 2011. Effects of selected root exudate components on soil bacterial communities. *FEMS Microbiol. Ecol.* 77: 600.
- Steer, J. and J.A. Harris. 2000. Shifts in the microbial community in rhizosphere and non-rhizosphere soils during the growth of *Agrostis stolonifera*. *Soil Biol. Biochem.* 32: 869-878. doi:[https://doi.org/10.1016/S0038-0717\(99\)00219-9](https://doi.org/10.1016/S0038-0717(99)00219-9).

- Tilman, D. and S. Pacala. 1993. The maintenance of species richness in plant communities. In: R. E. Ricklefs and D. Schluter, editors, *Species diversity in ecological communities: Historical and geographical perspectives*. University of Chicago Press, Chicago, Illinois. p. 13-25.
- Toju, H., A.S. Tanabe, S. Yamamoto and H. Sato. 2012. High-coverage ITS primers for the DNA-based identification of ascomycetes and basidiomycetes in environmental samples. *PLoS ONE* 7: e40863. doi:10.1371/journal.pone.0040863.
- Torsvik, V. and L. Øvreås. 2002. Microbial diversity and function in soil: From genes to ecosystems. *Curr. Opin. Microbiol.* 5: 240-245. doi:https://doi.org/10.1016/S1369-5274(02)00324-7.
- USGA. 2018. *USGA recommendations for a method of putting green construction*. United States Golf Association, Liberty Corner, NJ.
- van der Heijden, M.G., R.D. Bardgett and N.M. van Straalen. 2008. The unseen majority: Soil microbes as drivers of plant diversity and productivity in terrestrial ecosystems. *Ecology Letters* 11: 296-310. doi:10.1111/j.1461-0248.2007.01139.x.
- Vandenkoornhuyse, P., S.L. Baldauf, C. Leyval, J. Straczek and J.P.W. Young. 2002. Extensive fungal diversity in plant roots. *Science* 295: 2051-2051. doi:10.1126/science.295.5562.2051.
- Weisburg, W.G., S.M. Barns, D.A. Pelletier and D.J. Lane. 1991. 16S ribosomal DNA amplification for phylogenetic study. *J. Bacteriol.* 173: 697-703.
- Weiss, S., Z.Z. Xu, S. Peddada, A. Amir, K. Bittinger, A. Gonzalez, et al. 2017. Normalization and microbial differential abundance strategies depend upon data characteristics. *Microbiome* 5: 27. doi:10.1186/s40168-017-0237-y.
- White, T., T. Bruns, S. Lee and J. Taylor. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. *PCR Protocols: A Guide to Methods and Applications* 315-322.
- Whitelaw, M.A., T.J. Harden and G.L. Bender. 1997. Plant growth promotion of wheat inoculated with *Penicillium radicum* sp. Nov. *Soil Res.* 35: 291-300. doi:https://doi.org/10.1071/S96040.
- Wickham, H. 2009. *Ggplot2: Elegant graphics for data analysis*. Springer-Verlag New York. <http://ggplot2.org>.
- Yang, L., J. Xie, D. Jiang, Y. Fu, G. Li and F. Lin. 2008. Antifungal substances produced by *Penicillium oxalicum* strain py-1—potential antibiotics against plant

pathogenic fungi. *World J. Microbiol. Biotechnol.* 24: 909-915.
doi:10.1007/s11274-007-9626-x.

Yao, H., D. Bowman and W. Shi. 2011. Seasonal variations of soil microbial biomass and activity in warm- and cool-season turfgrass systems. *Soil Biol. Biochem.* 43: 1536-1543. doi:<https://doi.org/10.1016/j.soilbio.2011.03.031>.

Zhang, W., Z. Zhang, Y. Wang, D. Ai, P. Wen, Y. Zhu, et al. 2017. Effect of turfgrass establishment on soil microbiota using illumina sequencing. *Nat., Environ. Pollut. Technol.* 16: 679-686.

Chapter 3. Mapping Temporal Shifts in the Creeping Bentgrass Microbiome

ABSTRACT

For turfgrass managers there is a constant need for chemical inputs to maintain high quality stands of turfgrass. However, the indigenous turfgrass microbiome presents a potential reservoir of organisms that may be manipulated to increase plant health while decreasing the need for traditional management inputs. This project was developed to determine how bacterial and fungal populations of the creeping bentgrass (*Agrostis stolonifera* L. cv. “007”) microbiome fluctuate over time. Using a repeated measures design on replicate cone-tainers of creeping bentgrass planted into a 85% sand and 15% peat soil medium, bacterial and fungal communities were evaluated. Samples of foliage and the rhizosphere were taken at seedling emergence and at two, four, and six months post emergence. Environmental DNA was extracted, and bacterial 16S and fungal ITS amplicons were generated using PCR, and subsequently sequenced on the Illumina MiSeq as a 2 x 300 paired end run. Sequence outputs were quality filtered, amplicon sequence variants (ASVs) inferred, and diversity and statistical analyses performed using the R packages DADA2, phyloseq, and vegan. Sequencing runs generated 2.16×10^7 quality-filtered reads, which resulted in 8,811 bacterial and 1,221 fungal ASVs. Taxonomic profiling of bacterial and fungal communities showed a prevalence of Cyanobacteria, Proteobacteria, Sordariomycetes, and Dothideomycetes. Alpha diversity of bacterial communities increased from seedling emergence to two months and then stabilized. Lower bacterial alpha diversity was observed from foliage than in the rhizosphere. No significant differences were observed in fungal alpha diversity over time or across plant tissue. For both bacterial and fungal communities, ordination analyses

showed clustering by sampling time. These results show bacterial and fungal communities are evolving over time as the turf matures.

INTRODUCTION

Plant-associated microbial communities, or the microbiome, are complex entities that play a key role in determining plant health and productivity (Lambers et al., 2009; Berendsen et al., 2012). Through manipulation of the plant microbiome it is possible to reduce incidence of plant disease (Andrews, 1992), increase agricultural productivity (Bakker et al., 2012), and reduce chemical inputs (Adesemoye et al., 2009). There are several examples of improved plant growth as a direct result of microbiome manipulation. Inoculating tomatoes (*Solanum lycopersicum* L. cv. “Juliet”) with plant growth promoting rhizobacteria (*Bacillus amyloliquefaciens* IN937a and *B. pumilus* T4) and the arbuscular mycorrhizal fungi *Glomus intraradices* allowed for a 25% reduction in fertilizer rate without limiting plant growth (Adesemoye et al., 2009). Mendes and others observed that soils possessing higher abundance of specific bacterial taxa (i.e., Pseudomonadaceae, Burkholderiaceae, Xanthomonadaceae, and Lactobacillaceae) showed reduced incidence of crown and rhizosphere rot on sugar beets (*Beta vulgaris* L. cv. “Alligator”) caused by *Rhizoctonia solani* Kühn (2011). Additionally, disease suppression was partially transferred to conducive soils through the incorporation of small quantities of suppressive soil (Mendes et al., 2011).

Establishment of plant microbiomes is much like that of the disease triangle, in that it requires three components working together over time: 1) a competitive group of microbes; 2) a compatible plant host; 3) the right environment for establishing and

maintaining the plant-microbial relationship(s). The soil lying beneath plants is inhabited by an abundance of microbial life, with a gram of soil containing 10^8 - 10^9 bacteria and 10^5 - 10^6 fungi (Sylvia et al., 2005). Micallef and others showed that rhizosphere bacterial community succession progressed differently and in a repeatable manner when examining early stage development in two separate accessions of *Arabidopsis thaliana* L. (2009). Further analysis showed that communities converged as plants neared the end of their life cycle (~ 8 weeks in the study), which coincided with an expected decrease in rhizosphere exudate release, though root exudate quantities were not measured. Seasonal variations impact the environment and consequently, have been observed to impact plant associated microbial communities. Several warm- and cool-season turfgrasses were observed to have lower biomass in September and higher biomass in May and December, which may have been driven by competition for available N (Yao et al., 2011). Beirn et al. also showed significant changes in the rhizosphere bacterial and archaeal communities of a *Poa annua* L. putting green when sampling over a 12-month period (2016). Crouch et al. surveyed the rhizosphere bacterial communities before and after an extensive renovation of the soil and turfgrass at the National Mall in Washington D.C., and found that there was no appreciable change in the bacterial communities pre- or post-renovation (2017).

Plant management practices have the ability to alter host physiology in addition to the microenvironment. Research to understand how management practices impact the microbiome have produced mixed results. Gdanetz and Trail (2017) observed the whole wheat (*Triticum aestivum* L.) microbiome, both bacteria and fungi across all plant organs, to be unaffected when comparing growth under conventional or organic management

practice (2017), while Hartmann et al. previously found significant differences in rhizosphere bacterial and fungal communities of winter wheat and grass-clover rotations under long term organic and conventional management (2014). On a creeping bentgrass (*Agrostis stolonifera* L. cv. “A1”) putting green, Doherty et al. observed significant reductions in general bacterial, fungal, fluorescent *Pseudomonad*, and actinomycete populations in response to repeated fungicide applications over a two-year period (2017). In the first year of the study, fluazinam increased populations of actinomycetes and fluxapyroxad lowered populations of fluorescent *Pseudomonads* when compared to their non-treated controls (Doherty et al., 2017). Interestingly, in the second year, fluazinam and fosetyl-AI decreased actinomycete populations, fluazinam also reduced bacterial populations, while chlorothalonil and pyraclostrobin lowered fungal populations when compared to their respective non-treated controls (Doherty et al., 2017). Additional research is needed to further understand the plant-microbe interactions driving microbiome composition.

Our understanding of the turfgrass microbiome is limited, although recent research is showing that location plays a significant role in the microbiome composition (Beirn et al., 2016; Crouch et al., 2017; Elliott et al., 2008). One main goal of microbiome research is to utilize microbial groups in reducing agronomic inputs while simultaneously maintaining or improving plant health. However, it is essential to understand these developing microbial communities before we can hope to consistently manipulate the microbiome to our benefit. To that end this project was developed to map populations of the creeping bentgrass microbiome from seedling emergence through early

development, and to identify microbes that consistently associate with creeping bentgrass.

MATERIALS & METHODS

Growth Conditions. To minimize external influences on the turfgrass microbiome and ascertain temporal shifts in microbial populations driven by plant maturity, this experiment was conducted at the Research Plant Growth Facility at the University of Maryland, College Park, MD. Seeds of “007” creeping bentgrass were planted into replicate SC10 cone-tainers (Stuewe & Sons, Inc., Tangent, OR) filled with a soil medium of 85% sand and 15% peat (v:v). Plantings were then placed within a greenhouse and maintained until 6 months following seedling emergence. Experimental run 1 was initiated on 6 June 2016 and experimental run 2 was initiated on 10 August 2016. Greenhouse temperatures were maintained at 25°C and 23°C for experimental run 1 and 2, respectively. Irrigation was provided daily between the hours of 8 AM and 5:15 PM through a mister system within the greenhouse range every 15 min for 30 s, delivering 165 ml of H₂O per misting event. Nutrients were provided every 14 d in the form of 10 mL ½-strength Hoagland’s solution (Hoagland & Arnon, 1950).

Sample Collection and Processing. Using a repeated measures design, samples of foliage and rhizosphere were taken at emergence and at two, four, and six months post-emergence. To remove bulk soil, samples were shaken by hand and roots were combed through using flame-sterilized forceps. Foliar and rhizosphere samples were separated at the chlorophyll line at sampling using flame sterilized forceps and placed into autoclaved coin envelopes (#3, Staples, Framingham, MA). Samples were immediately placed on

ice to minimize microbial community degradation during transportation. Bulk soil was taken from each replicate cone-tainer at the conclusion of the experiment. All samples were stored at -20°C and processed within 24 h. Environmental DNA (eDNA), i.e., the DNA of all organisms present in a sample, was extracted from foliar samples using the Qiagen DNeasy Plant Mini Kit (Qiagen, Gaithersburg, MD). Samples were incubated overnight in solution AP1 (solution in DNeasy Plant Mini Kit) at 65° C (149° F), two 1.5 ml microcentrifuge tube capfuls of 450-600 µm glass beads (Acros Organics, Morris Plains, NJ) were added to each sample, subjected to 3 consecutive runs on a FastPrep-24 (MP Biomedicals, Solon, OH) at 4.5 m/s for 25 seconds, and extracted following the manufacturer's protocol. Rhizosphere and soil sample eDNA was extracted using the Qiagen PowerSoil kit (Qiagen). A modified protocol for low biomass soils was provided by the PowerSoil kit manufacturer to increase yields in high sand content root zones. An equal volume of 25:24:1 chloroform:phenol:isoamyl alcohol pH 8 (VWR, Radnor, PA) was used to replace 200 µl of the solution in the PowerSoil Bead tube, followed by adding 0.25 g of soil and 60 µl of solution C1. Vortexing was replaced with 3 runs on a FastPrep-24 at 4.5 m/s for 25 seconds. The manufacturer's protocol was then followed until the elution step where 60 µl of solution C6 was used for elution of eDNA.

DNA Manipulations. Quality and concentration of extracted eDNA were measured using a Nanodrop 1000 (Thermo Fisher Scientific, Waltham, MA) and a Qubit fluorometer (Life Technologies, Grand Island, NY), respectively. Extracted eDNA was then diluted to 1.5 ng/µl, based on Qubit measurements. Amplicons were generated using a two-step PCR process: first, specific regions of interest were PCR amplified utilizing primers with overhang adapters; second, an additional PCR step was utilized to

add Illumina P5 and P7 sequence adaptors. The bacterial 16S v3-v4 hypervariable region of ribosomal DNA was amplified using the primer pair Ba9F/Ba515Rmod1 (~500bp; Kittelmann et al., 2013; Weisburg et al., 1991). The fungal ITS2 region was amplified using the ITS3_KYO2/ITS4-R primer pair (~350bp; Toju et al., 2012; White et al., 1990). Prior to primer synthesis overhang adaptor sequences, used for integration of indices and Illumina sequencing adaptors, were added to the 5' end of each primer. Forward primers overhang sequences were 5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG-3', while reverse primer overhang sequences were 5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG-3'. Reverse primers were synthesized with 0-3 mixed sequence bases (where N is any nucleotide), and combined into an equimolar mixture for subsequent PCR reactions to increase sequencing diversity. All PCR reactions were performed using MangoTaq DNA Polymerase (BioLine, Taunton, MA) in 25 µL volumes containing 5X PCR buffer (BioLine), 0.2 mM of each dNTP (BioLine), 2 mM MgCl₂ (BioLine), and 10 µM of each primer. Cycle conditions were: 94°C for 2 min, followed by 30 cycles of 94°C for 1 min, 52°C for 45 s, 72°C for 45 s, followed by a final extension at 72°C for 5 min (Beirn et al., 2016). Amplicon production was confirmed with the QIAxcel Advanced system (Qiagen), then amplicons for each sample were pooled and purified using the ZR-96 DNA Clean & Concentrator™-5 (Zymo Research, Irvine, California).

Indices were added to the purified amplicon libraries using the Nextera XT index kit v2 (Illumina, San Diego, CA) to enable multiplexing of many samples in a single MiSeq cartridge. Indexing reactions were prepared in 40 µl volumes containing 5X PCR buffer (BioLine), 2 mM MgCl₂ (BioLine), 0.2 mM of each dNTP (BioLine), 5 µl of each

Nextera index primer (Illumina), and 2.5 U MangoTaq DNA polymerase. The indices were incorporated with the following cycling conditions: 72°C for 3 min, 95°C for 30 s, followed by 12 cycles of 95°C for 10 s, 55°C for 30 s, 72°C for 30 s, followed with a final extension at 72°C for 5 min (Beirn et al., 2016). Indexed libraries were cleaned using HighPrep PCR (MagBio Genomics, Gaithersburg, MD) with a modified protocol for targeted removal of any sequence fragments less than 200 bp. The QIAxcel Advanced (Qiagen) and Qubit fluorometer (Life Technologies) were used to determine fragment size and DNA concentration for each purified library. All libraries were then normalized to 4 nM and pooled into a single pooled amplicon library (PAL). PhiX control (Illumina) was spiked into the PAL at 30% final volume. The PAL and PhiX solution was denatured with an equal volume of 0.2N NaOH at room temperature for 5 min. Once denatured the solution was further diluted to 10 pM for optimal cluster density in the MiSeq run. Immediately prior to loading the MiSeq, the PAL and PhiX mixture was heat denatured at 95°C for 5 min, and subsequently put onto ice. Sequencing was performed as a paired-end (2x300) run on the Illumina MiSeq platform with a 600-cycle MiSeq v.3 Reagent Cartridge (Illumina). Two additional technical replicates for both experimental runs were performed identically from amplicon generation through sequencing on the Illumina MiSeq. All sequences were output in FASTQ format.

Sequence Analysis. Non-biological sequences were removed from paired-end sequences using cutadapt 1.9.1 (Martin, 2011). Within the cutadapt program, the “-p” option was utilized to parse paired-end files together, thereby maintaining matching reads between files for downstream analyses. Following the DADA2 1.6 pipeline (Callahan et al., 2016), paired-end files were quality filtered, denoised, joined, amplicon sequence

variants (ASVs) were inferred, and taxonomy assigned. Bacterial amplicon reads were trimmed to 275 bp to remove low quality tails. Fungal reads were joined without trimming or truncation to avoid masking the length variation within the ITS2 region. Sequences determined to be chimeric by DADA2 were removed from the dataset. Using a DADA2 formatted release of the GreenGenes v.13.8 database (Callahan, 2016;McDonald et al., 2012) and the UNITE general FASTA v.7.2 release (Kõljalg et al., 2013), taxonomy was assigned for bacteria and fungi, respectively. For ASVs not assigned taxonomy in DADA2, their sequences were subjected to a BLAST query. If identification was provided from BLAST, that taxonomy was added to the dataset. Any ASVs assigned Plantae, Protista, chloroplast, or mitochondria were removed from the analysis.

The R packages phyloseq (McMurdie and Holmes, 2013) and vegan (Oksanen et al., 2017) were utilized for diversity and statistical analyses within the R statistical computing environment v.3.4.2 (R Core Team, 2017). Unless otherwise stated, a p-value of ≤ 0.05 was considered significant. Graphics were generated using the ggplot2 package (Wickham, 2009), unless otherwise stated. Alpha diversity, or the species richness and evenness within samples, was measured using the Shannon diversity index. No rarefaction was performed for estimation of alpha diversity metrics, as these metrics are not sample size dependent (McMurdie and Holmes, 2014). Significant differences in alpha diversity between samples were determined using nonparametric Kruskal-Wallis test, with Benjamini-Hochberg correction for multiple pairwise comparisons applied using the dunn.test package (Dinno, 2017) in R.

Beta diversity, or the change in diversity between samples, was calculated using Bray-Curtis dissimilarity matrices, where large values indicate that samples are less similar and smaller values indicate that samples have similar community structure (Gardener, 2014). To account for variation in sequencing depth (>10X), reads were scaled for beta diversity measures (Weiss et al., 2017). Distance matrices were subjected to ordination analyses, permutational analysis of variance (PERMANOVA) for testing community centroids, and homoscedasticity tests to determine community variance. Ordinations were performed using phyloseq, and PERMANOVA and homogeneity of variance tests performed using the ‘adonis’ and ‘betadisper’ tests in vegan.

RESULTS

Sequencing Output. Sequencing of the bacterial 16S and fungal ITS amplicons resulted in 2.16×10^7 reads usable in downstream analyses. Only forward reads of 16S were used in analysis of bacterial communities as poor reverse read quality resulted in minimal overlap. The sequence reads contained 16,619 ASVs, with 11,454 bacterial and 5,165 fungal. Following removal of chimeras and contaminant ASVs, (i.e., chloroplast, mitochondria, Plantae, and Protista) 10,032 ASVs were identified, with 8,811 and 1,221 ASVs belonging to bacteria and fungi, respectively. Technical replicates were not different for each sample and were pooled together. Experimental runs are presented separately as analysis showed significance for both bacteria and fungi.

Diversity of Bacterial and Fungal Communities. For both bacterial and fungal alpha diversity, experimental run was not significant. Therefore, experimental runs were combined for alpha diversity metrics. Bacterial alpha diversity was lowest at seedling emergence, with no other differences between two, four, and six months (Table 6, Fig. 5A). Diversity of soil and rhizosphere bacterial communities was higher than the foliar bacterial communities (Fig. 5A). No significant differences were observed in the alpha diversity of fungal communities within the study (Table 6, Fig. 5B).

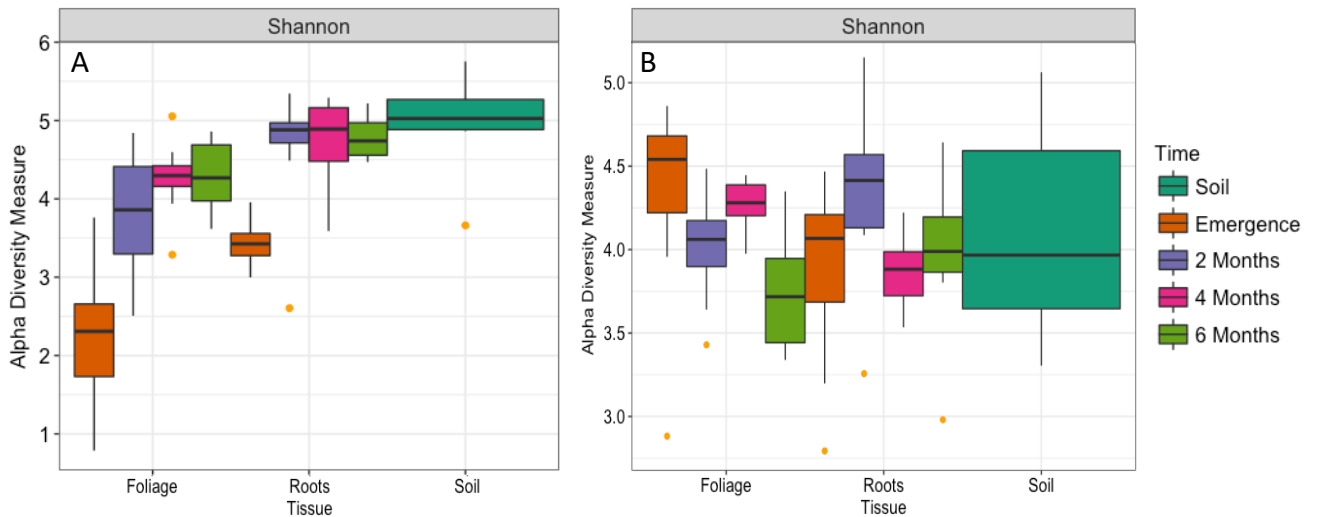


Figure 5. Shannon diversity measures, calculated using phyloseq in R, for A) bacterial and B) fungal communities associated with greenhouse grown creeping bentgrass. Higher values indicate higher overall species richness and evenness. Orange point indicate outliers.

Experimental run was significant for both bacterial and fungal beta diversity.

Within both experimental runs, PERMANOVA tests revealed significantly different centroids for both time and tissue sampled for bacterial and fungal communities (Table 7), however, only tissue sampled had significant tests of homoscedasticity in bacterial communities. Fungal communities had significant tests of homoscedasticity, except for sample time in the second experimental run (Table 7). Ordination of bacterial data revealed clustering by time sampled for both experimental runs (Fig. 6A). Similarly,

ordination of fungal data showed clustering by time sampled in both experimental runs (Fig. 6B).

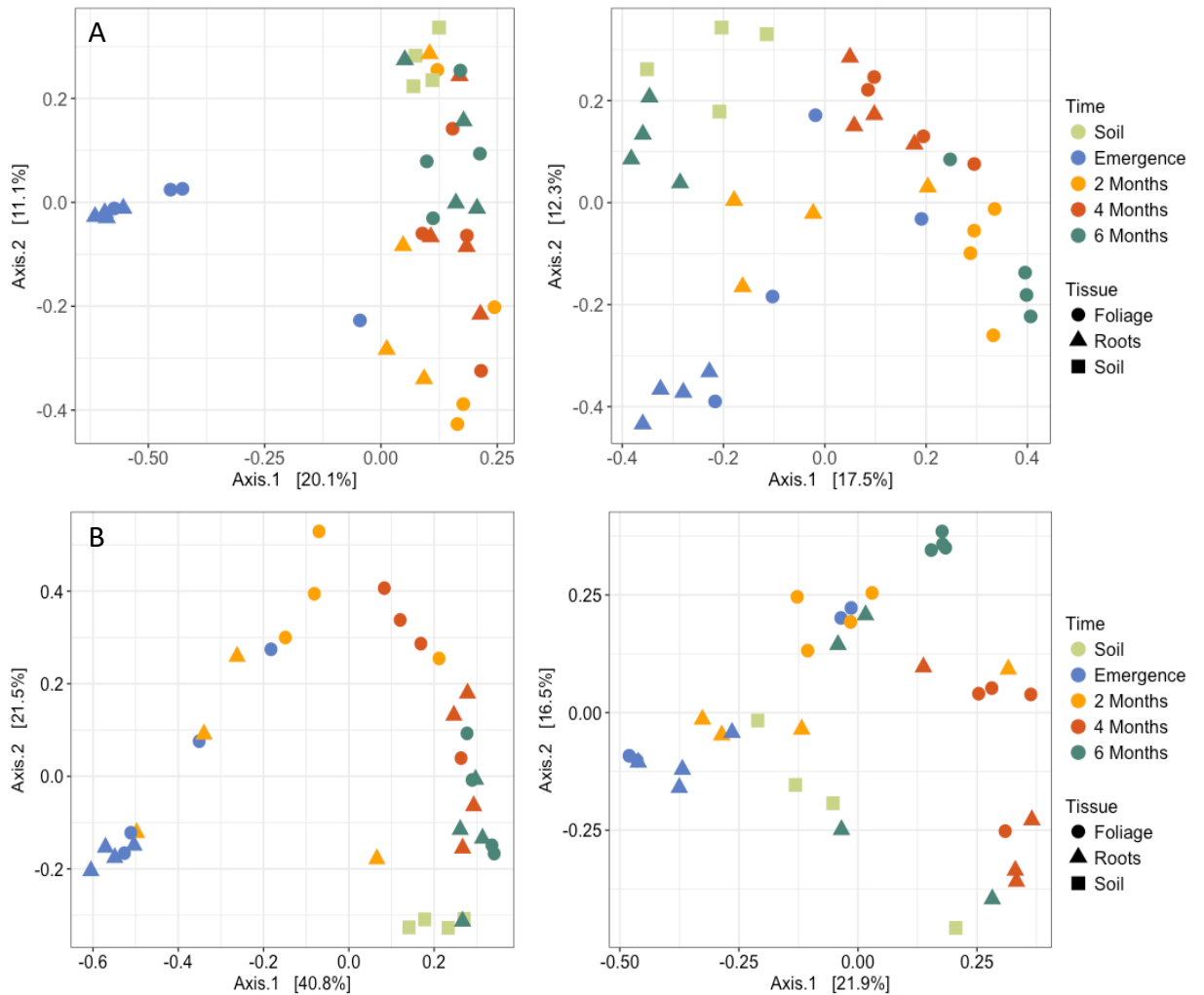


Figure 6. Principal component analysis, completed using phyloseq in R, of Bray-Curtis distance matrices, calculated in phyloseq, of A) bacterial and B) fungal communities of experimental run 1 (left) and experimental run 2 (right). Values in brackets indicate percent variation explained.

Taxonomic Composition of Amplicon Sequence Variants. Total bacterial ASVs

were predominantly made up of members of the Proteobacteria (41%) and Actinobacteria (15.2%). Only 5.9% of ASVs were not assigned taxonomy at the phylum level. At the genus level, taxonomic identification was not possible for 64.09% of the ASVs. The Proteobacteria were composed of Alphaproteobacteria (70%), with the next largest group

being the Gammaproteobacteria (10%). Actinobacteria were predominantly composed of the classes Actinobacteria (55%) and Acidimicrobiia (30%).

Fungal ASVs were predominantly composed of Sordariomycetes (22.5%), Dothideomycetes (18.6%), and Eurotiomycetes (14.3%). A large portion (i.e., 30.6%) of the total fungal ASVs could not be assigned taxonomy at the class level; however, when converted to relative abundances, these unassigned ASVs made up only 0.5% or less across samples throughout the course of the experiment (Fig. 7). The Sordariomycetes were predominantly composed of *Trichoderma* (30.5%), *Pleurophragmium* (11.6%), *Fusarium* (11.2%), and *Plectosphaerella* (10.5%). The most prevalent genus of the Dothideomycetes was *Curvularia* (14.9%), although 28.6% of Dothideomycetes could

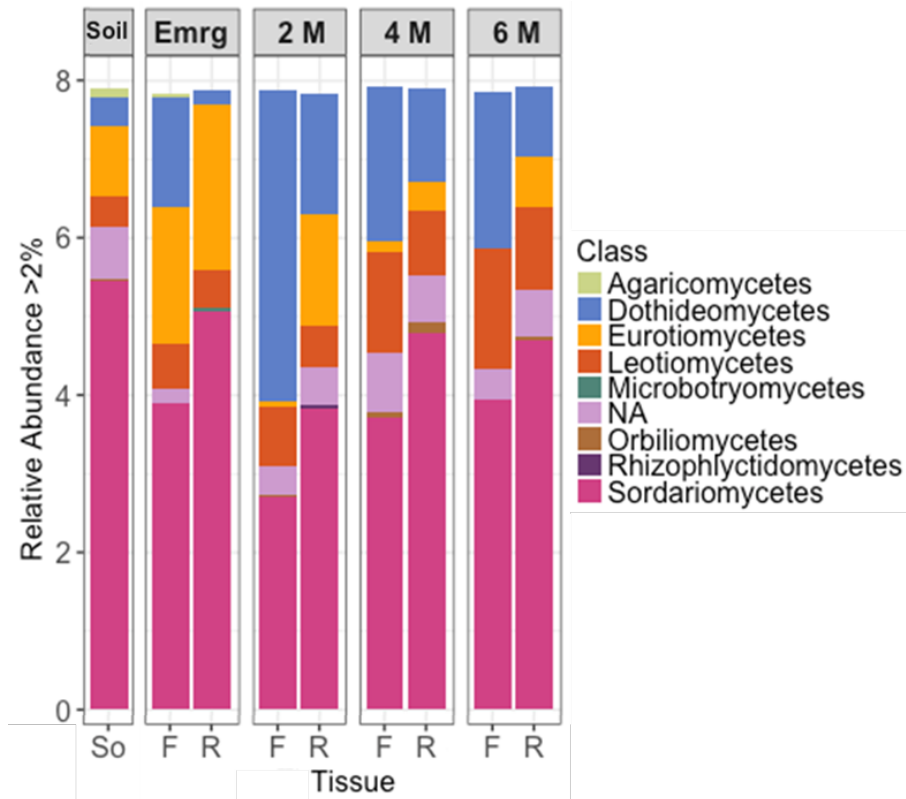


Figure 7. Relative abundances, calculated using phyloseq in R, of fungal classes associated with greenhouse grown creeping bentgrass. “F”, “R”, and “So” refer to “Foliar”, “Rhizosphere”, and “Soil” samples, respectively. “Emrg”, “2 M”, “4 M”, and “6 M” refer to “Emergence”, “2 Month”, “4 Month”, and “6 Month” sampling times, respectively.

not be identified at the genus level. Eurotiomycetes were predominantly composed of *Penicillium* (52%).

Changes in Taxonomy Across Plant Tissue and Time. Bacterial community abundance was dominated by Proteobacteria and Cyanobacteria over the six-month sampling period. As the turfgrass matured, the relative abundance of Cyanobacteria decreased from ~2.25% to ~1% (Fig. 8). The Cyanobacteria observed were predominantly members of the Nostocaceae family (Fig. 9). A high relative abundance (~2.25%) of *Burkholderia* was observed at seedling emergence, which decreased to <0.25% relative abundance at all subsequent time points (Fig. 10). *Erwinia* and *Dyella*

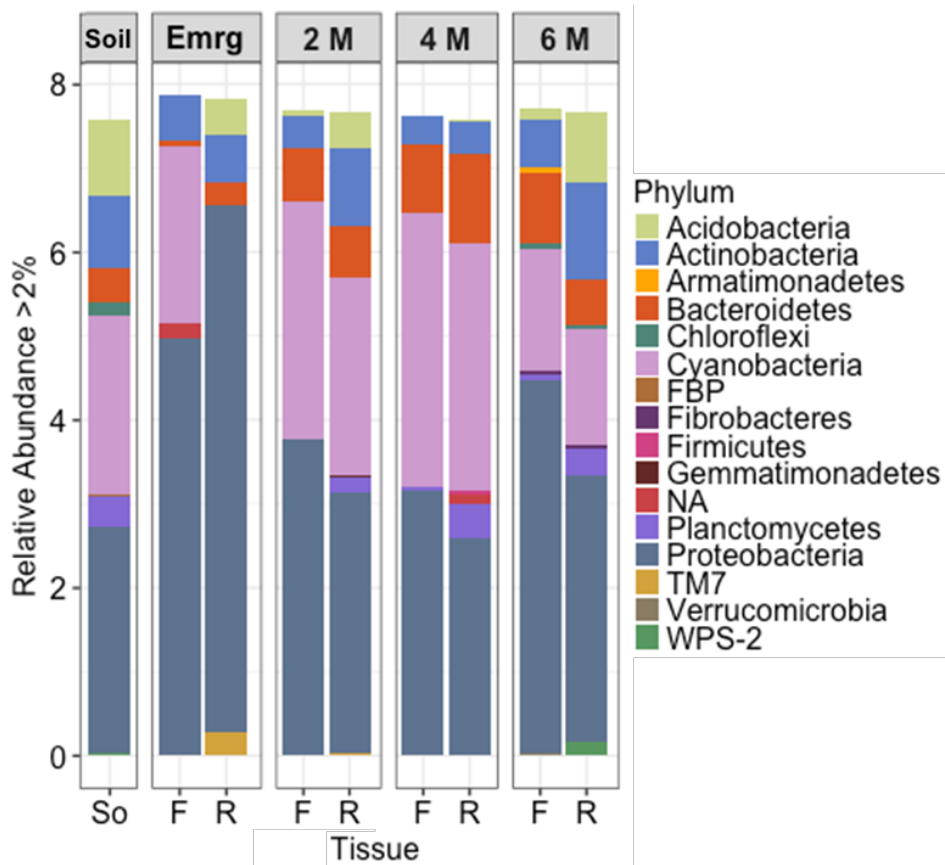


Figure 8. Relative abundances, calculated using phyloseq in R, of bacterial phyla associated with greenhouse grown creeping bentgrass. “F”, “R”, and “So” refer to “Foliar”, “Rhizosphere”, and “Soil” samples, respectively. “Emrg”, “2 M”, “4 M”, and “6 M” refer to “Emergence”, “2 Month”, “4 Month”, and “6 Month” sampling times, respectively.

were also observed at seedling emergence, but were undetectable and less abundant at additional sampling points (Fig. 10). Foliar samples had higher relative abundance of *Spingomonas* compared to rhizosphere and soil samples (Fig. 10).

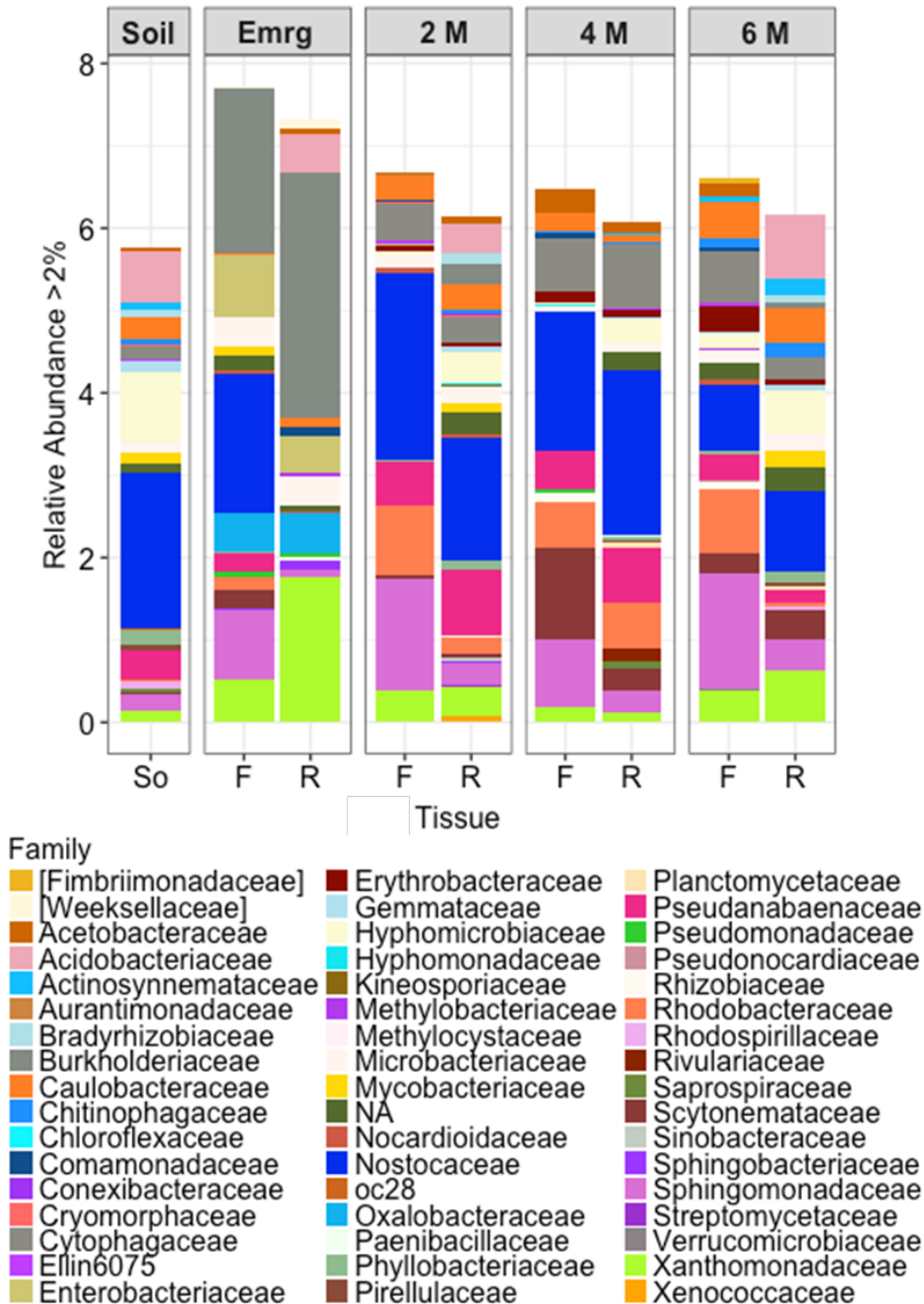


Figure 9. Relative abundances, calculated using phyloseq in R, of bacterial families associated with greenhouse grown creeping bentgrass. “F”, “R”, and “So” refer to “Foliar”, “Rhizosphere”, and “Soil” samples, respectively. “Emrg”, “2 M”, “4 M”, and “6 M” refer to “Emergence”, “2 Month”, “4 Month”, and “6 Month” sampling times, respectively.

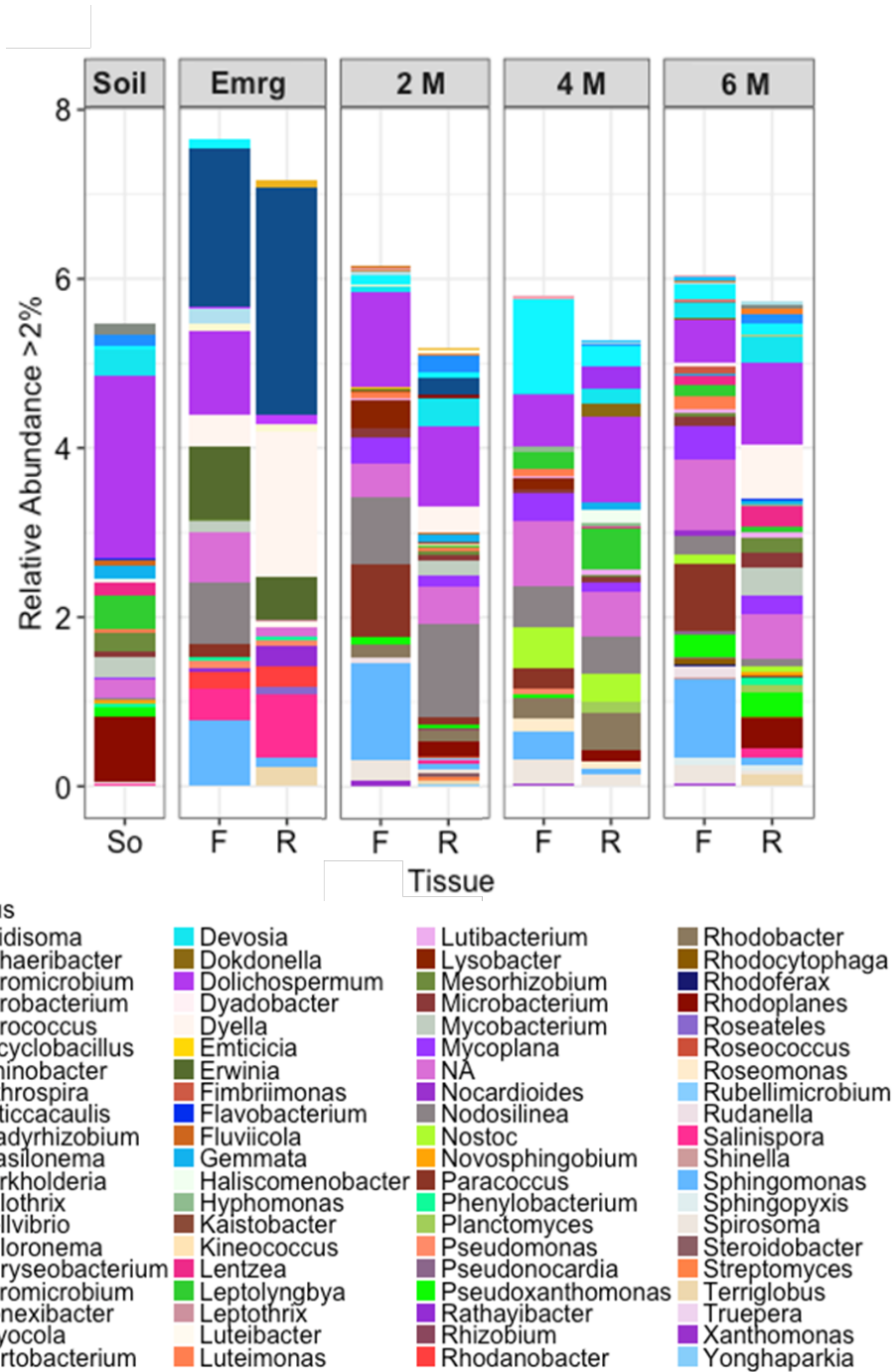


Figure 10. Relative abundances, calculated using phyloseq in R, of bacterial genera associated with greenhouse grown creeping bentgrass. “F”, “R”, and “So” refer to “Foliar”, “Rhizosphere”, and “Soil” samples, respectively. “Emrg”, “2 M”, “4 M”, and “6 M” refer to “Emergence”, “2 Month”, “4 Month”, and “6 Month” sampling times, respectively.

Apart from the foliar samples at 2-months post-emergence, fungal communities were dominated by Sordariomycetes, followed by Dothideomycetes and Eurotiomycetes (Fig. 7). The foliage at 2-months post-emergence had higher relative abundance of Dothideomycetes and lower relative abundance of Eurotiomycetes than all other samples (Fig. 7). Genus level taxonomy revealed more nuances in the fungal populations than class level taxonomy. Soil fungal communities were dominated by *Pleurophragmium* (~2.25% relative abundance) and *Trichoderma* (~1.25% relative abundance). At seedling emergence there was an increase of *Trichoderma* (3% and 5% relative abundance in foliage and rhizosphere, respectively) and minimal levels of *Pleurophragmium* (~<0.1% relative abundance) (Fig. 11). As time progressed *Trichoderma* continued to decrease until levels were below 0.25% relative abundance at six-months post-emergence (Fig. 11). The relative abundance of *Pleurophragmium* increased from 0-0.1% at emergence to 2-2.25% at six-months post-emergence (Fig. 11). There was an increase of *Curvularia* (~1.5-1.75% relative abundance) at two-months post emergence, followed by a continual decrease to ~0-0.25% relative abundance (Fig. 11). From 2 months forward the foliage consistently had about 1% relative abundance of *Plectosphaerella* (Fig. 11).

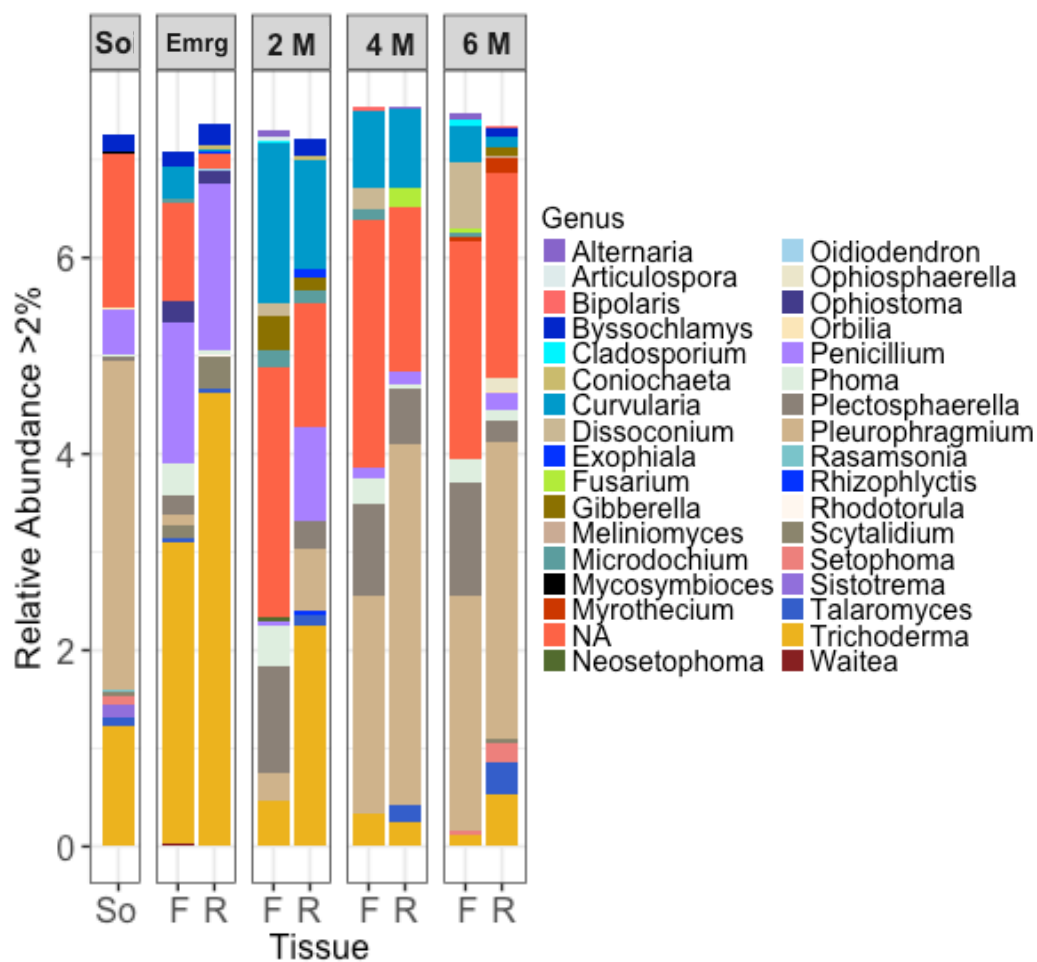


Figure 11. Relative abundances, calculated using phyloseq in R, of fungal genera associated with greenhouse grown creeping bentgrass. “F”, “R”, and “So” refer to “Foliar”, “Rhizosphere”, and “Soil” samples, respectively. “Emrg”, “2 M”, “4 M”, and “6 M” refer to “Emergence”, “2 Month”, “4 Month”, and “6 Month” sampling times, respectively.

Table 6. Microbial Community Alpha-Diversity Measures for Greenhouse Grown Creeping Bentgrass

		Alpha-Diversity [†]			
		Bacteria		Fungi	
Group 1	Group 2	Kruskal-Wallis chi ^{2‡}	p-value [§]	Kruskal-Wallis chi ²	p-value
Tissue		17.98	0.001***	0.43	0.810
Foliage	Roots	-3.00	0.002**	0.65	0.773
Foliage	Soil	-3.79	0.001***	0.15	0.439
Roots	Soil	-1.89	0.030*	-0.26	0.598
Time		37.73	0.001***	4.62	0.330
Emergence	2 Months	-3.50	0.006**	-0.12	0.453
Emergence	4 Months	-4.13	0.001***	0.72	0.394
Emergence	6 Months	-4.17	0.001***	1.79	0.183
2 Months	4 Months	-0.63	0.292	0.84	0.403
2 Months	6 Months	-0.67	0.312	1.91	0.281
4 Months	6 Months	-0.04	0.483	1.07	0.472

[†] Alpha diversity, calculated as Shannon's diversity index using phyloseq in R.

[‡] Chi² test statistic calculated through Kruskal-Wallis tests using dunn.test in R.

[§]p-values corrected using Benjamini-Hochberg correction for multiple comparisons.

* Significant at the 0.05 probability level.

** Significant at the 0.01 probability level.

*** Significant at the 0.001 probability level.

Table 7. Centroid and Dispersion Tests of Beta-Diversity[†] Metrics for Microbial Communities Associated with Greenhouse Grown Creeping Bentgrass

	Bacteria			
	Run 1		Run 2	
	PERMANOVA p-value [‡]	Dispersion p-value [§]	PERMANOVA p-value	Dispersion p-value
Tissue	0.022*	0.011*	0.001***	0.003**
Time	0.001***	0.873	0.001***	0.187
	Fungi			
Tissue	0.010**	0.001***	0.002**	0.005**
Time	0.001***	0.001***	0.001***	0.113

[†]Beta diversity metrics calculated as Bray-Curtis dissimilarity matrices using phyloseq in R.

[‡]Permutational analysis of variance p-value calculated using vegan in R.

[§]Dispersion tests used to test community variances, p-values calculated using vegan in R.

* Significant at the 0.05 probability level.

** Significant at the 0.01 probability level.

*** Significant at the 0.001 probability level.

DISCUSSION

The goal of this project was to obtain an understanding of community dynamics of the developing creeping bentgrass microbiome following seedling emergence, and to determine the core of the microbiome. Minimal shifts in bacterial community diversity were observed and no significant changes in fungal community diversity were evident over the six-month study. Consistent bacterial community dominance from the Proteobacteria, Cyanobacteria, and Soradiomycetes was observed; whereas, fungal communities were predominantly the Dothideomycetes. Ordination analyses revealed clustering by sample time, with sample time and tissue types all having significantly different centroids.

Bacterial communities in our study were consistently dominated by members of the Proteobacteria and Cyanobacteria. Within the Cyanobacteria, the Nostocaceae consistently dominated across sampled tissues and times. Members of the Nostocaceae are capable of fixing N in specialized cells called heterocysts (Komárek, 2016), and are the most heavily studied Cyanobacteria for their heterocyst glycolipid content (Gambacorta et al., 1998). Bacterial communities contained a large relative abundance (2-2.25%) of *Burkholderia* at emergence, yet these numbers diminished at subsequent sampling times. *Burkholderia* have been shown to produce antibiotic compounds (Pidot et al., 2014), and may prove useful combating plant diseases, but these compounds are often only produced under high cell concentrations or nutrient limited conditions (Haas and Keel, 2003). The Sphingomonadaceae, while exhibiting low relative abundance (~1%) can be antagonistic to plant pathogens, plant growth promoters, and bioremediators due to their capability to degrade xenobiotic and recalcitrant

(poly)aromatic compounds (Glaeser and Kampfer, 2014). Additional research is needed to understand how these bacteria maintain dominance in the microbial community in order to promote plant beneficial properties in the future.

Fungal communities in our study were dominated by *Trichoderma* at seedling emergence, yet were dominated by *Pleurophragmium* at 6 months. Many of the described species of *Pleurophragmium* are found as saprobes on decaying organic material (D'Souza and Bahat, 2012; Luttrell, 1964; Kedsueb et al., 2008). Additionally, some several genera are described as being hyperparasites of fungi within the Meliolales order (Luttrell, 1964). *Trichoderma* species are well known for their use as a biological control agents to combat plant disease. For example, in turfgrass systems *Trichoderma harzianum* 1295-22 has been used to reduce damage from *Sclerotinia homoeocarpa*, *Rhizoctonia solani*, and *Pythium graminicola* (Lo et al., 1997). Another isolate of *T. harzianum*, T39, has been observed to control *Botrytis cineria*, *Pseuoperonospora cubensis*, *S. sclerotiorum*, and *Sphaerotheca fusca* in cucumber under commercial greenhouse conditions (Elad, 2000).

Cladosporium and *Plectosphaerella* made up a smaller portion of the fungal community, but were still consistently present in plant tissue. The *Cladosporium* genus contains a wide variety of organisms, including saprobes (i.e., fungi that feed off non-living or decaying organic matter), fungicolous species (i.e., fungi growing on other fungi), and plant pathogens (Bensch et al., 2012). One of the most prominent members of the *Plectosphaerella* genus is *P. cucumerina*, which is a necrotrophic fungus that colonizes its host and causes cell death (Gamir et al., 2012). Taxonomic profiling reveals a microbiome inhabited by beneficial, detrimental, and neutral fungi. Focusing future

research on promoting relationships between plants and beneficial fungi is critical to improving plant health.

Over the course of the experiment the only change in alpha diversity was observed in bacterial communities, where diversity increased from seedling emergence to two months followed by a stabilization of diversity afterwards. Although, with this study being in a controlled environment (i.e., a greenhouse) some factors influencing the microbiome development may have been attenuated (e.g., reduced water stress from consistent watering, limited temperature fluctuations) or omitted (e.g., weather events, minimal inoculum introduced to microbiome). Investigations of soil microbial diversity over a turfgrass chronosequence (i.e., a set of sites that share similar attributes, but are of differing ages) observed that soil microbial community diversity, as measured through phospholipid fatty acid analysis and substrate use pattern, was similar at a depth of 0-5 cm for 1-, 6-, 23-, and 95-year old stands of turfgrass (Yao et al., 2006). Interestingly, the diversity at a sampling depth of 5-15cm was lower in the 1- and 6-year old turfgrass compared to the 2 older stands, which directly correlated to lower organic matter in the soil of the younger stands (Yao et al., 2006). However, community structure showed the 23- and 95- year old turfgrass stands diverging from the 1- and 6-year old turfgrass stands (Yao et al., 2006). Using next-generation sequencing technologies Beirn et al. (2016) showed an increase in bacterial and archaeal diversity over the course of a year in the rhizosphere of a 5-yr old *Poa annua* L. putting green. Increases in bacterial diversity have also been observed in wheat, as rhizosphere bacterial communities had higher diversity at flowering at seed development stages as compared to the vegetative growth stage (Gdanetz and Trail, 2017). Using the higher resolution acquired with next-

generation sequencing technologies we may be able to capture shifts in diversity previous methods missed.

Unlike bacterial diversity, fungal diversity exhibited no significant changes over the course of the study or across plant tissue. Consistent fungal diversity was also observed in wheat grown under field conditions (Gdanetz and Trail, 2017). Moreover, research on the agave (*Agave tequilana* L.) microbiome also similarities in fungal diversity of rhizosphere and phyllosphere samples (Coleman-Derr et al., 2016). Considering multiple studies, under both controlled and field conditions, found no differences in rhizosphere or phyllosphere fungal diversity, there may be a rapid systemic colonization of plant tissue following seedling emergence. Future research would benefit from utilizing multiple sampling points from seedling emergence to 24-48 hours post-emergence to capture the state of fungal colonization of the newly emerged plant tissue.

Ordination analyses revealed clustering of samples by the time when the samples were taken and tighter clustering as time progressed. This could be due to communities reaching an equilibrium with turfgrass maturity. In the 4- and 6-month samplings relative abundances of the Sphingomonadaceae, Cytophagaceae, Pseudanabeanaceae, Xanthomonadaceae, and *Pleurophragmium* appear to be stabilizing. Previous research examining *A. stolonifera* putting green rootzones in North Carolina showed that culturable communities of fluorescent pseudomonads, actinomycetes and Gram-negative bacteria became increasingly stable as the newly seeded putting greens matured over a 23-month period (Bigelow et al., 2002). Understanding what is driving this stabilization of microbiome populations could elucidate avenues for manipulating the microbiome to artificially select for desired microbes.

With one of the main goals of microbiome research being to increase plant-beneficial microbe interactions, several factors may need to be overcome in order to consistently achieve these interactions. Along with Bigelow et al. (2002) we observed a stabilization of bacterial communities as turfgrass plant matured. This could hinder or enhance establishment of introduced beneficial microorganisms. A stabilization could indicate niche fulfillment has occurred and the top competitors have emerged as the predominant microbiome members. Conversely, this stabilization could indicate reduced competition for an introduced organism to overcome. However, further research would benefit from testing introduced biological organisms across a multitude of environmental conditions and geographical regions, thus maximizing inference for the organisms' competitiveness. Additionally, by surveying the microbiome of a site it may be possible to supplement naturally occurring populations of a beneficial microorganism with an applied organism, thus resulting in a synergistic increase in the desired microorganisms. Although, for practitioners surveying the microbiome would be a costly and time-consuming endeavor, especially across large areas such as agricultural fields or all 18 fairways on a golf course. Therefore, development of targeted rapid testing methods would make this a more feasible implementation for practitioners.

In summary, this work provides a framework for next-generation sequencing analyses of the turfgrass microbiome. Our work illustrated that microbial communities associated with creeping bentgrass are diverse and resilient to change after seedling establishment. Diversity of bacterial communities increased following emergence, and fungal community diversity did not change over the course of the experiment. Dominant community members remained dominant over the six-month sampling period, and shifts

in taxonomic profiles were mainly driven by rarer species at low relative abundances. To our knowledge this is the first project aimed at mapping the creeping bentgrass microbiome from establishment. While we observed the same predominant bacterial and fungal members as field studies in both turf and additional graminaceous hosts, further research is needed to determine if these organisms are functioning in the same manner under field conditions.

LITERATURE CITED

- Adesemoye, A.O., H.A. Torbert and J.W. Kloepper. 2009. Plant growth-promoting rhizobacteria allow reduced application rates of chemical fertilizers. *Microb. Ecol.* 58: 921-929.
- Andrews, J.H. 1992. Biological control in the phyllosphere. *Annu. Rev. Phytopathol.* 30: 603-635.
- Bakker, M.G., D.K. Manter, A.M. Sheflin, T.L. Weir and J.M. Vivanco. 2012. Harnessing the rhizosphere microbiome through plant breeding and agricultural management. *Plant Soil* 360: 1-13. doi:10.1007/s11104-012-1361-x.
- Bartlett, M.D., I.T. James, J.A. Harris and K. Ritz. 2008. Size and phenotypic structure of microbial communities within soil profiles in relation to different playing areas on a UK golf course. *Eur. J. Soil Sci.* 59: 835-841. doi:10.1111/j.1365-2389.2008.01056.x.
- Beirn, L.A., J.W. Hempfling, C.J. Schmid, J.A. Murphy, B.B. Clarke and J.A. Crouch. 2016. Differences among soil-inhabiting microbial communities in *Poa annua* turf throughout the growing season. *Crop Sci.* doi:10.2135/cropsci2016.06.0463.
- Bensch, K., U. Braun, J.Z. Groenewald and P.W. Crous. 2012. The genus *Cladosporium*. *Studies in Mycology* 72: 1-401. doi:https://doi.org/10.3114/sim0003.
- Berendsen, R.L., C.M.J. Pieterse, and P.A.H.M. Bakker. 2012. The rhizosphere microbiome and plant health. *Trends in Plant Science* 17: 478-486. doi:https://doi.org/10.1016/j.tplants.2012.04.001
- Bigelow, C.A., D.C. Bowman and A.G. Wollum. 2002. Characterization of soil microbial population dynamics in newly constructed sand-based rootzones. *Crop Sci.* 42: 1611-1614. doi:10.2135/cropsci2002.1611.
- Buck, J.W. and L.L. Burpee. 2002. The effects of fungicides on the phylloplane yeast populations of creeping bentgrass. *Can. J. Microbiol.* 48: 522-529.
- Callahan, B.J. 2016. The RDP and Greengenes taxonomic training sets formatted for DADA2 [Data Set]. Zenodo. 10.5281/zenodo.158955.
- Callahan, B.J., P.J. McMurdie, M.J. Rosen, A.W. Han, A.J.A. Johnson and S.P. Holmes. 2016. DADA2: High-resolution sample inference from Illumina amplicon data. *Nat. Methods* 13: 581. doi:10.1038/neth.3869.
- Cole, M.A. and A.J. Turgeon. 1978. Microbial activity in soil and litter underlying bandane- and calcium arsenate-treated turfgrass. *Soil Biol. Biochem.* 10: 181-186. doi:https://doi.org/10.1016/0038-0717(78)90094-9.

- Coleman-Derr, D., D. Desgarences, C. Fonseca-Garcia, S. Gross, S. Clingenpeel, T. Woyke, et al. 2016. Plant compartment and biogeography affect microbiome composition in cultivated and native *Agave* species. *New Phytol.* 209: 798-811. doi:10.1111/nph.13697.
- Crouch, J.A., Z. Carter, A. Ismaiel and J.A. Roberts. 2017. The US National Mall microbiome: A census of rhizosphere bacteria inhabiting landscape turf. *Crop Sci.* 57: S-341-S-348. doi:10.2135/cropsci2016.10.0849.
- D'Souza, M.A., and D.J. Bahat. 2012. A new species of *Pleurophragmium* from India. *Mycotaxon.* 119: 477-482. doi:http://dx.doi.org/10.5248/119.477.
- Dinno, A. 2017. dunn.test: Dunn's test of multiple comparisons using rank sums. <https://CRAN.R-project.org/package=dunn.test>.
- Doherty, J.R., M. Botti-Marino, J.P. Kerns, D.F. Ritchie and J.A. Roberts. 2017. Response of microbial populations on the creeping bentgrass phyllosphere to periodic fungicide applications. *Plant Health Prog.* 18: 44-49. doi:http://dx.doi.org/10.1094/PHP-RS-16-0067.
- Elad, Y. 2000. Biological control of foliar pathogens by means of *Trichoderma harzianum* and potential modes of action. *Crop Prot.* 19: 709-714. doi:https://doi.org/10.1016/S0261-2194(00)00094-6.
- Elliott, M.L., E.A. Guertal and S. H.D. 2004. Rhizosphere bacterial population flux in golf course putting greens in the southeastern United States. *HortScience* 39: 1754-1758.
- Elliott, M.L., J.A. McNroy, K. Xiong, J.H. Kim, H.D. Skipper and E.A. Guertal. 2008. Taxonomic diversity of rhizosphere bacteria in golf course putting greens at representative sites in the southeastern United States. *HortScience* 43: 514-518.
- Frostegard, A., A. Tunlid and E. Baath. 2010. Use and misuse of PLFA measurements in soils. *Soil Biol. Biochem.* doi:10.1016/j.soilbio.2010.11.021.
- Gambacorta, A., E. Pagnotta, I. Romano, G. Sodano and A. Trincone. 1998. Heterocyst glycolipids from nitrogen-fixing cyanobacteria other than nostocaceae. *Phytochemistry* 48: 801-805. doi:https://doi.org/10.1016/S0031-9422(97)00954-0.
- Gamir, J., V. Pastor, M. Cerezo and V. Flors. 2012. Identification of indole-3-carboxylic acid as mediator of priming against *Plectosphaerella cucumerina*. *Plant Physiol. Biochem.* 61: 169-179. doi:https://doi.org/10.1016/j.plaphy.2012.10.004.

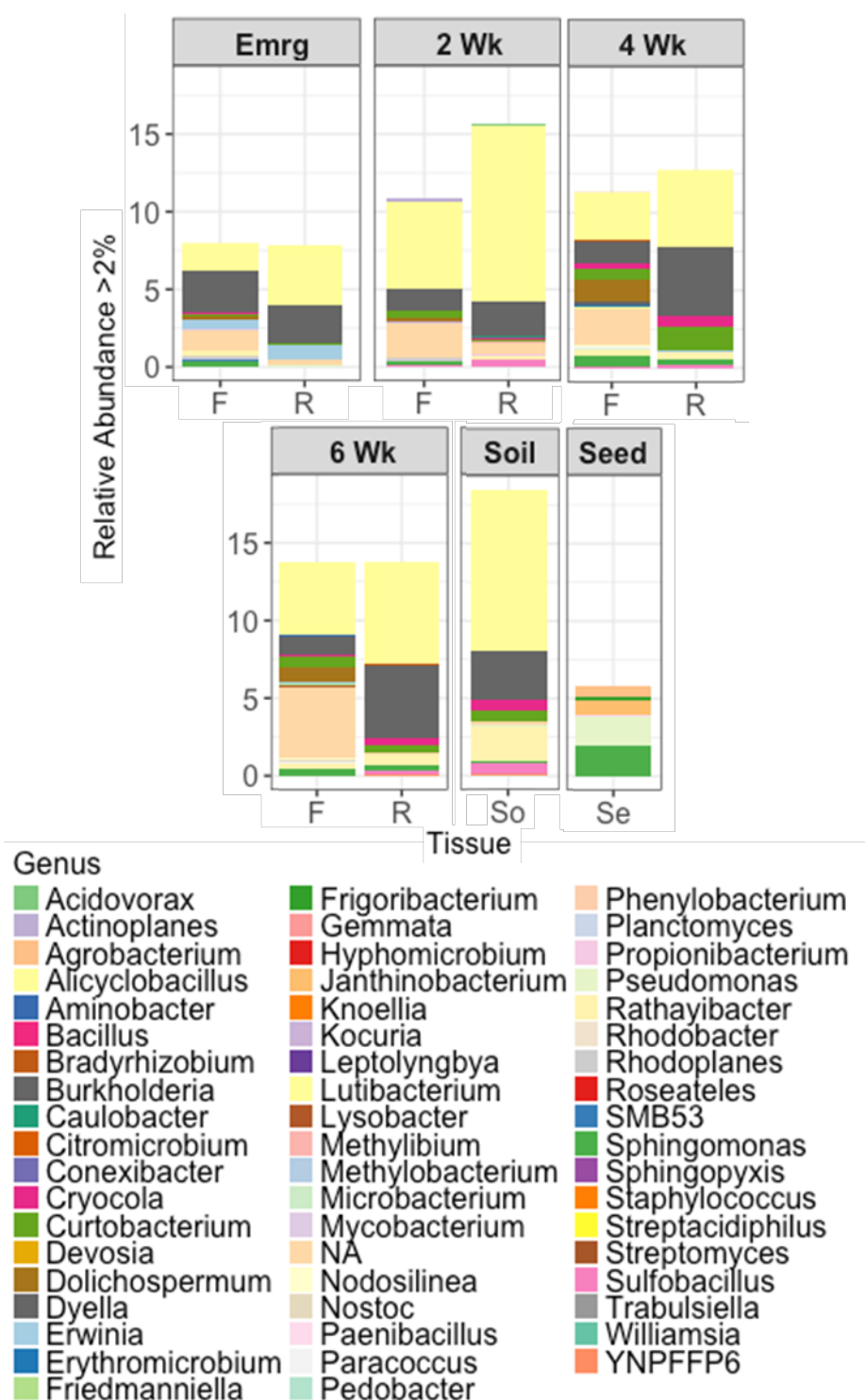
- Gardener, M. 2014. Community ecology: Analytical methods using R and Excel. Pelagic Publishing, Exeter, UK.
- Gdanetz, K. and F. Trail. 2017. The wheat microbiome under four management strategies, and potential for endophytes in disease protection. *Phytobiomes* 1: 158-168. doi:10.1094/PBIOMES-05-17-0023-R.
- Glaeser S.P. and P. Kämpfer. 2014. The family Sphingomonadaceae. In: Rosenberg E., DeLong E.F., Lory S., Stackebrandt E., Thompson F. (eds) *The Prokaryotes*. Springer, Berlin, Heidelberg.
- Hartmann, M., B. Frey, J. Mayer, P. Mäder and F. Widmer. 2014. Distinct soil microbial diversity under long-term organic and conventional farming. *ISME J.* 9: 1177. doi:10.1038/ismej.2014.210.
- Haas, D. and C. Keel. 2003. Regulation of antibiotic production in root-colonizing *Pseudomonas spp.* and relevance for biological control of plant disease. *Annu. Rev. Phytopathol.* 41: 117-153. doi:10.1146/annurev.phyto.41.052002.095656.
- Hoagland, D.R., and D.I. Arnon. 1950. The water-culture method for growing plants without soil. *California Agricultural Experiment Station Circular.* 347: 32.
- Kittelmann, S., H. Seedorf, W.A. Walters, J.C. Clemente, R. Knight, J.I. Gordon, et al. 2013. Simultaneous amplicon sequencing to explore co-occurrence patterns of bacterial, archaeal and eukaryotic microorganisms in rumen microbial communities. *PLoS ONE* 8: e47879. doi:10.1371/journal.pone.0047879.
- Kodseub, R., E.H.C. McKenzie, S. Lumyong, and K.D. Hyde. 2008. Diversity of saprobic fungi on Magnoliaceae. *Fungal Diversity.* 30: 37-53.
- Kõljalg, U., R.H. Nilsson, K. Abarenkov, L. Tedersoo, A.F.S. Taylor, M. Bahram, et al. 2013. Towards a unified paradigm for sequence-based identification of fungi. *Mol. Ecol.* 22: 5271-5277. doi:10.1111/mec.12481.
- Komárek, J. 2016. A polyphasic approach for the taxonomy of cyanobacteria: principles and applications. *Eur. J. Phycol.* 51: 346-353. doi:10.1080/09670262.2016.1163738.
- Lambers, H., C. Mougel, B. Jaillard and P. Hinsinger. 2009. Plant-microbe-soil interactions in the rhizosphere: An evolutionary perspective. *Plant Soil* 321:83-115. doi:10.1007/s11104-009-0042-x.
- Lo, C.T., E.B. Nelson and G.E. Harman. 1997. Improved biocontrol efficacy of *Trichoderma harzianum* 1295-22 for foliar phases of turf diseases by use of spray applications. *Plant Dis.* 81: 1132-1138. doi:10.1094/PDIS.1997.81.10.1132.

- Luttrell, E.S. 1964. Systematics of *Helminthosporium* and related genera. *Mycologia*. 56: 119-132. doi:10.2307/3756405.
- Martin, M. 2011. Cutadapt Removes Adapter Sequences from High-Throughput Sequencing Reads. *EMBNet.Journal* 17: 10-12.
- McDonald, D., M.N. Price, J. Goodrich, E.P. Nawrocki, T.Z. DeSantis, A. Probst, et al. 2012. An improved Greengenes taxonomy with explicit ranks for ecological and evolutionary analyses of bacteria and archaea. *ISME J.* 6: 610-618. doi:10.1038/ismej.2011.139.
- McMurdie, P.J. and S. Holmes. 2013. phyloseq: An R package for reproducible interactive analysis and graphics of microbiome consensus data. *PLoS ONE* 8: e61217.
- McMurdie, P.J. and S. Holmes. 2014. Waste not, want not: Why rarefying microbiome data is inadmissible. *PLoS Comput. Biol.* 10: e1003531. doi:10.1371/journal.pcbi.1003531.
- Mendes, R., M. Kruijt, I. de Bruijn, E. Dekkers, M. van der Voort, J.H.M. Schneider, et al. 2011. Deciphering the rhizosphere microbiome for disease-suppressive bacteria. *Science* 332: 1097-1100. doi:10.1126/science.1203980.
- Micallef, S.A., S. Channer, M.P. Shiaris and A. Colon-Carmona. 2009. Plant age and genotype impact the progression of bacterial community succession in the *Arabidopsis* rhizosphere. *Plant Signaling Behav.* 4: 777-780. doi:10.1093/jxb/erp053.
- Obasa, K., F.F. White, J. Fellers, M. Kennelly, S. Liu, B. Katz, et al. 2017. A dimorphic and virulence-enhancing endosymbiont bacterium discovered in *Rhizoctonia solani*. *Phytobiomes* 1: 14-23. doi:10.1094/PBIOMES-08-16-0005-R.
- Oksanen, J., F.G. Blanchet, M. Friendly, R. Kindt, P. Legendre, D. McGlinn, et al. 2017. vegan: Community Ecology Package. R package version 2.4-5, <https://CRAN.R-project.org/package=vegan>.
- Pham, V.H.T. and J. Kim. 2012. Cultivation of unculturable soil bacteria. *Trends Biotechnol.* 30: 475-484. doi:doi.org/10.1016/j.tibtech.2012.05.007.
- Pidot, S.J., S. Coyne, F. Kloss and C. Hertweck. 2014. Antibiotics from neglected bacterial sources. *Int. J. Med. Microbiol.* 304: 14-22. doi:<https://doi.org/10.1016/j.ijmm.2013.08.011>.
- R Core Team. 2017. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria, <https://www.R-project.org/>.

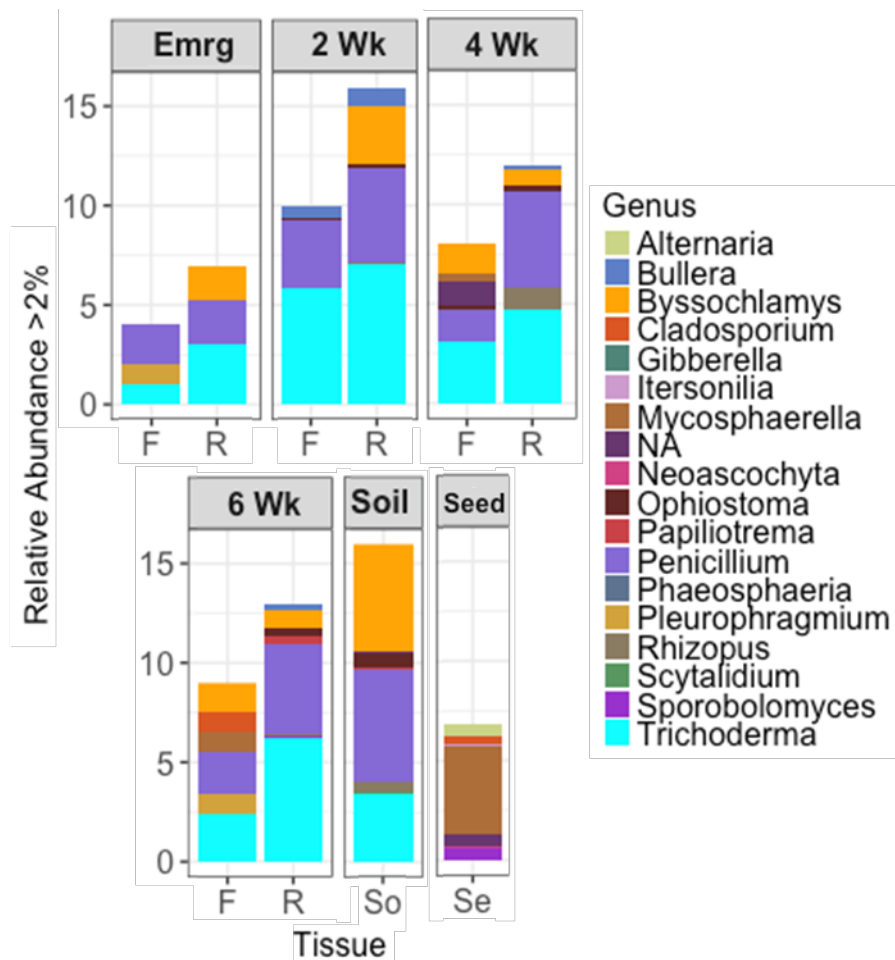
- Rastogi, G., J.J. Tech, G.L. Coaker and J.H.J. Leveau. 2010. A PCR-based toolbox for the culture-independent quantification of total bacterial abundances in plant environments. *J. Microbiol. Methods* 83: 127-132.
doi:<http://dx.doi.org/10.1016/j.mimet.2010.08.006>.
- Schoug, A., J. Fischer, H.J. Heipieper, J. Schnurer and S. Hakansson. 2008. Impact of fermentation pH and temperature on freeze-drying survival and membrane lipid composition of *Lactobacillus coryniformis* Si3. *J. Ind. Microbiol. Biotechnol.* 35: 175-181.
- Steer, J. and J.A. Harris. 2000. Shifts in the microbial community in rhizosphere and non-rhizosphere soils during the growth of *Agrostis stolonifera*. *Soil Biol. Biochem.* 32: 869-878. doi:[https://doi.org/10.1016/S0038-0717\(99\)00219-9](https://doi.org/10.1016/S0038-0717(99)00219-9).
- Sylvia, D.M., J.J. Fuhrmann, P.G. Hartel and D.A. Zuberer. 2005. Principles and applications of soil microbiology. 2 ed. Prentice Hall.
- Toju, H., A.S. Tanabe, S. Yamamoto and H. Sato. 2012. High-coverage ITS primers for the DNA-based identification of ascomycetes and basidiomycetes in environmental samples. *PLoS ONE* 7: e40863.
doi:10.1371/journal.pone.0040863.
- Torsvik, V. and L. Øvreås. 2002. Microbial diversity and function in soil: from genes to ecosystems. *Curr. Opin. Microbiol.* 5: 240-245.
doi:[https://doi.org/10.1016/S1369-5274\(02\)00324-7](https://doi.org/10.1016/S1369-5274(02)00324-7).
- Torsvik, V., K. Salte, R. Sørheim and J. Goksøyr. 1990. Comparison of phenotypic diversity and DNA heterogeneity in a population of soil bacteria. *Appl. Environ. Microbiol.* 56: 776-781.
- Weisburg, W.G., S.M. Barns, D.A. Pelletier and D.J. Lane. 1991. 16S ribosomal DNA amplification for phylogenetic study. *J. Bacteriol.* 173: 697-703.
- Weiss, S., Z.Z. Xu, S. Peddada, A. Amir, K. Bittinger, A. Gonzalez, et al. 2017. Normalization and microbial differential abundance strategies depend upon data characteristics. *Microbiome* 5: 27. doi:10.1186/s40168-017-0237-y.
- White, T., T. Bruns, S. Lee and J. Taylor. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. *PCR Protocols: A Guide to Methods and Applications* 315-322.
- Wickham, H. 2009. *ggplot2: Elegant Graphics for Data Analysis*. Springer-Verlag New York. <http://ggplot2.org>.

- Yao, H., D. Bowman and W. Shi. 2006. Soil microbial community structure and diversity in a turfgrass chronosequence: Land-use change versus turfgrass management. *Applied Soil Ecology* 34: 209-218. doi:<http://dx.doi.org/10.1016/j.apsoil.2006.01.009>.
- Yao, H., D. Bowman and W. Shi. 2011. Seasonal variations of soil microbial biomass and activity in warm- and cool-season turfgrass systems. *Soil Biol. Biochem.* 43: 1536-1543. doi:<https://doi.org/10.1016/j.soilbio.2011.03.031>.

APPENDIX 1



Supplementary Figure 1. Relative abundances, calculated using phyloseq in R, of bacterial genera associated with creeping bentgrass grown under sterile conditions over time. “F”, “R”, “So” and “Se” refer to “Foliar”, “Rhizosphere”, “Soil” and “Seed” samples, respectively. “Emrg”, “2 Wk”, “4 Wk”, “6 Wk” refer to “Emergence”, “2 Week”, “4 Week” and “6 Week” sampling times, respectively.



Supplementary Figure 2. Relative abundances, calculated using phyloseq in R, of fungal genera associated with creeping bentgrass grown under sterile conditions over time. “F”, “R”, “So” and “Se” refer to “Foliar”, “Rhizosphere”, “Soil” and “Seed” samples, respectively. “Emrg”, “2 Wk”, “4 Wk”, “6 Wk” refer to “Emergence”, “2 Week”, “4 Week” and “6 Week” sampling times, respectively.

LITERATURE CITED

- Adesemoye, A.O., H.A. Torbert and J.W. Kloepper. 2009. Plant growth-promoting rhizobacteria allow reduced application rates of chemical fertilizers. *Microb. Ecol.* 58: 921-929.
- Amann, R.I., W. Ludwig and K.H. Schleifer. 1995. Phylogenetic identification and *in situ* detection of individual microbial cells without cultivation. *Microbiol. Mol. Biol. Rev.* 59: 143-169.
- American Phytopathological Society. 2016. *Phytobiomes: A roadmap for research and translation*. American Phytopathological Society, St. Paul, MN.
- Andrews, J.H. 1992. Biological control in the phyllosphere. *Annu. Rev. Phytopathol.* 30: 603-635.
- Arias, M.E., J.A. Gonzalez-Perez, F.J. Gonzalez-Vila and A.S. Ball. 2005. Soil health -- a new challenge for microbiologists and chemists. *Int. Microbiol.* 8: 13-21.
- Bahram, M., S. Polme, U. Koljalg and L. Tedersoo. 2010. A single European aspen (*Populus tremula*) tree individual may potentially harbour dozens of *Cenococcum geophilium* ITS genotypes and hundreds of species of ectomycorrhizal fungi. *FEMS Microbiol. Ecol.* 75: 2.
- Bais, H.P., T.L. Weir, L.G. Perry, S. Gilroy and J.M. Vivanco. 2006. The role of root exudates in rhizosphere interactions with plants and other organisms. *Annu. Rev. Plant Biol.* 57: 233-266.
- Bakker, M.G., D.K. Manter, A.M. Sheflin, T.L. Weir and J.M. Vivanco. 2012. Harnessing the rhizosphere microbiome through plant breeding and agricultural management. *Plant Soil* 360: 1-13. doi:10.1007/s11104-012-1361-x.
- Bartlett, M.D., I.T. James, J.A. Harris and K. Ritz. 2008. Size and phenotypic structure of microbial communities within soil profiles in relation to different playing areas on a UK golf course. *Eur. J. Soil Sci.* 59: 835-841. doi:10.1111/j.1365-2389.2008.01056.x.
- Baxter, N.T., M.T. Ruffin, M.A. Rogers, and P.D. Schloss. 2016. Microbiota-based model improves the sensitivity of fecal immunochemical test for detecting colonic lesions. *Genome Med.* 8: 37. doi:10.1186/s13073-106-0290-3.
- Beirn, L.A., J.W. Hempfling, C.J. Schmid, J.A. Murphy, B.B. Clarke and J.A. Crouch. 2016. Differences among soil-inhabiting microbial communities in *Poa annua* turf throughout the growing season. *Crop Sci.* doi:10.2135/cropsci2016.06.0463.

- Bensch, K., U. Braun, J.Z. Groenewald and P.W. Crous. 2012. The genus *Cladosporium*. *Studies in Mycology* 72: 1-401. doi:<https://doi.org/10.3114/sim0003>.
- Berendsen, R.L., C.M.J. Pieterse, and P.A.H.M. Bakker. 2012. The rhizosphere microbiome and plant health. *Trends in Plant Science* 17: 478-486. doi:<https://doi.org/10.1016/j.tplants.2012.04.001>
- Berg, G. and K. Smalla. 2009. Plant species and soil type cooperatively shape the structure and function of microbial communities in the rhizosphere. *FEMS Microbiol. Ecol.* 68: 1-13. doi:10.1111/j.1574-6941.2009.00654.x.
- Berg, G., A. Erlacher and M. Grube. 2015. The edible plant microbiome: Importance and health issues. In: B. Lugtenberg, editor. *Principles of plant-microbe interactions: Microbes for sustainable agriculture*. Springer International Publishing, Cham. p. 419-426.
- Bertin, C., X.H. Yang and L.A. Weston. 2003. The role of root exudates and allelochemicals in the rhizosphere. *Plant Soil* 256: 67-83.
- Bigelow, C.A., D.C. Bowman and A.G. Wollum. 2002. Characterization of soil microbial population dynamics in newly constructed sand-based rootzones. *Crop Sci.* 42: 1611-1614. doi:10.2135/cropsci2002.1611.
- Buck, J.W. and L.L. Burpee. 2002. The effects of fungicides on the phylloplane yeast populations of creeping bentgrass. *Can. J. Microbiol.* 48: 522-529.
- Callahan, B.J. 2016. The RDP and Greengenes taxonomic training sets formatted for dada2 [data set]. Zenodo. 10.5281/zenodo.158955.
- Callahan, B.J., P.J. McMurdie, M.J. Rosen, A.W. Han, A.J.A. Johnson and S.P. Holmes. 2016. DADA2: High-resolution sample inference from Illumina amplicon data. *Nat. Methods* 13: 581. doi:10.1038/neth.3869.
- Callahan, B.J., P.J. McMurdie and S.P. Holmes. 2017. Exact sequence variants should replace operational taxonomic units in marker-gene data analysis. *ISME J.* 11: 2639. doi:10.1038/ismej.2017.119.
- Clay, K. 1987. Effects of fungal endophytes on the seed and seedling biology of *Lolium perenne* and *Festuca arundinacea*. *Oecologia* 73: 358-362. doi:10.1007/bf00385251.
- Cole, M.A. and A.J. Turgeon. 1978. Microbial activity in soil and litter underlying bandane- and calcium arsenate-treated turfgrass. *Soil Biol. Biochem.* 10: 181-186. doi:[https://doi.org/10.1016/0038-0717\(78\)90094-9](https://doi.org/10.1016/0038-0717(78)90094-9).

- Coleman-Derr, D., D. Desgarenes, C. Fonseca-Garcia, S. Gross, S. Clingenpeel, T. Woyke, et al. 2016. Plant compartment and biogeography affect microbiome composition in cultivated and native agave species. *New Phytol.* 209: 798-811. doi:10.1111/nph.13697.
- Compant, S., C. Clément and A. Sessitsch. 2010. Plant growth-promoting bacteria in the rhizo- and endosphere of plants: Their role, colonization, mechanisms involved and prospects for utilization. *Soil Biol. Biochem.* 42: 669-678. doi:http://dx.doi.org/10.1016/j.soilbio.2009.11.024.
- Crouch, J.A., Z. Carter, A. Ismaiel and J.A. Roberts. 2017. The us national mall microbiome: A census of rhizosphere bacteria inhabiting landscape turf. *Crop Sci.* 57: S-341-S-348. doi:10.2135/cropsci2016.10.0849.
- D'Souza, M.A., and D.J. Bahat. 2012. A new species of *Pleurophragmium* from India. *Mycotaxon.* 119: 477-482. doi:http://dx.doi.org/10.5248/119.477.
- de Souza, R.S.C., V.K. Okura, J.S.L. Armanhi, B. Jorrín, N. Lozano, M.J. da Silva, et al. 2016. Unlocking the bacterial and fungal communities assemblages of sugarcane microbiome. *Sci. Rep.* 6: 28774. doi:10.1038/srep28774.
- Delmotte, N., C. Knief, S. Chaffron, G. Innerebner, B. Roschitzki, R. Schlapbach, et al. 2009. Community proteogenomics reveals insights into the physiology of phyllosphere bacteria. *PNAS* 106: 16428-16433.
- Dinno, A. 2017. *Dunn.Test*: Dunn's test of multiple comparisons using rank sums. <https://CRAN.R-project.org/package=dunn.test>.
- Doherty, J.R., M. Botti-Marino, J.P. Kerns, D.F. Ritchie and J.A. Roberts. 2017. Response of microbial populations on the creeping bentgrass phyllosphere to periodic fungicide applications. *Plant Health Prog.* 18: 44-49. doi:http://dx.doi.org/10.1094/PHP-RS-16-0067.
- Donn, S., J.A. Kirkegaard, G. Perera, A.E. Richardson and M. Watt. 2015. Evolution of bacterial communities in the wheat crop rhizosphere. *Environ. Microbiol.* 17: 610-621. doi:10.1111/1462-2920.12452.
- Edwards, J., C. Johnson, C. Santos-Medellín, E. Lurie, N.K. Podishetty, S. Bhatnagar, et al. 2015. Structure, variation, and assembly of the root-associated microbiomes of rice. *PNAS* 112: E911-E920. doi:10.1073/pnas.1414592112.
- Elad, Y. 2000. Biological control of foliar pathogens by means of *Trichoderma harzianum* and potential modes of action. *Crop Prot.* 19: 709-714. doi:https://doi.org/10.1016/S0261-2194(00)00094-6.

- Elliot, M.L., E.A. Guertal and S. H.D. 2004. Rhizosphere bacterial population flux in golf course putting greens in the southeastern United States. *HortScience* 39: 1754-1758.
- Elliott, M.L., J.A. McInroy, K. Xiong, J.H. Kim, H.D. Skipper and E.A. Guertal. 2008. Taxonomic diversity of rhizosphere bacteria in golf course putting greens at representative sites in the southeastern United States. *HortScience* 43: 514-518.
- El-Tarabily, K.A. and K. Sivasithamparam. 2006. Non-streptomycete actinomycetes as biocontrol agents of soil-borne fungal plant pathogens and as plant growth promoters. *Soil Biol. Biochem.* 38: 1505-1520.
doi:<https://doi.org/10.1016/j.soilbio.2005.12.017>.
- Fadrosh, D.W., B. Ma, P. Gajer, N. Sengamalay, S. Ott, R.M. Brotman, et al. 2014. An improved dual-indexing approach for multiplexed 16s rRNA gene sequencing on the Illumina MiSeq platform. *Microbiome* 2: 6. doi:10.1186/2049-2618-2-6.
- Fisher, M.M., and E.W. Triplett. 1999. Automated approach for ribosomal intergenic spacer analysis of microbial diversity and its application for freshwater bacterial communities. *Appl. Environ. Microbiol.* 65: 4630-4636.
- Frostegard, A., A. Tunlid and E. Baath. 2010. Use and misuse of PLFA measurements in soils. *Soil Biol. Biochem.* doi:10.1016/j.soilbio.2010.11.021.
- Gambacorta, A., E. Pagnotta, I. Romano, G. Sodano and A. Trincone. 1998. Heterocyst glycolipids from nitrogen-fixing cyanobacteria other than Nostocaceae. *Phytochemistry* 48: 801-805. doi:[https://doi.org/10.1016/S0031-9422\(97\)00954-0](https://doi.org/10.1016/S0031-9422(97)00954-0).
- Gamir, J., V. Pastor, M. Cerezo and V. Flors. 2012. Identification of indole-3-carboxylic acid as mediator of priming against *Plectosphaerella cucumerina*. *Plant Physiol. Biochem.* 61: 169-179. doi:<https://doi.org/10.1016/j.plaphy.2012.10.004>.
- Gardener, M. 2014. *Community ecology: Analytical methods using R and Excel*. Pelagic Publishing, Exeter, UK.
- Gdanetz, K. and F. Trail. 2017. The wheat microbiome under four management strategies, and potential for endophytes in disease protection. *Phytobiomes* 1: 158-168. doi:10.1094/PBIOMES-05-17-0023-R.
- Geiser, D.M., C. Gueidan, J. Miadlikowska, F. Lutzoni, F. Kauff, V. Hofstetter, et al. 2006. Eurotiomycetes: Eurotiomycetidae and chaetothyriomycetidae. *Mycologia* 98: 1053-1064. doi:10.1080/15572536.2006.11832633.
- Gholami, A., S. Shahsavani and S. Nezarat. 2009. The effect of plant growth promoting rhizobacteria (pgpr) on germination, seedling growth and yield of maize.

- Gilbert, J.A., F. Meyer, J. Jansson, J. Gordon, N. Pace, J. Tiedje, et al. 2010. The earth microbiome project: Meeting report of the “1st EMP meeting on sample selection and acquisition” at Argonne National Laboratory October 6th 2010. *Stand. Genomic Sci.* 3: 249-253. doi:10.4056/aigs.1443528.
- Glaeser S.P. and P. Kämpfer. 2014. The family Sphingomonadaceae. In: Rosenberg E., DeLong E.F., Lory S., Stackebrandt E., Thompson F. (eds) *The Prokaryotes*. Springer, Berlin, Heidelberg.
- Haas, D. and C. Keel. 2003. Regulation of antibiotic production in root-colonizing *Pseudomonas spp.* and relevance for biological control of plant disease. *Annu. Rev. Phytopathol.* 41: 117-153. doi:10.1146/annurev.phyto.41.052002.095656.
- Hartmann, M., B. Frey, J. Mayer, P. Mäder and F. Widmer. 2014. Distinct soil microbial diversity under long-term organic and conventional farming. *ISME J.* 9: 1177. doi:10.1038/ismej.2014.210
- Henry, A., W. Doucette, J. Norton, S. Jones, J. Chard and B. Bugbee. 2006. An axenic plant culture system for optimal growth in long-term studies. *J Environ. Qual.* 35: 590-598. doi:10.2134/jeq2005.0127.
- Hoagland, D.R., and D.I. Arnon. 1950. The water-culture method for growing plants without soil. *California Agricultural Experiment Station Circular.* 347: 32.
- Hodges, C.F. 1990. The microbiology of non-pathogens and minor root pathogens in high sand content greens. *GCM* 48: 60-75.
- Hossain, M.M., F. Sultana, M. Kubota, H. Koyama and M. Hyakumachi. 2007. The plant growth-promoting fungus *Penicillium simplicissimum* gp17-2 induces resistance in *Arabidopsis thaliana* by activation of multiple defense signals. *Plant Cell Physiol.* 48: 1724-1736. doi:10.1093/pcp/pcm144.
- Howell, C.R. 2003. Mechanisms employed by trichoderma species in the biological control of plant diseases: The history and evolution of current concepts. *Plant Dis.* 87: 4-10. doi:10.1094/PDIS.2003.87.1.4.
- Hur, M., Y.W. Lim, J.J. Yu, S.U. Cheon, Y.I. Choi, S.-H. Yoon, et al. 2012. Fungal community associated with genetically modified poplar during metal phytoremediation. *Journal of Microbiology* 50: 910-915. doi:10.1007/s12275-012-2491-9.

- Jenkins, J.R., M. Viger, E.C. Arnold, Z.M. Harris, M. Ventura, F. Miglietta, et al. 2017. Biochar alters the soil microbiome and soil function: Results of next- generation amplicon sequencing across europe. *GCB Bioenergy* 9: 591-612. doi:doi:10.1111/gcbb.12371.
- Johnston-Monje, D., D.S. Lundberg, G. Lazarovits, V.M. Reis and M.N. Raizada. 2016. Bacterial populations in juvenile maize rhizospheres originate from both seed and soil. *Plant Soil* 405: 337-355. doi:10.1007/s11104-016-2826-0.
- Jongman, R.H.G, C.J.F. Ter Braak and O.F.R. Van Tongeren. 1995. Data analysis in community and landscape ecology. Cambridge University Press: Cambridge, UK.
- Karlsson, I., H. Friberg, C. Steinberg and P. Persson. 2014. Fungicide effects on fungal community composition in the wheat phyllosphere. *PLOS ONE* 9: e111786. doi:10.1371/journal.pone.0111786.
- Karp, M.A. and E.B. Nelson. 2004. Bacterial communities associated with creeping bentgrass in soil and sand rootzones. *USGA Turf Environ. Res. Online* 3: 1-19.
- Kembel, S.W. and R.C. Mueller. 2014. Plant traits and taxonomy drive host associations in tropical phyllosphere fungal communities. *Botany* 92: 303-311. doi:10.1139/cjb-2013-0194.
- Kennedy, A.C. and K.L. Smith. 1995. Soil microbial diversity and the sustainability of agricultural soils. *Plant Soil* 170: 75-86. doi:10.1007/bf02183056.
- Kinkel, L.L., M. Wilson and S.E. Lindow. 1996. Utility of microcosm studies for predicting phylloplane bacterium population sizes in the field. *Appl. Environ. Microbiol.* 62: 3413-3423.
- Kirk, P.M., G.C. Ainsworth, G.R. Bisby and C.A.B. International. 2008. Ainsworth & Bisby's dictionary of the fungi. CABI.
- Kittlmann, S., H. Seedorf, W.A. Walters, J.C. Clemente, R. Knight, J.I. Gordon, et al. 2013. Simultaneous amplicon sequencing to explore co-occurrence patterns of bacterial, archaeal and eukaryotic microorganisms in rumen microbial communities. *PLOS ONE* 8: e47879. doi:10.1371/journal.pone.0047879.
- Knief, C., N. Delmotte, S. Chaffron, M. Stark, G. Innerebner, R. Wassmann, et al. 2012. Metaproteogenomic analysis of microbial communities in the phyllosphere and rhizosphere of rice. *ISME J.* 6: 1378-1390. doi:10.1038/ismej.2011.192.
- Kodseub, R., E.H.C. McKenzie, S. Lumyong, and K.D. Hyde. 2008. Diversity of saprobic fungi on Magnoliaceae. *Fungal Diversity*. 30: 37-53.

- Kõljalg, U., R.H. Nilsson, K. Abarenkov, L. Tedersoo, A.F. Taylor, M. Bahram, et al. 2013. Towards a unified paradigm for sequence-based identification of fungi. *Mol. Ecol.* 22: 5271-5277. doi:10.1111/mec.12481.
- Komárek, J. 2016. A polyphasic approach for the taxonomy of cyanobacteria: principles and applications. *Eur. J. Phycol.* 51: 346-353. doi:10.1080/09670262.2016.1163738.
- Kopylova, E., J.A. Navas-Molina, C. Mercier, Z.Z. Xu, F. Mahé, Y. He, et al. 2016. Open-source sequence clustering methods improve the state of the art. *mSystems* 11. doi:10.1128/mSystems.00003-15.
- Kozdrój, J. and J.D. van Elsas. 2000. Application of polymerase chain reaction-denaturing gradient gel electrophoresis for comparison of direct and indirect extraction methods of soil DNA used for microbial community fingerprinting. *Biol. Fertil. Soils* 31: 372-378. doi:10.1007/s003749900182.
- Kwaśna, H., G.L. Bateman and E. Ward. 2010. Microbiota in wheat roots evaluated by cloning of ITS1 rDNA and sequencing. *J. Phytopathol.* 158: 278-287. doi:10.1111/j.1439-0434.2009.01595.x.
- Lambais, M.R., D.E. Crowley, J.C. Cury, R.C. Büll and R.R. Rodrigues. 2006. Bacterial diversity in tree canopies of the atlantic forest. *Science* 312: 1917-1917. doi:10.1126/science.1124696.
- Lambers, H., C. Mougel, B. Jaillard and P. Hinsinger. 2009. Plant-microbe-soil interactions in the rhizosphere: An evolutionary perspective. *Plant Soil* 321:83-115. doi:10.1007/s11104-009-0042-x.
- Leibold, M.A. and M.A. McPeck. 2006. Coexistence of the niche and neutral perspectives in community ecology. *Ecology* 87: 1399-1410. doi:10.1890/0012-9658(2006)87[1399:COTNAN]2.0.CO;2.
- Lenc, L., H. Kwaśna, C. Sadowski and A. Grabowski. 2015. Microbiota in wheat roots, rhizosphere and soil in crops grown in organic and other production systems. *J. Phytopathol.* 163: 245-263. doi:10.1111/jph.12313.
- Lindemann, J., D.C. Arny and C.D. Upper. 1984. Epiphytic populations of *Pseudomonas syringae* pv *syringae* on snap bean and nonhost plants and the incidence of bacterial brown spot disease in relation to cropping patterns. *Phytopathology* 74: 1329-1333.
- Lindow, S.E. and G.L. Andersen. 1996. Influence of immigration on epiphytic bacterial populations on navel orange leaves. *Appl. Environ. Microbiol.* 36: 2978-2987.

- Lindow, S.E., D.C. Arny and C.D. Upper. 1978. Distribution of ice nucleation-active bacteria on plants in nature. *Appl. Environ. Microbiol.* 36: 831-838.
- Lo, C.T., E.B. Nelson and G.E. Harman. 1997. Improved biocontrol efficacy of *Trichoderma harzianum* 1295-22 for foliar phases of turf diseases by use of spray applications. *Plant Dis.* 81: 1132-1138. doi:10.1094/PDIS.1997.81.10.1132.
- Luna, G.M., A. Dell'Anno and R. Danovaro. 2006. DNA extraction procedure: A critical issue for bacterial diversity assessment in marine sediments. *Environ. Microbiol.* 8: 308-320.
- Lundberg, D.S., S.L. Lebeis, S.H. Paredes, S. Yourstone, J. Gehring, S. Malfatti, et al. 2012. Defining the core *Arabidopsis thaliana* root microbiome. *Nature* 488: 86. doi:10.1038/nature11237.
- Luttrell, E.S. 1964. Systematics of Helminthosporium and related genera. *Mycologia.* 56: 119-132. doi:10.2307/3756405.
- Magan, N. and J. Lacey. 1986. The phyllosphere microflora of ripening wheat and effect of late fungicide applicaitons. *Ann. Appl. Biol.* 109: 117-128.
- Mancino, C.F., M. Barakat and A. Maricic. 1993. Soil and thatch microbial populations in an 80% sand : 20% peat creeping bentgrass putting green. *HortScience* 28: 189-191.
- Marchesi, J.R. and J. Ravel. 2015. The vocabulary of microbiome research: A proposal. *Microbiome* 3: 31. doi:10.1186/s40168-015-0094-5.
- Marilley, L. and M. Aragno. 1999. Phylogenetic diversity of bacterial communities differing in degree of proximity of lolium perenne and trifolium repens roots. *Applied Soil Ecology* 13: 127-136. doi:https://doi.org/10.1016/S0929-1393(99)00028-1.
- Martin, M. 2011. Cutadapt removes adapter sequences from high-throughput sequencing reads. *EMBNNet.Journal* 17: 10-12.
- Marsh, T.L. 1999. Terminal restriction fragment length polymorphism (T-RFLP): An emerging method for characterizing diversity among homologous populations of amplification products. *Curr. Opin. Microbiol.* 2: 323-327. doi:https://doi.org/10.1016/S1369-5274(99)80056-3.
- McDonald, D., M.N. Price, J. Goodrich, E.P. Nawrocki, T.Z. DeSantis, A. Probst, et al. 2012. An improved Greengenes taxonomy with explicit ranks for ecological and evolutionary analyses of bacteria and archaea. *ISME J.* 6: 610-618. doi:10.1038/ismej.2011.139.

- McMurdie, P.J. and S. Holmes. 2013. phyloseq: An R package for reproducible interactive analysis and graphics of microbiome consensus data. PLoS ONE 8: e61217.
- McMurdie, P.J. and S. Holmes. 2014. Waste not, want not: Why rarefying microbiome data is inadmissible. PLoS Comput. Biol. 10: e1003531. doi:10.1371/journal.pcbi.1003531.
- Mendes, L.W., E.E. Kuramae, A.A. Navarrete, J.A. van Veen and S.M. Tsai. 2014. Taxonomical and functional microbial community selection in soybean rhizosphere. ISME J. 8: 1577. doi:10.1038/ismej.2014.17.
- Mendes, R., M. Kruijt, I. de Bruijn, E. Dekkers, M. van der Voort, J.H.M. Schneider, et al. 2011. Deciphering the rhizosphere microbiome for disease-suppressive bacteria. Science 332: 1097-1100. doi:10.1126/science.1203980.
- Micallef, S.A., S. Channer, M.P. Shiaris and A. Colon-Carmona. 2009. Plant age and genotype impact the progression of bacterial community succession in the *Arabidopsis* rhizosphere. Plant Signaling Behav. 4: 777-780. doi:10.1093/jxb/erp053.
- Micallef, S.A., M.P. Shiaris and A. Colon-Carmona. 2009. Influence of *Arabidopsis thaliana* accessions on rhizobacterial communities and natural variation in root exudates. J. Exp. Bot. 60: 1729-1742. doi:10.1093/jxb/erp053.
- Mosca, S., M.G. Li Destri Nicosia, S.O. Cacciola and L. Schena. 2014. Molecular analysis of *Colletotrichum* species in the carposphere and phyllosphere of olive. PLoS ONE 9: e114031. doi:10.1371/journal.pone.0114031.
- Muyzer, G. and K. Smalla. 1998. Application of denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) in microbial ecology. Antonie van Leeuwenhoek 73: 127-141. doi:10.1023/a:1000669317571.
- Neri, F., M. Mari and S. Brigati. 2006. Control of *Penicillium expansum* by plant volatile compounds. Plant Pathol. 55: 100-105. doi:10.1111/j.1365-3059.2005.01312.x.
- Nettles, R., J. Watkins, K. Ricks, M. Boyer, M. Licht, L.W. Atwood, et al. 2016. Influence of pesticide seed treatments on rhizosphere fungal and bacterial communities and leaf fungal endophyte communities in maize and soybean. Applied Soil Ecology 102: 61-69. doi:https://doi.org/10.1016/j.apsoil.2016.02.008.
- Nunan, N., K. Wu, I.M. Young, J.W. Crawford and K. Ritz. 2003. Spatial distribution of bacterial communities and their relationships with the micro-architecture of soil. FEMS Microbiol. Ecol. 44: 203-215. doi:10.1016/S0168-6496(03)00027-8.

- O'Brien, H.E., J.L. Parrent, J.A. Jackson, J.-M. Moncalvo and R. Vigalys. 2005. Fungal community analysis by large-scale sequencing of environmental samples. *Appl. Environ. Microbiol.* 71: 5544-5550. doi:10.1128/AEM.71.9.5544-5550.2005.
- Obasa, K., F.F. White, J. Fellers, M. Kennelly, S. Liu, B. Katz, et al. 2017. A dimorphic and virulence-enhancing endosymbiont bacterium discovered in *Rhizoctonia solani*. *Phytobiomes* 1: 14-23. doi:10.1094/PBIOMES-08-16-0005-R.
- Ofek, M., M. Voronov-Goldman, Y. Hadar and D. Minz. 2014. Host signature effect on plant root-associated microbiomes revealed through analyses of resident vs. active communities. *Environ. Microbiol.* 16: 2157-2167. doi:10.1111/1462-2920.12228.
- Oksanen, J., F.G. Blanchet, M. Friendly, R. Kindt, P. Legendre, D. McGlinn, et al. 2017. Vegan: Community ecology package. R package version 2.4-5, <https://CRAN.R-project.org/package=vegan>.
- Palaniyandi, S.A., S.H. Yang, L. Zhang and J.-W. Suh. 2013. Effects of actinobacteria on plant disease suppression and growth promotion. *Appl. Microbiol. Biotechnol.* 97: 9621-9636. doi:10.1007/s00253-013-5206-1.
- Panke-Buisse, K., A.C. Poole, J.K. Goodrich, R.E. Ley and J. Kao-Kniffin. 2014. Selection on soil microbiomes reveals reproducible impacts on plant function. *ISME J.* 9: 980. doi:10.1038/ismej.2014.196
- Paterson, E., T. Gebbing, C. Abel, A. Sim and G. Telfer. 2007. Rhizodeposition shapes rhizosphere microbial community structure in organic soil. *New Phytol.* 173: 600-610. doi:10.1111/j.1469-8137.2006.01931.x.
- Peay, K.G., P.G. Kennedy and J.M. Talbot. 2016. Dimensions of biodiversity in the earth mycobiome. *Nat. Rev. Microbiol.* 14: 434. doi:10.1038/nrmicro.2016.59.
- Peiffer, J.A., A. Spor, O. Koren, Z. Jin, S.G. Tringe, J.L. Dangl, et al. 2013. Diversity and heritability of the maize rhizosphere microbiome under field conditions. *PNAS* 110: 6548-6553. doi:10.1073/pnas.1302837110.
- Pham, V.H.T. and J. Kim. 2012. Cultivation of unculturable soil bacteria. *Trends Biotechnol.* 30: 475-484. doi:doi.org/10.1016/j.tibtech.2012.05.007.
- Phytobiomes Initiative. 2015. What is the phytobiome?
<http://www.phytobiomes.org/about/Pages/What-is-the-Phytobiome.aspx>
- Pidot, S.J., S. Coyne, F. Kloss and C. Hertweck. 2014. Antibiotics from neglected bacterial sources. *Int. J. Med. Microbiol.* 304: 14-22.
doi:<https://doi.org/10.1016/j.ijmm.2013.08.011>.

- Pocheville, A. 2015. The ecological niche: History and recent controversies. In: T. Heams, P. Huneman and G. Lecointre, editors, Handbook of evolutionary thinking in the sciences. Springer, Dordrecht. p. 547-586.
- Prosser, J.I. 2002. Molecular and functional diversity in soil micro-organisms. *Plant Soil* 244: 9-17. doi:10.1023/a:1020208100281.
- R Core Team. 2017. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria, <https://www.R-project.org/>.
- Ranjard, L., F. Poly, J. Combrisson, A. Richaume, F. Gourbière, J. Thioulouse, et al. 2000. Heterogeneous cell density and genetic structure of bacterial pools associated with various soil microenvironments as determined by enumeration and DNA fingerprinting approach (risa). *Microb. Ecol.* 39: 263-272. doi:10.1007/s002480000032.
- Ranjard, L. and A. Richaume. 2001. Quantitative and qualitative microscale distribution of bacteria in soil. *Res. Microbiol.* 152: 707-716. doi:[http://dx.doi.org/10.1016/S0923-2508\(01\)01251-7](http://dx.doi.org/10.1016/S0923-2508(01)01251-7).
- Rastogi, G., G.L. Coaker and J.H.J. Leveau. 2013. New insights into the structure and function of phyllosphere microbiota through high- throughput molecular approaches. *FEMS Microbiol. Lett.* 348: 1-10. doi:doi:10.1111/1574-6968.12225.
- Rastogi, G., J.J. Tech, G.L. Coaker and J.H.J. Leveau. 2010. A PCR-based toolbox for the culture-independent quantification of total bacterial abundances in plant environments. *J. Microbiol. Methods* 83: 127-132. doi:<http://dx.doi.org/10.1016/j.mimet.2010.08.006>.
- Richamue, A., C. Steinberg, L. Jocteur-Monrozier and G. Faurie. 1993. Differences between direct and indirect enumeration of soil bacteria: The influence of soil structure and cell location. *Soil Biol. Biochem.* 25: 641-643.
- Roberts, J.A., B. Ma, L.P. Tredway, D.F. Ritchie and J.P. Kerns. 2017. Identification and pathogenicity of bacteria associated with etiolation and decline of creeping bentgrass golf course putting greens. *Phytopathology* 108: 23-30. doi:10.1094/PHYTO-01-17-0015-R.
- Rodríguez, M.A., N. Venedikian and A. Godeas. 2001. Fungal populations on sunflower (*helianthus annuus*) anthosphere and their relation to susceptibility or tolerance to *sclerotinia sclerotiorum* attack. *Mycopathologia* 150: 143-150. doi:10.1023/a:1010994317980.

- Rodriguez, R.J., J.F. White Jr, A.E. Arnold and R.S. Redman. 2009. Fungal endophytes: Diversity and functional roles. *New Phytol.* 182: 314-330. doi:10.1111/j.1469-8137.2009.02773.x.
- Ryu, C.-M., M.A. Farag, C.-H. Hu, M.S. Reddy, H.-X. Wei, P.W. Paré, et al. 2003. Bacterial volatiles promote growth in *Arabidopsis*. *PNAS* 100: 4927-4932. doi:10.1073/pnas.0730845100.
- Saleh-Lakha, S., M. Miller, R.G. Campbell, K. Schneider, P. Elahimanesh, M.M. Hart, et al. 2005. Microbial gene expression in soil: Methods, applications and challenges. *J. Microbiol. Methods* 63: 1-19. doi:http://doi.org/10.1016/j.mimet.2005.03.007.
- Schadt, C.W., A.P. Martin, D.A. Lipson and S.K. Schmidt. 2003. Seasonal dynamics of previously unknown fungal lineages in tundra soils. *Science* 301: 1359-1361. doi:10.1126/science.1086940.
- Schoug, A., J. Fischer, H.J. Heipieper, J. Schnurer and S. Hakansson. 2008. Impact of fermentation pH and temperature on freeze-drying survival and membrane lipid composition of *Lactobacillus coryniformis* s3. *J. Ind. Microbiol. Biotechnol.* 35: 175-181.
- Shade, A., P.S. McManus and J. Handelsman. 2013. Unexpected diversity during community succession in the apple flower microbiome. *mBio* 4: e00602-00612.
- Shi, S.J., A.E. Richardson, M. O'Callaghan, K.M. DeAngelis, E.E. Jones, A. Stewart, et al. 2011. Effects of selected root exudate components on soil bacterial communities. *FEMS Microbiol. Ecol.* 77: 600.
- Sigler, W.V. and R.F. Turco. 2002. The impact of chlorothalonil application on soil bacterial and fungal populations as assessed by denaturing gradient gel electrophoresis. *Applied Soil Ecology* 21: 107-118. doi:https://doi.org/10.1016/S0929-1393(02)00088-4.
- Smiley, R.W. and M.M. Craven. 1979. Microflora of turfgrass treated with fungicides. *Soil Biol. Biochem.* 11: 349-353. doi:https://doi.org/10.1016/0038-0717(79)90047-6.
- Steer, J. and J.A. Harris. 2000. Shifts in the microbial community in rhizosphere and non-rhizosphere soils during the growth of *agrostis stolonifera*. *Soil Biol. Biochem.* 32: 869-878. doi:https://doi.org/10.1016/S0038-0717(99)00219-9.
- Sylvia, D.M., J.J. Fuhrmann, P.G. Hartel and D.A. Zuberer. 2005. Principles and applications of soil microbiology. 2 ed. Prentice Hall.
- Tilman, D. and S. Pacala. 1993. The maintenance of species richness in plant communities. In: R. E. Ricklefs and D. Schluter, editors, *Species diversity in*

ecological communities: Historical and geographical perspectives. University of Chicago Press, Chicago, Illinois. p. 13-25.

- Toju, H., A.S. Tanabe, S. Yamamoto and H. Sato. 2012. High-coverage ITS primers for the DNA-based identification of ascomycetes and basidiomycetes in environmental samples. *PLOS ONE* 7: e40863. doi:10.1371/journal.pone.0040863.
- Torsvik, V. and L. Øvreås. 2002. Microbial diversity and function in soil: From genes to ecosystems. *Curr. Opin. Microbiol.* 5: 240-245. doi:[https://doi.org/10.1016/S1369-5274\(02\)00324-7](https://doi.org/10.1016/S1369-5274(02)00324-7).
- Torsvik, V., K. Salte, R. Sørheim and J. Goksøyr. 1990. Comparison of phenotypic diversity and DNA heterogeneity in a population of soil bacteria. *Appl. Environ. Microbiol.* 56: 776-781.
- Turnbaugh, P.J., R.E. Ley, M. Hamady, C. Fraser-Liggett, R. Knight and J.I. Gordon. 2007. The human microbiome project: Exploring the microbial part of ourselves in a changing world. *Nature* 449: 804.
- Turner, T.R., E.K. James and P.S. Poole. 2013. The plant microbiome. *Genome Biol.* 14: 209-219.
- USGA. 2018. USGA recommendations for a method of putting green construction. United States Golf Association, Liberty Corner, NJ.
- van der Heijden, M.G., R.D. Bardgett and N.M. van Straalen. 2008. The unseen majority: Soil microbes as drivers of plant diversity and productivity in terrestrial ecosystems. *Ecology Letters* 11: 296-310. doi:10.1111/j.1461-0248.2007.01139.x.
- Vandenkoornhuyse, P., S.L. Baldauf, C. Leyval, J. Straczek and J.P.W. Young. 2002. Extensive fungal diversity in plant roots. *Science* 295: 2051-2051. doi:10.1126/science.295.5562.2051.
- Velmourougane, K., D.P. Kumari, C.B. Prakasan and Jayarama. 2006. Microbial diversity of popular arabica and robusta selections. *Indian Coffee* 70: 27-31.
- von Wintzingerode, F., U.B. Gobel and E. Stackebrandt. 1997. Determination of microbial diversity in environmental samples: Pitfalls of PCR-based rRNA analysis. *FEMS Microbiol. Rev.* 21: 213-229.
- Vorholt, J.A. 2012. Microbial life in the phyllosphere. *Nat Rev Microbiol* 10: 828-840. doi:10.1038/nrmicro2910.
- Weisburg, W.G., S.M. Barns, D.A. Pelletier and D.J. Lane. 1991. 16S ribosomal DNA amplification for phylogenetic study. *J. Bacteriol.* 173: 697-703.

- Weiss, S., Z.Z. Xu, S. Peddada, A. Amir, K. Bittinger, A. Gonzalez, et al. 2017. Normalization and microbial differential abundance strategies depend upon data characteristics. *Microbiome* 5: 27. doi:10.1186/s40168-017-0237-y.
- Westcott, S.L. and P.D. Schloss. 2015. De novo clustering methods outperform reference-based methods for assigning 16s rRNA gene sequences to operational taxonomic units. *PeerJ* 3: e1487. doi:10.7717/peerj.1487.
- White, D.C. 1983. Analysis of microorganisms in terms of quantity and activity in natural environments. In: Slater, J.H., Whittenbury, R., Wimpenny, J.W.T. (eds.), *microbes in their natural environments*. Society of General Microbiology Symposium 34: 37-66.
- White, T., T. Bruns, S. Lee and J. Taylor. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. *PCR Protocols: A Guide to Methods and Applications* 315-322.
- Whitelaw, M.A., T.J. Harden and G.L. Bender. 1997. Plant growth promotion of wheat inoculated with *Penicillium radicum* sp. Nov. *Soil Res.* 35: 291-300. doi:<https://doi.org/10.1071/S96040>.
- Wickham, H. 2009. *Ggplot2: Elegant graphics for data analysis*. Springer-Verlag New York <http://ggplot2.org>.
- Yang, C.-H., D.E. Crowley, J. Borneman and N.T. Keen. 2001. Microbial phyllosphere populations are more complex than previously realized. *PNAS USA* 98: 3889-3894.
- Yang, L., J. Xie, D. Jiang, Y. Fu, G. Li and F. Lin. 2008. Antifungal substances produced by *Penicillium oxalicum* strain py-1—potential antibiotics against plant pathogenic fungi. *World J. Microbiol. Biotechnol.* 24: 909-915. doi:10.1007/s11274-007-9626-x.
- Yao, H., D. Bowman and W. Shi. 2006. Soil microbial community structure and diversity in a turfgrass chronosequence: Land-use change versus turfgrass management. *Applied Soil Ecology* 34: 209-218. doi:<http://dx.doi.org/10.1016/j.apsoil.2006.01.009>.
- Yao, H., D. Bowman and W. Shi. 2011. Seasonal variations of soil microbial biomass and activity in warm- and cool-season turfgrass systems. *Soil Biol. Biochem.* 43: 1536-1543. doi:<https://doi.org/10.1016/j.soilbio.2011.03.031>.
- Zhang, W., Z. Zhang, Y. Wang, D. Ai, P. Wen, Y. Zhu, et al. 2017. Effect of turfgrass establishment on soil microbiota using illumina sequencing. *Nat., Environ. Pollut. Technol.* 16: 679-686.

Zhu, S., J.M. Vivanco and D.K. Manter. 2016. Nitrogen fertilizer rate affects root exudation, the rhizosphere microbiome and nitrogen-use-efficiency of maize. *Applied Soil Ecology* 107: 324-333.
doi:<https://doi.org/10.1016/j.apsoil.2016.07.009>.