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RESEARCH

Differences among Soil-Inhabiting Microbial Communities in *Poa annua* Turf throughout the Growing Season

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ABSTRACT

There is increasing interest in understanding plant-associated microbial communities and their impact on plant health. However, research has been limited to major agronomic systems and little is known about the resident microorganisms in economically important specialty crops, such as turfgrass. In this study, we generated a community-wide inventory of the archaea and bacteria that inhabit the soil of *Poa annua* L. putting green turf at five time points over a 1-yr period. Next-generation sequencing of the nuclear ribosomal DNA 16S revealed 1.5×10^5 unique operational taxonomic units from 25 pooled soil samples. Seventeen archaeal taxa were identified at the species level, 53% of which were members of the Crenarchaeota. Proteobacteria was the most abundant bacterial phylum, comprising 36% of the 442 taxa present. Distance analysis and analysis of similarities revealed that the archaeal and bacterial communities clustered according to sample date, with samples collected during warm months grouping separately from those collected during cool months. Clustering was less distinct when plotted using detrended correspondence analysis. These data indicate that microbial community structure varied throughout the growing season, possibly due to seasonal changes in temperature and/or other environmental factors. This research also shows that *P. annua* putting green turf supports a diverse microbial community despite management practices that include frequent pesticide applications.

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Abbreviations: DCA, detrended correspondence analysis; OTU, operational taxonomic unit; PCR, polymerase chain reaction.

Soil-inhabiting microorganisms are vital constituents of all ecosystems and contribute to numerous important biological and chemical processes. In agricultural systems, soil-inhabiting microbes are essential for maintaining plant productivity, controlling nutrient release, and suppressing plant pathogenic microorganisms (Arias et al., 2005; van der Heijden et al., 2008; Mendes et al., 2011). There is currently considerable interest within the scientific community to learn how these microbes respond to anthropogenic inputs in the environment. In particular, understanding how resident microorganisms respond to routine agronomic practices, such as soil cultivation or chemical inputs, is just one component of an increasingly popular phytobiome initiative that seeks to understand whether microbial assemblages might be manipulated to promote crop health and sustainability, reduce the need for fertilizer and chemical inputs, and increase plant yield in agricultural settings (Bakker et al., 2012). For example, Mendes et al. (2011) recently showed that the entire soil microbiome is responsible for suppressing disease caused by the fungus *Rhizoctonia solani* Kühn in *Beta vulgaris* L. fields in Norway, and that this suppression could be transferred to soils that were conducive to disease. Similarly, analysis of microbial communities in desert farming systems revealed that

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the resident microflora can confer drought tolerance to *Capsicum annuum* L. (Marasco et al., 2012).

Research examining microbial communities in agriculture has been primarily limited to major cropping systems that have significant global economic impact, especially staple food crops needed to feed expanding human populations worldwide (e.g., Pérez-Piqueres et al., 2006; Fliessbach et al., 2009). Conversely, little is known about the microbial communities inhabiting specialty horticultural crops cultivated for fruit, flower, or vegetable production or plants cultivated as components of the aesthetic environment, such as turfgrass and landscape plants. This is surprising, given that turfgrass alone encompasses more than 20.2 million ha of land in the United States (National Turfgrass Federation, 2009) and is represented by an industry with an estimated value of US$40 billion annually (National Turfgrass Federation, 2009). Given the economic importance of the turf industry and the high level of inputs typically required to maintain healthy specialty turf such as golf course putting greens, understanding how resident microbes in this system are affected by routine management practices is an essential first step towards selecting microbial communities that promote healthy and sustainable turf.

Changes in land use practices can dramatically alter soil properties and, in turn, may affect resident soil microbial communities (Lauber et al., 2008). For example, when comparing irrigated and nonirrigated agricultural sites and annual or never-tilled perennial grasslands, Steenwerth et al. (2002) found that each study site possessed a specific microbial community that could be used to identify land use type. Similar differences in microbial community structure associated with land use practices have been documented from grazed pastures in the European Alps. In this environment, a more homogeneous microbial community was found across regions compared with native grasslands, further supporting that land use may be an important driver of such communities (Geremia et al., 2015). As such, it is also likely that the resident microbial community associated with cultivated turfgrass may be distinct from that observed in other cropping systems. The thatch layer (an organic layer composed of living and dead plant material between the soil surface and aboveground vegetation) in *Agrostis stolonifera* L. putting green turf has been shown to contain more bacteria, fungi, and actinomycetes than the soil at lower depths. In addition, the microbial populations in these locations were similar to those observed in native soils (Mancino et al., 1993). When compared with less intensely managed sites, such as fairways and roughs, *Poa annua* L. putting greens were found to contain fewer microbes (reported as phospholipid fatty acid profiles), suggesting that the regular management practices commonly employed on putting greens may negatively impact the resident microbial community (Bartlett et al., 2008). Still, there have been no large-scale, comprehensive analyses of the resident microbial communities in turfgrass putting greens to date.

Seasonality is also known to affect overall microbial population counts and enzymatic function in bacterial populations, as well as decrease metabolic activity in perennial grassland ecosystems and agricultural soils during winter months (Dunfield and Germida, 2003; Cho et al., 2008; Guicharnaud et al., 2010). However, little is known about the impact of seasonality on microbial populations in cultivated turfgrass. Much of what is known about seasonal effects on turf-associated microorganisms is based on early studies conducted using culture-based methods targeting specific bacteria, rather than analyses of entire communities. For example, in a *Cynodon dactylon* (L.) Pers. putting green, population counts of one rhizosphere bacterium [*Stenotrophomonas maltophilia* (Hugh) Palleroni & Bradbury] was significantly different on one sampling date over a 2-yr period (Elliott and Des Jardin 1999). Similarly, molecular surveys of microbial enzymes in turf have provided important insight into seasonal changes of microbial activity but often did not identify the organisms associated with these changes. For example, Yao et al. (2011) observed seasonal fluxes in microbial respiration and enzymatic activities in the soil of six cultivated cool- and warm-season turfgrass species [cool-season species: *Festuca arundinacea* Schreb., *Poa pratensis* L., and *A. stolonifera*; warm-season species: *Eremochloa ophiuroides* (Munro) Hack., *Zoysia japonica* Steud., and *C. dactylon*] sampled over 9 mo in North Carolina. Together, these studies suggest that, to thoroughly characterize the resident microbial community in turfgrasses, sampling should be performed throughout the year, as seasonal fluxes may influence community dynamics.

Golf course putting greens, with their input-intensive management practices and perennial nature, represent a unique environment unlike any cropping system in traditional agriculture. Characterizing microbial communities that inhabit the soil of turfgrass putting greens throughout the year would provide valuable information regarding the microorganisms that reside within this highly managed system and would offer insight on how management practices may potentially be utilized to select for microbial communities with beneficial properties. With the increasing accessibility of advanced high-throughput molecular technologies, such as next-generation metabarcode sequencing, it is now possible to rapidly identify the entire cohort of resident microorganisms in putting greens. To this end, the objective of this study was to identify the archaeal and bacterial communities inhabiting the soil of *P. annua* putting green turf at five time points over the course of 12 mo.

MATERIALS AND METHODS

General Field Maintenance

A 12-mo field trial was initiated in 2014 on a 5-yr-old monostand of *P. annua* turf maintained as a putting green at the Rutgers

Horticultural Research Farm No. 2 in North Brunswick, NJ (40°28′ N, 74°25′ W). Turf was grown on a sand-topdressed mat layer (40 mm deep) overlaying a Nixon sandy loam (fine-loamy, mixed, semiactive, mesic Type Hapludult) with a pH of 5.9. Kiln-dried, medium-coarse silica sand was applied at a rate of 0.15 L m^{-2} and brushed in with a coco mat every 14 d.

Turf was mowed daily with a triplex greens mower (Models 3000–04350 or 3150–04357, Toro Co., Bloomington, MN) at a bench setting of 3.2 mm with clippings collected. Turf was irrigated with overhead irrigation and a handheld syringe hose to maintain moderately dry conditions typical of golf course putting greens in the northeastern United States. Urea (CH_4N_2O) was applied to the entire study at 4.9 kg N ha^{-1} on 14 and 26 Apr., 22 Sep., and 6 Oct. 2014, with supplemental nitrogen (N) fertilization applied as treatments during summer months. On sampling dates, soil was collected prior to the application of N treatments. Additional macro- (calcium, magnesium, potassium, and phosphorous) and micronutrients were applied as needed, based on soil test results.

A disease management program to suppress fungal diseases such as summer patch [caused by *Magnaporthiopsis poae* (Landsch. & N. Jacks.) J. Luo & N. Zhang], brown patch (caused by *Rhizoctonia solani* Kühn), dollar spot (caused by *Sclerotinia homoeocarpa* F.T. Bennett), and brown ring patch (caused by *Waitea circinata* Warcup & Talbot) was in place throughout the duration of the study. In 2014, dollar spot was preventatively controlled with vinclozolin [3-(3, 5-dichlorophenyl)-5-ethenyl-5-methyl-2, 4-oxazolidinedione] at 1.5 kg a.i. ha^{-1} or boscalid {3-pyridinecarboxamide, 2-chloro-N-[4'-chloro(1,1'-biphenyl)-2-yl]} at 0.4 kg a.i. ha^{-1} every 14 d from 25 Apr. to 23 Aug. 2014. Fluoxastrobin {[(1E)-[2-[[6-(2-chlorophenoxy)-5-fluoro-4-pyrimidinyl] oxy]phenyl]-5,6-dihydro-1,4,2-dioxazin-3-yl) methanone-O-methyloximewere} at 0.44 kg a.i. ha^{-1} or flutolanil [N-(3-isopropoxyphenyl)-2-(trifluoromethyl) benzamide] at 6.4 kg a.i ha^{-1} was applied biweekly between 1 Apr. and 6 May 2014 to control brown ring patch. Flutolanil at 6.4 kg a.i. ha^{-1} every 14 d was used to suppress brown patch between 11 June and 9 Aug. 2014. Summer patch was controlled with azoxystrobin [methyl (E)-2-{2-[6-(2-cyanophenoxy) pyrimidin-4-yloxy]phenyl}-3-methoxyacrylate] at 3 kg a.i. ha^{-1} on 14 May and 11 June 2014. Chlorothalonil (tetrachloroisophthalonitrile) was applied at 12.63 kg a.i. ha^{-1} on 26 Sept. and 6 Oct. 2014 to suppress algae. In 2015, only boscalid at 0.4 kg a.i. ha^{-1} and fluoxastrobin at 0.44 kg a.i. ha^{-1} were applied on 14 and 19 May 2015, respectively, to prevent spring diseases. As a result, turfgrass diseases were not observed during the duration of this study.

The growth regulators ethephon [(2-chloroethyl) phosphonic acid] and trinexapac-ethyl [4-(cyclopropyl-α-hydroxy-methylene)-3,5-dioxocyclohexanecarboxylic acid ethylester] were applied as a mixture at 3.3 and 0.05 kg a.i. ha^{-1}, respectively, on 4 and 21 Apr. and 6 May 2014 and 2, 7, and 27 May 2015 to suppress seed heads, followed by weekly applications of trinexapac-ethyl at 0.05 kg a.i. ha^{-1} from 14 May to 12 Nov. 2014 and on 4 June 2015 to restrict vertical growth.

Chlorantraniliprole {3-bromo-N-[4-chloro-2-methyl-6-[(methylamino)carbonyl]phenyl]-1-(3-chloro-2-pyridinyl)-1H-pyrazole-5-carboxamide} was sprayed at 0.15 kg a.i. ha^{-1} on 14 May 2014 and bifenthrin {2-Methyl-3-phenylphenyl) methyl (1*S*,3*S*)-3-[(*Z*)-2-chloro-3,3,3-trifluoroprop-1-enyl]-2,2-dimethylcyclopropane-1-carboxylate} at 0.12 kg a.i. ha^{-1} on 25 May 2015 to control annual bluegrass weevils (*Listronotus maculicollis* Dietz).

Moss and crabgrass were controlled with the herbicides carfentrazone-ethyl {ethyl 2-chloro-3-[2-chloro-5-[4-(difluoromethyl)-3-methyl-5-oxo-1,2,4-triazol-1-yl]-4-fluorophenyl]propanoate} at 0.03 kg a.i ha^{-1} and fluazifop-P-butyl {butyl (*R*)-2-[4-[[5-(trifluoromethyl)-2-pyridinyl]oxy]phenoxy] propanoate} at 0.21 kg a.i. ha^{-1} on 14, 20, 27, and 30 Oct. and 12 Nov. 2014. All products (fungicides, growth regulators, insecticides, and herbicides) were applied uniformly to the study site.

Experimental Area and Soil Sampling

Turf plots used for soil sampling measured 1.8 by 1.8 m. Soil samples were obtained from each of five N treatments, which provided a range of N rates that are typically applied on golf course putting greens in the United States: (1) no N, (2) 4.9 kg N ha^{-1} every 7 d, (3) 4.9 kg N ha^{-1} every 14 d, (4) 4.9 kg N ha^{-1} every 28 d, or (5) 9.8 kg ha^{-1} every 7 d. Nitrogen was applied as a foliar spray containing urea with a walk-behind spray boom (model I-1575D, The Broyhill Co., Dakota City, NE) equipped with five extended-range flat fan nozzles (model XR8003VS; TeeJet® Technologies) that delivered 815 L ha^{-1} water at 379 kPa using a gasoline-powered backpack sprayer (model SHR-210, ECHO Inc., Lake Zurich, IL). Nitrogen treatments were applied every 7, 14 or 28 d, from 7 May through 8 Sept. 2014, and 5 through 26 May 2015.

Sampling occurred over the course of two growing seasons over a period of 12 months. Specific sampling dates were as follows: 11 June, 25 July, and 27 Aug. 2014 and 16 Apr. and 3 June 2015. The samples obtained on each date served as replicates to evaluate changes in archaeal and bacterial communities over the course of 12 mo and were not intended to evaluate community changes due to N treatment. Soil samples were taken 3 d after N applications, except for the 16 Apr. 2015 sampling date, which had no N applications since 8 Sept. 2014. No fungicides were sprayed at least 11 d prior to sampling. Soil was sampled by taking four 12.7-mm diameter by 50.8-mm depth soil cores (the approximate depth of *P. annua* roots during much of the growing season) from four replicated plots for each of the five levels of N using a custom-designed soil probe. Plots were sampled within a 30- by 45-cm region located in the center of the plots. The same plots were sampled on each date, with care exercised to avoid prior sampling holes. If a sample was collected that contained mostly topdressing sand, it was discarded and a new sample was taken. Soil cores were stored on ice immediately following removal and screened through a 2.5-mm sieve before DNA extractions. Sieving removed all plant debris and large soil particulates, leaving the surrounding bulk soil for analysis. Corresponding air and soil temperatures for each sampling date are listed in Table 1.

DNA Manipulations

The PowerSoil DNA Isolation Kit (Mo-Bio, Carlsbad, CA) was used for all genomic DNA extractions. Twelve blank DNA extractions (no sample included) were performed to serve as controls throughout the extraction and polymerase chain reaction (PCR) processing. The PowerSoil protocol was modified to improve DNA yield and quality because the standard manufacturer's protocol

Table 1. Average air and soil temperature for North Brunswick.

Sampling date	Air temperature			Soil temperature†	
	Mean	Min.	Max.	Min.	Max.
			°C		
11 June 2014‡	20	13	27	17	19
25 July 2014§	24	17	28	22	23
27 Aug. 2014§	23	15	28	21	22
16 Apr. 2015§	11	4	17	8	9
3 June 2015‡	16	11	20	17	19

† Soil temperature measured at 10.16-cm depth in turfgrass stand.

‡ Average temperatures for 11 June 2014 and 3 June 2015 sampling dates are only reported from 1 June 2014 or 2015 until the date of sampling (11 June 2014 or 3 June 2015, respectively).

§ Temperatures are averaged across all days in each respective sampling month.

consistently resulted in poor DNA yields (<10 ng μl^{-1}), possibly due to the high sand content and minimal organic matter in our samples. In the PowerSoil Bead tube, 200 μL of bead solution was replaced with an equal volume of phenol:chloroform:isoamyl alcohol pH 8 (Fisher Scientific, Pittsburgh, PA), then 0.5 g of soil and 60 μL of Solution C1 were added. Tubes were shaken in a BioSpec bead-beater (BioSpec Products, Bartlesville, OK) on the medium setting for 1 min. The remaining steps of the PowerSoil protocol were followed according to manufacturer's recommendation for low biomass soil.

Amplicon libraries for Illumina sequencing were generated using a two-step PCR process: (i) amplification of the region-of-interest using targeted PCR primers that included overhang adaptor sequences and (ii) the addition of unique barcode indices and Illumina P5 and P7 sequence adapters. Organism-specific genomic DNA ribosomal markers were PCR amplified from bacteria using the Ba9F/Ba515Rmod1 primer pair for the 16S rDNA (~500 bp; Weisburg et al., 1991; Kittelmann et al., 2013) and from archaea using the Ar915aF/Ar1386R primer pair for the 16S rDNA (~500 bp; Skillman et al., 2004; Watanabe et al., 2004). Unique overhang adaptor sequences were added to the 5′ end of each primer set to allow attachment of indices and Illumina sequencing adapters: forward primer = TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG; reverse primer = GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG. Reverse primers were synthesized in four different versions, with the addition of zero to three mixed sequence bases (where N is any nucleotide) between the overhang adaptor and the locus-specific sequence to introduce sequence complexity. A complete list of PCR primers used in this study is available in Table 2. Polymerase chain reactions were performed using MangoTaq DNA Polymerase (BioLine, Taunton, MA) in 25 μL volumes containing 5x PCR buffer, 2 mM $MgCl_2$, 0.2 mM of each deoxynucleotide triphosphate, and 10 nM of each primer. Equal volumes of all four reverse primers were used to create a 10 nM working stock. The cycling conditions were as follows: 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. Following PCR, amplicons were visualized on a 0.8% agarose gel and the four amplicons from each sample were pooled. Pooled amplicons were purified using the DNA (PCR) Clean and Concentrator Kit (Zymo Research, Irvine, CA).

Sequencing libraries were prepared using MangoTaq DNA Polymerase (BioLine, Taunton, MA) in 40 μL volumes containing 5x PCR buffer, 2 mM $MgCl_2$, 0.2 mM of each deoxynucleotide triphosphate, and 5 μL of each Nextera index primer (Illumina, San Diego, CA) to allow sample multiplexing. The cycling conditions were as follows: 72°C for 3 min, 95°C for 30s, followed by 12 cycles of 95°C for 10 s, 55°C for 30 s, 72°C for 30 s, followed by a final extension at 72°C for 5 min. Libraries were purified using the Zymo DNA (PCR) Clean and Concentrator Kit and quantified using the QIAxcel System (QIAGEN, Gaithersburg, MD) and Qubit fluorometer (Life Technologies, Grand Island, NY). Purified libraries were normalized to 4 nM and pooled into a single sample. To increase library complexity, a phiX control (Illumina) and an indexed genomic DNA library of the fungus *Colletotrichum graminicola* (Ces.) G.W. Wilson was spiked into the same run. Pooled libraries were denatured, diluted to 18 pM, and sequenced as paired-end reads on Illumina's MiSeq platform using a 600 cycle MiSeq version 3 Reagent Kit. To generate further data, two additional sequencing runs were performed as paired-end reads on the MiSeq platform with 600-cycle chemistry, except pooled libraries were diluted to 12 pM. Sequencing data from all runs was output in fastq format.

Bioinformatic Analyses

The QIIME pipeline version 1.9.1 (Caporaso et al., 2010) was used for initial filtering and data analyses of binned datasets, with all steps performed on an Amazon EC2 image (Amazon machine image number: ami-1918ff72). Forward and reverse Illumina sequence reads were stitched together using fastq-join in the ea-utils package (Aronesty 2011). Sequence reads were only assembled if there were no base differences in the overlap region. Following assembly, barcodes were removed and pooled reads were separated into individual samples with the split_libraries_fastq.py script. Only sequences with a quality score >20 and zero ambiguous bases were retained. Assembled sequences were checked for chimeras using the 64-bit version of USEARCH (Edgar et al., 2011). Any sequences identified as chimeric were removed from the dataset.

Table 2. Polymerase chain reaction (PCR) primers used in this study.

Primers	Description	Sequence	Reference
		5′ to 3′	
Ar915aF	Archaea 16S rDNA forward primer†	AGGAATTGGCGGGGGAGCAC	Watanabe et al., 2004
Ar1386R	Archaea 16S rDNA reverse primer‡	GCGGTGTGTGCAAGGAGC	Skillman et al., 2004
Ba9F	Bacterial 16S rDNA forward primer‡	GAGTTTGATCMTGGCTCAG	Weisburg et al., 1991
Ba515Rmod1	Bacterial 16S rDNA reverse primer‡	CCGCGGCKGCTGGCAC	Kittelmann et al., 2013

† Forward overhang adapter TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG was appended to the 5′end of all forward primers

‡ Reverse overhang adapter GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG-(N, NN, NNN) was appended to the 5′ end of all reverse primers. Reverse primers were synthesized in four different versions, with the addition of zero to three mixed sequence bases (N) between the overhang adapter and the locus-specific sequence to introduce sequence complexity.

A 97% sequence similarity threshold was used to cluster sequences into operational taxonomic units (OTUs) with a de novo picking strategy. Following OTU picking, any OTU represented in the datasets by just one sequence was removed, as these can represent sequencing errors and overestimate diversity metrics (Kunin et al., 2010). Archaea and bacteria OTUs were classified to the lowest possible taxonomic rank (domain through species) with the RDP Classifier 2.2 (Wang et al., 2007) and the Greengenes database version 13_8 (McDonald et al., 2012; Werner et al., 2012)

Data-parsing scripts developed in C++ were used to remove any OTUs that could not be identified using the Greengenes database. Taxonomic assignments discussed herein represent the lowest rank that could be determined for a given OTU. Some bacteria are described as belonging to so-called "candidate divisions," which are lineages of organisms that carry no formal taxonomic rank through the International Code of Nomenclature of Bacteria. These candidate divisions are defined solely through environmental DNA sequence data in the absence of a cultured representative (Hugenholtz et al., 1998).

Data Analyses

Unless otherwise specified, data analyses were performed in the R statistical computing environment (R Core Team, 2015). Where calculated, a p-value ≤ 0.05 was considered significant for all analyses. Detrended correspondence analysis (DCA) was conducted using the vegan package (Oksanen et al., 2013). Rarified datasets were used for all subsequent analyses to account for varying sequencing depth, where the multiple_rarefactions.py QIIME script was used to rarify sequences to a depth of 4500 (100-step increments, 10 replicates). The Shannon index was used to assess microbial diversity across all samples on the local scale (α diversity).

Nonparametric two-way t tests utilizing Monte Carlo permutations were performed to determine whether there were significant differences in α diversity metrices between samples. Differentiation in diversity values between pairs of samples (β diversity) was calculated using Bray–Curtis dissimilarity matrices. Large dissimilarity values indicate that samples are not similar to one another in microbial composition, versus smaller values that indicate similar community structure (Gardener, 2014). Analysis of similarities (ANOSIM) was used to test if samples within categories were more similar to one another than samples in different categories using 999 permutations across the dissimilary matrices. Rank abundance plots were produced using the plot_rank_abundance_graph.py script in QIIME. Rank abundance curves provide a way to visualize OTU abundance between samples (Hughes et al., 2001). Taxa are ordered from most to least abundant on the x axis, and the abundance of each type is plotted on the y axis (Hughes et al., 2001). A steep slope indicates a community where taxa are not very evenly distributed, as high-ranking taxa (left on the x axis) have much higher abundance (Hughes et al., 2001). Cluster-based neighbor-joining trees were generated from the Bray–Curtis distance matrices in QIIME.

Data Availability

All sequence data is available under accession number SRP063317 in NCBI's Sequence Read Archive.

RESULTS AND DISCUSSION

Sequence Data

Three independent runs of Illumina sequencing of the PCR amplicons generated for all 25 samples (five pooled N treatments collected for the five time points, one library) produced 3.84×10^7 total reads with an average length of 331 bp (run 1 = 1.06×10^7 reads, run 2 = 8.92×10^6 reads, run 3 = 1.00×10^7 reads). A total of 2.00×10^7 reads passed quality filtering (run 1 = 1.00×10^7 reads, run 2 = 8.53×10^6 reads, run 3 = 9.52×10^6 reads). After read stitching and demultiplexing, an average of 1.31×10^5 sequences per sample were generated from the combined three runs. The average size of the stitched sequences was 278 bp. From the complete set of sequences, 18.9% were identified as chimeras and removed from the dataset, leaving a final dataset of 1.27×10^7 sequences for microbial community analyses.

Sequencing of blank control samples from the PowerSoil DNA extraction kit generated a kitome (kit microbiome) that included 34 bacterial taxa. Removal of singletons from the kitome eliminated 11 bacterial taxa. The remaining 23 bacterial taxa represented five phyla: Acidobacteria (three taxa), Actinobacteria (four taxa), Chloroflexi (one taxon), Firmicutes (four taxa), and Proteobacteria (11 taxa). Kitome percent abundance averaged across all samples ranged from 1.31×10^{-3} (most abundant, *Pseudomonas* sp.) to 1.26×10^{-5} (least abundant, *Enhydrobacter* sp.). The kitome taxa were removed from all downstream analyses by quality filtering.

Rarefaction curves of the sequence datasets plateaued for all 25 samples, indicating that microbial diversity was adequately captured from the sampling (Supplemental Fig. 1). Rank abundance plots revealed a similar slope for all samples collected on each of the five sampling dates for archaea and bacteria (Supplemental Fig. 2) indicating that, within each respective sample, microbial populations from different sampling months display similar richness (number of taxa) and evenness (proportions of taxa).

In total, 1.50×10^5 OTUs were identified from the samples, of which 1.03×10^5 were archaea and bacteria. The remaining 4.66×10^4, 2.65×10^{-5}, and 3.09×10^{-6} OTUs represented fungi, plant, and protist DNA, respectively. Archaea and bacteria were identified from all samples. The large number of OTUs identified here, in just 12.5 g of soil, was equal to or greater than that described from many agricultural or natural ecosystems. For example, 1.03×10^5 archaeal and bacterial OTUs were identified in this study, while approximately 3.3×10^4 and 5.3×10^4 OTUs have been reported for *B. vulgaris* and *Solanum tuberosum* L. agroecosystems, respectively (Inceoglu et al., 2011; Mendes et al., 2011). In the Amazonian forest, the Mojave Desert, and the Konza Prairie, 1.0×10^4 archaeal and bacterial OTUs have been reported from 10 g of soil (Fierer et al., 2007).

Community Diversity

Two pairwise comparisons of microbial α diversity (species richness and abundance) between sampling dates were significant (Table 3). Samples collected on 3 June 2015 exhibited significantly higher archaea and bacteria community diversity compared with the 11 June 2014 (p = 0.03) and 16 Apr. 2015 (p = 0.01) sampling dates (Shannon index 10.93 vs. 10.46 and 10.60, respectively). These data indicate that archaea and bacteria diversity does change over time, a finding that has also been reported in conventional and organic agricultural systems (Lauber et al., 2013); thus, to accurately assess α diversity in a turfgrass site, soil samples should be taken over time. The diversity metrics observed in this system are comparable with those observed from the soil of a range of biomes (e.g., the polar desert, a hot desert, the arctic tundra, temperate grasslands, tropical forests, temperate deciduous forests, coniferous forests, and boreal forests) (Fierer et al., 2012b).

Archaea Community Composition

In total, four archaeal phyla were identified, with total average abundance as follows: Crenarchaeota 0.58%, Euryarchaeota 0.07%, an unidentified archaeal phyla 0.04%, and Parvarchaeota 0.01% (data not shown). The Crenarchaeota, which is a widely distributed phylum of archaea best known for their thermophyllic or hyperthermophyllic lifestyles, made up 53% of all archaea identified. The most abundant Crenarchaeota class was the Thaumarchaeota (data not shown), a group of chemolithoautotrophic ammonia-oxidizers that are thought to play a major role in global N and carbon cycling (Leininger et al., 2006). Members of the Euryarchaeota phylum, which is made up a diverse group of methanogens, thermophiles, and halobacteria, was also present at relatively high frequency within the archaea dataset, comprising 29% of the total. Within the Euryarchaeota, the class Methanobacteria was the most frequently identified at 23%. The Parvarchaeota, a relatively new archaeal phylum populated by just two species—Candidatus *Micrarchaeum* and C. *Parvarchaeum* (Rinke et al., 2013)—made up 12% of the archaea. Averaged across all samples, all archaeal phyla were present during each sampling month (Table 4).

The frequency and widespread presence of the Crenarchaeota suggests an important role for these microbes in the turfgrass system. Crenarchaeota are the most abundant phyla of ammonia-oxidizing microorganisms in terrestrial habitats, where they can reduce ammonia to nitrite for use by the plant (Leininger et al., 2006). Recently, many ammonia-oxidizing archaea have been shown to possess urease genes, possibly as a way to facilitate nitrification (Alonso-Saez et al., 2012). In addition, gene expression studies have also shown increased expression of *amoA*, the archaeal gene involved in the first step of ammonia oxidation, in the presence of urea (Lu et al., 2012), the N source used on our study. This suggests that microbes within the Crenarchaeota are capable of utilizing urea to begin ammonia oxidation. Further investigations of how ammonia-oxidizing archaea affect the N cycle in turfgrass putting greens will be necessary to fully understand how such organisms might be used to improve N use efficiency in cultivated turfgrasses. While additional data is required to evaluate the function of these microbes, their identification serves as a starting point for future research investigating the link between microbial function and plant health in the turfgrass ecosystem.

Bacteria Community Composition

A complete list of the 33 bacterial phyla identified can be found in Table 4. The two most abundant bacterial phyla were the Proteobacteria (8.14%) and Acidobacteria (7.75%). The remaining 31 bacterial phyla were present in abundances <1%. Within the Proteobacteria, average abundance of the four classes identified was as follows: Alphaproteobacteria 53%, Betaproteobacteria 20%, Gammaproteobacteria 16%, and Deltaproteobacteria 11% (data not shown). Of particular interest was the overall abundance of Rhizobiales and Burkholderiales, representing 46% of all Alphaproteobacteria and 50% of all Betaproteobacteria. Bacteria in both of these orders have been reported as having a beneficial effect on plants, either

Table 3. Pairwise comparison of Shannon diversity indices for archaea and bacteria communities, grouped by treatment and sampling date. Comparisons were performed using nonparametric two-way *t* tests. Data is from a depth of 1000 sequences sample^{-1}.

Sampling date		Factor 1 Shannon index mean†	Factor 1 SD	Factor 2 Shannon index mean†	Factor 2 SD	*t* statistic	*p*-value
11 June 2014	25 July 2014	10.46	0.34	10.72	0.13	–1.41	1.00
11 June 2014	27 Aug. 2014	10.46	0.34	10.83	0.14	–2.02	0.58
11 June 2014	16 Apr. 2015	10.46	0.34	10.60	0.19	–0.74	1.00
11 June 2014	3 June 2015	10.46	0.34	10.93	0.11	2.60	0.03
25 July 2014	27 Aug. 2014	10.72	0.13	10.83	0.14	–1.20	1.00
25 July 2014	16 Apr. 2015	10.72	0.13	10.60	0.19	1.00	1.00
25 July 2014	3 June 2015	10.72	0.13	10.93	0.11	2.42	0.52
27 Aug. 2014	16 Apr. 2015	10.83	0.14	10.60	0.19	1.95	1.00
27 Aug. 2014	3 June 2015	10.83	0.14	10.93	0.11	1.08	1.00
16 Apr. 2015	3 June 2015	10.60	0.19	10.93	0.11	2.94	0.01

† Shannon index as log base 2 output from QIIME.

Table 4. Archaeal and bacterial abundance, averaged by sampling month. Values can range from zero to one. Taxonomic groups reflect the lowest possible assigned nomenclature.

Taxonomic groups		Sampling date				
		11 June 2014	25 July 2014	27 Aug. 2014	16 Apr. 2015	3 June 2015
Archaea	Unassigned archaea	2.83–04	5.19E–04	4.66E–04	4.51E–04	4.89E–04
	Parvarchaeota	3.12E–04	6.14E–05	1.21E–04	5.94E–05	8.40E–05
	Crenarchaeota	7.77E–03	3.68E–03	5.56E–03	4.31E–03	7.64E–03
	Euryarchaeota	1.48E–04	1.03E–03	1.40E–03	3.77E–04	3.75E–04
Bacteria	Unassigned bacteria	3.75E–04	6.88E–04	4.92E–04	4.01E–04	4.66E–04
	Thermi	0	0	0	7.02E–06	1.48E–05
	Acidobacteria	6.32E–02	9.10E–02	7.33E–02	6.61E–02	9.39E–02
	Actinobacteria	5.54E–03	1.05E–02	6.06E–03	8.95E–03	1.15E–02
	Armatimonadetes	2.29E–04	4.00E–04	4.30E–04	3.28E–04	5.32E–04
	Bacteroidetes	2.03E–03	3.72E–03	4.01E–03	2.85E–03	6.33E–03
	BHI80–139	0	0	1.87E–05	0	0
	Chlorobi	3.59E–05	3.10E–04	1.42E–04	4.75E–05	1.25E–04
	Chloroflexi	6.77E–03	9.44E–03	8.15E–03	6.74E–03	1.20E–02
	Cyanobacteria	2.25E–03	2.79E–03	1.92E–03	4.20E–03	3.23E–03
	Elusimicrobia	5.24E–05	2.91E–04	2.47E–04	7.31E–05	1.18E–04
	FBP	1.03E–04	2.05E–04	2.27E–04	4.96E–05	2.80E–04
	FCPU426	4.34E–06	4.10E–05	8.58E–05	0	2.15E–05
	Fibrobacteres	6.77E–05	2.63E–04	3.84E–04	1.55E–04	3.57E–04
	Firmicutes	1.41E–04	2.74E–04	2.97E–04	8.47E–04	3.41E–04
	Gemmatimonadetes	7.17E–04	1.02E–03	1.09E–03	9.82E–04	8.04E–04
	GN02	1.78E–04	6.42E–04	5.88E–04	1.51E–04	2.93E–04
	Kazan-3B-28	8.69E–06	1.74E–05	0	0	0
	MVP-21	9.09E–05	1.06E–04	7.07E–05	1.71E–04	6.10E–05
	Nitrospirae	9.88E–05	2.29E–04	2.16E–04	3.70E–05	2.28E–04
	OD1	2.18E–03	7.20E–03	7.29E–03	2.63E–03	2.75E–03
	OP11	1.13E–02	4.76E–03	5.84E–03	6.45E–03	6.66E–03
	OP3	1.59E–05	6.77E–05	6.02E–05	0	0
	Planctomycetes	1.91E–03	5.37E–03	4.64E–03	2.58E–03	4.64E–03
	Proteobacteria	5.51E–02	1.02E–01	7.96E–02	7.00E–02	1.00E–01
	SBR1093	0	1.18E–05	0	0	6.75E–06
	Spirochaetes	4.68E–05	1.13E–04	7.07E–05	6.17E–06	5.00E–05
	SR1	2.39E–03	4.21E–05	4.73E–05	1.59E–04	4.39E–04
	Tenericutes	2.59E–05	2.68E–05	1.20E–05	3.21E–05	3.97E–05
	TM6	1.64E–04	8.34E–04	5.76E–04	2.05E–04	6.03E–04
	TM7	3.38E–03	7.51E–03	5.07E–03	8.66E–03	5.68E–03
	TPD-58	7.23E–06	0	1.20E–05	1.81E–05	6.82E–06
	Verrucomicrobia	9.92E–04	5.70E–03	4.79E–03	1.86E–03	2.46E–03
	WPS-2	1.35E–04	1.77E–04	1.90E–04	7.09E–05	2.17E–04

through N fixation, plant growth promotion, or pathogen suppression (Hayat et al., 2010).

Within Acidobacteria, members of the Acidobacteriales were most abundant, representing 32% of all Acidobacteria. Within the Actinobacteria, Actinomycetales, a group of known antibiotic producers (Lazzarini et al., 2000,) was most abundant, accounting for 72% of all Actinobacteria identified. Acidobacteria are also abundant in many other soil ecosystems, including agricultural fields and native grasslands (Dunfield and Germida, 2003; Cho et al., 2008; Guicharnaud et al., 2010; Fierer et al., 2012a), indicating that manmade ecosystems share similarities with natural sites. Twenty-seven of the bacteria phyla were present in all sampling months (Table 4).

In total, 114 bacterial genera were identified from the 25 samples, plus an additional nine candidate genera of bacteria (Table 5). Fourteen of the 114 total bacterial genera identified here have also been reported from the rhizosphere of *A. stolonifera* and *C. dactylon* putting greens in the southeastern United States (Elliott et al., 2008). Three of these genera, *Brevibacterium*, *Burkholderia*, and *Pseudomonas*, possess interesting metabolic capabilities. For example, members of the Burkholderiales and Actinomycetales represent bacterial groups known to produce antibiotics (Lazzarini et al., 2000; Pidot et al., 2014); thus, they may possess compounds for fighting plant diseases. In fact, bacteria in the Pseudomonadaceae and Burkholderaceae, like those identified in this study, have been

Table 5. Bacterial genera identified in *Poa annua* putting green soil, not including candidate divisions.

Bacterial genera				
Acutodesmus	*Cellulomonas*	*Janthinobacterium*	*Nitrospira*	*Rhodovastum*
Aerococcus	*Chitinophaga*	*Kaistia*	*Nostoc*	*Roseomonas*
Afifella	*Chryseobacterium*	*Kaistobacter*	*Novosphingobium*	*Rubrivivax*
Agrobacterium	*Chthoniobacter*	*Kineococcus*	*Ochrobactrum*	*Rudanella*
Anaeromyxobacter	*Clostridium*	*Kouleothrix*	*Parvibaculum*	*Sediminibacterium*
Aquicella	*Couchioplanes*	*Labrys*	*Pasteuria*	*Singulisphaera*
Asteroleplasma	*Dechloromonas*	*Lautropia*	*Pedomicrobium*	*Sphingobium*
Asticcacaulis	*Deinococcus*	*Legionella*	*Pedosphaera*	*Sphingomonas*
Azospirillum	*Desulfovibrio*	*Leptolyngbya*	*Phaeospirillum*	*Sphingopyxis*
Bacillus	*Devosia*	*Limnohabitans*	*Phenylobacterium*	*Spirochaeta*
Balneimonas	*Dokdonella*	*Luteolibacter*	*Phormidium*	*Spirosoma*
Bdellovibrio	*Dolichospermum*	*Magnetospirillum*	*Pilimelia*	*Steroidobacter*
Belnapia	*Exiguobacterium*	*Mesorhizobium*	*Pirellula*	*Streptomyces*
Blastomonas	*Fimbriimonas*	*Methylibium*	*Planctomyces*	*Sulfuritalea*
Bosea	*Flavisolibacter*	*Methylobacterium*	*Plesiocystis*	*Tatlockia*
Bradyrhizobium	*Flavobacterium*	*Methylopila*	*Propionicimonas*	*Telmatospirillum*
Brevibacterium	*Fluviicola*	*Methylosinus*	*Prosthecobacter*	*Terriglobus*
Burkholderia	*Frankia*	*Microbacterium*	*Pseudomonas*	*Terrimonas*
Candidatus *Koribacter*	*Friedmanniella*	*Microcoleus*	*Pseudonocardia*	*Thermomonas*
Candidatus *Liberibacter*	*Gemmata*	*Microlunatus*	*Ralstonia*	*Uliginosibacterium*
Candidatus *Solibacter*	*Gemmatimonas*	*Mogibacterium*	*Ramlibacter*	*Virgisporangium*
Candidatus *Xiphinematobacter*	*Geobacter*	*Mycobacterium*	*Rhizobium*	*Zoogloea*
Caulobacter	*Hyphomicrobium*	*Mycoplana*	*Rhodoplanes*	

associated with disease-suppressive soils in *B. vulgaris* fields and other agricultural systems where their presence has been shown to suppress *R. solani* (Mendes et al., 2011). *Pseudomonas* species have also been successfully used as biocontrol agents in *Gossypium hirsutum* L., *Nicotiana tabacum* L., *Raphanus sativus* L., and *S. tuberosum* (reviewed by Weller, 2007) or as plant-growth promotors in *Oryza sativa* L. (Noori and Saud, 2012). A similar effect has been seen in *Triticum aestivum* L. (wheat), where Pseudomonads have been used to suppress take-all disease caused by the fungus *Gaeumannomyces graminis* var. *tritici* J. Walker (Weller et al., 1988; reviewed in Weller et al., 2002). The identification of organisms capable of behaving as antagonists in our study is promising, and investigations targeting these specific organisms should be pursued in future research.

Seasonal Patterns

Seasonality was found to influence both archaeal and bacterial communities in our study. For example, soil samples from 25 July and 27 Aug. 2014 sampling dates (the two hottest months, Table 1) exhibited the most similar OTU frequencies and abundance values (β diversity) to one another and were grouped in the same clade (Fig. 1). Immediately adjacent to this group, in the neighbor-joining tree, were all samples collected on 11 June 2014 (another very hot period in 2014, Table 1), except for one, which grouped in a clade with two samples from 3 June 2015 and one sample from 16 Apr. 2015. The remaining 3 June 2015 and 16 Apr. 2015 samples each formed their own clades. When β diversity was analyzed by sampling month, the null hypothesis that all microbial populations are the same was rejected ($p = 0.001$, test statistic = 0.600), despite exhibiting similar α diversity profiles (Shannon index).

Clustering based on sample date was less distinct when plotted using DCA, which showed that archaea and bacteria samples from 25 July 2014, 27 Aug. 2014, 16 Apr. 2015, and 3 June 2015 grouped closely to one another on both *x* and *y* axes (Fig. 2). Samples from 11 June 2014 were more removed from all other samples, grouping farther right on the *x* axis and less clustered to one another on the *y* axis.

The sample clustering patterns observed here are not surprising, as bacterial populations are known to vary in response to temperature in perennial ecosystems (Dunfield and Germida, 2003; Cho et al., 2008), and changing temperatures are known to impact metabolic activities in microorganisms (Guicharnaud et al., 2010). Interestingly, species identified from samples of archaea and bacteria collected during the hot summer months in the current study (25 July and 27 Aug. 2014; average air and soil temperatures = 23.5 and 22.0°C, respectively) typically grouped together, as did species from samples collected in the cooler months (3 June 2015 and 16 Apr. 2015; average air and soil temperatures = 13.5 and 13.3°C, respectively). However, seasonality, although influential, is not the only factor that affects microbial diversity and species composition in turfgrass systems. For example, despite sharing identical average soil temperatures, samples collected from 11 June 2014 and 3 June 2015 did not cluster together (Fig. 1). A culture-based analysis of bacterial populations in *A. stolonifera* putting greens in Alabama and North Carolina

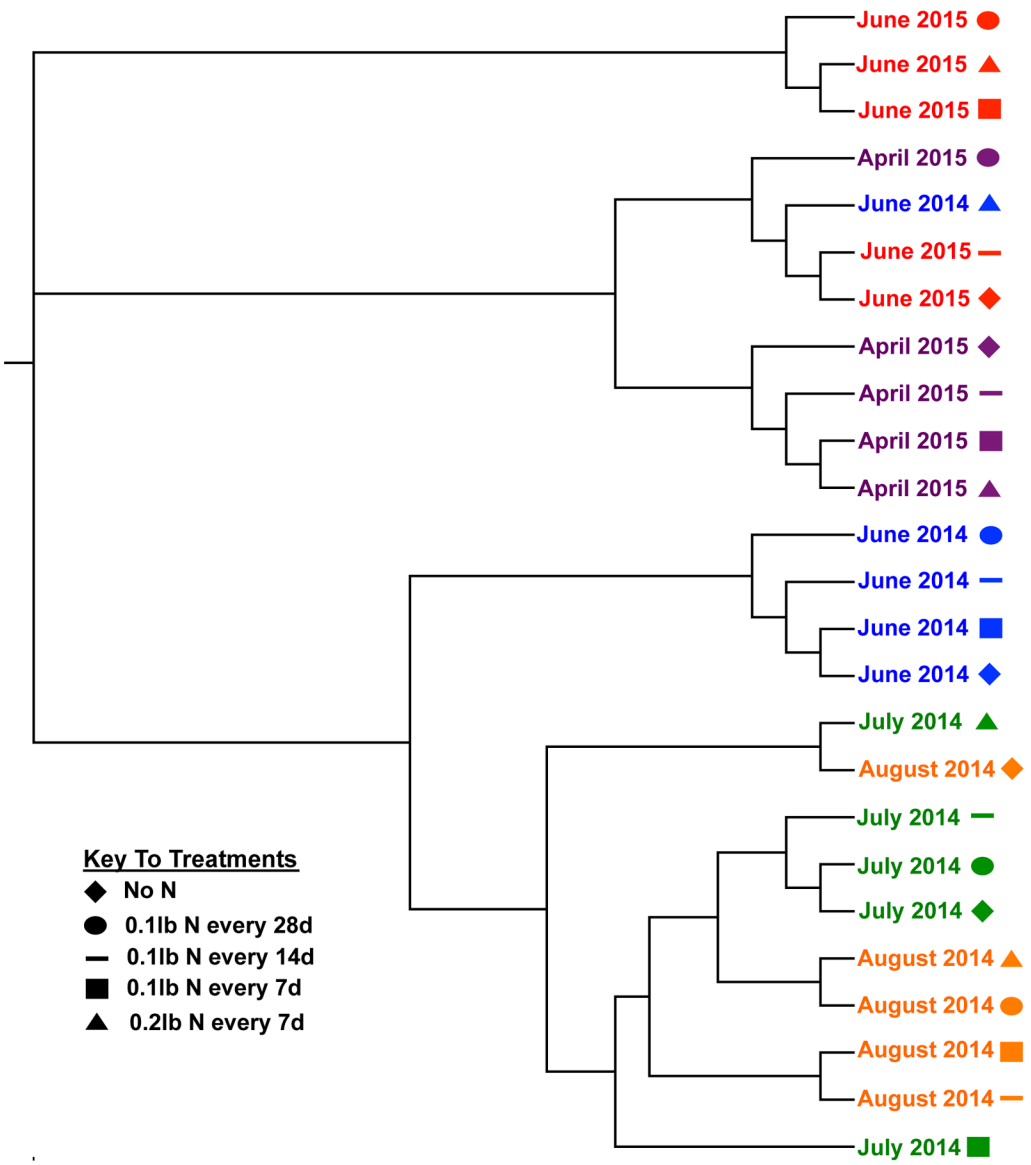

Fig. 1. Neighbor-joining tree generated from the Bray–Curtis dissimilarity matrix for archaea and bacteria. Samples are divided by sampling date.

and *C. dactylon* greens in Florida and South Carolina, sampled over 4 yr, revealed significant differences in bacterial counts due to sampling date, turfgrass host, and site location (Elliott et al., 2004). In their studies, however, sampling date represented less than 10% of the observed differences overall, with site location and host plant accounting for most of the variation (Elliott et al., 2004).

The influence of site location on microbial communities is interesting and can inherently be tied to a range of factors, including soil type, microclimate, and turf management practices. The four sites examined by Elliott et al. (2004) were all maintained differently (two for research studies and two as golf courses), a factor that could have contributed to the underlying differences in microbial communities between those sites. For 4 yr prior to the initiation of our research, our study site was maintained as a monostand of *P. annua* but was not used for research purposes and therefore received less mechanical and chemical inputs than that of a traditional *P. annua* putting green located on a golf course. At the initiation of our study in May 2014, the site started to be maintained under conditions common to those employed on golf course putting greens in the northeastern United States (e.g., daily mowing, biweekly topdressing, increased fertility, and pesticide applications). This factor, rather than seasonality, might offer an alternate explanation of why the 11 June 2014 and 3 June 2015 samples did not cluster together in the distance tree, despite identical soil temperatures, although the unusually cold winter of 2014 to 2015 may also have contributed to the disparity between these sampling dates. Similarly, the timing of the high-input putting green management protocol could have led to the closer proximity of archaeal and bacterial samples in DCA plots over time. It is possible that management practices employed to maintain a healthy *P. annua* putting green provide conditions that select for distinct microbial communities, and that these communities will continue to undergo change and potentially reach an equilibrium state as the duration of management practices increases. Such a

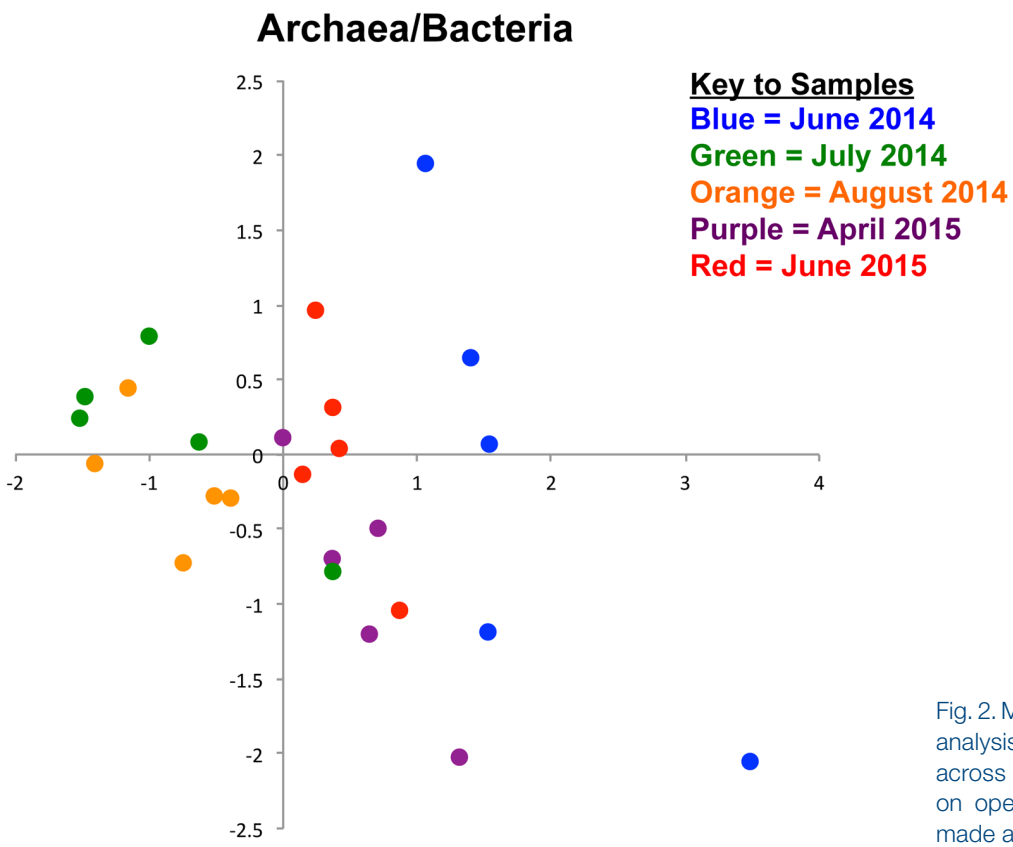

Fig. 2. Multivariate detrended correspondence analysis of archaea and bacteria communities across 25 sample sites. Analyses are based on operational taxonomic unit assignments made at the genus level.

scenario was documented in newly established *A. stolonifera* greens in North Carolina, where several microbial populations became stable with age over the course of a 23-mo experiment, including fluorescent pseudomonads, actinomycetes, and Gram-negative bacteria (Bigelow et al., 2002). Conventional agricultural tillage sites have been shown to possess different microbial communities compared with the same sites maintained under organic practices (Crecchio et al., 2004); thus, the influence of management practices in golf course putting greens may be equally important. Regardless of whether the sampling clustering patterns observed in this study can be attributed to seasonality or the increased influence of management practices accumulating over time, our study demonstrates that multiple samples must be taken over time to adequately reflect the resident microbial community at a given site.

CONCLUSION

The primary objective of this study was to identify the resident archaeal and bacterial community in the soil of *P. annua* putting green turf throughout the growing season. This study represents the first next-generation sequence based analysis of microbial communities in *P. annua* putting green turf, highlighting a wide array of microorganisms spanning two kingdoms of life, many of which have been reported in other agricultural and natural ecosystems. Of course, caution should be exercised when making comparisons across ecosystems, as different methodologies were used to identify the microbial communities in these studies. Nevertheless, it is important to note that the soil of highly maintained *P. annua* putting green turf supports thousands of microorganisms, indicating that this is not as inhospitable an environment as generally assumed (Elliott et al., 2008). It is not known whether archaea and bacteria shared between natural systems and highly managed anthropogenic landscapes such as *P. annua* putting greens are functioning in the same manner; however, using the results from this study, researchers can now target specific microbial taxa and begin investigating microbial function, as well as the role that these communities play in biogeochemical cycling, feedback mechanisms, and overall turfgrass sustainability and health.

Conflict of Interest

The authors declare there to be no conflict of interest.

Supplemental Material Available

Supplemental material for this article is available online.

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