RESEARCH ARTICLE

One-time nitrogen fertilization shifts switchgrass soil microbiomes within a context of larger spatial and temporal variation

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Abstract

Soil microbiome responses to short-term nitrogen (N) inputs remain uncertain when compared with previous research that has focused on long-term fertilization responses. Here, we examined soil bacterial/archaeal and fungal communities pre- and post-N fertilization in an 8 year-old switchgrass field, in which twenty-four plots received N fertilization at three levels (0, 100, and 200 kg N ha^{-1} as NH_4NO_3) for the first time since planting. Soils were collected at two depths, 0–5 and 5–15 cm, for DNA extraction and amplicon sequencing of 16S rRNA genes and ITS regions for assessment of microbial community composition. Baseline assessments prior to fertilization revealed no significant pre-existing divergence in either bacterial/archaeal or fungal communities across plots. The one-time N fertilizations increased switchgrass yields and tissue N content, and the added N was nearly completely removed from the soil of fertilized plots by the end of the growing season. Both bacterial/archaeal and fungal communities showed large spatial (by depth) and temporal variation (by season) within each plot, accounting for 17 and 12–22% of the variation as calculated from the Sq. root of PERMANOVA tests for bacterial/archaeal and fungal community composition, respectively. While N fertilization effects accounted for only ~4% of overall variation, some specific microbial groups, including the bacterial genus *Pseudonocardia* and the fungal genus *Archaeorhizomyces*, were notably repressed by fertilization at 200 kg N ha^{-1}. Bacterial groups varied with both depth in the soil profile and time of sampling, while temporal variability shaped the fungal community more significantly than vertical heterogeneity in the soil. These results suggest that short-term effects of N fertilization are significant but subtle, and other sources of variation will need to be carefully accounted for study designs including multiple intra-annual sampling dates, rather than one-time "snapshot" analyses that are common in the literature. Continued analyses of these trends over time with

fertilization and management are needed to understand how these effects may persist or change over time.

Introduction

Cultivation of dedicated bioenergy crops is of interest to sustain long-term energy supplies [1]. The International Energy Agency predicts that biofuels could satisfy more than a quarter of world needs for transportation energy by 2050 [2]. Switchgrass (*Panicum virgatum* L.) has been a prominent candidate as an energy crop due to its high biomass yield, low maintenance and limited-input requirements [3, 4], and high adaptability to marginal sites [5]. Such characteristics may allow switchgrass crops to be used to reclaim degraded or abandoned agricultural lands while reserving fertile lands for food production [6]. With its well-developed and deep rooting systems, switchgrass may also improve belowground carbon storage and nutrient acquisition [7], and potentially modulate the diversity of below-ground and plant-associated microbiomes.

Soil microbial communities play fundamental roles in terrestrial ecosystems, such as regulating the decomposition of organic matter as well as driving nutrient cycles and energy flow [8, 9]. To this end, these soil microbiomes have considerable effects on soil quality and agricultural sustainability [10]. However, soil management with fertilizer additions may shift soil microbial abundances, composition, and functions by affecting soil physical and chemical characteristics [11] as well as perturbing plant-microbe interactions and symbioses [12]. For example, laboratory studies showed that N addition depresses soil microbial activity, microbial biomass, and enzyme activities by shifting the metabolic capabilities of soil bacterial communities toward the decomposition of more labile soil carbon pools [13]. In addition, nutrient inputs have been shown to shift the composition of soil microbial communities in consistent ways in grasslands across the globe with reduced average genome sizes of microbial community members following nutrient amendment, leading to decreased relative abundances of some important microbial functional groups, such as methanogenic archaea, oligotrophic bacteria and mycorrhizal fungi [14]. Several reasons may account for such microbial responses to fertilization. Long-term fertilization may cause soil acidification, and thus alter soil microbial diversity and composition [11, 15]. Additionally, nutrient amendments may have direct effects on organic matter decomposition, leading to changes in the quantity and quality of resources available for microbes, and therefore reshape microbial community structure based on their substrate utilization and allocation patterns [16]. However, our understanding of the mechanisms of N fertilization effects on microbial communities are mostly based on long-term fertilization experiments, in which potentially interacting edaphic soil properties are often significantly altered by soil management over time. Although transient nutrient enrichment effects upon terrestrial microbial C and N processes have been reported [17–19], our understanding of the immediate response of the below-ground microbial community to N inputs is still limited, and sometimes inconsistent with results of long-term experimental data [20–22]. Additional research is thus necessary to evaluate microbial community dynamics and their interactions with nutrient cycling under the no-, low- or periodic fertilization regimes that would be optimal for sustainable perennial bioenergy crop production scenarios [23, 24].

Besides soil nutrient availability, soil microbial distributions are influenced by a wide range of soil characteristics, such as soil pH, substrate quantity and quality, moisture and oxygen levels, nearly all of which vary with soil depth [25] and over seasons [26]. Soil depth and sampling

time in a growing season thus influence patterns of spatial and temporal community variation [27, 28]. Compared to top soils, subsurface soils have higher mineral content, less aeration, and lower organic carbon availability. Thus, microbial biomass and diversity typically decrease rapidly with depth in the soil profile [29]. Often, most variability in microbial community composition occurs in surface soils, while deeper soils have more similar communities regardless of soil management [25]. Seasonal variability also has a large influences on microbial communities [30]. For example, seasonal changes in temperature and soil moisture can directly shape microbial communities [31, 32]. Moreover, seasonal changes in plant growth and allocation can indirectly affect microbial communities through changes in soil C inputs [33, 34]. Lauber et al. [26] investigated the temporal variability of bacterial communities in different ecosystems, showing that most of the temporal variation in bacterial composition within an agricultural field could be explained by soil moisture and temperature variation. Given these previous studies, it is possible that the shifting spatial and temporal patterns of soil microbial communities may overwhelm short-term soil nitrogen management effects and needs to be accounted for in such assessments.

Here, we used high-throughput barcoded amplicon sequencing of ribosomal RNA marker genes to assess short-term effects of one-time N fertilization on the spatio-temporal variation of soil microbial communities in an 8 year-old switchgrass field, over two soil depths and across four sampling seasons. We hypothesized that (1) one-time nutrient inputs could significantly change above-ground plant yields and substrate quality, and thus re-shape soil bacterial and fungal communities, but that short term N effects would be modest compared to existing spatio-temporal variation, (2) bacterial and fungal composition would differ spatially and temporally, but the response of these communities to the N-fertilization would be taxon specific.

Materials and methods

Site characterization, experimental design and plant and soil sampling

The experiment was established in an eight year-old switchgrass field near the Heritage Center, located on US DOE land, in Oak Ridge, Tennessee, USA (35.9255 N, 84.3947 W) adjacent to the ORNL Meteorology Network tower "L" with a 25+ year record of data available online (http://metweb.ornl.gov—Fig 1). The 10-year mean annual temperature and annual precipitation at the site were 14.1°C and 1436 mm, respectively. The field was in pasture and hay rotations when taken over by US Army in the 1940s during the Manhattan Project. However, due to proximity of floodplain of the Clinch River and Poplar Creek, the land was never developed and instead maintained as wildlife habitat and riparian buffer in a field of mixed grasses and forbs, using a combination of mowing and prescribed burning for management. In 2009, under contract for UT Institute of Agriculture and Genera Energy, the site was cleared, seeded with an 'Alamo' switchgrass variety, and subsequently managed for switchgrass production. After the initial contract expired in 2012 when Genera Energy changed the focus of their local efforts, the site has remained in switchgrass, but has again been managed as buffer and wildlife habitat and maintained only with periodic prescribed fire and mowing. In mid-December 2016, the switchgrass field was mowed to a 10-cm stubble height and twenty four plots (5 m × 5 m) were set up including three N fertilization levels (0, 100, and 200 kg N ha^{-1}) with eight replicates based on a complete randomized design (Fig 1). A 2.5-m inter-plot "alley way" is periodically mowed to allow access and separate the plots between treatments and replicates (Fig 1). Just before spring emergence of the switchgrass (March 30, 2017) commercial ammonium nitrate (34% nitrogen) was hand-applied to fertilizer-treatment plots with N fertilization levels of 100, and 200 kg N ha^{-1}. Post-emergence (June 20, 2017), all plots were treated with Garlon 3A herbicide as prescribed by the manufacturer to help control broadleaf weeds. After
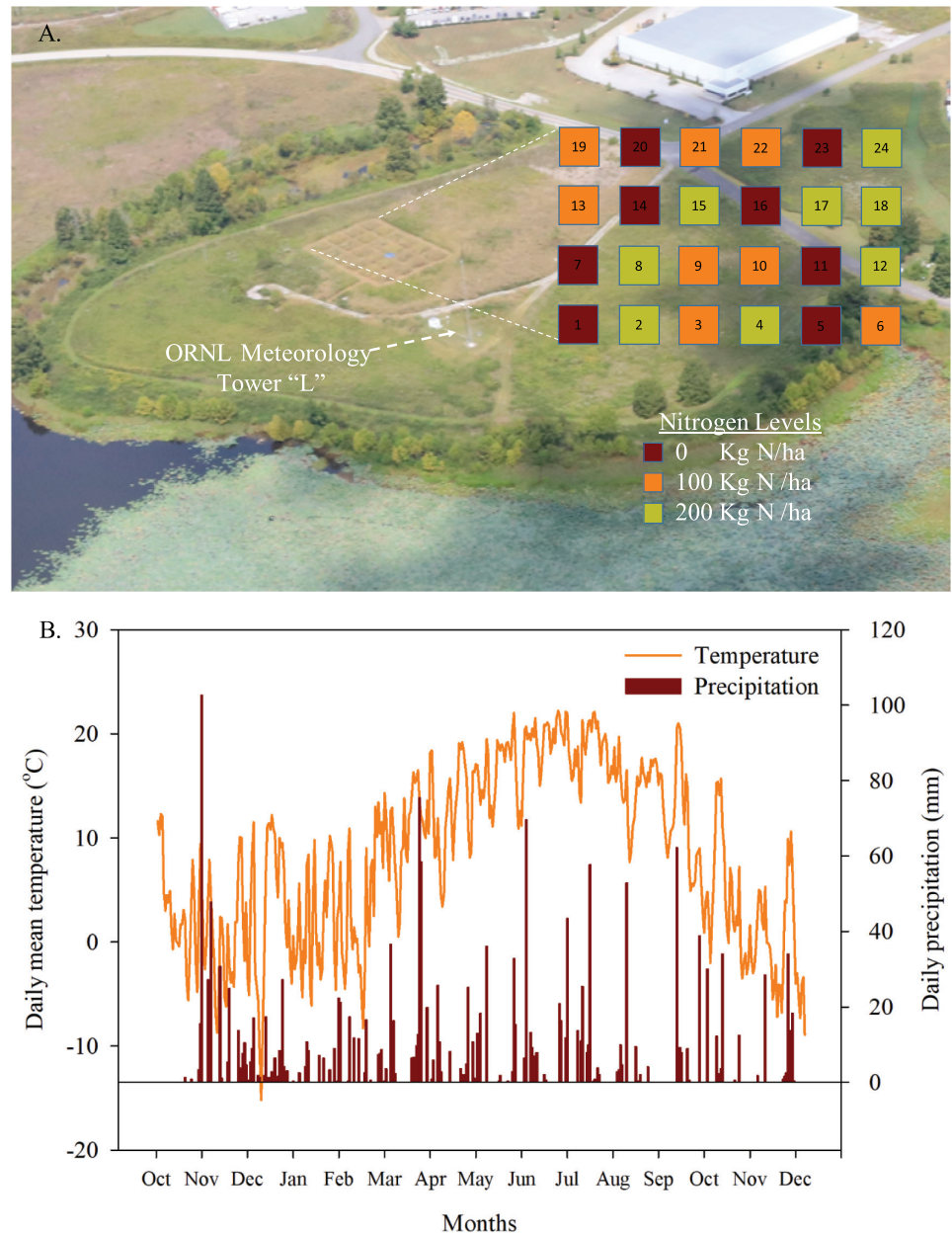
Fig 1. A. An ariel photograph from August 2018 showing the layout of twenty four plots (5 m × 5 m) with three N fertilization levels (0, 100, and 200 kg N ha^{-1}) and eight replicates based on a complete randomized design. B. Daily mean temperature and precipitation (November 2016 to December 2017) from the adjacent meteorological tower in Oak Ridge, Tennessee, USA.

fall senescence (November 13, 2017) above-ground biomass of switchgrass was measured using a sickledrat to harvest all aboveground biomass from a 0.1 m^2 area at four randomly chosen locations in each plot [33]. The four samples of aboveground biomass were pooled by plot in paper bags, oven dried at 70˚C, and weighed to determine dry mass per unit area. A subsample of the plant material was then ground into powder using a laboratory mill before total C and N were determined by dry combustion method using a Perkin-Elmer 2400 CHN analyzer (Perkin-Elmer Corporation, Norwalk, CT, USA).

For soil DNA and chemical characterization, four sets of soil samples were collected across seasons including Winter 2016 (December 16, 2016 –Pre-fertilization), Spring 2017 (April 5, 2017 –Post-fertilization/emergence), Summer 2017 (July 5, 2017 –Mid-growing season), and Late Fall 2017 (November 15, 2017—Senescence). At each sampling event, soil cores (2.5 cm diameter ×15 cm height) were collected randomly from each plot and separated into two depth increments of 0–5 and 5–15 cm as preliminary tests had showed that soil of 0–5 cm had greater amount of soil DNA than 5–15 cm, and that soil sampling of depths below 15 cm were inconsistent across plots due to a rocky layer underlying the plots in the southwest corner of the experiment. Soils collected in Winter 2016 (before N fertilization) were used to optimize soil methods for microbiomes and metagenomes assessments, and assess whether there were any systematic pre-existing differences across plots in the experiment. Soils collected following N fertilization, *i.e.*, Spring 2017, Summer 2017, and Late fall 2017, were used to compare microbial communities among N treatments. Soil collected for all four seasonal sampling dates were used to assess how soil depth and sampling season affected microbial communities across all three nitrogen treatments. All soil samples for microbial analyses were transported on dry ice to the lab and stored at -80˚ C prior to soil DNA extraction. Total C and N were determined on samples collected in the Summer of 2017 using the dry combustion method and soil inorganic N (NH_4^+-N and NO_3^--N) was analyzed using a FIA QuikChem 8000 autoanalyzer (Lachat Instruments, Loveland, CO, USA). Generally, 0–5 cm soils had an average soil pH of 5.8, 4.5% total soil C, 0.2% total soil N, 10.2 mg kg^{-1} NH_4^+, and 1.3 mg kg^{-1} NO_3^-, while 5–15 cm soil had a significantly lower average soil pH of 5.2, 1.8% total soil C, 0.03% total soil N, 1.8 mg kg^{-1} NH_4^+, and 0.3 mg kg^{-1} NO_3^-.

DNA extraction, rRNA gene amplicon sequencing, and metagenomic sequencing

Approximately 10 g of soil from each sample was homogenized in a mortar and pestle with liquid N_2, and soil DNA was extracted from a 0.25 g aliquot of the soil sample using the MoBio DNeasy PowerSoil Kit (Qiagen, Carlsbad, CA, USA) according to the manufacturer's instructions. DNA concentrations were determined and purity was confirmed by the ratio of absorbance at 260 and 280 nm (1.70–1.90) using a NanoDrop 1000 spectrophotometer (NanoDrop Products, Wilmington, DE, USA).

A two-step PCR approach was used to barcode tag templates with frameshifting nucleotide primers for amplicon sequencing [35] with some modifications previously described [36]. To increase phylogenetic coverage for community analysis of bacteria, archaea, and fungi, a group of nine forward and six reverse primers for bacteria and archaea, and another group of eleven forward and seven reverse primers for fungi, mixed at equal concentration of 0.5 μM were used to target 16S rRNA V4 region and fungal ITS2 rRNA, respectively [36]. Primary PCR was conducted for 5 cycles of 1 min at 95 ˚C, 2 min at 50 ˚C, and 1 min at 72 ˚C, followed by a final elongation of 5 min at 72 ˚C. This PCR product was then cleaned up using Agencourt AMPure beads (Agencourt Bioscience, Beverly, MA, USA) and eluted in 21 μL of nuclease-free water. To tag amplicons with barcoded reverse primers and forward primers, 20 μL of purified DNA fragments from the primary PCRs were added to 50 μL secondary PCR assays, which were initiated at 95 ˚C for 45 sec, followed by 32 cycles of 15 sec at 95 ˚C, 30 sec at 60 ˚C, and 30 sec at 72 ˚C, followed by a final elongation of 30 sec at 72 ˚C. The use of separate tagging reactions can help reduce heterodimers because PCR clean-up more efficiently removes shorter primers [35]. Up to 96 secondary PCR products were then pooled based on agarose gel band intensity, followed by a second clean-up with Agencourt AMPure beads (Agencourt Bioscience, Beverly, MA, USA) using 0.7–1 of bead-to-DNA ratios. The mixtures

of the purified 16S rRNA gene or ITS amplicon fragments were then paired-end sequenced on Illumina MiSeq platform (250×2 paired end, v2 chemistry) (Illumina, San Diego, CA, USA) using a 9 pM amplicon concentration. For all amplicon sequencing data, any sample with fewer sequence reads than 10000 for 16S and 5000 for ITS, respectively, was removed and re-sequenced to ensure the minimum sequencing depths.

To examine microbial community potential and function prior at the site and make sure that there were no preexisting differences in either microbial community or functional profiles across N plots prior to N fertilization, DNA extracted from soil samples collected in Winter 2016 were pooled to form four composite samples, and metagenomic sequence data were generated and compared to the results of functional predictions by PICRUSt (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States) [37]. Specifically, for each of two soil depths, DNA from No. 1–12 and 13–24 plots was pooled together, respectively (two depths × two replicates). Shotgun metagenomes were prepared using Nextera XT sequencing libraries (Illumina, San Diego, CA) according to the manufacture's recommendations using 500ng of DNA (15031942 v03). Final libraries were validated on an Agilent Bioanalyzer (Agilent, Santa Clara, CA) using a DNA7500 chip and concentration was determined on an Invitrogen Qubit (Waltham, MA) with the broad range double stranded DNA assay. Barcoded libraries were pooled and prepared for sequencing following the manufactures recommended protocol (15039740v09, Standard Normalization). One paired end sequencing run (2 x 300) was completed on an Illumina MiSeq instrument (Illumina, San Diego, CA) using v3 chemistry. The sequencing depths obtained were similar among four samples (10,069,906–11,652,583 reads per sample).

Bioinformatic and statistical analyses

Forward and reverse primers were trimmed with Cutadapt [38]. Paired-end sequencing data were then joined and demultiplexed using QIIME [39] with quality filter at Phred > 19. Chimeras of trimmed and filtered sequences were identified and removed using a usearch method in QIIME. Operational taxonomic units (OTUs) with 97% identity were picked with the open reference algorithm and usearch61 otu-picking method. Taxonomy was assigned using the RDP (Ribosomal Database Project) taxonomy-assignment method [40] against the most recent version of Greengenes database (13.8) for 16S rRNA sequencing data and UNITE database (12.11) for ITS sequencing data. All global singletons were removed from the dataset. The 16S and ITS OTUs were further analyzed for alpha and beta diversity using QIIME. For alpha diversity analysis, all sequences were rarefied to the depths of 10000 for 16S and 5000 for ITS, respectively. Bray-Curtis distance metrics were applied for analyzing beta diversity. The beta diversity of both amplicon and metagenomic sequencing was based on relative abundance, which can only be related to the community structure and potential functions, but cannot be directly interpreted to ecosystem functions. To test whether bacterial community functional traits could be accurately predicted from 16S rRNA genes, we used PICRUSt [37] for comparison with 4 metagenome libraries described below. The MiSeq sequences were deposited on NCBI Sequence Read Archive (SRA) database under the BioProject accession number of PRJNA512218.

Soil metagenome sequences were uploaded to Rapid Annotation using Subsystems Technology for Metagenomes (MG-RAST; http://metagenomics.anl.gov) [41] under project accession number mgp22000, and annotated using the RefSeq database for taxonomic assignment and the SEED Subsystems database for functional classification (maximum e-value cutoff was $1e^{-5}$, minimum identity cutoff was 60%, and minimum alignment length was 50).

One-way analysis of variance (ANOVA) of a completely randomized design (SAS 9.3, SAS Institute Inc. Cary, NC, USA) was used to assess significant differences in above-ground yields and plant C/N contents among N fertilization levels. A three-factor ANOVA of a completely randomized design was used to analyze microbial alpha diversity and the abundances of microbial taxonomic groups among the three N fertilization levels, two soil depths, and four seasonal sampling dates. Microbial beta diversity was compared using a three-factor PEMA-NOVA method (N fertilization levels, soil depths, and sampling season) with 9999 permutations conducted in PRIMER (Plymouth Routines in Multivariate Ecological Research Statistical Software, v7.0.13, PRIMER-E Ltd, UK). A RELATE analysis was also performed to evaluate the relatedness between bacterial and fungal beta diversity by calculating a Spearman's Rho correlation coefficient in PRIMER. The DistLM (distance-based linear model) function in PRIMER was used to evaluate the associations of above-ground yields and plant C/N contents with bacterial and fungal beta diversity [42]. Heat maps were constructed using HeatMapper [43] to represent all taxonomic groups at genus level that differed significantly ($P < 0.05$) among three N fertilization levels, two soil depths, and four sampling dates. Venn's diagrams were also constructed to visualize how many significantly affected bacterial/archaeal and fungal genera were shared between the factors of soil depth and sampling date using Venny 2.1.0 [44]. Additionally, Pearson's correlation coefficients were examined to further evaluate relationships between the relative abundances of taxa and N fertilization rates.

Results

Spatial variation in microbial community structure and function pre-nitrogen addition

Both 16S rRNA gene and ITS region amplicon sequencing revealed no significant pre-existing differences in alpha or beta diversity in either the bacterial/archaeal or fungal communities across the 24 plots in this switchgrass field before N fertilization, however diverse bacterial/archaeal and fungal taxa were observed (Fig 2). Bacterial communities varied significantly by depth ($P < 0.05$) with the 0–5 cm soil layer having greater Planctomycetes (8%), Bacteroidetes (7%), and Verrucomicrobia (5%), but less abundance of Proteobacteria (34%), Chloroflexi (5%), and Gemmatimonadetes (2%) than the deeper layers (Fig 2). Surprisingly, fungal phyla level analyses did not show any differences between the soil depths examined in these switchgrass soils.

Shotgun metagenomes also showed high taxonomic and functional diversity in the switchgrass field (S1 Fig). However, when phylogenetic assignments of the metagenome reads were compared to the relative abundance in 16S rRNA gene amplicon analyses, soil metagenomes indicated significant differences in the datasets across several of the dominant phyla. For example, there was a 45% increase in Proteobacteria, a 15-fold increase in Firmicutes, and a 2-fold increase in Cyanobacteria in the shotgun metagenomes when compared to 16S rRNA gene amplicon analyses. Other phyla, such as Acidobacteria, Planctomycetes, and Chloroflexi, were reduced by as much as 52–63% in soil metagenomes when compared to 16S rRNA gene amplicon analyses from the same samples and dates. Soil metagenome predicted functional gene profiles were compared to those predicted from PICRUST-based analysis of 16S rRNA gene amplicon data and also indicated significantly different profiles (S2 and S3 Figs). As metagenome-based analyses of all replicate samples on all dates was impractical, and PICRUST-based analyses of seasonal functional gene patterns and responses to fertilization were not consistent with metagenome analyses, these approaches were not pursued further for this study.
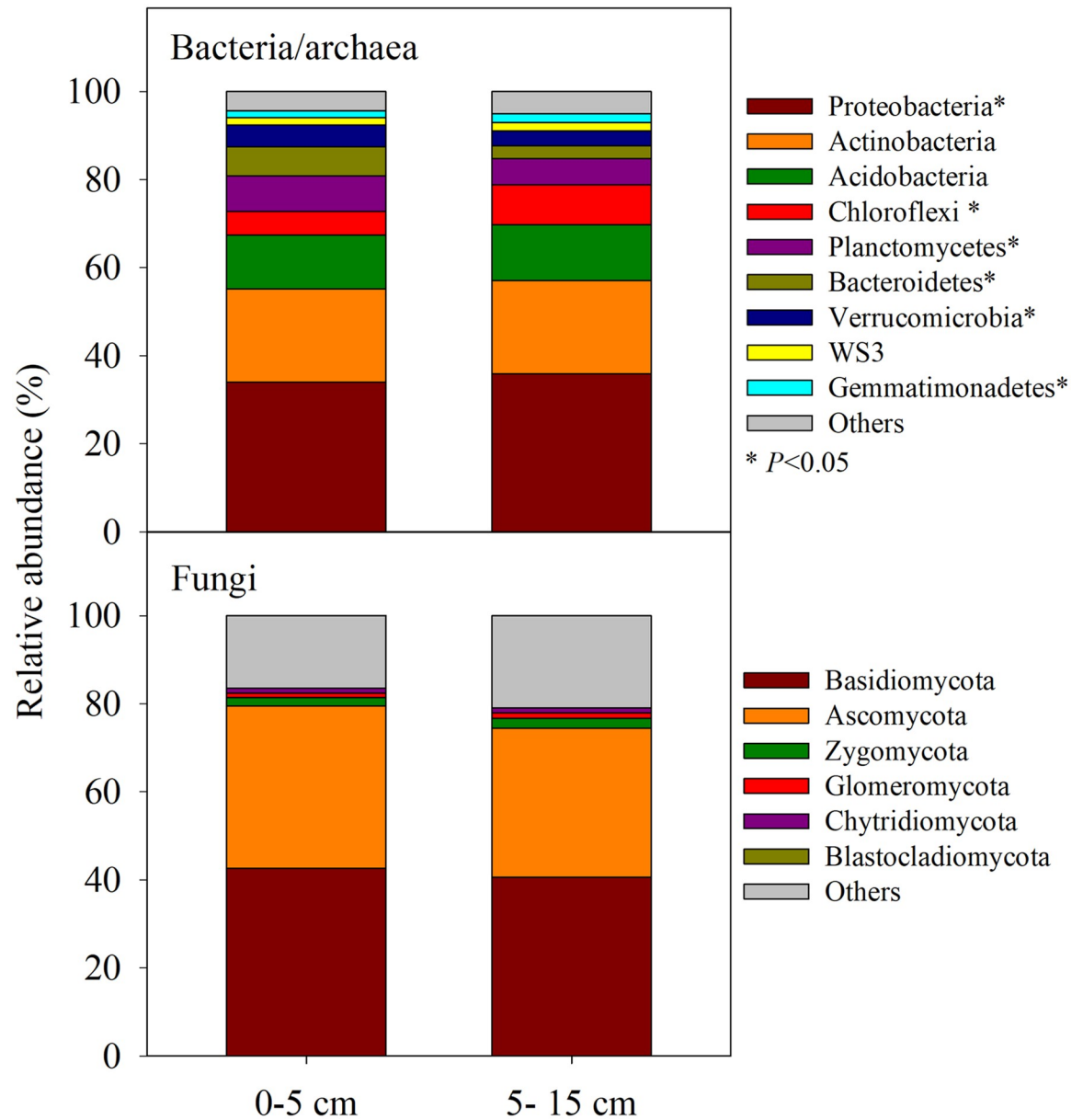
Fig 2. Relative abundances of bacterial/archaeal and fungal dominant phyla (average abundances > 1%) affected by two soil depths (0–5 and 5–15 cm). Asterisks indicate significant difference at α = 0.05 between two soil depths.

https://doi.org/10.1371/journal.pone.0211310.g002

Microbial alpha and beta diversity post-nitrogen addition

Although neither bacterial/archaeal nor the fungal alpha diversity were significantly affected by N fertilization levels, both the community richness (Chao1 index) and diversity (Shannon index) showed significant spatio-temporal changes ($P < 0.05$) (Fig 3). Between the two soil depths, the 0–5 cm layer had significantly higher Chao1 richness and Shannon evenness indices in both the bacterial/archaeal and fungal communities compared to the 5–15 cm layer ($P < 0.05$). In analyses of seasonal variation, Chao1 diversity showed a similar pattern. Spring 2017 had lower richness in the bacterial/archaeal community, while Winter 2016 and Fall 2017 had significantly greater richness in fungal communities ($P < 0.05$). Shannon diversity indices
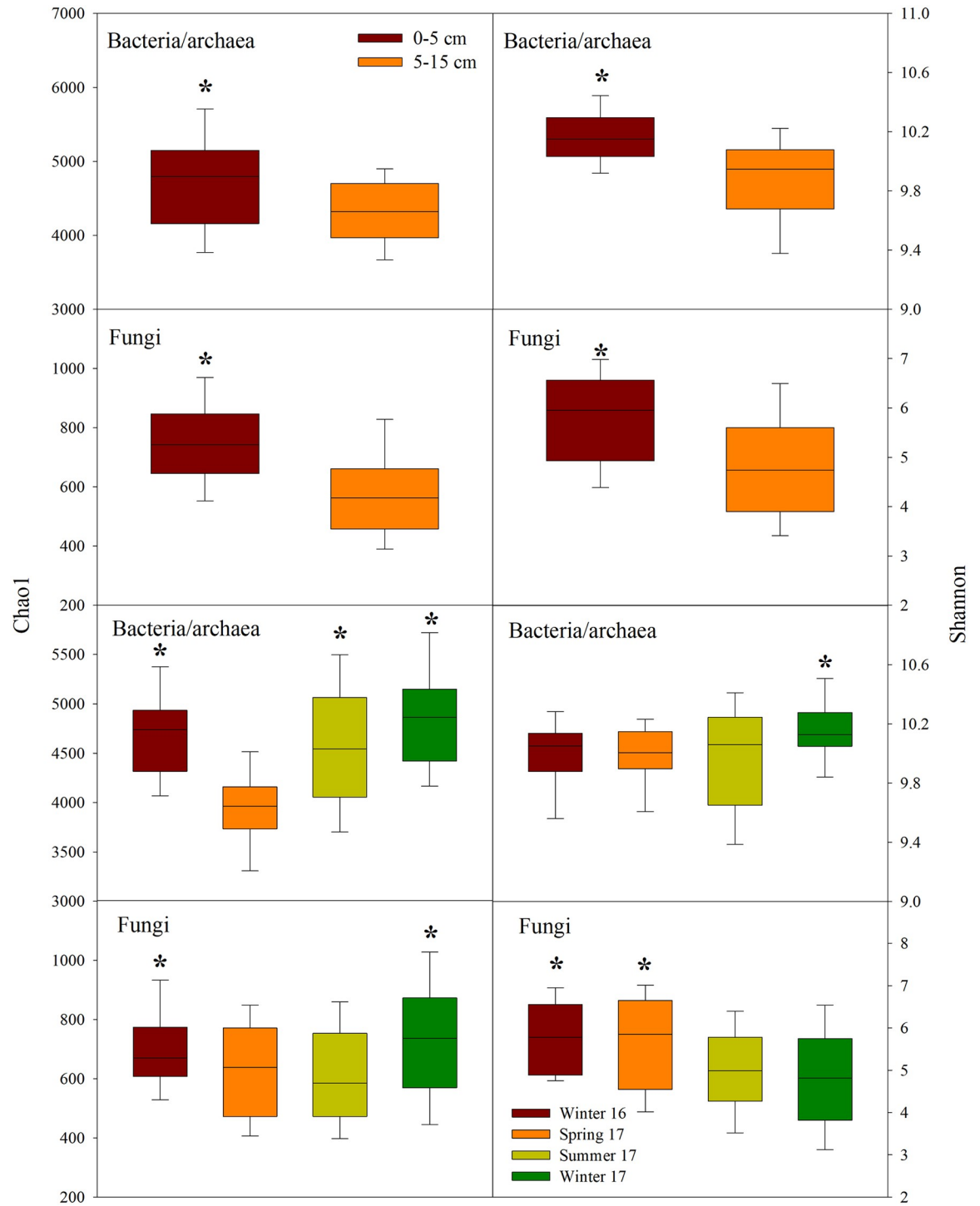
Fig 3. Box plots showing Chao1 richness and Shannon diversity of bacterial/archaeal and fungal communities affected by two soil depths (0–5 and 5–15 cm) and four sampling seasons (Winter 2016, Spring, Summer, and Fall 2017). Sequence depths were 10000 for 16S and 5000 for ITS. Asterisks indicate significant difference at α = 0.05 between two soil depths or among four sampling seasons.

https://doi.org/10.1371/journal.pone.0211310.g003

Table 1. Three-factor PERMANOVA results for differences in bacterial/archaeal and fungal community structure affected by three N fertilization levels (0, 100, and 200 kg N ha^{-1}), two soil depths (0–5 and 5–15 cm), and three sampling seasons following N input (Spring, Summer, and Fall 2017).

Source	df	SS	MS	PS-F	P(perm)	UP	Estimate	Sq.root
Bacteria/Archaea								
N	2	4010	2005	1.44	0.0384	9873	12.9	3.6
Depth	1	21742	21742	15.68	0.0001	9886	282.7	16.8
Season	2	31772	15886	11.46	0.0001	9884	302.1	17.3
N×Depth	2	1825	913	0.66	0.9879	9869	-19.7	-4.4
N×Season	4	5071	1268	0.91	0.6992	9809	-7.4	-2.7
Depth×Season	2	9614	4807	3.47	0.0001	9793	142.5	11.9
N×Depth×Season	4	3729	932	0.67	0.9995	9805	-56.8	-7.5
Res	126	1.8E+05	1386					
Total	143	2.5E+05						
Fungi								
N	2	8995	4497	1.29	0.0319	9808	21.5	4.6
Depth	1	14453	14453	4.17	0.0001	9859	152.6	12.4
Season	2	54894	27447	7.92	0.0001	9808	499.6	22.4
N×Depth	2	3897	1949	0.56	1.0000	9803	-63.2	-8.0
N×Season	4	14744	3686	1.06	0.3351	9720	13.8	3.7
Depth×Season	2	19295	9647	2.78	0.0001	9793	257.6	16.1
N×Depth×Season	4	9534	2383	0.69	1.0000	9704	-135.2	-11.6
Res	126	4.4E+05	3465					
Total	143	5.6E+05						

df, degrees of freedom; SS, sum of squares; MS, mean squares; PS-F, pseudo-F value; P(perm), permutation P-value based on 9999 permutations; UP, unique values of test statistic obtained under permutation; Estimate, estimated component of variation; Sq.root, square root of the estimated component of variation.

https://doi.org/10.1371/journal.pone.0211310.t001

showed significant divergence across seasons ($P < 0.05$), and the bacterial/archaeal community was more evenly distributed in Fall 2017, whereas the fungal community was more uneven in Summer and Fall 2017 than the other two sampling seasons ($P < 0.05$).

PERMANOVA tests showed that short-term application of N fertilizers could explain a small but significant amount of the variation in bacterial/archaeal and fungal community composition ($P < 0.05$) (Table 1 and Fig 4). Together, N fertilization effects could explain 3.4% of variation in bacterial/archaeal and 4.4% of fungal community variation calculated from the Sq. root of the PERMANOVA analyses (Table 1). However, the spatio-temporal variation (depth and season) were more significant than N effects for bacterial, archaeal and fungal communities ($P < 0.0001$) (Table 1 and Fig 4). Soil depth and sampling season explained approximately 16.8 and 17.3% of bacterial/archaeal community variation, respectively, and 12.4 and 22.4% of fungal community change, respectively (Table 1), thus indicating relatively small short-term effects of N fertilization on microbial communities when compared to the spatio-temporal variation. In addition, RELATE analyses further confirmed that bacterial/archaeal community structures were significantly related to the fungal community (Rho = 0.218, $P < 0.01$), suggesting that the patterns of spatio-temporal variation were generally similar in both bacterial/archaeal and fungal community distributions among tested plots and seasons.

Microbial taxonomic composition post-nitrogen addition

Because N addition levels had no interaction with soil depth and sampling season (Table 1), N effects on microbial phylogenetic composition were assessed across both sampling depths and seasons (Fig 5). Generally, N fertilization caused significant differences in the recovered genus
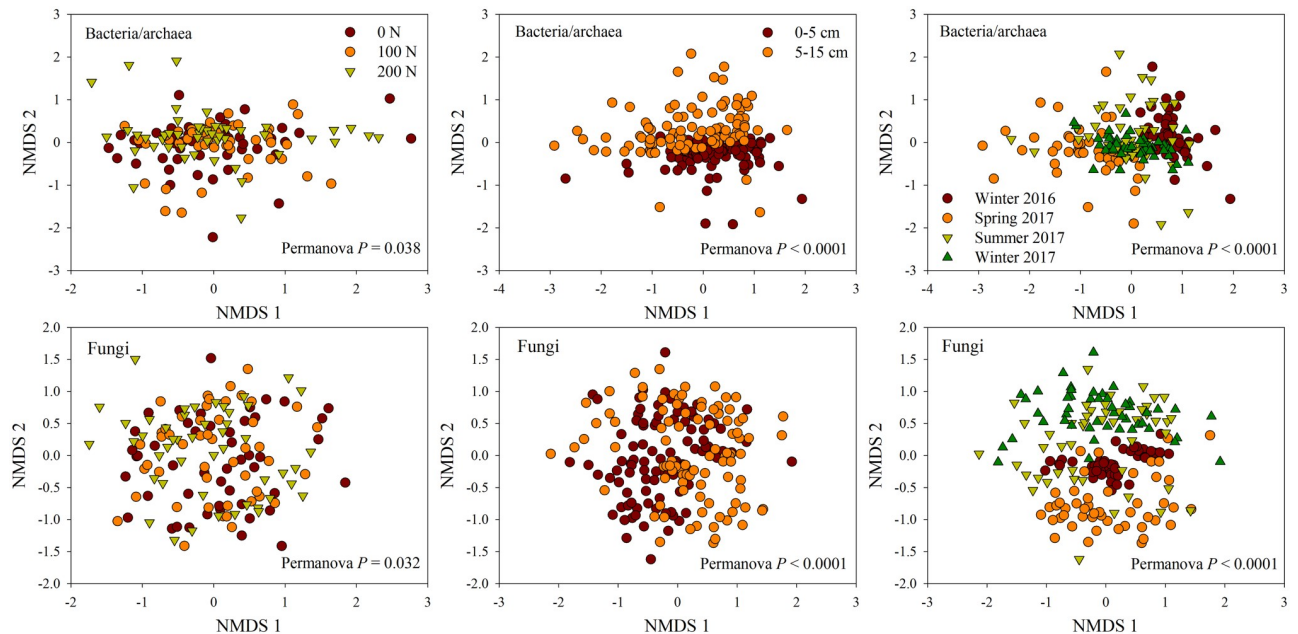
Fig 4. Non-metric multidimensional scaling (NMDS) analysis of bacterial/archaeal and fungal communities affected by three N fertilization levels (0, 100, and 200 kg N ha^{-1}), two soil depth (0–5 and 5–15 cm), and four sampling seasons (Winter 2016, Spring, Summer, and Fall 2017). PERMANOVA P values were also given.

https://doi.org/10.1371/journal.pone.0211310.g004

level composition for prominent members of the bacterial/archaeal (6%) and the fungal (5%) communities, respectively (relative abundance > 0.01%) (Fig 5). Specifically, for bacterial/archaeal community composition, N inputs at 200 kg N ha^{-1} were associated with significantly reduced relative abundances of *Salinibacterium* and *Pseudonocardia* (Actinobacteria), *Caldilinear* (Chloroflexi), and *Desulfobulbus* (Proteobacteria), but increased *Sorangium* (Proteobacteria) ($P < 0.05$) abundance, indicating that these taxonomic groups were significantly altered by the addition of synthetic N fertilizers. In the fungal community profiles, the application of N fertilizers at either 100 or 200 kg N ha^{-1} significantly decreased the proportion of *Archaeorhizomyces* (Ascomycota), as well as *Crepidotus* and *Uthatobasidium* (Basidiomycota) ($P < 0.05$).

Both soil depth and sampling season resulted in more significant alteration to bacterial/archaeal community composition than N applications (Fig 6). For example, 81% bacterial taxonomic groups at the genus level (with relative abundance > 0.1%) differed significantly between 0–5 and 5–15 cm of soil layers ($P < 0.05$), and significant variation occurred even at the phylum level. Generally, the 0–5 cm soil layer had a greater abundance of the phyla of Bacteroidetes, Planctomycetes, and Verrucomicrobia, whereas the phyla Chloroflexi, Nitrospirae, and Proteobacteria dominated the 5–15 cm soil layer ($P < 0.05$) (Fig 6). Sampling season also caused significant changes in bacterial community composition with ~80% of bacterial genera significantly altered ($P < 0.05$) (Fig 6), mostly in the prominent phyla of Acidobacteria, Actinobacteria, Bacteroidetes, and Verrucomicrobia, suggesting that these taxonomic groups were most responsive to temporal changes.

In the fungal community, only 54% prominent genera (of relative abundance >0.1%) showed significant changes between two soil depths, in which members of the phyla of Ascomycota, Chytridiomycota, and Glomeromycota were more prevalent in upper soil layer of 0–5 cm ($P < 0.05$) (Fig 7). Approximately 90% of the prominent fungal taxonomic groups
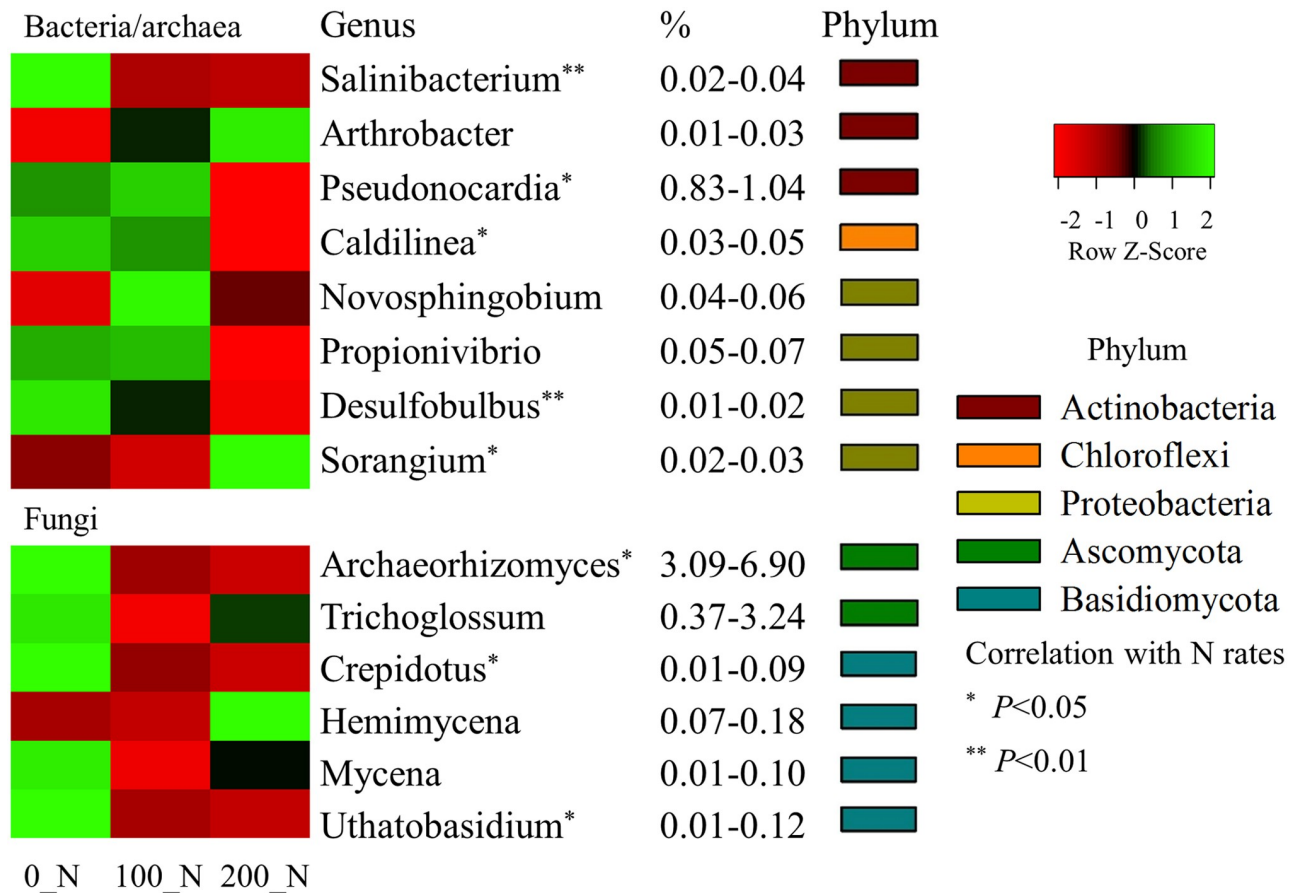
Fig 5. A heat map of relative abundance of bacterial/archaeal and fungal dominant taxonomic groups at genus level (average abundances > 0.1%) that were significantly affected by three N fertilization levels (0, 100, and 200 kg N ha^{-1}). Asterisks indicate significant Spearman correlations of taxonomic abundance with N fertilization levels at α = 0.05.

classified at the genus level (relative abundance > 0.1%) also significantly varied over sampling seasons ($P < 0.05$) (Fig 7).

Venn diagrams were used to better visualize these changes of bacterial/archaeal and fungal taxonomic groups affected by soil depth and sampling season (Fig 8). In bacteria, 61% of significantly affected genera were shared by the two factors of soil depth and sampling season, illustrating that most bacterial groups that differed between depths also responded to temporal change. In fungi, many more fungal taxonomic groups significantly varied across the four seasonal samples than with the soil depth (Fig 8), again indicating that temporal variation affected fungal community composition more significantly than spatial variation.

Microbial community associations with switchgrass yields and plant C/N contents

Compared to the control plots, N fertilization of plots at 100 and 200 kg N ha^{-1} increased switchgrass yields by 43% and 171%, respectively (Table 2). In addition, N inputs also significantly increased plant tissue N, but reduced relative C content and C/N ratios ($P < 0.05$) as measured at the end of the growing season. However, soil inorganic N analyses, including NH_3^+-N and NO_3^--N, of summer samples showed no difference among the three N fertilization levels (data not shown), indicating that applied N fertilizers were almost completely
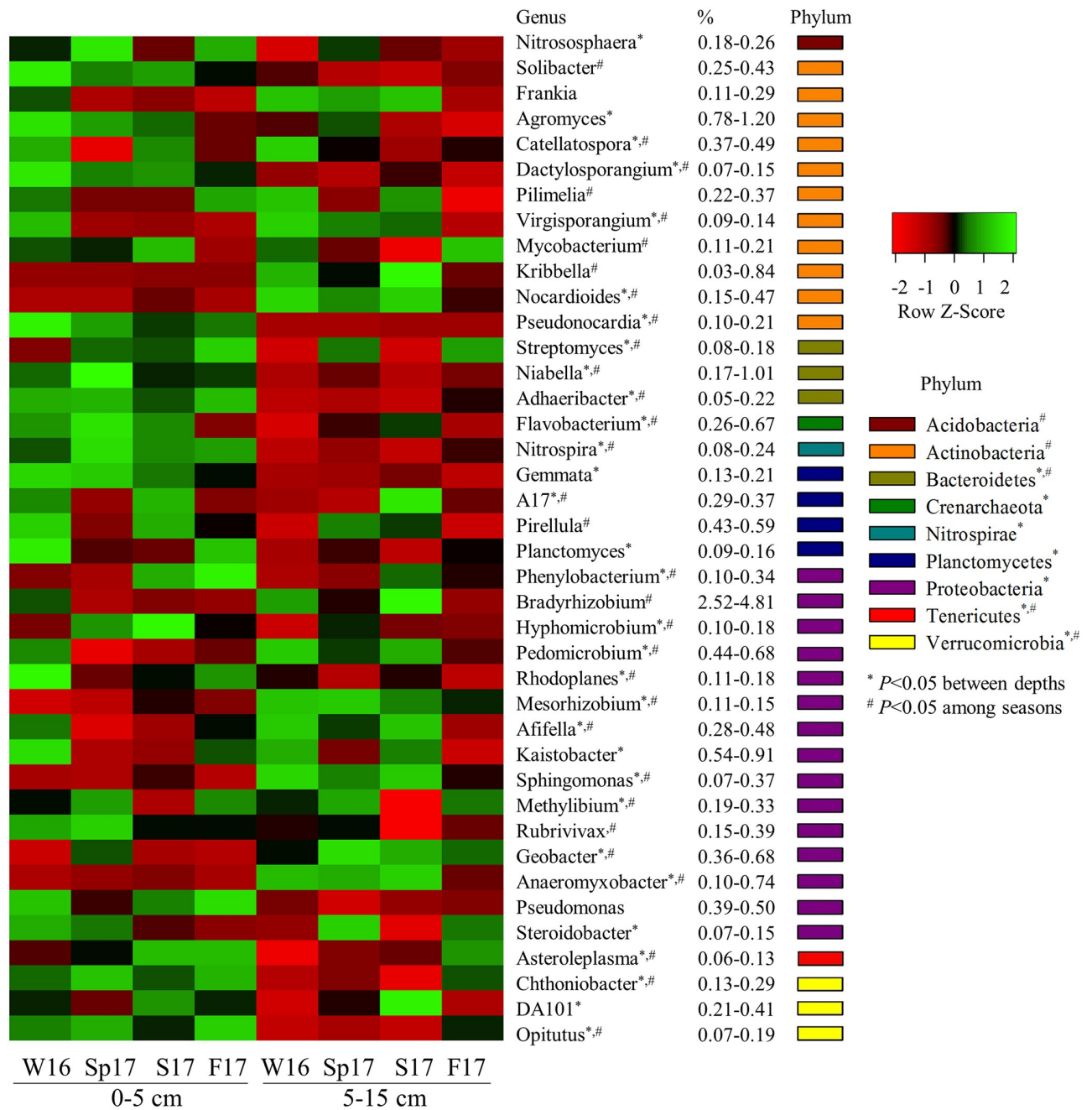
Fig 6. A heat map of relative abundance of bacterial/archaeal dominant taxonomic groups at genus level (average abundances > 0.1%) that were significantly variable between two soil depths (0–5 and 5–15 cm) and over four sampling seasons (Winter 2016, Spring, Summer, and Fall 2017). Asterisks indicate significant difference between two soil depths (0–5 and 5–15 cm) at α = 0.05. Number signs indicate significant difference over four sampling seasons (Winter 2016, Spring, Summer, and Fall 2017) at α = 0.05.

https://doi.org/10.1371/journal.pone.0211310.g006

depleted in soil within approximately four months following N inputs. The DistLM analysis showed that switchgrass yields were significantly correlated with the community structure of bacteria/archaea and fungi, but explained only a small portion of variation, *i.e.*, 2.6%, 1.2%, in bacterial/archaeal and fungal profiles, respectively ($P < 0.01$) (Table 2), suggesting a small but
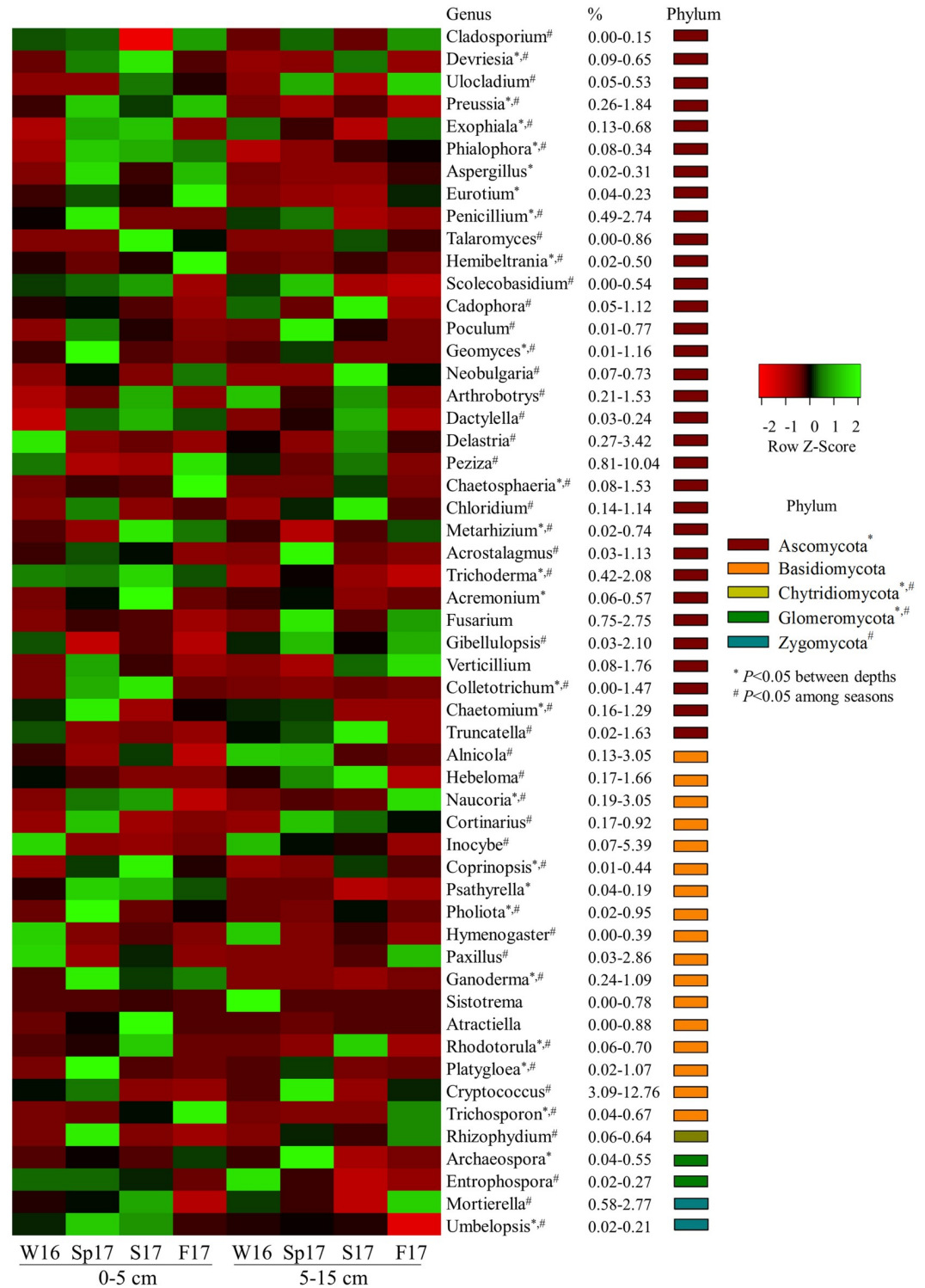
Fig 7. A heat map of relative abundance of fungal dominant taxonomic groups at genus level (average abundances > 0.1%) that were significantly variable between two soil depths (0–5 and 5–15 cm) and over four sampling seasons (Winter 2016, Spring, Summer, and Fall 2017). Asterisks indicate significant difference between two soil depths (0–5 and 5–15 cm) at α = 0.05. Number signs indicate significant difference over four sampling seasons (Winter 2016, Spring, Summer, and Fall 2017) at α = 0.05.

https://doi.org/10.1371/journal.pone.0211310.g007
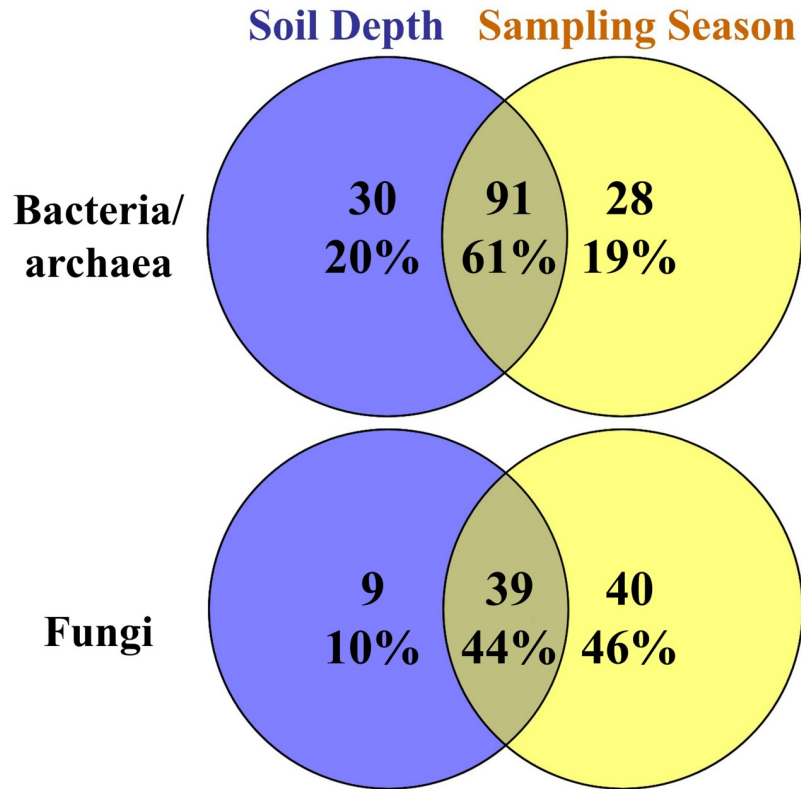
Fig 8. Venn's diagrams showing significantly affected bacterial/archaeal and fungal dominant taxonomic groups at genus level (average abundances > 0.1%) shared between the factors of soil depth and sampling season.

significant correlation between above-ground switchgrass growth and below-ground micro-biomes through N fertilization.

Discussion

Short-term N effects on microbial communities

Long-term N inputs, whether through fertilization or atmospheric deposition, can alter microbial composition and diversity through N-induced soil acidification and fertility decline [11]. Many long-term studies have reported that N fertilization not only reduces below-ground

Table 2. Above-ground biomass yields and plant C/N contents of switchgrass affected by N fertilization levels as well as their association with community structure of bacteria/archaea and fungi by marginal test of DistLM. Different letters within each column indicate significant effects by N fertilization levels at $\alpha = 0.05$. The *, **, and *** indicate significant DistLM relationship at $\alpha = 0.05$, 0.01, and 0.001, respectively.

	Yield (Mg Ha^{-1})	C (%)	N (%)	C/N
N level (kg N ha^{-1})				
0	29.6 b	50.43 a	0.28 a	189.46 a
100	42.4 ab	49.70 b	0.83 b	74.73 b
200	80.1 a	49.47 b	0.98 b	60.62 b
Proportion of explained variation				
Bacteria/archaea	2.6%***	1.2%*	1.0%	0.6%
Fungi	1.2%**	1.0%	1.0%	0.8%

biodiversity but also shifts bacterial composition at the phylum level, for groups such as Proteobacteria, Acidobacteria and Actinobacteria [45–49]. Field studies focusing on short-term effects of N fertilization on microbial communities however are limited in number for comparison. In our study, one-time fertilization did not affect the richness and diversity of soil microbial communities, but caused more limited structural changes in both bacterial/archaeal and fungal community composition (Fig 4). Our work suggests that some phylogenetic groups of bacteria and fungi might quickly react either to N inputs directly or through indirect plant associated mechanisms, even when soil properties are not significantly modified by short-term N fertilization. These small but subtle N effects were consistent across the two soil depths and three sampling times after the N inputs, because there was no significant interaction between N and depth/season (Table 1). We also observed that the one-time N amendment appeared to directly repress some bacterial and fungal groups based on the negative relationships of relative abundance with N levels, for example, bacterial genus *Pseudonocardia* and fungal genus *Archaeorhizomyces* (Fig 5). However, we did not observe any changes in organism classifications that might be traditionally associated with ammonia or nitrate transformations (e.g., nitrification or denitrification).

The *Pseudonocardia* is a common endophytic Actinomycete frequently isolated from host plant tissues [50], which has been reported to achieve associative nitrogen fixation without the formation of nodules [51] and protect their hosts against soil-borne pathogenic infection through producing antibiotics or siderophores [52, 53]. As a free living diazotrophic Actinomycete, it has also been reported to be prominent in nutrient limited environments [54, 55] or low-input agro-ecosystems [56, 57], due to its low requirement for N. Based on sequencing of 16S rRNA genes, it was also found that *Pseudonocardia* OTUs were reduced in the fertilized plant rhizospheres of Canola (*Brassica napus*) [58]. Our results support that the relative abundances of *Pseudonocardia* are significantly and negatively associated with N fertilization (Fig 5), and suggest that even short-term N inputs might acutely suppress this associative nitrogen fixer in switchgrass cultivated lands.

The *Archaeorhizomyces* are an ancient class of ubiquitous soil fungi [59], which are neither mycorrhizal nor pathogenic, but may be root endophytic or free-living saprophytes [60]. This group was first discovered in tundra soils [61] using rRNA-based sequencing, but was only isolated into culture more recently [59]. Very little is definitively known about the physiology and ecology of this group and this knowledge comes from only one extant isolate of what is a broad class of organisms. Much of our knowledge of this group accordingly only comes from molecular microbial ecology studies like this one, examining changes in these organisms under different conditions. For example, by investigating how organic matter accumulation and forest fertility influences fungal community composition, it was found from ITS rRNA gene analyses that *Archaeorhizomyces* dominated the root-associated Ascomycetes and their abundance significantly correlated with a fertility gradient in European boreal forests [62]. Moreover, it has been shown that the relative abundance of *Archaeorhizomyces* in grasslands is greatly stimulated by amendment of the biofertilizers *Trichoderma* [63] and correlations between soil properties and fungal abundance suggested that soil P availability (rather than N) may be a controlling factor for *Archaeorhizomyces* relative abundance. However, in our study, inorganic N fertilization significantly reduced the relative abundance of *Archaeorhizomyces*, which was one of the dominant groups of Ascomycota present in our study at 3.1–6.9% relative abundance (Fig 5). Further studies on the ecology of these diverse fungi are clearly needed through both additional rRNA gene amplicon studies in natural systems, as well as the isolation of additional representatives for ecophysiological analyses.

Spatial heterogeneity in microbial communities

Several studies have reported that soil bacterial and fungal diversity levels can either decrease [25, 64, 65], remain unchanged [27, 66, 67] or increase [68] with soil depth. We consistently observed reduced community richness and diversity in 5–15 cm compared to 0–5 cm soil layers for both bacterial/archaeal and fungal communities (Fig 3). Since plant residue serves as a key carbon source for soil microbes, the vertical distribution of microbial communities is likely to reflect the different available organic matter content with soil depths for microbial decomposers [65]. For example, the surface soil may have more easily decomposable carbon directly derived from crop residues, with more diverse groups of microbes able to access the labile organic materials in this niche [69] whereas subsurface soils may harbor relatively more recalcitrant carbon sources or be more dependent on root inputs. We also observed less soil C and N in 5–15 cm soil layers, further suggesting nutrient levels may be among the factors driving these depth related patterns in diversity.

Compared to the small amount of community variation attributable to N addition, we observed more significant shifts in both bacterial/archaeal and fungal community composition between soil depths. Generally, Bacteroidetes, Planctomycetes, and Verrucomicrobia were more abundant in the 0–5 cm soil layer. This spatial differentiation of the dominant bacterial groups by soil depth was consistent to many previous studies. For example, it has been shown that bacterial community composition was significantly altered at different soil depths, which was associated primarily with a decline of Bacteroidetes with depth [25]. Others have also reported that Verrucomicrobia exhibit higher relative abundance in the surface soils [27, 68]. In contrast, our results showed that the 5–15 cm soil layer had greater abundance in the phyla of Chloroflexi, Nitrospirae, and Proteobacteria. Similarly, it is also demonstrated that as soil depth increased, the relative abundance of Proteobacteria increased and it became the dominant bacterial group in subsoil [66]. Though the overall Proteobacteria were more abundant in 5–15 cm soils, the class Betaproteobacteria was most abundant in 0–5 cm, which was also found in other study [70]. Similar to our results, others have also reported that Chloroflexi [67, 68] and Nitrospirae [71] increase in abundance with soil depth.

In this study, we observed that fungal community also showed strong vertical distribution patterns in the major groups, such as Ascomycota, Chytridiomycota, and Glomeromycota, which were more abundant in top 0–5 cm soil layer; however, compared to bacterial community, there were overall fewer fungal taxonomic groups that differed between soil depths (Figs 6 and 7). Several studies have highlighted the ecological significance of vertically distinct fungal communities. For example, using pyrosequencing of ITS amplicon, others have found a decrease in relative abundance of Ascomycota with increasing soil depth, whereas Zygomycota showed the opposite trend [64]. At finer taxonomic scales, it was reported that Sordariomycetes of the phylum Ascomycota decrease with soil depth [71], and this pattern was similar in our study. Others have also shown overall fungal communities pattern were highly variable with soil depth, where deeper soil have some distinct fungal groups, but significantly less overall diversity [65] similar to what we observe here.

Temporal variation in microbial communities

We found significant temporal changes in alpha diversity in both bacterial and fungal communities (Fig 3). Although the evaluation of chronosequences of microbial community has been of interest to study in switchgrass cultivation [72], our results showed a significant temporal variation of both bacterial and fungal communities even in one year. Considering such variation is important as these significant temporal effects may override the changes from N additions. Only by applying a well replicated design and accounting for temporal and depth

variation in our study, could we thoroughly assess the differences between the N-free controls and N fertilization treatments. It has previously been shown that bacterial community alpha diversity varies more substantially than beta diversity over time, and in this case exceeded the variability between land-use types [26], which attributed to rhizodeposition as a controlling factor on soil bacterial diversity. Our data show that seasonal variation is found in most dominant phylogenetic groups of bacteria, such as Acidobacteria, Actinobacteria, Bacteroidetes, Chloroflexi, and Verrucomicrobia (Fig 6). Interestingly Acidobacteria, Bacteroidetes, Betaproteobacteria, Deltaproteobacteria, Gammaproteobacteria, and Verrucomicrobia all had similar seasonal patterns, which were opposite to those of Actinobacteria, Chloroflexi, and Alphaproteobacteria. That dominant bacterial phyla, such as Actinobacteria and Betaproteobacteria, shift seasonally to temporal patterns has also been reported previously [66]. In addition, it has been shown that seasonal dynamics often appear to be coherent within taxonomic lineages, in which Acidobacteria and Proteobacteria are more prevalent in summer, whereas Actinobacteria and Chloroflexi increase in winter [73].

In our experiment, the fungal phyla Chytridiomycota, Glomeromycota, and Zygomycota, were also found to vary significantly over the different seasonal sampling dates (Fig 7). It has been reported that Ascomycota and Glomeromycota increase in summer, whereas Basidiomycota are dominant in winter [74] or alternatively that Ascomycota, Basidiomycota, and Zygomycota are variable from spring to winter [75]. Changes in litter decomposition and phytosynthate allocation likely also contribute to the seasonal variations of fungal community [76] in addition to the direct effects of soil moisture and temperature [26]. However, while season is important in overall community structure, contrary to the above, we did not observe distinct seasonal changes in the overall dominant patterns of Ascomycota or Basidiomycota in our study and in any case such patterns are likely to be ecosystem specific.

Conclusions

With the aid of high-throughput 16S rRNA gene and ITS region amplicon sequencing, we found highly diverse and dynamic communities across this 8 year-old switchgrass field. The one-time application of N fertilization significantly stimulated switchgrass growth and N uptake, and subtly but significantly shifted below-ground bacterial and fungal communities, with the bacterial genus *Pseudonocardia* and *Archaeorhizomyces* fungi negatively responsive to N inputs. However, these shifts took place within the context of much larger spatial and temporal variation in the microbial community. Only by using a highly replicated study, and thoroughly accounting large spatial and seasonal fluctuations in microbial communities, could we account for these subtle differences. This reinforces the importance of robust sampling designs and should caution against overinterpretation of studies based on one-time sampling events. Follow-up studies should aim at obtaining a better understanding of the ecological and physiological mechanisms of responses to N fertilization by these microbes and how these may influence ecosystem functions.

Supporting information

S1 Fig. Relative abundances of soil metagenomes annotated in RefSeq database (RefSeq), and functional classification annotated in SEED Subsystems database (SEED Subsystems) in composite soils sampled at two soil depths (0–5 and 5–15 cm) in Winter 2016. Asterisks indicate significant difference at $\alpha = 0.05$ between two soil depths.
(TIF)

S2 Fig. Non-metric multidimensional scaling (NMDS) analysis of 24 putative functions at KEGG level 1 predicted by PICRUSt based on 16S rRNA amplicons compared with 4 soil metagenomes annotated in KO database (KEGG level 1) using soils sampled in Winter 2016. PERMANOVA *P* values were also given.
(TIF)

S3 Fig. A linear discriminant analysis effect size (LEfSe) method showing dominant functional groups (average abundances > 1%) that were significantly different between soil metagenomes annotated in KO database by MG-RAST and putative functions in KEGG predicted by PICRUSt based on 16S rRNA amplicons using soils sampled in Winter 2016. From the center outward, each circle represents the KEGG level 1, 2, and 3, respectively. The functional groups with significant differences are labeled by red color (MG-RAST) or green colors (PICRUSt).
(TIF)

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References

1. Demirbas A. Political, economic and environmental impacts of biofuels: A review. Applied Energy. 2009; 86, Supplement 1(0):S108–S17.

2. Jagger A. Biofuels for transport in 2050. Biofuels, Bioproducts and Biorefining. 2011; 5(5):481–5.

3. Chen H, Dai Z, Jager HI, Wullschleger SD, Xu J, Schadt CW. Influences of nitrogen fertilization and climate regime on the above-ground biomass yields of miscanthus and switchgrass: A meta-analysis. Renewable and Sustainable Energy Reviews. 2019; 108:303–11.

4. Wang Z, Smyth TJ, Crozier CR, Gehl RJ, Heitman AJ. Yield and Nitrogen Removal of Bioenergy Grasses as Influenced by Nitrogen Rate and Harvest Management in the Coastal Plain Region of North Carolina. BioEnergy Research. 2018; 11(1):44–53.

5. Fike JH, Pease JW, Owens VN, Farris RL, Hansen JL, Heaton EA, et al. Switchgrass nitrogen response and estimated production costs on diverse sites. Gcb Bioenergy. 2017; 9(10):1526–42.

6. Gopalakrishnan G, Cristina Negri M, Snyder SW. A novel framework to classify marginal land for sustainable biomass feedstock production. Journal of environmental quality. 2011; 40(5):1593–600. https://doi.org/10.2134/jeq2010.0539 PMID: 21869522

7. Toma YO, FernÁNdez FG, Sato S, Izumi M, Hatano R, Yamada T, et al. Carbon budget and methane and nitrous oxide emissions over the growing season in a Miscanthus sinensis grassland in Tomako-mai, Hokkaido, Japan. GCB Bioenergy. 2011; 3(2):116–34.

8. Nazir R, Warmink JA, Boersma H, Van Elsas JD. Mechanisms that promote bacterial fitness in fungal-affected soil microhabitats. FEMS Microbiology Ecology. 2010; 71(2):169–85. https://doi.org/10.1111/j.1574-6941.2009.00807.x PMID: 20002182

9. Nielsen U, Ayres E, Wall D, Bardgett R. Soil biodiversity and carbon cycling: a review and synthesis of studies examining diversity–function relationships. European Journal of Soil Science. 2011; 62(1):105–16.

10. Van Der Heijden MG, Bardgett RD, Van Straalen NM. The unseen majority: soil microbes as drivers of plant diversity and productivity in terrestrial ecosystems. Ecology letters. 2008; 11(3):296–310. https://doi.org/10.1111/j.1461-0248.2007.01139.x PMID: 18047587

11. Dai Z, Su W, Chen H, Barberán A, Zhao H, Yu M, et al. Long-term nitrogen fertilization decreases bacterial diversity and favors the growth of Actinobacteria and Proteobacteria in agro-ecosystems across the globe. Global change biology. 2018.

12. Vimal SR, Singh JS, Arora NK, Singh S. Soil-plant-microbe interactions in stressed agriculture management: a review. Pedosphere. 2017; 27(2):177–92.

13. Ramirez KS, Craine JM, Fierer N. Consistent effects of nitrogen amendments on soil microbial communities and processes across biomes. Global Change Biology. 2012; 18(6):1918–27.

14. Leff JW, Jones SE, Prober SM, Barberán A, Borer ET, Firn JL, et al. Consistent responses of soil microbial communities to elevated nutrient inputs in grasslands across the globe. Proceedings of the National Academy of Sciences. 2015; 112(35):10967–72.

15. Rousk J, Bååth E, Brookes PC, Lauber CL, Lozupone C, Caporaso JG, et al. Soil bacterial and fungal communities across a pH gradient in an arable soil. The ISME journal. 2010; 4(10):1340. https://doi.org/10.1038/ismej.2010.58 PMID: 20445636

16. Bardgett RD, Lovell RD, Hobbs PJ, Jarvis SC. Seasonal changes in soil microbial communities along a fertility gradient of temperate grasslands. Soil Biology and Biochemistry. 1999; 31(7):1021–30.

17. Tlustos P, Willison T, Baker J, Murphy D, Pavlikova D, Goulding K, et al. Short-term effects of nitrogen on methane oxidation in soils. Biology and Fertility of Soils. 1998; 28(1):64–70.

18. Wang Q, Wang S, Liu Y. Responses to N and P fertilization in a young Eucalyptus dunnii plantation: microbial properties, enzyme activities and dissolved organic matter. Applied Soil Ecology. 2008; 40(3):484–90.

19. Wang C, Long R, Wang Q, Liu W, Jing Z, Zhang L. Fertilization and litter effects on the functional group biomass, species diversity of plants, microbial biomass, and enzyme activity of two alpine meadow communities. Plant and Soil. 2010; 331(1–2):377–89.

20. Jang I, Lee S, Zoh K-D, Kang H. Methane concentrations and methanotrophic community structure influence the response of soil methane oxidation to nitrogen content in a temperate forest. Soil Biology and Biochemistry. 2011; 43(3):620–7.

21. Deslippe JR, Egger KN, Henry GH. Impacts of warming and fertilization on nitrogen-fixing microbial communities in the Canadian High Arctic. FEMS microbiology ecology. 2005; 53(1):41–50. https://doi.org/10.1016/j.femsec.2004.12.002 PMID: 16329928

22. Piceno Y, Lovell C. Stability in natural bacterial communities: I. Nutrient addition effects on rhizosphere diazotroph assemblage composition. Microbial ecology. 2000; 39(1):32–40. PMID: 10790515

23. Karp A, Shield I. Bioenergy from plants and the sustainable yield challenge. New Phytologist. 2008; 179(1):15–32. https://doi.org/10.1111/j.1469-8137.2008.02432.x PMID: 18422906

24. McLaughlin SB, Kszos LA. Development of switchgrass (Panicum virgatum) as a bioenergy feedstock in the United States. Biomass and Bioenergy. 2005; 28(6):515–35.

25. Eilers KG, Debenport S, Anderson S, Fierer N. Digging deeper to find unique microbial communities: the strong effect of depth on the structure of bacterial and archaeal communities in soil. Soil Biology and Biochemistry. 2012; 50:58–65.

26. Lauber CL, Ramirez KS, Aanderud Z, Lennon J, Fierer N. Temporal variability in soil microbial communities across land-use types. The ISME journal. 2013; 7(8):1641. https://doi.org/10.1038/ismej.2013.50 PMID: 23552625

27. Bai R, Wang J-T, Deng Y, He J-Z, Feng K, Zhang L-M. Microbial community and functional structure significantly varied among distinct types of paddy soils but responded differently along gradients of soil depth layers. Frontiers in microbiology. 2017; 8:945. https://doi.org/10.3389/fmicb.2017.00945 PMID: 28611747

28. Dong H-Y, Kong C-H, Wang P, Huang Q-L. Temporal variation of soil friedelin and microbial community under different land uses in a long-term agroecosystem. Soil Biology and Biochemistry. 2014; 69:275–81.

29. Will C, Thürmer A, Wollherr A, Nacke H, Herold N, Schrumpf M, et al. Horizon-specific bacterial community composition of German grassland soils, as revealed by pyrosequencing-based analysis of 16S rRNA genes. Applied and environmental microbiology. 2010; 76(20):6751–9. https://doi.org/10.1128/AEM.01063-10 PMID: 20729324

30. Lewandowski TE, Forrester JA, Mladenoff DJ, Stoffel JL, Gower ST, D'Amato AW, et al. Soil microbial community response and recovery following group selection harvest: Temporal patterns from an experimental harvest in a US northern hardwood forest. Forest Ecology and Management. 2015; 340:82–94.

31. Kramer S, Marhan S, Haslwimmer H, Ruess L, Kandeler E. Temporal variation in surface and subsoil abundance and function of the soil microbial community in an arable soil. Soil Biology and Biochemistry. 2013; 61:76–85.

32. Cregger MA, Schadt CW, McDowell NG, Pockman WT, Classen AT. Response of the soil microbial community to changes in precipitation in a semiarid ecosystem. Applied and environmental Microbiology. 2012; 78(24):8587–94. https://doi.org/10.1128/AEM.02050-12 PMID: 23023755

33. Stevenson BA, Hunter DW, Rhodes PL. Temporal and seasonal change in microbial community structure of an undisturbed, disturbed, and carbon-amended pasture soil. Soil Biology and Biochemistry. 2014; 75:175–85.

34. DeBruyn JM, Bevard DA, Essington ME, McKnight JY, Schaeffer SM, Baxter HL, et al. Field-grown transgenic switchgrass (Panicum virgatum L.) with altered lignin does not affect soil chemistry, microbiology, and carbon storage potential. Gcb Bioenergy. 2017; 9(6):1100–9.

35. Lundberg DS, Yourstone S, Mieczkowski P, Jones CD, Dangl JL. Practical innovations for high-throughput amplicon sequencing. Nature methods. 2013; 10(10):999. https://doi.org/10.1038/nmeth.2634 PMID: 23995388

36. Cregger M, Veach A, Yang Z, Crouch M, Vilgalys R, Tuskan G, et al. The Populus holobiont: dissecting the effects of plant niches and genotype on the microbiome. Microbiome. 2018; 6(1):31. https://doi.org/10.1186/s40168-018-0413-8 PMID: 29433554

37. Langille MG, Zaneveld J, Caporaso JG, McDonald D, Knights D, Reyes JA, et al. Predictive functional profiling of microbial communities using 16S rRNA marker gene sequences. Nature biotechnology. 2013; 31(9):814–21. https://doi.org/10.1038/nbt.2676 PMID: 23975157

38. Martin M. Cutadapt removes adapter sequences from high-throughput sequencing reads. EMBnet journal. 2011; 17(1):pp. 10–2.

39. Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, et al. QIIME allows analysis of high-throughput community sequencing data. Nature Methods. 2010; 7(5):335–6. https://doi.org/10.1038/nmeth.f.303 PMID: 20383131

40. Wang Q, Garrity GM, Tiedje JM, Cole JR. Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. Applied and environmental microbiology. 2007; 73 (16):5261–7. https://doi.org/10.1128/AEM.00062-07 PMID: 17586664

41. Meyer F, Paarmann D, D'Souza M, Olson R, Glass EM, Kubal M, et al. The metagenomics RAST server–a public resource for the automatic phylogenetic and functional analysis of metagenomes. BMC bioinformatics. 2008; 9(1):386.

42. Anderson M. DISTLM v. 5: a FORTRAN computer program to calculate a distance-based multivariate analysis for a linear model. Department of Statistics, University of Auckland, New Zealand. 2004; 10:2016.

43. Babicki S, Arndt D, Marcu A, Liang Y, Grant JR, Maciejewski A, et al. Heatmapper: web-enabled heat mapping for all. Nucleic acids research. 2016; 44(W1):W147–W53. https://doi.org/10.1093/nar/gkw419 PMID: 27190236

44. Oliveros J. An interactive tool for comparing lists with Venn's diagrams (2007–2015).

45. Zhou J, Guan D, Zhou B, Zhao B, Ma M, Qin J, et al. Influence of 34-years of fertilization on bacterial communities in an intensively cultivated black soil in northeast China. Soil Biology and Biochemistry. 2015; 90:42–51.

46. Xun W, Zhao J, Xue C, Zhang G, Ran W, Wang B, et al. Significant alteration of soil bacterial communities and organic carbon decomposition by different long-term fertilization management conditions of extremely low-productivity arable soil in South China. Environmental microbiology. 2016; 18(6):1907–17. https://doi.org/10.1111/1462-2920.13098 PMID: 26486414

47. Ling N, Chen D, Guo H, Wei J, Bai Y, Shen Q, et al. Differential responses of soil bacterial communities to long-term N and P inputs in a semi-arid steppe. Geoderma. 2017; 292:25–33.

48. Coolon JD, Jones KL, Todd TC, Blair JM, Herman MA. Long-term nitrogen amendment alters the diversity and assemblage of soil bacterial communities in tallgrass prairie. PLoS One. 2013; 8(6):e67884. https://doi.org/10.1371/journal.pone.0067884 PMID: 23840782

49. Pan Y, Cassman N, de Hollander M, Mendes LW, Korevaar H, Geerts RH, et al. Impact of long-term N, P, K, and NPK fertilization on the composition and potential functions of the bacterial community in grassland soil. FEMS microbiology ecology. 2014; 90(1):195–205. https://doi.org/10.1111/1574-6941. 12384 PMID: 25046442

50. Hamedi J, Mohammadipanah F. Biotechnological application and taxonomical distribution of plant growth promoting actinobacteria. Journal of industrial microbiology & biotechnology. 2015; 42(2):157–71.

51. Valdés M, Pérez N-O, Estrada-de Los Santos P, Caballero-Mellado J, Pena-Cabriales JJ, Normand P, et al. Non-Frankia actinomycetes isolated from surface-sterilized roots of Casuarina equisetifolia fix nitrogen. Applied and environmental microbiology. 2005; 71(1):460–6. https://doi.org/10.1128/AEM.71. 1.460-466.2005 PMID: 15640222

52. Janso JE, Carter GT. Biosynthetic potential of phylogenetically unique endophytic actinomycetes from tropical plants. Applied and environmental microbiology. 2010; 76(13):4377–86. https://doi.org/10. 1128/AEM.02959-09 PMID: 20472734

53. Wang R, Zhang H, Sun L, Qi G, Chen S, Zhao X. Microbial community composition is related to soil biological and chemical properties and bacterial wilt outbreak. Scientific Reports. 2017; 7(1):343. https:// doi.org/10.1038/s41598-017-00472-6 PMID: 28336973

54. Zhang Y-Q, Liu H-Y, Chen J, Yuan L-J, Sun W, Zhang L-X, et al. Diversity of culturable actinobacteria from Qinghai–Tibet plateau, China. Antonie Van Leeuwenhoek. 2010; 98(2):213–23. https://doi.org/10. 1007/s10482-010-9434-4 PMID: 20361256

55. De Mandal S, Chatterjee R, Kumar NS. Dominant bacterial phyla in caves and their predicted functional roles in C and N cycle. BMC microbiology. 2017; 17(1):90. https://doi.org/10.1186/s12866-017-1002-x PMID: 28399822

56. Yin C, Mueth N, Hulbert S, Schlatter D, Paulitz TC, Schroeder K, et al. Bacterial communities on wheat grown under long-term conventional tillage and no-till in the Pacific Northwest of the United States. Phytobiomes. 2017; 1(2):83–90.

57. Dorr de Quadros P, Zhalnina K, Davis-Richardson A, Fagen JR, Drew J, Bayer C, et al. The effect of tillage system and crop rotation on soil microbial diversity and composition in a subtropical acrisol. Diversity. 2012; 4(4):375–95.

58. Monreal C, Zhang J, Koziel S, Vidmar J, González M, Matus F, et al. Bacterial community structure associated with the addition of nitrogen and the dynamics of soluble carbon in the rhizosphere of canola (Brassica napus) grown in a Podzol. Rhizosphere. 2018; 5:16–25.

59. Rosling A, Cox F, Cruz-Martinez K, Ihrmark K, Grelet G-A, Lindahl BD, et al. Archaeorhizomycetes: unearthing an ancient class of ubiquitous soil fungi. Science. 2011; 333(6044):876–9. https://doi.org/10. 1126/science.1206958 PMID: 21836015

60. Rosling A, Timling I, Taylor DL. Archaeorhizomycetes: Patterns of distribution and abundance in soil. Genomics of Soil-and Plant-Associated Fungi: Springer; 2013. p. 333–49.

61. Schadt CW, Martin AP, Lipson DA, Schmidt SK. Seasonal dynamics of previously unknown fungal lineages in tundra soils. Science. 2003; 301(5638):1359–61. https://doi.org/10.1126/science.1086940 PMID: 12958355

62. Kyaschenko J, Clemmensen KE, Karltun E, Lindahl BD. Below-ground organic matter accumulation along a boreal forest fertility gradient relates to guild interaction within fungal communities. Ecology letters. 2017; 20(12):1546–55. https://doi.org/10.1111/ele.12862 PMID: 29057614

63. Zhang F, Huo Y, Cobb AB, Luo G, Zhou J, Yang G, et al. Trichoderma biofertilizer links to altered soil chemistry, altered microbial communities, and improved grassland biomass. Frontiers in microbiology. 2018; 9.

64. Santalahti M, Sun H, Jumpponen A, Pennanen T, Heinonsalo J. Vertical and seasonal dynamics of fungal communities in boreal Scots pine forest soil. FEMS microbiology ecology. 2016; 92(11):fiw170.

65. Schlatter DC, Kahl K, Carlson B, Huggins DR, Paulitz T. Fungal community composition and diversity vary with soil depth and landscape position in a no-till wheat-based cropping system. FEMS microbiology ecology. 2018.

66. Bevivino A, Paganin P, Bacci G, Florio A, Pellicer MS, Papaleo MC, et al. Soil bacterial community response to differences in agricultural management along with seasonal changes in a Mediterranean region. PLoS One. 2014; 9(8):e105515. https://doi.org/10.1371/journal.pone.0105515 PMID: 25144665

67. Steven B, Gallegos-Graves LV, Belnap J, Kuske CR. Dryland soil microbial communities display spatial biogeographic patterns associated with soil depth and soil parent material. FEMS microbiology ecology. 2013; 86(1):101–13. https://doi.org/10.1111/1574-6941.12143 PMID: 23621290

68. Mueller RC, Belnap J, Kuske CR. Soil bacterial and fungal community responses to nitrogen addition across soil depth and microhabitat in an arid shrubland. Frontiers in microbiology. 2015; 6:891. https://doi.org/10.3389/fmicb.2015.00891 PMID: 26388845

69. Boer Wd, Folman LB, Summerbell RC, Boddy L. Living in a fungal world: impact of fungi on soil bacterial niche development. FEMS microbiology reviews. 2005; 29(4):795–811. https://doi.org/10.1016/j.femsre.2004.11.005 PMID: 16102603

70. Lüdemann H, Arth I, Liesack W. Spatial changes in the bacterial community structure along a vertical oxygen gradient in flooded paddy soil cores. Applied and Environmental Microbiology. 2000; 66 (2):754–62. https://doi.org/10.1128/aem.66.2.754-762.2000 PMID: 10653747

71. Ko D, Yoo G, Yun S-T, Jun S-C, Chung H. Bacterial and fungal community composition across the soil depth profiles in a fallow field. Journal of Ecology and Environment. 2017; 41(1):34.

72. Walker LR, Wardle DA, Bardgett RD, Clarkson BD. The use of chronosequences in studies of ecological succession and soil development. Journal of Ecology. 2010; 98(4):725–36.

73. Kuffner M, Hai B, Rattei T, Melodelima C, Schloter M, Zechmeister-Boltenstern S, et al. Effects of season and experimental warming on the bacterial community in a temperate mountain forest soil assessed by 16S rRNA gene pyrosequencing. FEMS microbiology ecology. 2012; 82(3):551–62. https://doi.org/10.1111/j.1574-6941.2012.01420.x PMID: 22670891

74. Aoki Y, Fujita K, Shima H, Suzuki S. Survey of annual and seasonal fungal communities in Japanese Prunus mume orchard soil by next-generation sequencing. Advances in Microbiology. 2015; 5(13):817.

75. Kim CS, Nam JW, Jo JW, Kim S-Y, Han J-G, Hyun MW, et al. Studies on seasonal dynamics of soil-higher fungal communities in Mongolian oak-dominant Gwangneung forest in Korea. Journal of Microbiology. 2016; 54(1):14–22.

76. Voříšková J, Brabcová V, Cajthaml T, Baldrian P. Seasonal dynamics of fungal communities in a temperate oak forest soil. New Phytologist. 2014; 201(1):269–78. https://doi.org/10.1111/nph.12481 PMID: 24010995