

1 One-time Nitrogen Fertilization Shifts Switchgrass Soil Microbiomes within a Context of Larger
2 Spatial and Temporal Variation

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32 **Abstract**

33 Soil microbiome responses to short-term nitrogen (N) inputs within the context of existing
34 spatio-temporal variability remain uncertain. Here, we examined soil bacterial and fungal
35 communities pre/post-N fertilization in an 8 year-old switchgrass field, in which twenty-four
36 plots received N fertilization at three levels (0, 100, and 200 kg N ha⁻¹ as NH₄NO₃) for the first
37 time since planting. Soils were collected at two depths, 0-5 and 5-15 cm, for DNA extraction and
38 amplicon sequencing of 16S rRNA genes and ITS regions, and soil metagenomic analysis.
39 Baseline assessment prior to fertilization revealed no pre-existing differences in either bacterial
40 or fungal communities across plots. The one-time N fertilization increased switchgrass yields
41 and tissue N content, and the added N was nearly completely removed from the soil of fertilized
42 plots by the end of the growing season. Both bacterial/archaeal and fungal communities showed
43 large spatial (by depth) and temporal variation (by season) within each plot, accounting for 17
44 and 12-22 % of the variation in bacterial/archaeal and fungal community composition,
45 respectively. While N fertilization effects accounted for only ~4% of overall variation, some
46 specific microbial groups, including the bacterial genus *Pseudonocardia* and the fungal genus
47 *Archaeorhizomyces*, were notably repressed by fertilization at 200 kg N ha⁻¹. Bacterial groups
48 varied with both depth in the soil profile and time of sampling, while temporal variability shaped
49 the fungal community more significantly than vertical heterogeneity in the soil. Thus, variability
50 within the field might override the changes induced by N addition. Continued analyses of these
51 trends over time with fertilization and management are needed to understand whether these
52 transient effects change over time.

53

54 **Introduction**

55 Cultivation of dedicated bioenergy crops is of interest to sustain long-term energy supplies [1].
56 The International Energy Agency predicts that biofuels could satisfy more than a quarter of
57 world needs for transportation energy by 2050 [2]. Switchgrass (*Panicum virgatum* L.) has been
58 a prominent candidate as an energy crop due to its high biomass yield, low maintenance and
59 limited-input requirements [3], and high adaptability to marginal sites [4]. Such characteristics
60 may allow switchgrass for its use to reclaim degraded or abandoned agricultural lands while
61 reserving fertile lands for food production [5]. With its well developed and deep rooting systems,
62 switchgrass may also improve belowground carbon storage and nutrient acquisition [6] and
63 potentially moderate the diversity of below-ground and plant-associated microbiomes. Thus, how
64 switchgrass cultivation affects soil microbial communities and their interaction with crop yields
65 needs further investigation to understand the long-term ecosystem consequences and
66 sustainability of the cultivation of perennial crops, such as switchgrass.

67 Soil microbial communities play fundamental roles in terrestrial ecosystems, such as
68 regulating the decomposition of organic matter as well as driving nutrient cycles and energy flow
69 [7, 8]. To this end, these microbiomes have considerable effects on soil quality and agricultural
70 sustainability [9]. However, soil management with fertilizer additions may shift soil microbial
71 abundance and composition as well as functions by affecting soil physical and chemical
72 characteristics [10]. For example, laboratory studies showed that N addition depresses soil
73 microbial activity, microbial biomass, and enzyme activities by shifting the metabolic
74 capabilities of soil bacterial communities toward the decomposition of more labile soil carbon
75 pools [11]. In addition, nutrient inputs have been shown to shift the composition of soil microbial
76 communities in consistent ways in grasslands across the globe with reduced average genome

77 sizes of microbial communities following nutrient amendment, leading to decreased relative
78 abundances of some important microbial functional groups, such as methanogenic archaea,
79 oligotrophic bacteria and mycorrhizal fungi [12]. Several reasons may account for such
80 microbial responses to fertilization. Fertilization may cause soil acidification, and thus alter soil
81 microbial diversity and composition [10, 13]. Additionally, nutrient amendments may have direct
82 effects on organic matter decomposition, leading to changes in the quantity and quality of
83 resources available for microbes, and therefore reshape microbial community structure based on
84 their substrate utilization preferences [14]. However, our understanding of the mechanisms of N
85 fertilization effects on microbial communities are mostly based on long-term fertilization, in
86 which edaphic soil properties have likely been significantly altered by soil management over
87 time. Although transient nutrient enrichment effects upon terrestrial microbial C and N processes
88 have been reported [15-17], our understanding of the immediate response of the below-ground
89 microbial community to N inputs is still limited, and sometimes inconsistent with results of long-
90 term experimental data [18-20]. Additional research is thus necessary to evaluate microbial
91 community dynamics and their interactions with nutrient cycling under the no-, low- or periodic
92 fertilization regimes that would be optimal for sustainable perennial bioenergy crop production
93 scenarios [21, 22].

94 Besides soil nutrient availability, soil microbial distribution is influenced by a wide range of
95 soil characteristics, such as soil pH, substrate quantity and quality, moisture and oxygen levels,
96 nearly all of which could typically change with soil depth [23] and vary over seasons [24]. Soil
97 depth and measuring time in a growing season thus influence patterns of spatial and temporal
98 community variation [25, 26]. Compared to top soil, subsurface soils have higher mineral
99 content, less aeration, and lower organic carbon availability. Thus, microbial biomass and

100 diversity typically decrease rapidly with depth in the soil profile [27]. Often, most variability in
101 microbial community composition occurs in surface soils, while deeper soils have more similar
102 communities regardless of soil management [23]. Seasonal variability also has a large influence
103 on microbial communities [28]. For example, seasonal changes of temperature and soil moisture
104 can directly shape microbial communities [29, 30]. Moreover, seasonal changes in plant growth
105 and allocation can indirectly affect soil C inputs [31, 32]. Lauber et al. [24] investigated the
106 temporal variability of bacterial communities in different ecosystems, showing that most of the
107 temporal variation in bacterial composition within an agricultural field could be explained by soil
108 moisture and temperature variations. Given these previous studies, it is possible that the shifting
109 spatial and temporal patterns of soil microbial communities may overwhelm short-term soil
110 nitrogen management effects and needs to be accounted for in such assessments.

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

119

120 **Materials and Methods**

121 **Site characterization, experimental design and plant and soil sampling**

122 The experiment was established in an eight year-old switchgrass field near the Heritage Center,
123 located on US DOE land, in Oak Ridge, Tennessee, USA (35.9255 N, 84.3947 W). The 10-year
124 mean annual temperature and annual precipitation at the site were 14.1°C and 1436 mm,
125 respectively. The field was in pasture and hay rotations when taken over by DOE in the 1940s
126 during the Manhattan Project. However, due to proximity of floodplain of the Clinch River and
127 Poplar Creek, the land was never developed and instead maintained as wildlife habitat and
128 riparian buffer as a field of mixed grasses and forbs, using a combination of mowing and
129 prescribed burning. In 2009, under contract for UT Institute of Agriculture and Genera Energy,
130 the site was cleared, seeded, and subsequently managed for switchgrass production. After the
131 contract expired in 2012, the site remained in switchgrass, but has again been managed as buffer
132 and wildlife habitat, and maintained only with periodic prescribed fire and mowing. In mid-
133 December 2016, the switchgrass field was mowed to a 10-cm stubble height and twenty four
134 plots (5 m × 5 m) were set up including three N fertilization levels (0, 100, and 200 kg N ha⁻¹)
135 with eight replicates based on a complete randomized design (Fig. 1). A 2.5-m inter-plot “alley
136 way” was periodically mowed to allow access and separate the plots between treatments and
137 replicates. Just before spring emergence of the switchgrass (March 30, 2017) commercial
138 ammonium nitrate (34% nitrogen) was hand-applied to fertilizer-treated plots with N fertilization
139 levels of 100, and 200 kg N ha⁻¹. Post-emergence (June 20, 2017), all plots were treated with
140 Garlon 3A herbicide as prescribed by the manufacture to help control broadleaf weeds. After fall
141 senescence (November 13, 2017) above-ground biomass of switchgrass was measured [33] using
142 a sickledrat to harvest all aboveground biomass from a 0.1 m² area at four randomly chosen
143 locations in each plot. The four samples of aboveground biomass were pooled by plot in paper
144 bags, oven dried at 70° C, and weighed to determine dry mass per unit area. A subsample of the

145 plant material was then ground into powder using a laboratory mill before total C and N were
146 determined by dry combustion method using a Perkin-Elmer 2400 CHN analyzer (Perkin-Elmer
147 Corporation, Norwalk, CT, USA).

148 For soil DNA and chemical characterization, four sets of soil samples were collected across
149 seasons including Winter 2016 (December 16, 2016), Spring 2017 (April 5, 2017), Summer 2017
150 (July 5, 2017), and Late Fall 2017 (November 15, 2017). At each sampling event, soil cores (2.5
151 cm diameter × 15 cm height) were collected randomly from each plot and separated into two
152 depth increments of 0-5 and 5-15 cm. Soils collected in Winter 2016 (before N fertilization) were
153 used to assess soil microbiomes and metagenomes, and check whether there was systematic pre-
154 existing differences of microbial communities across plots. Soils collected following N
155 fertilization, *i.e.*, Spring 2017, Summer 2017, and Late fall 2017, were used to compare
156 difference in microbial communities among N treatments. Soil collected for all four seasonal
157 samplings was used to assess how soil depth and sampling seasons affected microbial
158 communities across all three nitrogen treatments. All soil samples for microbial analyses were
159 transported on dry ice to the lab and stored at -80° C prior to soil DNA extraction. Total C and N
160 were determined on samples collected in the Summer of 2017 using the dry combustion method
161 and soil inorganic N ($\text{NH}_4^+\text{-N}$ and $\text{NO}_3^-\text{-N}$) was analyzed using a FIA QuikChem 8000
162 autoanalyzer (Lachat Instruments, Loveland, CO, USA). Generally, 0-5 cm soils had 4.5% soil
163 total C, 0.2% soil total N, 10.2 mg kg⁻¹ NH_4^+ , and 1.3 mg kg⁻¹ NO_3^- , while 5-15 cm soil had
164 significantly lower 1.8% soil total C, 0.03% soil total N, 1.8 mg kg⁻¹ NH_4^+ , and 0.3 mg kg⁻¹
165 NO_3^- .

166

167 **DNA extraction, rRNA gene amplicon sequencing, and metagenomic sequencing**

168 Approximately 10 g of soil from each sample was homogenized in a mortar and pestle with
169 liquid N₂, and soil DNA was extracted from a 0.25 g aliquot of the soil sample using the MoBio
170 DNeasy PowerSoil Kit (Qiagen, Carlsbad, CA, USA) according to the manufacturer's
171 instructions. DNA concentrations were determined and purity was confirmed by the ratio of
172 absorbance at 260 and 280 nm (1.70-1.90) using a NanoDrop 1000 spectrophotometer
173 (NanoDrop Products, Wilmington, DE, USA).

174 A two-step PCR approach was used to barcode tag templates with frameshifting nucleotide
175 primers for amplicon sequencing [34] with some modifications previously described [35]. To
176 increase phylogenetic coverage for community analysis of bacteria, archaea, and fungi, a group
177 of nine forward and six reverse primers for bacteria and archaea, and another group of eleven
178 forward and seven reverse primers for fungi, mixed at equal concentration of 0.5 μM were used
179 to target 16S rRNA V4 region and fungal ITS2 rRNA, respectively [35]. Primary PCR was
180 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
181 elongation of 5 min at 72 °C. This PCR product was then cleaned up using Agencourt AMPure
182 beads (Agencourt Bioscience, Beverly, MA, USA) and eluted in 21 μL of nuclease-free water.
183 To tag amplicons with barcoded reverse primers and forward primers, 20 μL of purified DNA
184 fragments from the primary PCRs were added to 50 μL secondary PCR assays, which were
185 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
186 at 72 °C, followed by a final elongation of 30 sec at 72 °C. The use of separate tagging reactions
187 can help reduce heterodimers because PCR clean-up more efficiently removes shorter primers
188 [34]. Up to ninety six secondary PCR products were then pooled based on agarose gel band
189 intensity, followed by a second clean-up with Agencourt AMPure beads (Agencourt Bioscience,
190 Beverly, MA, USA) using 0.7-1 of bead-to-DNA ratios. The mixtures of the purified 16S rRNA

191 gene or ITS amplicon fragments were then paired-end sequenced on Illumina Miseq platform
192 (250×2 paired end, v2 chemistry) (Illumina, San Diego, CA, USA) using a 9 pM amplicon
193 concentration.

194 To examine microbial community potential and function prior at the site, DNA extracted
195 from soil samples collected in Winter 2016 were pooled to form four composite samples.
196 Specifically, for each of two soil depths, DNA from No. 1-12 and 13-24 plots was pooled
197 together, respectively (two depth × two replicates). Shotgun metagenomes were prepared using
198 Nextera XT sequencing libraries (Illumina, San Diego, CA) according to the manufacture's
199 recommendations using 500ng of DNA (15031942 v03). Final libraries were validated on an
200 Agilent Bioanalyzer (Agilent, Santa Clara, CA) using a DNA7500 chip and concentration was
201 determined on an Invitrogen Qubit (Waltham, MA) with the broad range double stranded DNA
202 assay. Barcoded libraries were pooled and prepared for sequencing following the manufactures
203 recommended protocol (15039740v09, Standard Normalization). One paired end sequencing run
204 (2 x 300) was completed on an Illumina MiSeq instrument (Illumina, San Diego, CA) using v3
205 chemistry.

206 **Bioinformatic and Statistical Analyses**

207 Forward and reverse primers were trimmed with Cutadapt [36]. Paired-end sequencing data
208 were then joined and demultiplexed using QIIME [37] with quality filter at Phred > 19. Chimeras
209 of trimmed and filtered sequences were identified and removed using a usearch method in
210 QIIME. Operational taxonomic units (OTUs) with 97% identity were picked with the open
211 reference algorithm and usearch61 otu-picking method. Taxonomy was assigned using the RDP
212 (Ribosomal Database Project) taxonomy-assignment method [38] against the most recent version
213 of Greengenes database (13.8) for 16S rRNA sequencing data and UNITE database (12.11) for

214 ITS sequencing data. All global singletons were removed from the dataset. The 16S and ITS
215 OTUs were further analyzed for alpha and beta diversity using QIIME. Metrics for analyzing
216 beta diversity were Bray-Curtis distance. Bacterial community functional traits were predicted
217 using PICRUSt (Phylogenetic Investigation of Communities by Reconstruction of Unobserved
218 States) [39]. The Miseq sequences were deposited on NCBI Sequence Read Archive (SRA)
219 database under the BioProject accession number of PRJNA512218.

220 Soil metagenome sequences were uploaded to Rapid Annotation using Subsystems
221 Technology for Metagenomes (MG-RAST; <http://metagenomics.anl.gov>) [40] under project
222 accession number mgp22000, and annotated using the RefSeq database for taxonomic
223 assignment and the SEED Subsystems database for functional classification (maximum e-value
224 cutoff was $1e^{-5}$, minimum identity cutoff was 60%, and minimum alignment length was 50).

225 One-way analysis of variance (ANOVA) of a completely randomized design (SAS 9.3, SAS
226 Institute Inc. Cary, NC, USA) was used to assess significant differences in above-ground yields
227 and plant C/N contents among N fertilization levels. A three-factor ANOVA of a completely
228 randomized design was used to analyze microbial alpha diversity and the abundances of
229 microbial taxonomic groups among the three N fertilization levels, two soil depths, and four
230 seasonal samplings. Microbial beta diversity was compared using a three-factor PEMANOVA
231 method (N fertilization levels, soil depths, and sampling season) with 9999 permutations
232 conducted in PRIMER (Plymouth Routines in Multivariate Ecological Research Statistical
233 Software, v7.0.13, PRIMER-E Ltd, UK). A RELATE analysis was also performed to evaluate
234 the relatedness between bacterial and fungal beta diversity by calculating Spearman's Rho
235 correlation coefficient in PRIMER. The DistLM (distance-based linear model) function in
236 PRIMER was used to evaluate the associations of above-ground yields and plant C/N contents

237 with bacterial and fungal beta diversity [41]. Heat maps were constructed using HeatMapper [42]
238 to represent all taxonomic groups at genus level that differed significantly ($P < 0.05$) among
239 three N fertilization levels, two soil depths, and four sampling times. Venn's diagrams were also
240 constructed to visualize how many significantly affected bacterial/archaeal and fungal genera
241 were shared between the factors of soil depth and sampling time using Venny 2.1.0 [43].
242 Additionally, Pearson's correlation coefficients were examined to further evaluate relationships
243 between the relative abundances of taxa and N fertilization rates.

244

245 **Results**

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

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

256 Shotgun metagenomes also showed high taxonomic and functional diversity in the
257 switchgrass field (Fig. S1). However, when phylogenetic assignments of the metagenome reads
258 were compared to the relative abundance in 16S rRNA gene amplicon analyses, soil
259 metagenomes indicated significant differences in the datasets across several of the dominant

260 phyla. There was a 45% increase in Proteobacteria, a 15-fold increase in Firmicutes, and a 2-fold
261 increase in Cyanobacteria in the shotgun metagenomes when compared to 16S rRNA gene
262 amplicon analyses. Other phyla, such as Acidobacteria, Planctomycetes, and Chloroflexi, were
263 reduced by 52-63% in soil metagenomes when compared to 16S rRNA gene amplicon analyses
264 from the same samples and dates. Soil metagenome predicted functional gene profiles were
265 compared to those predicted from PICRUST-based analysis of 16S rRNA gene amplicon data
266 and indicated significantly different profiles (Fig. S2). As a result, PICRUST-based analyses of
267 seasonal functional gene patterns and responses to fertilization were not pursued further.

268

269 **Microbial alpha and beta diversity post-nitrogen addition**

270 Although neither bacterial/archaeal nor the fungal alpha diversity were significantly affected by
271 N fertilization levels, both the community richness (Chao1 index) and diversity (Shannon index)
272 showed significant spatio-temporal changes ($P < 0.05$) (Fig. 3). Between the two soil depths, the
273 0-5 cm layer had significantly higher Chao1 richness and Shannon evenness indices in both the
274 bacterial/archaeal and fungal communities compared to the 5-15 cm layer ($P < 0.05$). In analyses
275 of seasonal variation, Chao1 diversity showed a similar pattern. Spring 2017 had lower richness
276 in the bacterial/archaeal community, while Winter 2016 and Fall 2017 had significantly greater
277 richness in fungal communities ($P < 0.05$). Shannon diversity indices showed significant
278 divergence across seasons ($P < 0.05$), and the bacterial/archaeal community was more evenly
279 distributed in Fall 2017, whereas the fungal community was more uneven in Summer and Fall
280 2017 than the other two sampling seasons ($P < 0.05$).

281 Permanova tests showed that short-term application of N fertilizers caused significant
282 variation in bacterial/archaeal and fungal community composition ($P < 0.05$) (Table 1 and Fig.

283 4). Together, N fertilization effects could explain 3.4% of variation in bacterial/archaeal and
284 4.4 % of fungal community variation (Table 1). However, the spatio-temporal variation (depth
285 and season) were more significant than N effects for bacterial, archaeal and fungal communities
286 ($P < 0.0001$) (Table 1 and Fig. 4). Soil depth and sampling season contributed to approximately
287 16.8 and 17.3% of bacterial/archaeal community variation, respectively, and 12.4 and 22.4 % of
288 fungal community change, respectively (Table 1), thus indicating relatively slight short-term
289 effects of N fertilization on microbial communities when compared to the spatio-temporal
290 variation. In addition, RELATE analyses further confirmed that bacterial/archaeal community
291 structures were significantly related to the fungal community (Rho = 0.218, $P < 0.01$),
292 suggesting that the patterns of spatio-temporal variation were generally similar in both bacterial
293 and fungal community distributions among tested plots and seasons.

294

295 **Microbial taxonomic composition post-nitrogen addition**

296 Because N level factors had no interaction with soil depth and sampling season (Table 1), N
297 effects on microbial phylogenetic composition were assessed across both sampling depths and
298 seasons (Fig. 5). Generally, N fertilization caused significant differences in the recovered genus
299 level composition for prominent members of the bacterial/archaeal (6%) and the fungal (5%)
300 communities, respectively (relative abundance $> 0.01\%$) (Fig. 5). Specifically, for
301 bacterial/archaeal community composition, N input at 200 kg N ha⁻¹ significantly reduced the
302 relative abundance of *Salinibacterium* and *Pseudonocardia* (Actinobacteria), *Caldilinear*
303 (Chloroflexi), and *Desulfobulbus* (Proteobacteria), but increased *Sorangium* (Proteobacteria) (P
304 < 0.05), indicating that these taxonomic groups were significantly altered by the synthetic N
305 fertilizers. In the fungal community profiles, application of N fertilizers at 100 or 200 kg N ha⁻¹

306 significantly decreased the proportion of *Archaeorhizomyces* (Ascomycota), as well as
307 *Crepidotus* and *Uthatabasidium* (Basidiomycota) ($P < 0.05$).

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

319 In the fungal community, only 54% prominent genera (of relative abundance $> 0.1\%$)
320 showed a significant changes between two soil depths, in which members of the phyla of
321 Ascomycota, Chytridiomycota, and Glomeromycota were more prevalent in top soil layer of 0-5
322 cm ($P < 0.05$) (Fig. 7). Approximately 90% of the prominent fungal taxonomic groups classified
323 at the genus level (relative abundance $> 0.1\%$) significantly varied over sampling seasons ($P <$
324 0.05) (Fig. 7).

325 Venn diagrams were used to better visualize these changes of bacterial/archaeal and fungal
326 taxonomic groups affected by soil depth and sampling season (Fig. 8). In bacteria, there were
327 61% of significantly affected genera shared by two factors of soil depth and sampling season,
328 showing that most bacterial groups that differed between depths also responded to temporal

329 change. In fungi, many more fungal taxonomic groups significantly varied across the four
330 seasonal samples than depth difference (Fig. 8), indicating that temporal variation affected fungal
331 community composition more significantly than spatial variation.

332

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

334 Compared to the control plots, N fertilization at 100 and 200 kg N ha⁻¹ increased switchgrass
335 yields by 43% and 171%, respectively (Table 2). In addition, N inputs also significantly
336 increased plant N, but reduced relative C content and C/N ratios ($P < 0.05$) as measured at the
337 end of the growing season. The DistLM analysis showed that switchgrass yields were
338 significantly correlated with the community structure of bacteria/archaea and fungi, but
339 explained only a small portion of variation, *i.e.*, 2.6%, 1.2%, in bacterial/archaeal and fungal
340 profiles, respectively ($P < 0.01$) (Table 2), suggesting a small but significant correlation between
341 above-ground switchgrass growth and below-ground microbiomes through N fertilization.

342

343 **Discussion**

344 **Short-term N effects on microbial communities**

345 Long-term N input can alter microbial composition and diversity, mainly due to N-induced soil
346 acidification and fertility decline [10]. Many long-term studies have reported that N fertilization
347 not only reduces below-ground biodiversity but also shifts bacterial composition at the phylum
348 level, for groups such as Proteobacteria, Acidobacteria and Actinobacteria [44-48]. Field studies
349 focusing on short-term effects of N fertilization on microbial communities however are limited
350 in number for comparison. In our study, one-time fertilization did not affect the richness and
351 diversity of soil microbial communities, but caused structural changes in both bacterial/archaeal

352 and fungal community composition (Fig. 4). Our work suggests that some phylogenetic groups
353 of bacteria and fungi might quickly react to N inputs, even when soil properties are not
354 significantly modified by short-term N fertilization. These N effects were consistent across two
355 soil depths and four sampling seasons because there was no significant interaction between N
356 and depth/season (Table 1). We also observed that the one-time N amendment appeared to
357 directly repress some bacterial and fungal groups based on the negative relationship of relative
358 abundance with N levels, for example bacterial genus *Pseudonocardia*, and fungal genus
359 *Archaeorhizomyces* (Fig. 5).

360 *Pseudonocardia* is a common endophytic Actinomycete frequently isolated from host plant
361 tissues [49], which has been reported to achieve associative nitrogen fixation without the
362 formation of nodules [50] and protect their hosts against soil-borne pathogenic infection through
363 producing antibiotics or siderophores [51, 52]. As a free living diazotrophic Actinomycete, it has
364 also been reported to be prominent in nutrient limited environments [53, 54] or low-input agro-
365 ecosystems [55, 56], due to its low requirement for N. Based on sequencing of 16S rRNA genes,
366 it was also found that *Pseudonocardia* OTUs were reduced in the fertilized plant rhizosphere of
367 Canola (*Brassica napus*) [57]. Our results support that the relative abundances of
368 *Pseudonocardia* are significantly and negatively associated with N fertilization (Fig. 5),
369 suggesting that even short-term N inputs might acutely suppress this associative nitrogen fixer in
370 switchgrass cultivated lands.

371 The *Archaeorhizomyces* are an ancient class of ubiquitous soil fungi [58], which are neither
372 mycorrhizal nor pathogenic, but may be root endophytic or free-living saprophytes [59]. This
373 group was first discovered in tundra soils [60] using rRNA-based sequencing, but was only
374 isolated into culture more recently [58] and very little is definitively known about the physiology

375 and ecology of this group of organisms and this knowledge comes only from only one extant
376 isolate of the broad class of organisms. By investigating how organic matter accumulation and
377 forest fertility influences fungal community composition, it was found from ITS rRNA gene
378 analyses that *Archaeorhizomyces* dominated root-associated Ascomycetes and there abundance
379 significantly correlated with a fertility gradient in European boreal forests [61]. Moreover, it has
380 been shown that the relative abundance of *Archaeorhizomyces* in grasslands is greatly stimulated
381 by amendment of the biofertilizers *Trichoderma* [62] and correlations between soil properties
382 and fungal abundance suggested that soil P availability (rather than N) may be a controlling
383 factor for *Archaeorhizomyces* relative abundance. However, in our study, inorganic N
384 fertilization significantly reduced the relative abundance of *Archaeorhizomyces*, which was one
385 of the dominant groups of the in Ascomycota present in our study at 3.1-6.9% relative abundance
386 (Fig. 5). Further studies on the ecology of these diverse fungi are clearly needed through both
387 additional rRNA gene amplicon studies in natural systems, as well as the isolation of additional
388 representatives for ecophysiological analyses.

389

390 **Spatial heterogeneity in microbial communities**

391 Several studies have reported that soil bacterial and fungal diversity levels can either decrease
392 [23, 63, 64], remain unchanged [25, 65, 66] or increase [67] with soil depth. We consistently
393 observed reduced community richness and diversity in 5-15 cm compared to 0-5 cm soil layers
394 for both bacterial/archaeal and fungal communities (Fig. 3). Since plant residue serves as a key
395 carbon source for soil microbes, the vertical distribution of microbial communities is likely to
396 reflect the different available organic matter content with soil depths for microbial decomposers
397 [64]. For example, the surface soil may have more easily decomposable carbon directly derived

398 from crop residues, with more diverse groups of microbes able to access the labile organic
399 materials in this niche [68] whereas subsurface soils may harbor relatively more recalcitrant
400 carbon sources or be more dependent on root inputs. We also observed less soil C and N in 5-15
401 cm soil layers, further suggesting nutrient levels may be among the factors driving these depth
402 related patterns in diversity.

403 Compared to the small amount of community variation attributable to N addition, we
404 observed more significant shifts in both bacterial/archaeal and fungal community composition
405 between soil depths. Generally, Bacteroidetes, Planctomycetes, and Verrucomicrobia were more
406 abundant in the 0-5 cm soil layer. This spatial differentiation of the dominant bacterial groups by
407 soil depth was consistent to many previous studies. For example, it has been shown that bacterial
408 community composition was significantly altered at different soil depths, which was associated
409 primarily with a decline of Bacteroidetes with depth [23]. Others have also reported that
410 Verrucomicrobia exhibit higher relative abundance in the surface soils [25, 67]. In contrast, our
411 results showed that the 5-15 cm soil layer had greater abundance in the phyla of Chloroflexi,
412 Nitrospirae, and Proteobacteria. Similarly, it is also demonstrated that as soil depth increased, the
413 relative abundance of Proteobacteria increased and it became the dominant bacterial group in
414 subsoil [65]. Though the overall Proteobacteria were more abundant in 5-15 cm soils, the class
415 Betaproteobacteria was most abundant in 0-5 cm, which was also found in other study [69].
416 Similar to our results, others have also reported that Chloroflexi [66, 67] and Nitrospirae [70]
417 increase in abundance with soil depth.

418 In this study, we observed that fungal community also showed strong vertical distribution
419 patterns in the major groups, such as Ascomycota, Chytridiomycota, and Glomeromycota, which
420 were more abundant in top 0-5 cm soil layer; however, compared to bacterial community, there

421 were overall fewer fungal taxonomic groups that differed between soil depths (Fig. 6 and 7).
422 Several studies have highlighted the ecological significance of vertically distinct fungal
423 communities. For example, using pyrosequencing of ITS amplicon, others have found a decrease
424 in relative abundance of Ascomycota with increasing soil depth, whereas Zygomycota showed
425 the opposite trend [63]. At finer taxonomic scales, it was reported that Sordariomycetes of the
426 phylum Ascomycota decrease with soil depth [70], and this pattern was similar in our study.
427 Others have also shown overall fungal communities pattern were highly variable with soil depth,
428 where deeper soil have some distinct fungal groups, but significantly less overall diversity [64]
429 similar to what we observe here.

430

431 **Temporal variation in microbial communities**

432 We found significant temporal changes in alpha diversity in both bacterial and fungal
433 communities (Fig. 3). Similarly, it has been shown that bacterial community alpha diversity
434 varied more substantially than beta diversity over time, and in this case exceeded the variability
435 between land-use types [24]. Thus, it was suggested that temporal differences in rhizodeposition
436 may be a controlling factor to affect soil bacterial diversity. Our data show that seasonal
437 variation is found in most dominant phylogenetic groups of bacteria, such as Acidobacteria,
438 Actinobacteria, Bacteroidetes, Chloroflexi, and Verrucomicrobia (Fig. 6). Interestingly
439 Acidobacteria, Bacteroidetes, Betaproteobacteria, Deltaproteobacteria, Gammaproteobacteria,
440 and Verrucomicrobia all had similar seasonal patterns, which were opposite to those of
441 Actinobacteria, Chloroflexi, and Alphaproteobacteria. That dominant bacterial phyla, such as
442 Actinobacteria and Betaproteobacteria, shift with seasonally to temporal patterns has been
443 reported previously [65]. In addition, it has been shown that seasonal dynamics often appear to

444 be coherent within taxonomic lineages, in which Acidobacteria and Proteobacteria are more
445 prevalent in summer, whereas Actinobacteria and Chloroflexi increase in winter [71].

446 In our experiment, the fungal phyla Chytridiomycota, Glomeromycota, and Zygomycota,
447 were also found to vary significantly over the different seasonal sampling times (Fig. 7).
448 Similarly, it was reported that the number of fungal species belonging to Ascomycota and
449 Glomeromycota increase in summer, whereas Basidiomycota were dominant in winter [72] or
450 that Ascomycota, Basidiomycota, and Zygomycota are variable from spring to winter [73]. It has
451 been suggested that changes in litter decomposition and phytosynthate allocation contribute to
452 the seasonal variations of fungal community [74] as well as the direct effects of soil moisture and
453 temperature [24]. However, contrary to this we did not observe distinct seasonal changes in the
454 overall dominance patterns of Ascomycota or Basidiomycota in our study.

455

456 **Conclusions**

457 With the aid of high-throughput 16S rRNA gene and ITS region amplicon sequencing, we found
458 highly diverse and dynamic communities across this 8 year-old switchgrass field. The one-time
459 application of N fertilization significantly stimulated switchgrass growth and N uptake, and
460 subtly but significantly shifted below-ground bacterial and fungal communities, with the
461 bacterial genus *Pseudonocardia* and *Archaeorhizomyces* fungi negatively responsive to N inputs.
462 However, these shifts took place within the context of much larger spatial and temporal variation
463 in the microbial community. These large spatial and seasonal fluctuations in microbial
464 communities reinforce the importance of robust sampling designs and should caution against
465 overinterpretation of studies based on one-time sampling events. Further studies should aim at

466 studying the ecological and physiological mechanisms of responses to N fertilization by these
467 microbes and how these may influence ecosystem functions.

468

469 **Data Availability Statement**

470 The 16S rRNA gene and ITS region amplicon sequences were deposited on NCBI Sequence
471 Read Archive (SRA) database under the BioProject accession number of PRJNA512218. Soil
472 metagenome sequences were uploaded to Rapid Annotation using Subsystems Technology for
473 Metagenomes (MG-RAST; <http://metagenomics.anl.gov>) under project accession number
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475

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480

481 **Competing Interest**

482 Co-author Dafeng Hui is an Academic Editor for PLoSOne. This does not alter the authors'
483 adherence to all the PLoSOne policies on sharing data and materials.

484

485 **Author Contributions**

486 Conceived and designed the experiments: HC CWS. Performed the experiments: HC ZKY DY
487 RHM SJL DMK DH CWS. Analyzed the data: HC CWS. Contributed to the writing of the
488 manuscript: HC MAC RLH GW FEL CWS.

489

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702
703

704 **Table 1.** Three-factor Permanova results for differences in bacterial/archaeal and fungal
 705 community structure affected by three N fertilization levels (0, 100, and 200 kg N ha⁻¹), two soil
 706 depths (0-5 and 5-15 cm), and three sampling seasons following N inputs (Spring, Summer, and
 707 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						

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

712

713 **Table 2.** Above-ground biomass yields and plant C/N contents of switchgrass affected by N
714 fertilization levels as well as their association with community structure of bacteria/archaea and
715 fungi by marginal test of DistLM. Different letters within each column indicate significant
716 effects by N fertilization levels at $\alpha=0.05$. The *, **, and *** indicate significant DistLM
717 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%

718

719

720 **Figure captions**

721 **Fig. 1.** A Google map showing twenty four plots ($5\text{ m} \times 5\text{ m}$) of three N fertilization levels (0,
722 100, and 200 kg N ha^{-1}) with eight replicates based on a complete randomized design.

723 **Fig. 2.** Relative abundances of bacterial/archaeal and fungal dominant phyla (average
724 abundances $> 1\%$) affected by two soil depths (0-5 and 5-15 cm). Asterisks indicate significant
725 difference at $\alpha = 0.05$ between two soil depths.

726 **Fig. 3.** Box plots showing Chao1 richness and Shannon diversity of bacterial/archaeal and fungal
727 communities affected by two soil depths (0-5 and 5-15 cm) and four sampling seasons (Winter
728 2016, Spring, Summer, and Fall 2017). Sequence depths were 10000 for 16S and 5000 for ITS.
729 Asterisks indicate significant difference at $\alpha = 0.05$ between two soil depths or among four
730 sampling seasons.

731 **Fig. 4.** Non-metric multidimensional scaling (NMDS) analysis of bacterial/archaeal and fungal
732 communities affected by three N fertilization levels (0, 100, and 200 kg N ha^{-1}), two soil depth
733 (0-5 and 5-15 cm), and four sampling seasons (Winter 2016, Spring, Summer, and Fall 2017).
734 Permanova P values were also given.

735 **Fig. 5.** A heat map of relative abundance of bacterial/archaeal and fungal dominant taxonomic
736 groups at genus level (average abundances $> 0.1\%$) that were significantly affected by three N
737 fertilization levels (0, 100, and 200 kg N ha^{-1}). Asterisks indicate significant Spearman
738 correlations of taxonomic abundance with N fertilization levels at $\alpha = 0.05$.

739 **Fig. 6.** A heat map of relative abundance of bacterial/archaeal dominant taxonomic groups at
740 genus level (average abundances $> 0.1\%$) that were significantly variable between two soil
741 depths (0-5 and 5-15 cm) and over four sampling seasons (Winter 2016, Spring, Summer, and
742 Fall 2017). Asterisks indicate significant difference between two soil depths (0-5 and 5-15 cm) at

743 $\alpha = 0.05$. Number signs indicate significant difference over four sampling seasons (Winter 2016,
744 Spring, Summer, and Fall 2017) at $\alpha = 0.05$.

745 **Fig. 7.** A heat map of relative abundance of fungal dominant taxonomic groups at genus level
746 (average abundances $> 0.1\%$) that were significantly variable between two soil depths (0-5 and
747 5-15 cm) and over four sampling seasons (Winter 2016, Spring, Summer, and Fall 2017).

748 Asterisks indicate significant difference between two soil depths (0-5 and 5-15 cm) at $\alpha = 0.05$.

749 Number signs indicate significant difference over four sampling seasons (Winter 2016, Spring,
750 Summer, and Fall 2017) at $\alpha = 0.05$.

751 **Fig. 8.** Venn's diagrams showing significantly affected bacterial/archaeal and fungal dominant
752 taxonomic groups at genus level (average abundances $> 0.1\%$) shared between the factors of soil
753 depth and sampling season.

754 **Supporting Information**

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

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

763

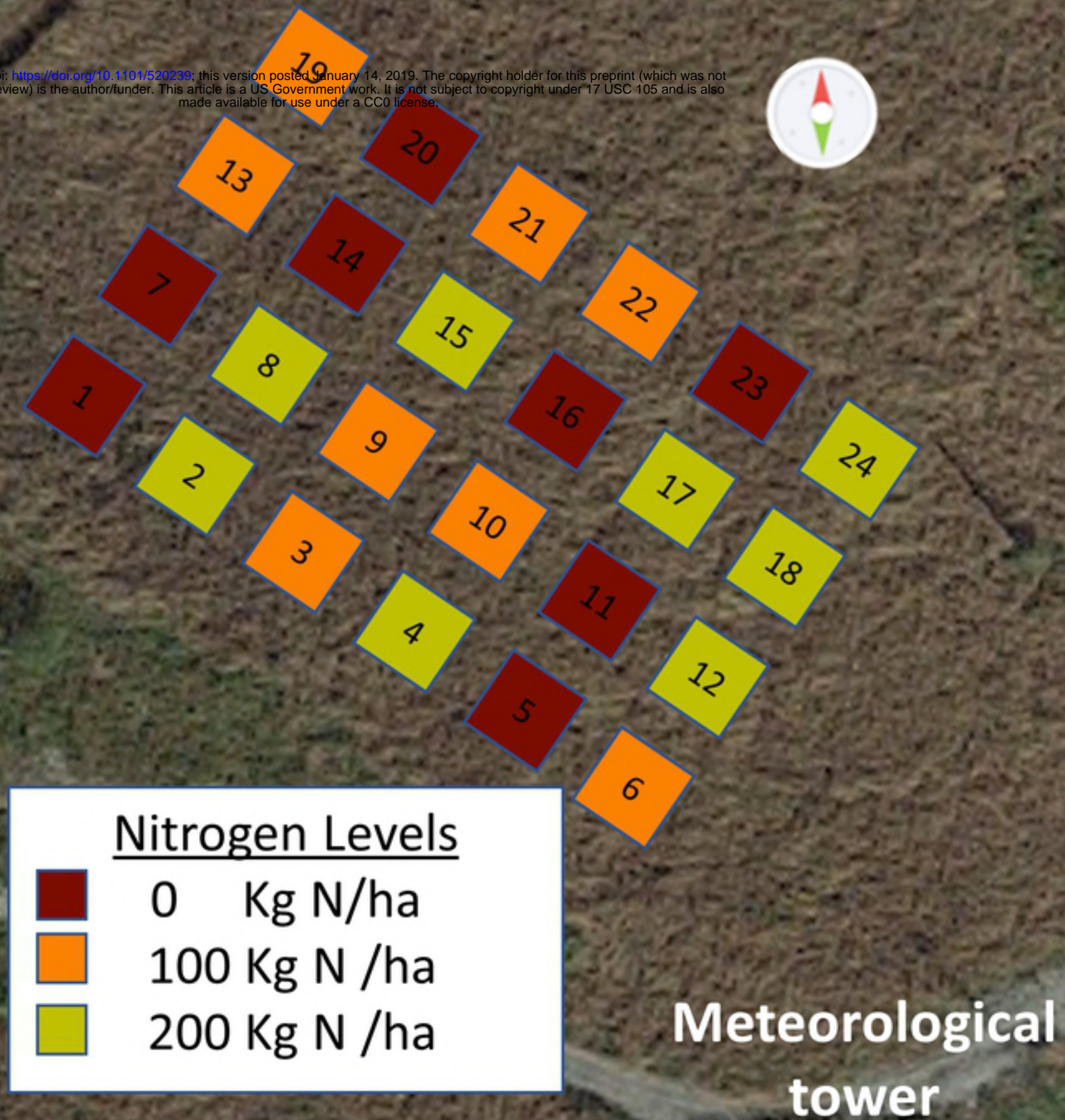


Figure 1

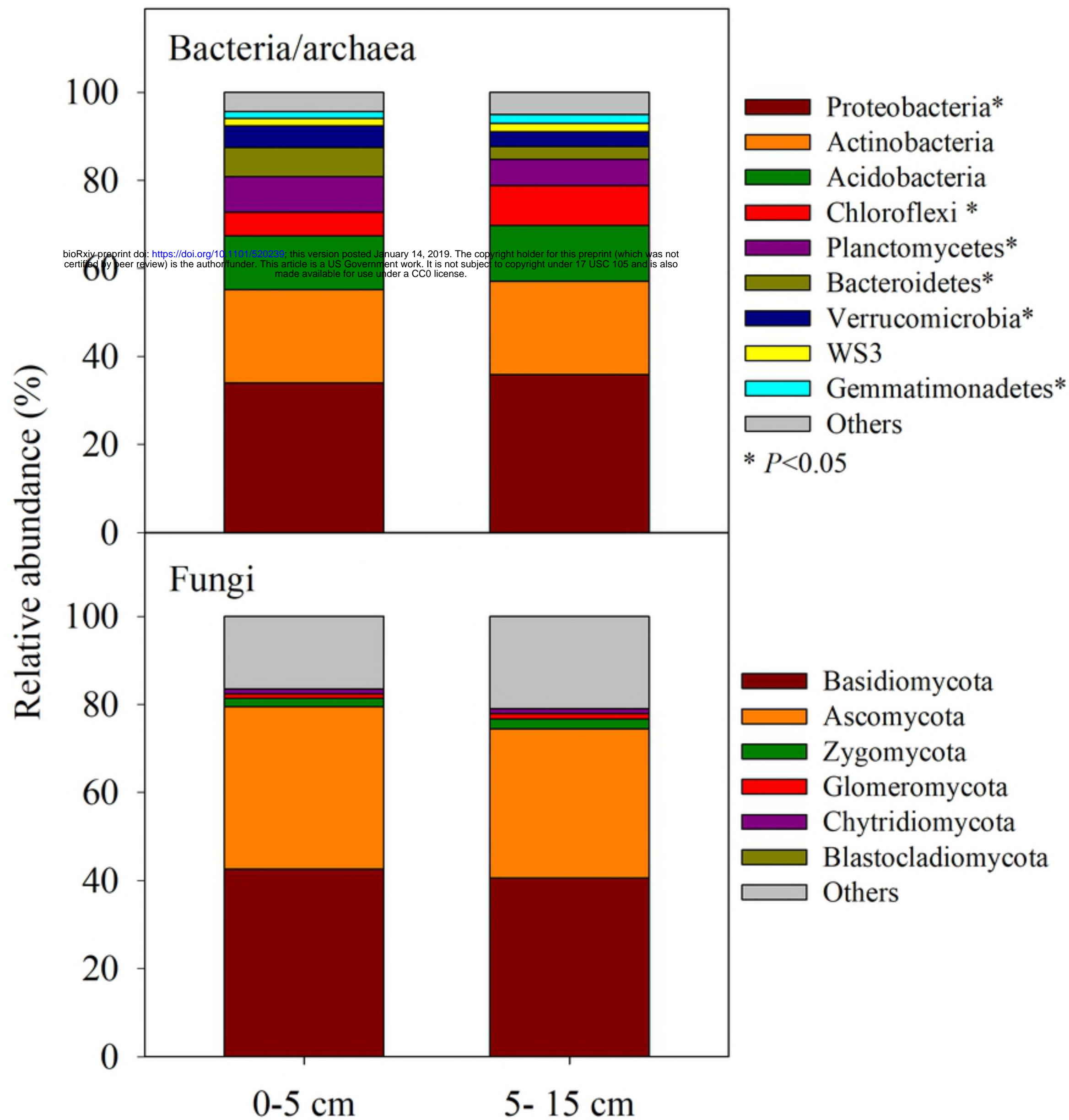


Figure 2

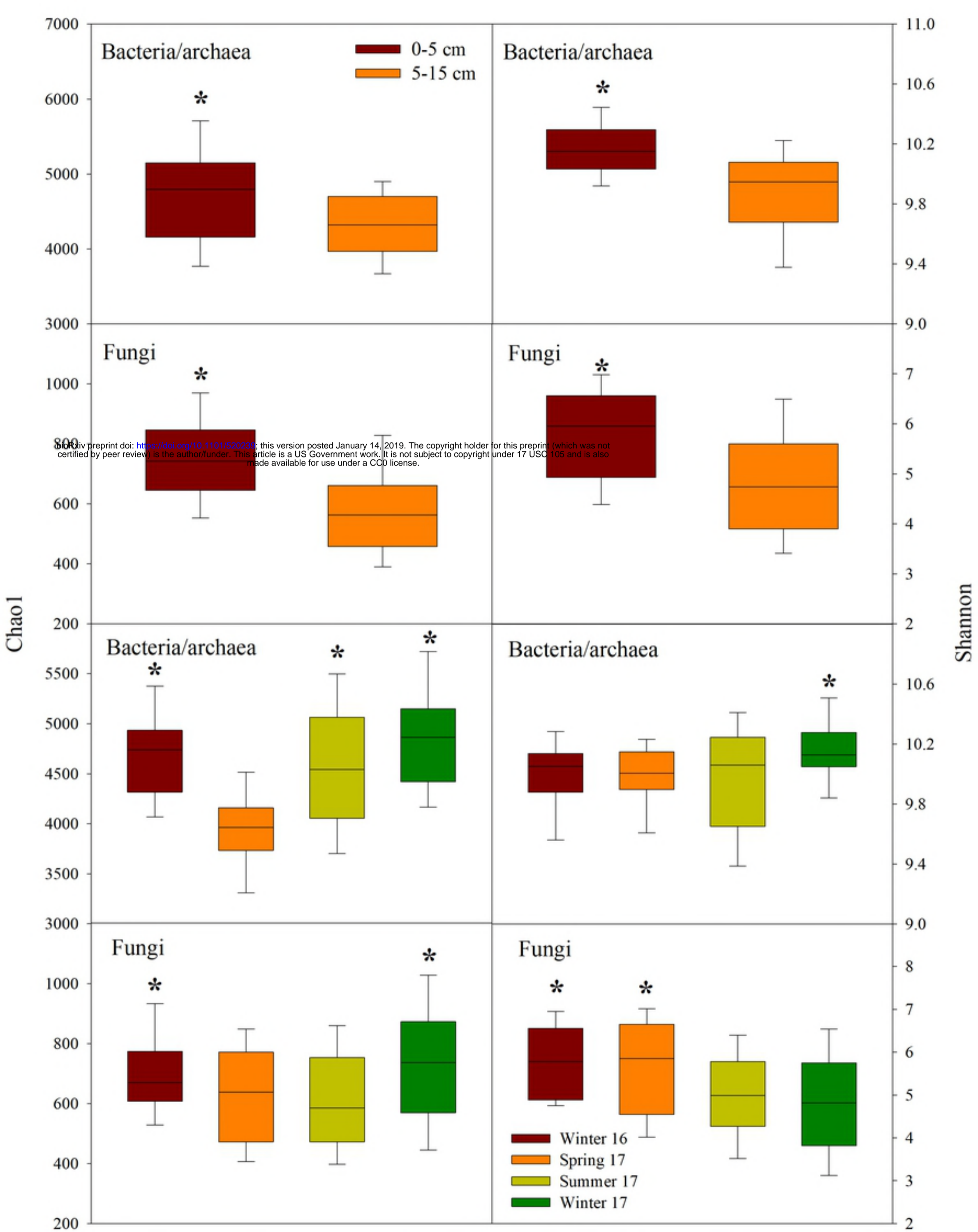


Figure 3

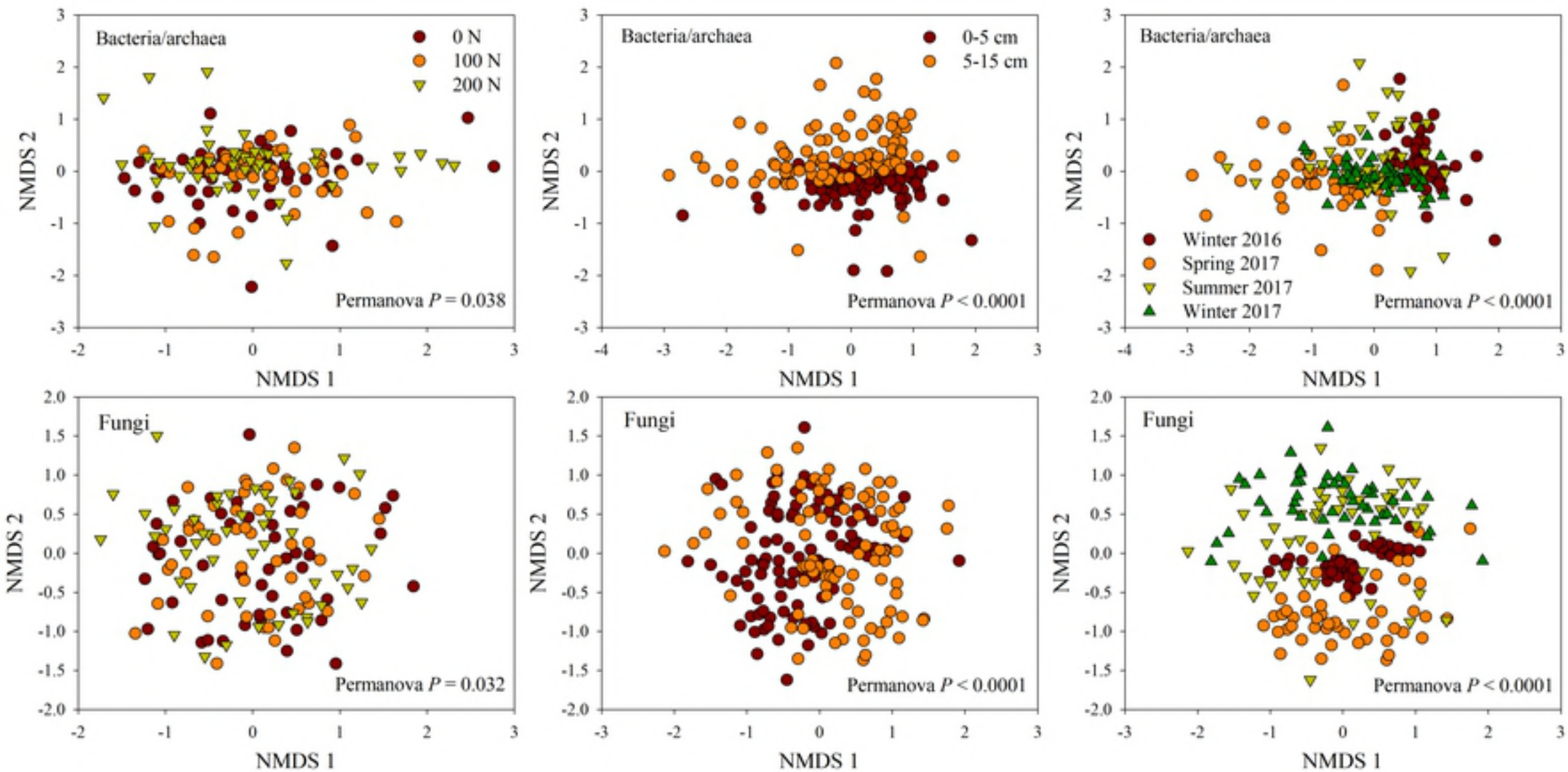


Figure 4

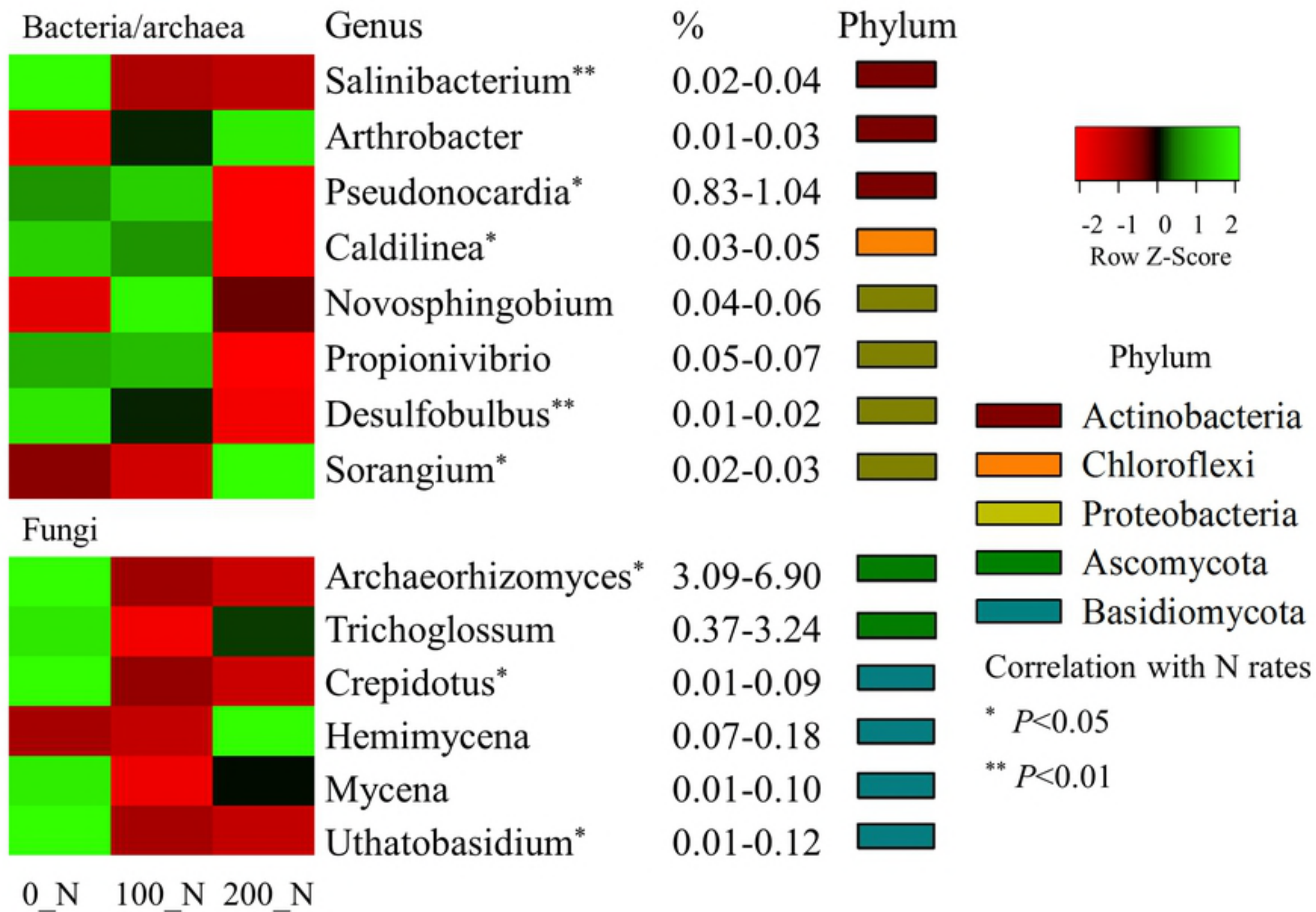


Figure 5

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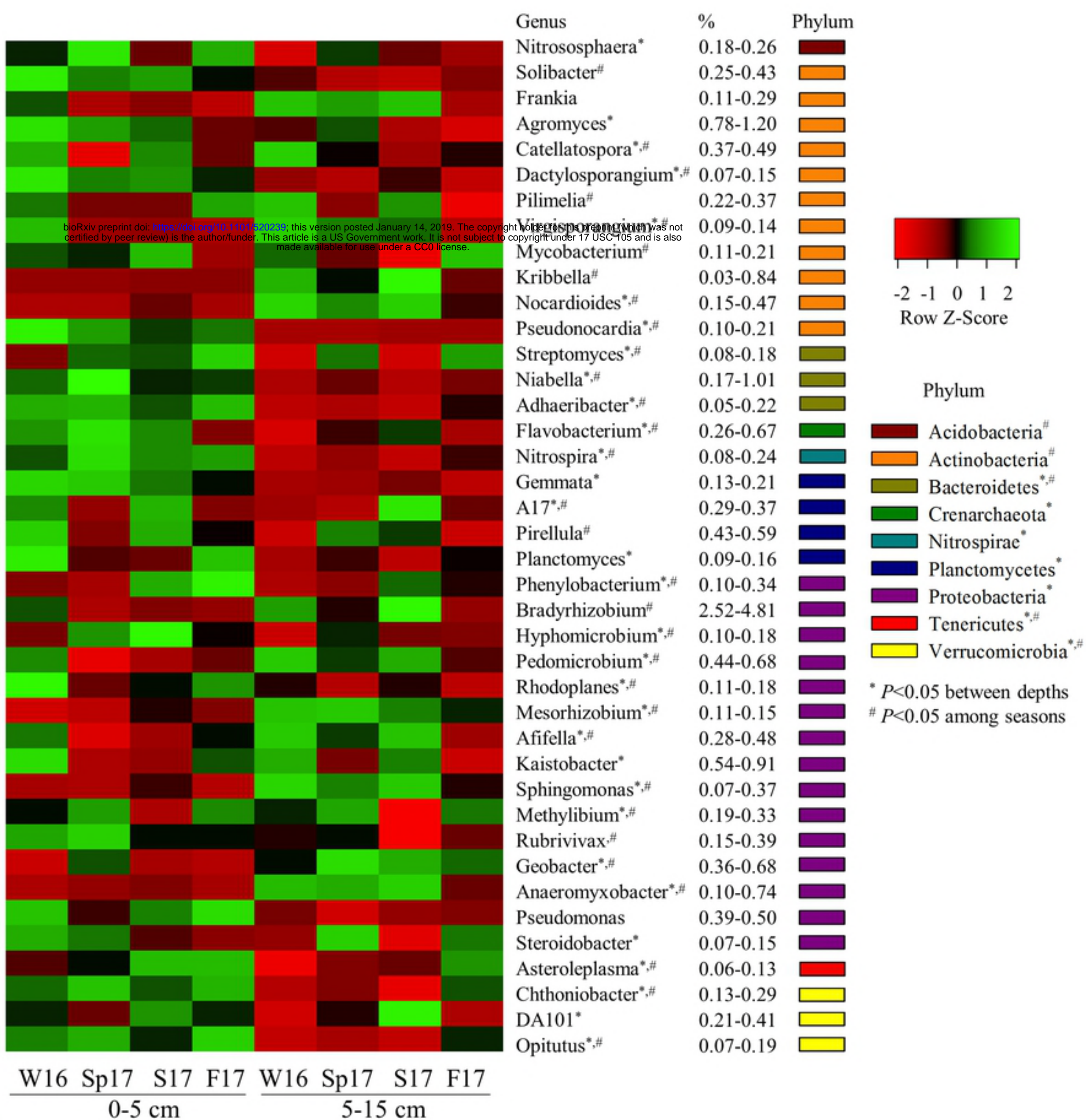


Figure 6

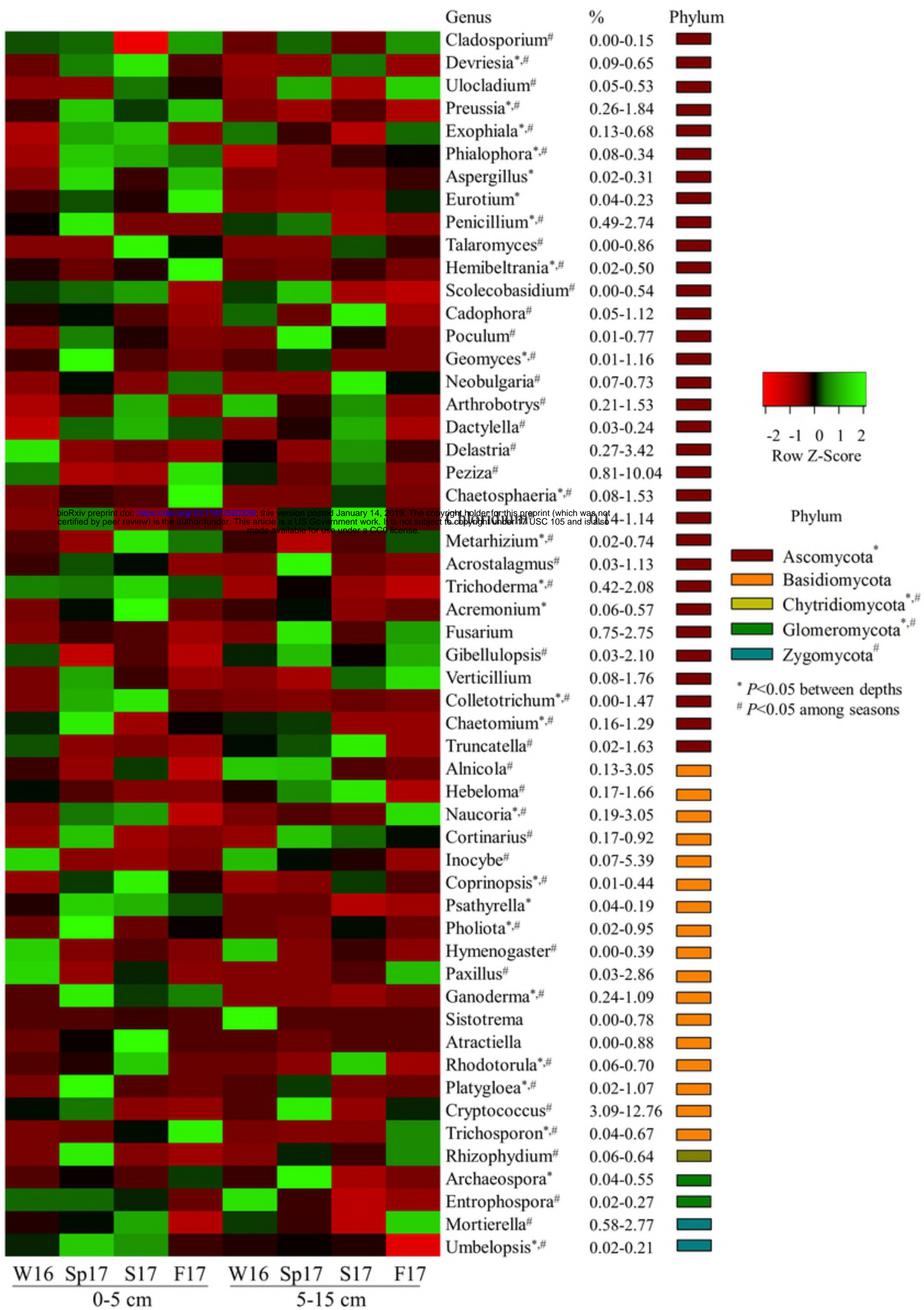


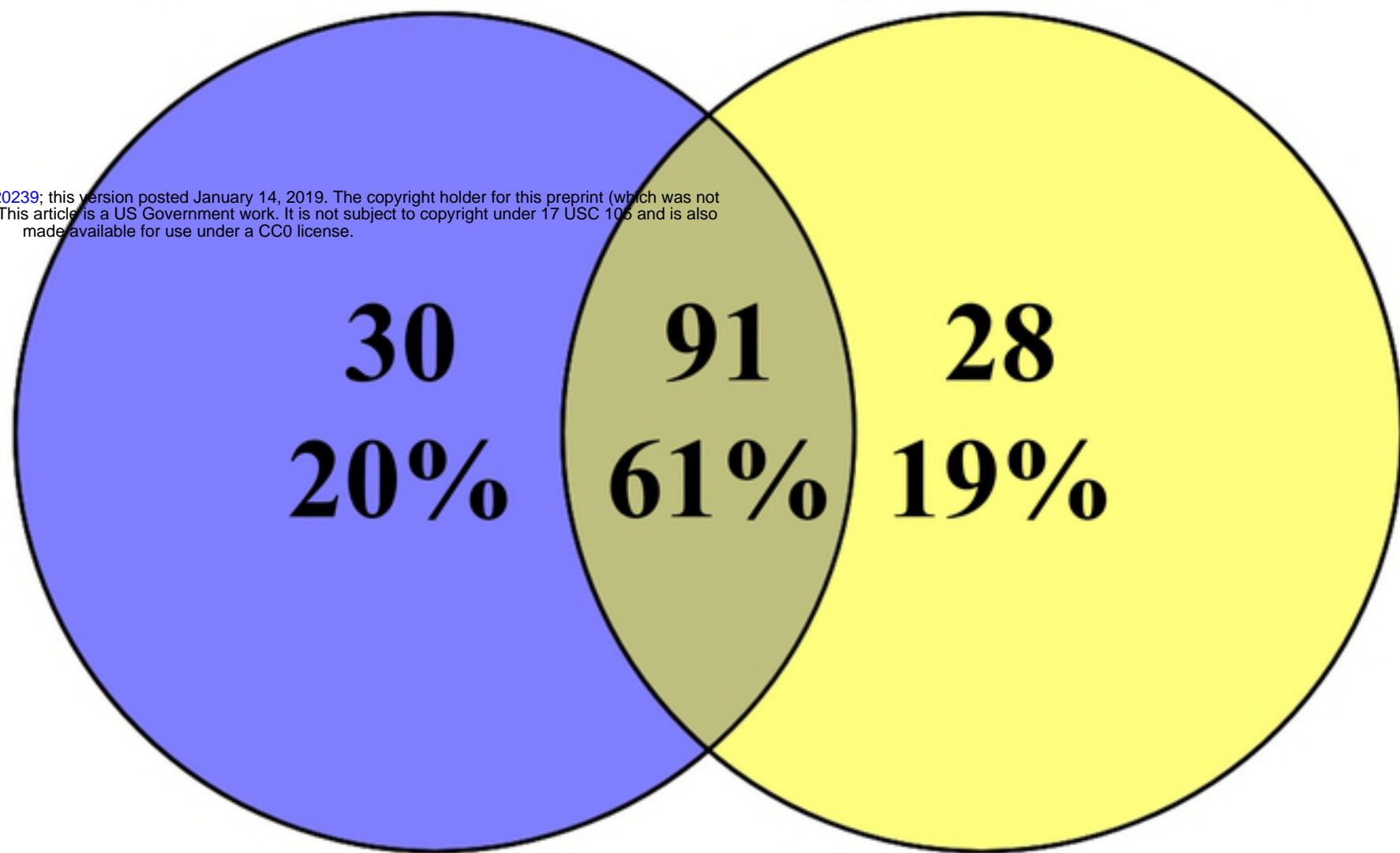
Figure 7

Soil Depth

Sampling Season

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Bacteria/ archaea



Fungi

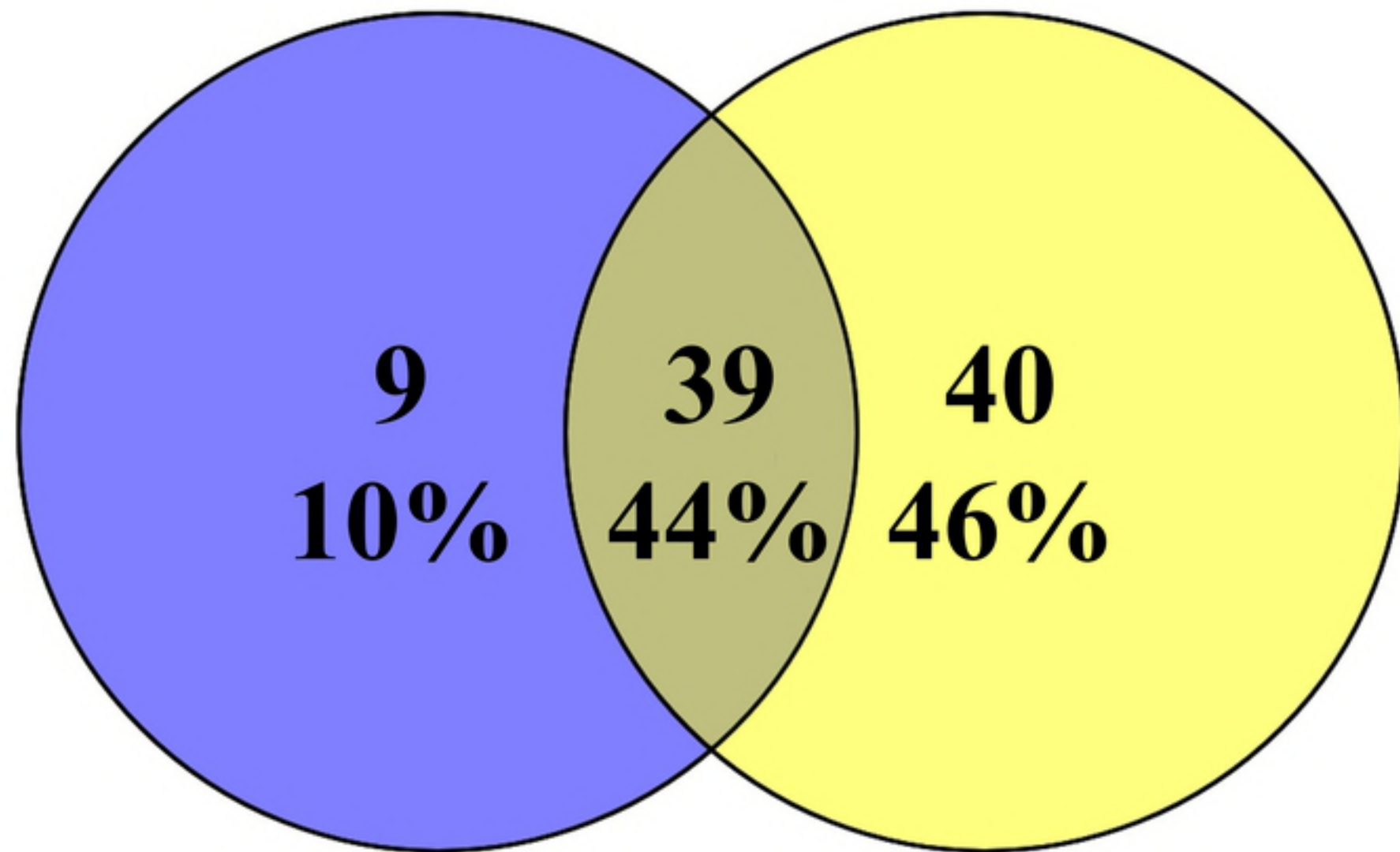


Figure 8