# Soil microbial communities in diverse agroecosystems exposed to glyphosate

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**Conflict of interest**

The authors declare they have no conflict of interest.

**Abstract**

In spite of glyphosate's wide use in agriculture, questions remain about effects of the herbicide on soil microbial communities. Conflicting scientific literature reports divergent results; from no observable effect of glyphosate to the enrichment of common agricultural pathogens such as *Fusarium*. We conducted a comprehensive field-based study to compare treatments that did and did not receive foliar application of glyphosate spray. The study included two field sites, Maryland and Mississippi; two crops, soybean and corn; four site years, 2013 and 2014; and a variety of organic and conventional farming systems. Using amplicon sequencing, the prokaryotic (16S rRNA) and fungal (ITS) communities were described along with chemical and physical properties of the soil. Sections of corn and soy roots were plated to screen for the presence of plant pathogens. Geography, farming system, and seasonal progression were significant factors determining composition of fungal and bacterial communities. Plots treated with or without glyphosate did not differ in overall microbial community composition after controlling for these factors. No differential effect of glyphosate treatment was found in the relative abundance of organisms such as *Fusarium* spp. or putative growth-promoting bacteria *Pseudomonas* spp.

## Introduction

Providing food for the exponentially growing global human population [1] requires agricultural productivity to double by the year 2050 [2]. Thirty six percent of the Earth's potential agricultural land is already under production, and much of the remaining land is considered marginal and susceptible to degradation when put under intensive management [3]. External inputs for nutritional supplementation and pest control are significant production costs and non-point sources of pollution that negatively impact human and environmental health. Thus, to continue feeding the world population, farmers need new approaches to increase agricultural productivity while simultaneously mitigating negative environmental impacts [2, 4].

Introduction of genetically modified glyphosate-resistant (GR) crops has transformed agroecosystems across the globe by increasing adoption of no-till agriculture where weeds are controlled chemically rather than by tillage [5]. Glyphosate interrupts the shikimate biosynthesis pathway [6], which is responsible for the production of aromatic amino acids and other key components of cell metabolism. The shikimate pathway is found in bacteria, fungi, algae, plants and some protozoans, although not in animals. Glyphosate competitively binds to the enzyme 5-enolpyruvylshikimate 3-phosphate synthase (EPSPS) and is known to be lethal to most species of plants and a large proportion fungi. However, some microbes are resistant to glyphosate due to rapid metabolism of glyphosate or to a GR form of EPSPS. Once this biosynthetic pathway is blocked, plants die due to metabolic disruption. Even at sub lethal application rates [7, 8] glyphosate can weaken a plant's hypersensitive response enough that a pathogen is able to infect and kill the plant. In the absence of a pathogen the plant may have a stunted appearance for a few weeks but then recover.

Plants have been shown to exude glyphosate from their roots within 24 hrs of foliar application [9]. Glyphosate strongly binds to some soil components, making it rather immobile in most soil types [10]. Its tight binding to soil contributes to its weak phytotoxicity to plants as a soil applied herbicide. The episodic exudation of glyphosate may have indirect effects on the soil microbial community, and these changes may be important to the long term sustainability of agroecosystems, but patterns or changes in the microbial community are difficult to detect in the context of seasonality, changing crop species, and geographic locations.

Concerns have been raised about increased pathogen loads and suppression of beneficial organisms associated with glyphosate use [11, 12]. There are two mechanisms by which glyphosate could enrich the soil for plant pathogens: 1) pathogens can attack glyphosate-susceptible weeds that succumb to the herbicide, the dying biomass of which then acts as refugia for subsequent crop infestation (green bridge) or 2) pathogens can gain a "foothold" in a glyphosate-resistant plant due to reduced immune response from alterations in the shikimate

76 pathway, resulting in a non-lethal infection that allows the pathogen to propagate. A review of all
77 GR crops by Hammerschmidt [13] determined there is no conclusive evidence that glyphosate
78 increases the susceptibility of GR crops to disease. Another review [14] challenges this
79 conclusion. For example, several studies have observed that GR beets and soybean have
80 increased susceptibility to pathogens when glyphosate is applied at recommended rates [14–
81 16]. One study found no effect of glyphosate on disease induction in GR beets until rates
82 exceeded normal field application rates by one order of magnitude [17]. However, other studies
83 with GR crops have found no influence of glyphosate on disease [18], as well as instances of
84 fungicidal activity against plant pathogens, especially rusts (reviewed by Duke [19]).
85 Two key studies have substantiated the glyphosate-pathogen-enrichment hypothesis,
86 finding over long study periods that glyphosate repeatedly increases the rate of colonization of
87 crops by *Fusarium* (presumed to be a pathogenic strain), while decreasing the abundance of
88 fluorescent *Pseudomonas* bacteria (taken as putative beneficial organisms) in the soil [11, 16].
89 These studies are often cited as conclusive evidence that long-term use of glyphosate increases
90 the pathogen load and decreases the abundance of growth promoting bacteria in soils. Both
91 studies applied culture-based methodology to quantify these microbial groups, with molecular
92 analysis of the ribosomal internal transcribed spacer region (ITS) for fungi; however, the
93 identification techniques employed were not sufficiently discriminatory to distinguish pathogenic
94 and beneficial genotypes for either group. Studies using culture-free methodology to
95 characterize microbial communities have failed to detect substantial glyphosate effects on
96 pathogen abundance [20, 21]. The key to conclusive determination of glyphosate effect on
97 microbial communities of GR crops is to carefully compare glyphosate sprayed and non-sprayed
98 treatments within an agronomic context. Farming systems, soil factors, crop varieties,
99 glyphosate legacy and application rates can all impact the behavior of glyphosate and its
100 interaction with the crop and soil microbiome [22].

101 We conducted a field-scale study to observe the effects of glyphosate on the soil
102 microbiome and plant health for corn and soybean GR varieties. Specifically, we tested the
103 hypothesis glyphosate changes the composition of the soil microbiome when controlling for
104 differences in soils, seasonal time points, and farming systems. Furthermore, we tested the
105 hypothesis that *Fusarium* spp. sequence abundance or culturable numbers would increase due
106 to glyphosate treatment. Our study includes six farming systems and a total of 12 site years,
107 representing agricultural practices as implemented on working farms. Our study targeted both
108 naïve soil microbiomes that have not been exposed to glyphosate and those exposed to
109 glyphosate annually. High throughput sequencing was used to generate bacterial and archaeal
110 16S rRNA profiles and fungal ITS profiles.

111 **Materials and Methods**

112 **Description of field sites and experimental design:** The study was conducted for two years
113 at two United States Department of Agriculture, Agricultural Research Service (USDA-ARS)
114 sites: the Sustainable Agricultural Systems Laboratory, Beltsville, MD and the Crop Production
115 Systems Research Unit, Stoneville, MS.

116 The Beltsville site is managed as part of a USDA-Long Term Agricultural Research site
117 typical of the mid-Atlantic region and described previously [23, 24]. We conducted the study in
118 two conventional farming systems include one using a chisel plow for primary tillage (CT) and
119 one under no-tillage management (NT). These two systems rely on mineral fertilizers,
120 herbicides, and other pesticides as needed to manage a corn-rye cover crop-soybean-
121 wheat/soybean rotation. One organic farming system is a three-year corn (*Zea maize*) -rye
122 (*Cereale secale*) cover crop -soybean (*Glycine max*) -wheat (*Triticum aestivum*)/legume (*Vicia*
123 *villosa*) rotation (Org3). The second organic farming system is a six-year crop rotation (Org6) in
124 which alfalfa (*Medicago sativa*), a perennial crop in place for three years of the rotation,
125 replaces the vetch present in Org3. The organic systems rely on legumes, poultry litter and
126 $K_2SO_4$ to supply crop nutrients in accordance to soil test results and local regulations. A

127 moldboard plow and/or a chisel plow is used for primary tillage in the organic systems. Weed
128 control in the organic systems included use of a rotary hoe and between row cultivation after
129 crops were planted.

130 In Stoneville the experiment was conducted in two adjacent fields, one with a legacy of
131 glyphosate use, the other with no glyphosate history. The field with a history of glyphosate use
132 had GR soybean and cotton (*Gossypium hirsutum*) grown in rotation for the last 15 years prior
133 to the experiment. The field without glyphosate history had been maintained for weed biology
134 studies in a cogongrass [*Imperata cylindrica* (L.) Beauv.] monoculture with no herbicides applied
135 for 12 years prior to the experiment. Field preparation included killing the cogongrass with
136 repeated tillage, planting non-GR soybean and non-GR corn for one season prior to the current
137 field experiment, and flail mowing at maturity.

138 The experiment was conducted during both the corn and soybean phases of crop
139 rotations at both sites. At each site the following glyphosate treatments were established: a GR
140 cultivar with no glyphosate applied and the same GR cultivar with glyphosate applied at 0.87 kg
141 $ha^{-1}$ twice at 5 and 7 weeks after planting. Each plot was four rows (4.6 m) wide and 6.1 m
142 long. Soybean cultivar USG Allen (GR) was planted at 350,000 seeds $ha^{-1}$ and the corn cultivar
143 DKC 65-17 RR2 (GR) was planted at 30,000 seeds $ha^{-1}$. In Beltsville the corn or soybean plots
144 are each a phase of the main plot rotation which is farming system (NT-18yr, CT-18yr, Org3-
145 none or Org6-none) in this experimental design each phase of the rotation is considered a split-
146 plot of the main plot which is cropping system. At both sites four replicates of each factor and
147 level were established. All plots were hoed by hand periodically throughout the season to keep
148 them weed-free.

149 In October of each year, corn was harvested with an Almaco small plot combine
150 (Almaco, Nevada, IA); grain yield was estimated at 15.5% moisture from the two center rows of
151 the 6.1 m plots. In 2013 the soybean was harvested with a Almaco small plot combine and in

2014 the soybean were hand harvested and threshed from 3.05 m of the two center rows. Dry weights were calculated at 13.5% moisture.

**Soil Baseline Characteristics**

Beltsville soils are Coastal Plain silty loam Ultisols, consisting primarily of Christiana, Keyport, Matapeake and Mattapex soil map units. The soils at the Stoneville site were a silt loam typic Endoqualfs dominated by Dundee soil map units. At planting, soil samples from the top 15-cm depth were collected from each plot by combining soil from six or more cores (7.5 cm diameter and 15 cm depth) sampled in a semi-random pattern in a given plot. Samples were air-dried and sieved to 2 mm. The cores were collected on a diagonal line between the second and third crop rows, 3 m from each end of a given plot. Soil samples were analyzed by the Agricultural Analytical Services Laboratory at Pennsylvania State University for: pH, organic matter (OM), CEC, P, K, Mg, Ca, S, B, Zn, Mn, Fe, Cu, As, Al, Ba, Cd, Co, Cr, Ni, Pb, Se, and Sr. pH was determined in a 1:1 water dilution, OM was determined by mass loss on combustion and CEC was determined using the methods of Ross, D. and Q. Ketterings [25]. Mehlich 3 extractions were used to quantify Ca, Mg, and K; all other metals are expressed as total sorbed using the EPA 3050 method [26].

**Rhizosphere Soil and Root Sampling**

At the V3 to V4 crop growth stage (4 to 6 weeks after planting) and one day prior to glyphosate application six plants and root-associated soil were excavated from each plot by removing soil monoliths with 15 cm radius from stem and 15 cm deep using surface sterilized sharpshooter shovels. This time point is referred to as “PRE-spray.” Soil monoliths were placed on a sieve and soil around the root ball was gently removed by shaking and passed through a 2 mm sieve; this soil was considered bulk soil. Soil adhering to roots after this procedure (considered rhizosphere soil) was brushed onto a 2 mm sieve using a camel hair brush. Roots were brushed thoroughly, yet not so the integrity of the root surface was compromised. The rhizosphere samples from the six plants were pooled and 5 g were added to a 15 ml falcon tube

178 containing 10 ml of MoBio LifeGuard nucleic acid preservation solution. The contents of the
179 tubes were mixed and frozen at -80 °C. Plants were placed at 4 °C until processed further.
180 Approximately twenty days after glyphosate was applied to the GR corn and soybean
181 plots (at growth stage R2 to R3) the soil monolith sampling was repeated in the same plots.
182 Samples were labeled "POST-spray." Roots and adhering soils were collected and processed
183 the same as for PRE-spray samples. At each site, sampling was determined by the
184 developmental stage of the crop plants and was not constrained by Julian calendar dates.

185 **Identification of Endophytes from Roots**

186 Two-centimeter sections of root were cut at random sixteen times from each of six fresh
187 root systems for each treatment. The total wet weight of the 16 sections was recorded. Sections
188 were surface sterilized for 2 minutes in 1.25% sodium hypochlorite, followed by three rinses in
189 sterile distilled water. Sections were blotted dry on sterile paper towel and eight root sections
190 were placed on a plate containing Komada's Medium [27]. Plated roots were incubated in
191 ambient light at room temperature until colonies emerged. Fungal mycelium and spores from
192 emerging colonies were sampled and examined on a Nikon E60 microscope and identified to
193 genus, or to broader morphological group, based on taxonomic features. Colonies of typical
194 morphology were plated onto minimal media to induce sporulation for further identification.
195 Colonies not producing spores were characterized as "non-sporulating." Polymerase chain
196 reaction (PCR) screens for ITS followed by cloning and sequencing were conducted on over
197 384 colonies of typical morphology to validate microscopic identification. The methods followed
198 those described in (Chung et al. 2008). Sequences were quality checked and aligned using the
199 DNAStar suite of software (DNAStar, Madison, WI, USA), and identified using the basic local
200 alignment search tool and GenBank nucleotide data bank from the National Center for
201 Biotechnology Information, Bethesda, MD, USA (http://www.ncbi.nlm.nih.gov/. Accessed fall,
202 2014).

203 **Illumina Sequencing Library Preparation from Rhizosphere Soils**

204 Rhizosphere and bulk soils preserved in LifeGuard at -80 °C were thawed and 800 µl of
205 each slurry was processed using a PowerSoil-htp 96 Well Soil DNA Isolation Kit (MoBio
206 Laboratories Inc, Solana Beach, CA) according to the manufacturer's recommendations. DNA
207 was quantified and quality verified using a NanoDrop 2000 spectrophotometer (Thermo Fisher
208 Scientific, Pittsburgh, PA). 16S metagenome sequencing was conducted according to the
209 Illumina protocol Library Preparation Manual Part# 1504423 Rev. B, Illumina Inc.,
210 www.illumina.com. Five µl of cleaned adapter amplicon product for each sample were used for
211 index PCR using the Nextera XT Index Kit (Part# FC-131-1002; 16S Metagenomic Sequencing
212 Library Preparation Manual Part# 1504423 Rev. B, Illumina Inc., www.illumina.com). Index PCR
213 products were cleaned according to the Illumina protocol (16S Metagenomic Sequencing
214 Library Preparation Manual Part# 1504423 Rev. B, Illumina Inc., www.illumina.com), and 2 µl
215 aliquots per sample from each 96-well PCR plate were pooled for the final Illumina library. For
216 analysis, one-hundred µl of 10 nM solutions of each library pool were frozen and shipped on dry
217 ice for analysis on an Illumina MiSeq system at the Center for Genome Research and
218 Bioinformatics (CGRB), Oregon State University, Corvallis, OR. For the Beltsville site, where a
219 total of four cropping systems were also sampled, a total of 512 samples were sequenced. For
220 the Stoneville site 256 samples were sequenced.

221 **Bioinformatics and Statistical Analysis**

222 *Sequence filtering and trimming*. Reads were returned from CGRB after initial quality
223 control with standard Illumina workflows, including quality filtering and adapter trimming. Raw
224 data is available via the AgData commons NUMBER. Scripts used in subsequent steps can be
225 found at "https://github.com/rmkepler/FSP_script_repository". Prior to joining paired ends and
226 taxonomy assignment, forward and reverse primers were removed and sequences quality
227 trimmed (-q 22) at the 3-prime end using Cutadapt (version 1.8.3). Reads lacking primer
228 sequences or shorter than 75 bp before trimming were discarded.

229 *Assembly and taxonomy assignment.* The R package Dada2 [28] was used for paired
230 end assembly and taxonomy assignment. The command “filterandtrim” was used to remove
231 sequences with an expected error rate greater than two, and any sequences containing “N”
232 values (unreadable bases). Error rates were estimated for forward and reverse reads. Filtered
233 reads were then dereplicated with the “derepFastq” command. Dereplicated sequences were
234 denoised with the “dada” command and then paired ends were merged. Chimeric sequences
235 were removed with the command “removeBimeraDenovo”. Taxonomy was assigned to the
236 chimera-free table of sequences with the dada2 implementation of the RDP classifier [29]. The
237 UNITE database (version 7.2) [30] was used as the reference for identification of fungal ITS
238 sequence variants, and silva (release 132) [31] was used for prokaryotes.
239 *Community analysis.* We transformed community count data into relative abundance,
240 then calculated Bray-Curtis dissimilarity. Principal components analysis (PCA) was applied to
241 the Bray-Curtis dissimilarity matrix using the ordinate function of the vegan package v. 2.4 [32]
242 as implemented in phyloseq v. 1.22.2 [33] for both fungal and prokaryotic barcodes.
243 After subsetting by crop and site, richness and evenness were estimated from rarefied
244 datasets of the raw sequence counts using vegan. DESeq2 v. 1.18.1 [34] was used to produce
245 variance stabilized datasets [35]. Bray-Curtis dissimilarity for each sample was used for PCA.
246 We used PERMANOVA to determine significance of main effects and interactions between the
247 following factors: farming system, soil zone, glyphosate treatment, sampling date, and year. The
248 farming system factor had 4 categories for Beltsville (CT, NT, Org3 and Org6) and 2 for
249 Stoneville (NT_none, NT_15yr). All other factors had two categories at both sites: soil zone
250 (bulk and rhizosphere); year (2013, 2014); glyphosate treatment (spray, no spray); sampling
251 date (PRE glyphosate application, and POST glyphosate application). A repeated measures
252 model based on the plot ID was used.
253 The effect of glyphosate treatment on microbial communities was tested with the
254 Wilcoxon signed-rank test of differences between dates as implemented in the longitudinal plug-

255 in for Qiime2 [36]. The test was applied separately for three measures of richness: observed,
256 Shannon's and Simpson's.

257 *Differentially abundant taxa.* Tests for differentially abundant taxa in response to
258 glyphosate treatment were conducted in DESeq2 using likelihood ratio tests after subsetting
259 fungal and prokaryotic data by site, crop, and farming system. The test compared a full model
260 including group, sampling date terms, as well as an interaction term, where group is defined as
261 the combination of farming system and glyphosate treatment (e.g. Org3_spray) and sampling
262 date corresponds to PRE-spray and POST-spray sampling events. The full model was
263 compared to a reduced model lacking the interaction term. Thus, significant interaction terms
264 would indicate sampling date and glyphosate application interacted to be important predictors of
265 microbial abundance. This was tested for every fungal and bacterial taxon identified. Datasets
266 with untransformed counts were used as the starting data, which were then variance-stabilized
267 during testing.

268 **Results**

269 From sequencing analysis a total of 68,964 unique fungal and 72,454 unique prokaryotic
270 sequence variants were identified across all samples. Beltsville and Stoneville shared 13,964
271 bacterial and 5,740 fungal taxa. Stoneville featured 62,985 and 29,780 bacterial and fungal
272 taxa, respectively. Beltsville featured 41,538 and 44,924 unique bacterial and fungal taxa,
273 respectively. Fungal richness was higher for all Beltsville farming systems, compared with
274 Stoneville, with the exception of the Shannon's and Simpson's diversity metrics for Org_3
275 (Figure 1A). Conversely, prokaryotic diversity was greater for Stoneville in all measures (Figure
276 1B).

277 Principal component analysis showed that Beltsville and Stoneville communities were
278 distinct (Figure 1C & D). Permanova analysis of relative abundance for fungi and prokaryotes
279 revealed that site was the most significant factor accounting for Bray-Curtis dissimilarity
280 distances in fungi and prokaryotes ($p$ = 0.001 in both cases. Fungal $R^2$ = 0.19, Prokaryote $R^2$ =

281 0.16 ; supplemental data). Differences between the Stoneville and Beltsville microbial
282 communities were driven by differences in edaphic factors. Soil chemical characteristics differed
283 between the two sites (Canonical Discrimination Analysis, p<0.001, $R^2$ = 0.99), and between
284 cropping systems (Canonical Discrimination Analysis p<0.001, $R^2$ = 0.99). Soil in Stoneville was
285 significantly higher in pH and the cations Arsenic (As), Barium (Ba) and Strontium (Anova,
286 p<0.001), whereas Beltsville soil contained significantly more Phosphorous (P), Lead (Pb),
287 Sulfur (S), Cromium (Cr), Iron (Fe) and OM (Anova, p<0.001) (Figure 1E). In order to increase
288 power to detect local effects of glyphosate treatment, we analyzed sites and crop treatments
289 separately.

290 Farming system was the largest driver of fungal community structure regardless of crop
291 (Figure 2 & 3) in both Beltsville (Permanova; corn: $p$ = 0.001, $R^2$ = 0.16; soybean; $p$ = 0.001, $R^2$
292 = 0.16) and Stoneville (Permanova; corn: $p$ = 0.001, $R^2$ = 0.24; soybean; $p$ = 0.001, $R^2$ = 0.23).
293 Year of sampling was also significant but explained less variance in both Beltsville (corn: $p$ =
294 0.001, $R^2$ = 0.046; soybean; $p$ = 0.001, $R^2$ = 0.043) and Stoneville (corn: $p$ = 0.001, $R^2$ = 0.051;
295 soybean; $p$ = 0.001, $R^2$ = 0.052). No significant interaction was noted between sampling date
296 and glyphosate treatment ($p$ = 0.488 and 0.296 for corn and soybean, respectively).
297 Rhizosphere and bulk soil samples were also not significantly different (Supplemental data) for
298 any crop or site. Likelihood ratio tests of taxon abundance in DESeq2 also confirmed no
299 glyphosate treatment; the sampling date-glyphosate treatment interaction did not significantly
300 increase the explanatory power of the model for any taxon (supplementary data), regardless of
301 crop or farming system.

302 Farming system was also a driver of prokaryote community structure in Beltsville
303 (Permanova; corn: $p$ = 0.001, $R^2$ = 0.096; soybean; $p$ = 0.001, $R^2$ = 0.09) and Stoneville
304 (Permanova; corn: $p$ = 0.001, $R^2$ = 0.21; soybean; $p$ = 0.001, $R^2$ = 0.16).Farming explained less
305 variation in Beltsville prokaryotic communities (Figure 2), than in Stoneville (Figure 3). The year
306 term explained a lesser amount of variance for Beltsville (corn: $p$ = 0.001, $R^2$ = 0.096; soybean;

307 $p = 0.001$, $R^2 = 0.086$) and Stoneville (corn: $p = 0.001$, $R^2 = 0.051$; soybean; $p = 0.001$, $R^2 =$
308 0.069). The interaction between glyphosate with sampling date was not significant for either
309 crop (Supplemental data). Likelihood ratio tests of taxon abundance in DESeq2 also confirmed
310 no glyphosate treatment; the sampling date-glyphosate treatment interaction did not significantly
311 increase the explanatory power of the model for any taxon (supplementary data), regardless of
312 crop or farming system (supplementary data).

313 Wilcoxon rank sum tests showed several instances where species richness differed
314 significantly between the PRE and POST sampling dates (Figure 4, Supplemental Data);
315 however, differences were observed in both glyphosate and no-glyphosate treatments,
316 indicating this is a seasonality effect, and not due to glyphosate exposure. In Beltsville, corn and
317 soybean differed in their response over the two dates. Prokaryote richness for corn in every
318 Beltsville farming system was significantly different between the two dates. This trend was also
319 observed, but to a lesser degree in fungal communities, with half of the treatments differing
320 significantly for both spray and no spray treatments. Fungal communities did not differ
321 seasonally in the Beltsville soybean plots, and fungal species richness was unaffected by
322 sampling date for both corn and soybean in the Stoneville samples.

323 The root endophyte screening required analysis of over 6100 root segments and
324 identified over 2400 fungal colonies. Significantly more colony forming units (CFU)s were
325 observed in 2013 than in 2014 at the Beltsville site ($p<0.0003$), but no differences in the number
326 of CFUs were observed between years at the Stoneville site. A total of 384 of the typical
327 morphotypes were ITS amplicon sequenced, resulting in 11 identified dominant taxa: *Fusarium*
328 *sp*., *Macrophomina sp.*, *Alternaria sp.*, *Cladosporium sp*., *Penicillium sp.*, *Zygomycota sp*.,
329 *Trichoderma sp*., and *Epicoccum sp*. There was no significant difference in abundance of
330 *Fusarium sp*. or the other taxa in the glyphosate sprayed and unsprayed plots, regardless of
331 site, crop, or year ($p>0.07$) (Figure 4b).

There was no significant difference in corn yield among systems or among glyphosate application treatments for either 2013 or 2014 (Table 1). Corn yields were not significantly different from the county averages for all systems with a mean among systems of 9339.4 kg/ha. In 2013 an error occurred while using the small plot combine and beans harvested from different replicates were mixed, rendering the data unusable. In 2014 soybean yields were similar to the county averages with a mean of 2326.5 kg/ha. There was no significant difference in yield across farming systems, and no effect of glyphosate treatment on yield (Table 1)

**Discussion**

The structure of prokaryote and fungal communities among farming systems and between sampling dates were not driven by glyphosate use. Instead, tillage and carbon inputs appear to be the primary drivers of soil microbiome structure. For instance, even though the Beltsville site had a common history of no-till management prior to 1996, microbial communities today are easily differentiated by farming system. Differences in management have effects that extend beyond microbial taxa to include nematodes [37], as well as soil organic matter and phosphorous concentrations, greenhouse gas emissions, and total energetic costs of the farming system [24, 38, 39].

The absence of glyphosate effects in naïve soil communities suggests that typical application rates of glyphosate do not alter the overall microbial community. Existing literature suggests most microbial communities are susceptible to disturbance, although bias against reporting of no treatment could affect this view [40]. Understanding the factors contributing to resistance of microbes in agroecosystems remains an important goal [41]. In the current study resilience to glyphosate spray could be linked to several factors. Some bacterial and fungal species are known to metabolize glyphosate, and the presence of these organisms may protect susceptible species [42, 43]. Studies reporting effects of glyphosate on soil microbes often use higher concentrations of the herbicide than the approved rate, which may overwhelm buffering

358 by resistant members. Concentration-dependent effects of glyphosate on soil microbial
359 respiration and biomass have been reported and are consistent with reports on other
360 agrochemicals, showing only transient effects at recommended application rates [44].
361 Greenhouse studies with GR wheat conducted in the Pacific Northwest found only minor
362 effects of glyphosate on microbial communities, and determined site was a major driver of soil
363 microbial community structure [20, 21]. While these studies did detect effects of glyphosate on
364 the prevalence of a few microbial taxa, they applied glyphosate at twice the recommended rate,
365 increasing the likelihood that the microbial community experienced a significant effect. These
366 methodological differences may account for the detection of an effect on the abundance of
367 some taxa after glyphosate exposure where none was detected here, and ultimately increase
368 confidence in our finding that glyphosate has minimal effect on the microbial community when
369 applied at the recommended rate.
370 The Beltsville and Stoneville sites differ in soil chemistry and physical characteristics
371 (OM, pH). Soil microbial communities in these soils also differ considerably between sites
372 (Figure 1), with Beltsville having higher overall fungal richness and Stoneville having higher
373 prokaryotic richness (Figure 1). The higher richness of fungi and prokaryotes in Beltsville NT
374 plots relative to the other Beltsville management types receiving tillage is consistent with
375 previous studies, and may be due to the spatial heterogeneity that develops over time in the
376 absence of tillage [45]. However, in spite of differences in microbial communities between sites
377 and among management histories, fungal and prokaryotic richness were unaffected by
378 applications of glyphosate in all management and crop treatments.
379 Community richness changed across the growing season regardless of glyphosate
380 concentration (Figure 4). These results are similar to those of Hart et al. [46] in which the GR
381 corn and its genetically close isoline were grown for one season in Canada and the microbial
382 community compared by TRFLP with and without glyphosate application, although this study
383 could not have tested the long term legacy of glyphosate application. This study also found that

384 seasonality was a significant controlling factor in microbial community structure with and without
385 glyphosate under field conditions.

386 Previous culture-based work has found that *Fusarium* abundance increased and
387 *Pseudomonas* abundance decreased with glyphosate use [11]. In those studies, *Fusarium* were
388 presumed to be pathogenic while *Pseudomonas* were presumed to be symbionts. However, our
389 metabarcoding and culture data failed to detect an effect of glyphosate on the abundance of any
390 *Fusarium* or *Pseudomonas* spp. And while both barcoding and culture surveys detected other
391 pathogens, none responded to glyphosate (Supplementary Data). Our results are consistent
392 with previous metabarcoding studies [20, 21].

393 It is important to note that the ITS and 16s gene loci fail to resolve diversity at an
394 adequate level to differentiate pathogenic genotypes from closely related non-pathogenic
395 genotypes [47, 48]. For example, several species of *Metarhizium* known to occur at this site [49]
396 were not represented in the samples from this study. Most likely pathogenic strains were not
397 detected in this study. However, even if they are not identified to the strain level, pathogenic
398 species contribute to the relative abundance of their constituent OTU, and we did not detect any
399 change in total numbers of *Fusarium* spp. OTUs associating with crops due to glyphosate
400 application. This holds true for other genera of pathogenic fungi such as *Alterneria* spp. and
401 *Macrophomina* spp. (supplementary data). It should also be noted that while *Pseudomonas* spp.
402 are often taken to be inherently beneficial, there are at least a few confirmed pathogens [48];
403 and the type of beneficial function may differ substantially across strains. Regardless, as with
404 fungi, no *Pseudomonas* spp. changed in prevalence as a result of glyphosate treatment.

405 We also found no reductions in yield by glyphosate application on GR corn or GR
406 soybean in fields with a long history of glyphosate use or with no history of glyphosate use [50,
407 51]. In a similar study with GR sweet corn, there was even a slight increase in yield associated
408 with glyphosate application [52], which could have been due to hormesis, a phenomenon with

409 non-phytotoxic doses of glyphosate [53]. Lack of effects on yields are consistent with no
410 substantial detrimental effects on rhizosphere microbes.

411 Although glyphosate is widely used across the globe, relatively few studies have
412 investigated the effect of this herbicide on soil microbial communities in cropping systems with
413 and without a legacy of glyphosate application. This work provides an important contribution into
414 determining the effect of glyphosate on bacterial and fungal communities found in soils. No
415 changes due to glyphosate, coupled with a trend towards higher species richness in no-till plots,
416 suggests this widely employed management practice is not at risk of altering soil microbial
417 communities in a negative manner. However, increased glyphosate application rates in
418 response to evolution of resistant weeds could alter this conclusion. Whether the species
419 richness of no-till systems translates to increases in ecosystem function supportive of crop
420 productivity remains to be fully elucidated.

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424 **Conflict of interest**

425 The authors declare they have no conflict of interest.

426

427

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564
565

566 Figure 1. Principal component analyses of site chemistry, bacterial and fungal communities for
567 sites in Beltsville, MD and Stoneville, MS. A) Chemical analysis of all plots in the first year of this
568 study. B) Bray-Curtis dissimilarity of prokaryotic communities from all samples after rarefaction
569 to a depth of 20,000 reads per sample. C) Bray-Curtis dissimilarity of fungal communities for all
570 samples after relative abundance transformation of total counts.
571
572 Figure 2. Principal component analyses of microbial communities in Beltsville, MD, partitioned
573 by crop. Prokaryotic community data drawn from a dataset rarefied to 20,000 reads per sample.
574 Fungal data has been variance stabilized with negative binomial transformation in DESeq2.
575
576 Figure 3. Principal component analyses of microbial communities in Stoneville, MS, partitioned
577 by crop. Prokaryotic community data drawn from a dataset rarefied to 20,000 per sample.
578 Fungal data has been variance stabilized with negative binomial transformation in DESeq2.
579
580 Figure 4. Change in Shannon's richness of rarefied data across sampling dates in no-spray and
581 spray treatments. Stars on each plot are for raw (*) and false discovery rate corrected (**) p-
582 values less than 0.05 from Wilcoxon signed-rank test of differences between dates. Years are
583 pooled, although graphed separately. Red points and line represent mean richness
584
585 Figure 5. Abundance of *Fusarium* isolates +/- standard deviation. A) corn. B) soy roots. Colors
586 follow those used in Figures 2 & 3.
587

**Corn yield at 15.5% $H_2O$ (kg/ha)**

| | 2013 | | | | 2014 | | | |
|---|---|---|---|---|---|---|---|---|
| | DKC 65-17 RR2 | | DKC 65-17 RR2+**Gly** | | DKC 65-17 RR2 | | DKC 65-17 RR2+**Gly** | |
| **System** | **LS Means** | | **LS Means** | | **LS Means** | | **LS Means** | |
| CT | 9535.55 | n.s. | 8847.79 | n.s. | 9797.60 | n.s. | 8966.96 | n.s. |
| NT | 9780.09 | n.s. | 9140.77 | n.s. | 10756.83 | n.s. | 11122.74 | n.s. |
| ORG3 | 9209.83 | n.s. | 7634.06 | n.s. | 10225.41 | n.s. | 9496.73 | n.s. |
| ORG6 | 8832.09 | n.s. | 8509.80 | n.s. | 7627.45 | n.s. | 8184.97 | n.s. |

**Soybean Yield at 13.5% $H_20$ (kg/ha)**

| | 2013 | | 2014 | | | |
|---|---|---|---|---|---|---|
| | **Allen** | **Allen+Gly** | **Allen** | | **Allen+Gly** | |
| **System** | **LS Means** | **LS Means** | **LS Means** | | **LS Means** | |
| CT | -- | -- | 2437.60 | n.s. | 2159.60 | n.s. |
| NT | -- | -- | 2264.00 | n.s. | 2016.00 | n.s. |
| ORG3 | -- | -- | 2314.50 | n.s. | 2906.50 | n.s. |
| ORG6 | -- | -- | 2290.00 | n.s. | 2733.00 | n.s. |

Table 1. Corn and Soybean yield (kg/ha) for glyphosate treated or untreated plots in chisel till (CT), no-till (NT), Organic 3 yr. rotation (Org3) or Organic 6 yr. rotations (Org6). Comparison of means was calculated within each system for the glyphosate resistant genotype either treated with Glyphosate (Gly) or not. In 2013 an error in microplot harvesting resulted in mixing of treated and untreated plots therefore making the yield data un-usable.

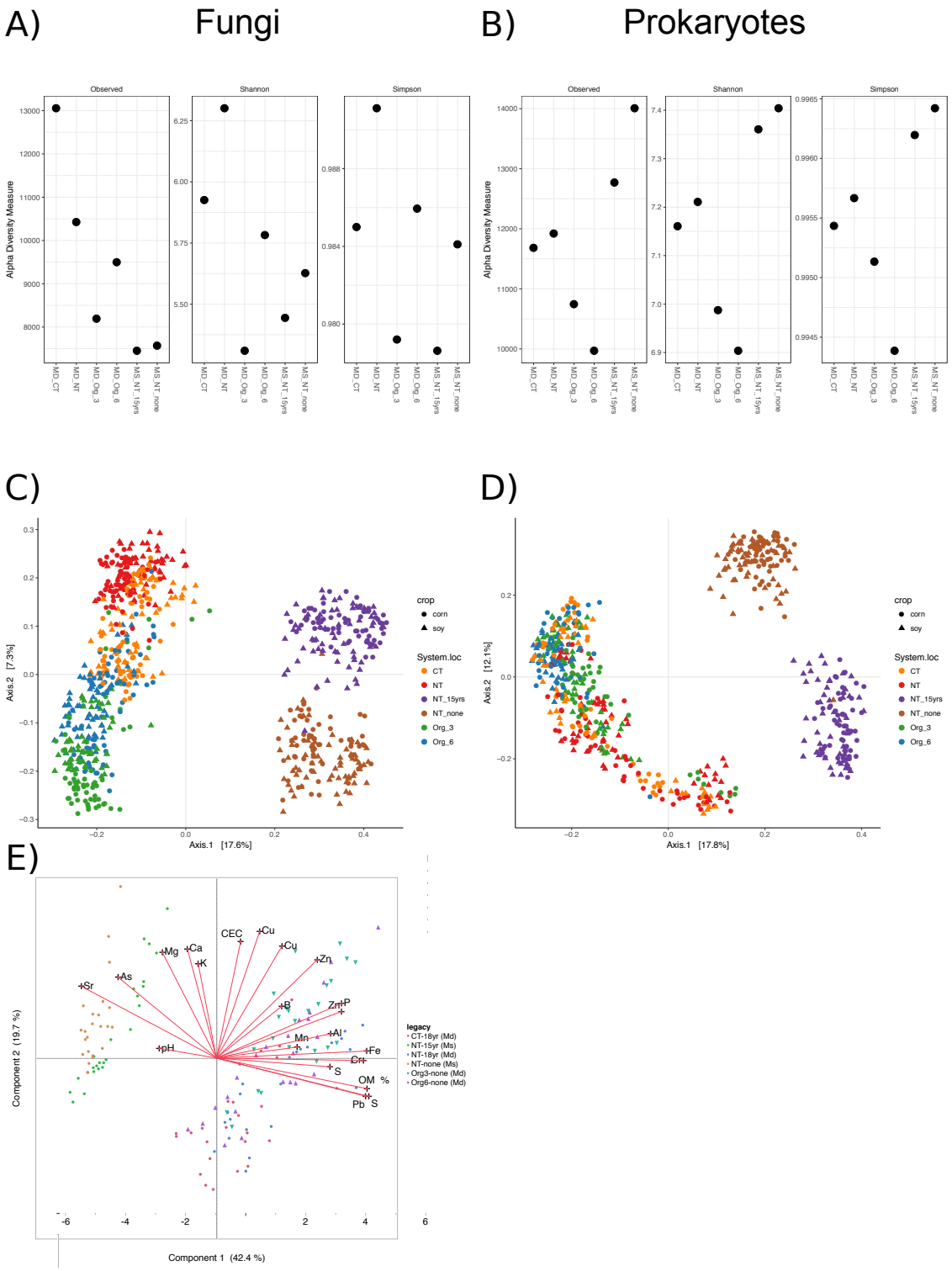

A)
Fungi
B)
Prokaryotes
Observed
Shannon
Simpson
Alpha Diversity Measure
MD_CT
MD_NT
MD_Org_3
MD_Org_6
MS_NT_15yrs
MS_NT_none
C)
D)
crop
corn
soy
System.loc
CT
NT
NT_15yrs
NT_none
Org_3
Org_6
Axis.1 [17.6%]
Axis.2 [7.3%]
Axis.1 [17.8%]
Axis.2 [12.1%]
E)
CEC
Cu
Mg
Ca
K
As
Sr
Zn
B
Zn
P
Al
Mn
pH
Fe
Cr
S
OM %
Pb
S
legacy
CT-18yr (Md)
NT-15yr (Ms)
NT-18yr (Md)
NT-none (Ms)
Org3-none (Md)
Org6-none (Md)
Component 1 (42.4 %)
Component 2 (19.7 %)

Figure 1

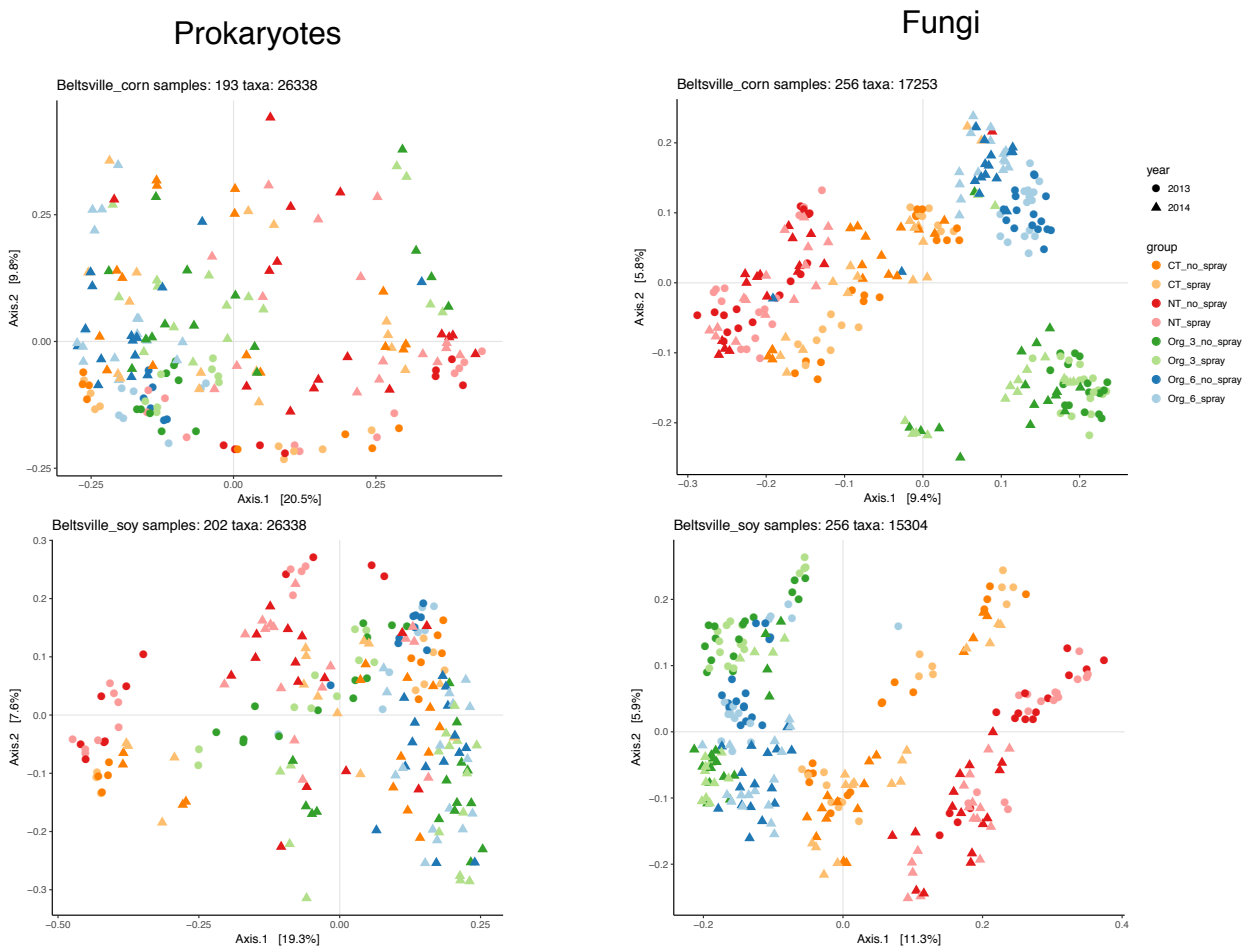

Prokaryotes
Fungi
Beltsville_corn samples: 193 taxa: 26338
Axis.1 [20.5%]
Axis.2 [9.8%]
Beltsville_corn samples: 256 taxa: 17253
Axis.1 [9.4%]
Axis.2 [5.8%]
Beltsville_soy samples: 202 taxa: 26338
Axis.1 [19.3%]
Axis.2 [7.6%]
Beltsville_soy samples: 256 taxa: 15304
Axis.1 [11.3%]
Axis.2 [5.9%]
year
2013
2014
group
CT_no_spray
CT_spray
NT_no_spray
NT_spray
Org_3_no_spray
Org_3_spray
Org_6_no_spray
Org_6_spray

Figure 2

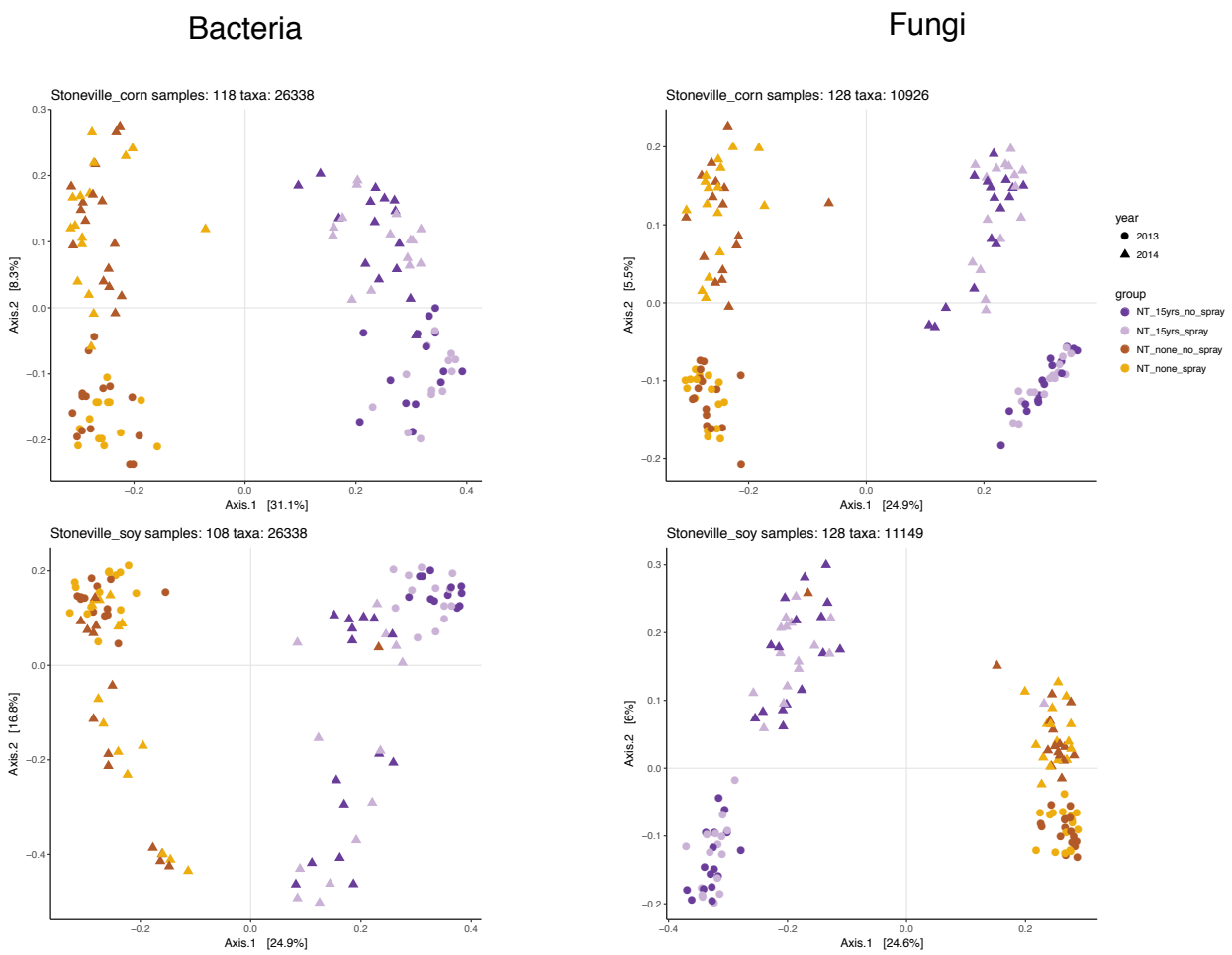
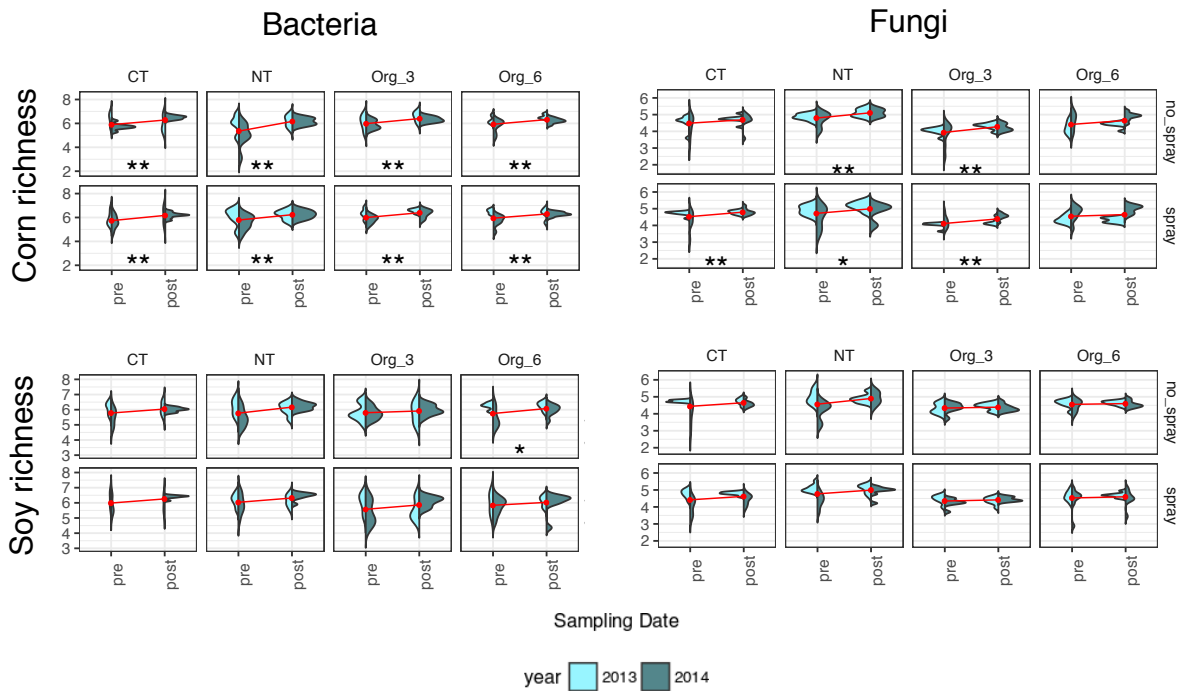

Bacteria
Fungi
Stoneville_corn samples: 118 taxa: 26338
Axis.2 [8.3%]
Axis.1 [31.1%]
Stoneville_corn samples: 128 taxa: 10926
Axis.2 [5.5%]
Axis.1 [24.9%]
Stoneville_soy samples: 108 taxa: 26338
Axis.2 [16.8%]
Axis.1 [24.9%]
Stoneville_soy samples: 128 taxa: 11149
Axis.2 [6%]
Axis.1 [24.6%]
year
2013
2014
group
NT_15yrs_no_spray
NT_15yrs_spray
NT_none_no_spray
NT_none_spray

Figure 3




601

602 Figure 4

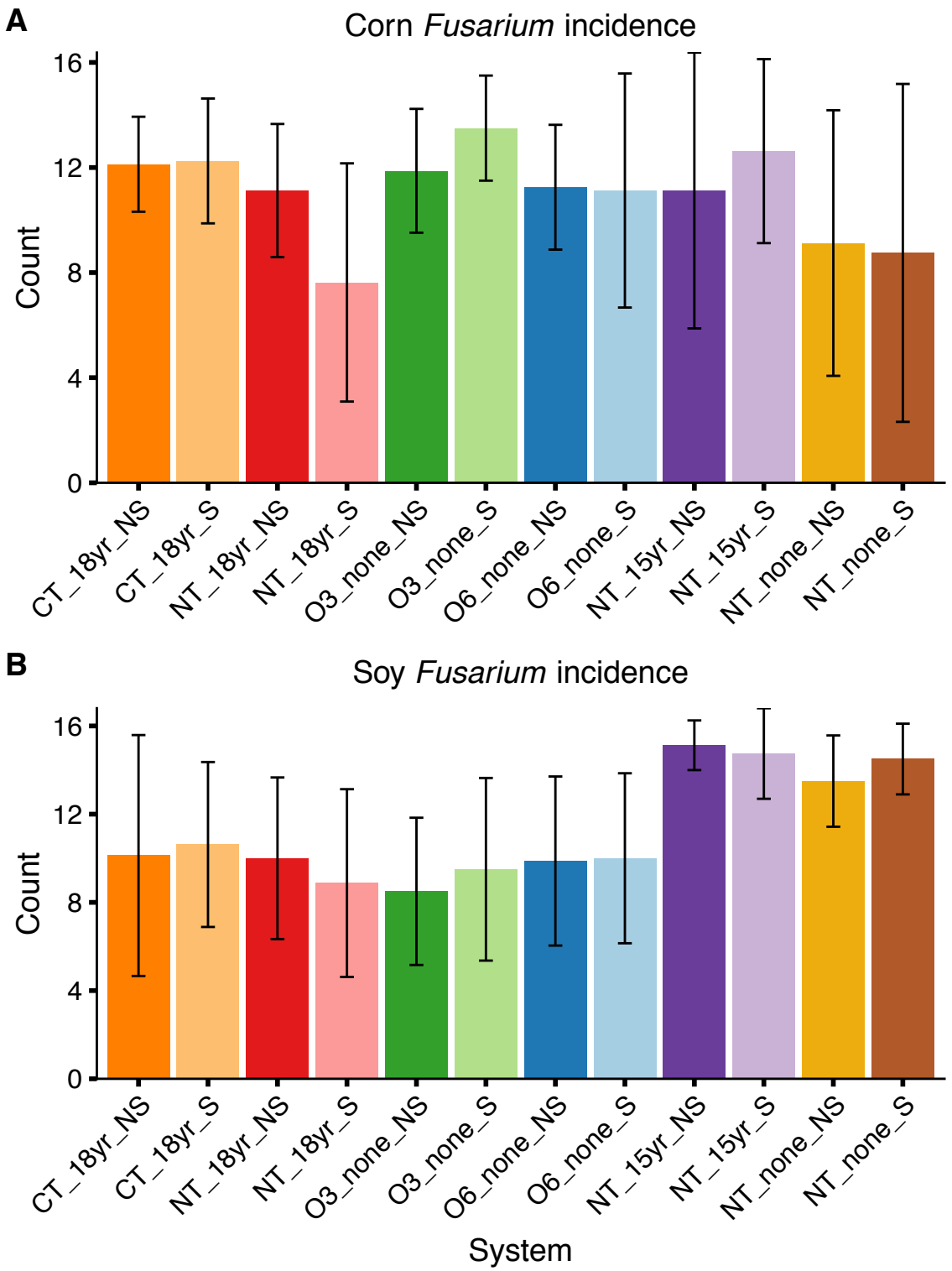

A
Corn *Fusarium* incidence
Count
16
12
8
4
0
CT_18yr_NS
CT_18yr_S
NT_18yr_NS
NT_18yr_S
O3_none_NS
O3_none_S
O6_none_NS
O6_none_S
NT_15yr_NS
NT_15yr_S
NT_none_NS
NT_none_S
B
Soy *Fusarium* incidence
Count
16
12
8
4
0
CT_18yr_NS
CT_18yr_S
NT_18yr_NS
NT_18yr_S
O3_none_NS
O3_none_S
O6_none_NS
O6_none_S
NT_15yr_NS
NT_15yr_S
NT_none_NS
NT_none_S
System

Figure 5