Soil microbial communities in diverse agroecosystems exposed to glyphosate

Ryan M. Kepler[1], Dietrich Epp-Schmidt[2], Stephanie A. Yarwood[2], Krishna N. Reddy[3], Stephen O. Duke[4], Carl A. Bradley[5], Martin Williams[6], Jeffery Buyer[1], Michel Cavigelli[1], Jude E. Maul[1]

[1]Sustainable Agricultural Systems Laboratory, USDA-ARS
10300 Baltimore Ave, bldg. 001
Beltsville, MD 20705

[2]Environmental Science and Technology Department, University of Maryland,
1204 HJ Patterson Hall
College Park, MD 20742, USA

[3]Crop Production Systems Research Unit, USDA-ARS
P.O. Box 350, Stoneville, Mississippi 38776

[4]Natural Products Utilization Research Unit, USDA-ARS
P.O. Box 1848, University, Mississippi 38677

[5]Department of Plant Pathology, University of Kentucky Research and Education Center
Princeton, KY 42445

[6]
1102 S. Goodwin Avenue
Urbana, Illinois 61801

Corresponding author: Jude Maul. Jude.Maul@ARS.USDA.GOV. phone: 301-504-9068

Conflict of interest

The authors declare they have no conflict of interest.

Abstract

In spite of its wide use in agriculture, questions remain about the effects of glyphosate 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 locations, 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 the significant factors determining composition of fungal and bacterial communities. Plots treated with and 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 bind to so 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]. 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

75 pathway, resulting in a non-lethal infection that allows the pathogen to propagate. A review of all
76 GR crops by Hammerschmidt [12] determined there is no conclusive evidence that glyphosate
77 increases the susceptibility of GR crops to disease. Another review [13] challenges this
78 conclusion. For example, several studies have observed that GR beets and soybean have
79 increased susceptibility to pathogens when glyphosate is applied at recommended rates [13–
80 15]. One study found no effect of glyphosate on disease induction in GR beets until rates
81 exceeded normal field application rates by one order of magnitude [16]. However, other studies
82 with GR crops have found no influence of glyphosate on disease [17], as well as instances of
83 fungicidal activity against plant pathogens, especially rusts (reviewed by Duke [18]).
84 Two key studies have substantiated the glyphosate-pathogen-enrichment hypothesis,
85 finding over long study periods that glyphosate repeatedly increases the rate of colonization of
86 crops by *Fusarium* (presumed to be a pathogenic strain), while decreasing the abundance of
87 fluorescent *Pseudomonas* bacteria (taken as putative beneficial organisms) in the soil [11, 15].
88 These studies are often cited as conclusive evidence that long-term use of glyphosate increases
89 the pathogen load and decreases the abundance of growth promoting bacteria in soils. Both
90 studies applied culture-based methodology to quantify these microbial groups, with minimal
91 molecular analysis of the ribosomal internal transcribed spacer region (ITS); however, the
92 identification techniques employed were not sufficiently discriminatory to distinguish pathogenic
93 and beneficial genotypes for either group. Studies using culture-free methodology to
94 characterize microbial communities have failed to detect substantial glyphosate effects on
95 pathogen abundance [19, 20]. The key to conclusive determination of glyphosate effect on
96 microbial communities of GR crops is to carefully compare glyphosate sprayed and non-sprayed
97 treatments within an agronomic context. Farming systems, soil factors, crop varieties,
98 glyphosate legacy and application rates can all impact the behavior of glyphosate and its
99 interaction with the crop and soil microbiome [21].

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

110 **Materials and Methods**

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

115 The Beltsville site is managed as part of a USDA-Long Term Agricultural Research site
116 typical of the mid-Atlantic region and described previously [22, 23]. We conducted the study in
117 two conventional systems include one using a chisel plow for primary tillage (CT) and one under
118 no-tillage management (NT). These two systems rely on mineral fertilizers, herbicides and other
119 pesticides as needed to manage a corn-rye cover crop-soybean-wheat/soybean rotation. One
120 organic system is a three-year corn (*Zea maize*) -rye (*Cereale secale*) cover crop -soybean
121 (*Glycine max*) -wheat (*Triticum aestivum*)/legume (*Vicia villosa*) rotation (Org3), and the second
122 is a six-year crop rotation (Org6) in which alfalfa (*Medicago sativa*), a perennial crop in place for
123 three years of the rotation, replaces the vetch present in Org3. The organic systems rely on
124 legumes, poultry litter and K_2SO_4 to supply crop nutrients in accordance to soil test results and
125 local regulations. A moldboard plow and/or a chisel plow is used for primary tillage in the

organic systems. Weed control in the organic systems included use of a rotary hoe and between row cultivation after crops were planted. In the text the systems in Beltsville are described as NT-18yr (Gly history), CT-18yr (Gly history), Org3-none or Org6-none.

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

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

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

151 2014 the soybean were hand harvested and threshed from 3.05 m of the two center rows. Dry
152 weights were calculated at 13.5% moisture.

153 **Soil Baseline Characteristics**

154 Beltsville soils are Coastal Plain silty loam Ultisols, consisting primarily of Christiana,
155 Keyport, Matapeake and Mattapex soil map units. The soils at the Stoneville site were a silt
156 loam typic Endoqualfs dominated by Dundee soil map units. At planting, soil samples from the
157 top 15-cm depth were collected from each plot by combining soil from six or more cores (7.5 cm
158 diameter and 15 cm depth) sampled in a semi-random pattern in a given plot. Samples were air-
159 dried and sieved to 2 mm. The cores were collected on a diagonal line between the second and
160 third crop rows, 3 m from each end of a given plot. Soil samples were analyzed by the
161 Agricultural Analytical Services Laboratory at Pennsylvania State University for: pH, organic
162 matter (OM), CEC, P, K, Mg, Ca, S, B, Zn, Mn, Fe, Cu, As, Al, Ba, Cd, Co, Cr, Ni, Pb, Se, and
163 Sr. pH was determined in a 1:1 water dilution, OM was determined by mass loss on combustion
164 and CEC was determined using the methods of Ross, D. and Q. Ketterings [24]. Mehlich 3
165 extractions were used to quantify Ca, Mg, and K; all other metals are expressed as total sorbed
166 using the EPA 3050 method [25].

167 **Rhizosphere Soil and Root Sampling**

168 At the V3 to V4 plant growth stage (4 to 6 weeks after planting) and one day prior to
169 glyphosate application six plants and root-associated soil were excavated from each plot by
170 removing soil monoliths with 15 cm radius from stem and15 cm deep using surface sterilized
171 sharpshooter shovels. This time point is referred to as “PRE-spray.” Soil monoliths were placed
172 on a sieve and soil around the root ball was gently removed by shaking and passed through a 2
173 mm sieve; this soil was considered bulk soil. Soil adhering to roots after this procedure
174 (considered rhizosphere soil) was brushed onto a 2 mm sieve using a camel hair brush. Roots
175 were brushed thoroughly, yet not so the integrity of the root surface was compromised. The
176 rhizosphere samples from the six plants were pooled and 5 g were added to a 15 ml falcon tube

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

184 **Identification of Endophytes from Roots**

185 Two centimeter sections of root were cut at random sixteen times from each of six fresh
186 root systems for each treatment. The total wet weight of the 16 sections was recorded. Sections
187 were surface sterilized for 2 minutes in 1.25% sodium hypochlorite, followed by three rinses in
188 sterile distilled water. Sections were blotted dry on sterile paper towel and eight root sections
189 were placed on a plate containing Komada's Medium [26]. Plated roots were incubated in
190 ambient light at room temperature until colonies emerged. Fungal mycelium and spores from
191 emerging colonies were sampled and examined on a Nikon E60 microscope and identified to
192 genus, or to broader morphological group, based on taxonomic features. Colonies of typical
193 morphology were plated onto minimal media to induce sporulation for further identification.
194 Colonies not producing spores were characterized as "non-sporulating." Polymerase chain
195 reaction (PCR) screens for ITS followed by cloning and sequencing were conducted on over
196 384 colonies of typical morphology to validate microscopic identification. The methods followed
197 those described in (Chung et al. 2008). Sequences were quality checked and aligned using the
198 DNAStar suite of software (DNAStar, Madison, WI, USA), and identified using the basic local
199 alignment search tool and GenBank nucleotide data bank from the National Center for
200 Biotechnology Information, Bethesda, MD, USA (http://www.ncbi.nlm.nih.gov/. Accessed fall,
201 2014).

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

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

220 **Bioinformatics and Statistical Analysis**

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

228 *Assembly and taxonomy assignment.* The R package Dada2 [27] was used for paired
229 end assembly and taxonomy assignment. The command “filterandtrim” was used to remove
230 sequences with an expected error rate greater than two, and any sequences containing “N”
231 values (unreadable bases). Error rates were estimated for forward and reverse reads. Filtered
232 reads were then dereplicated with the “derepFastq” command. Dereplicated sequences were
233 denoised with the “dada” command and then paired ends were merged. Chimeric sequences
234 were removed with the command “removeBimeraDenovo”. Taxonomy was assigned to the
235 chimera-free table of sequences with the dada2 implementation of the RDP classifier [28]. The
236 UNITE database (version 7.2) [29] was used as the reference for identification of fungal ITS
237 sequence variants, and silva (release 132) [30] was used for prokaryotes.
238 *Community analysis.* We transformed community count data into relative abundance,
239 then calculated Bray-Curtis dissimilarity. Principal components analysis (PCA) was applied to
240 the Bray-Curtis dissimilarity matrix using the ordinate function of the vegan package v. 2.4 [31]
241 as implemented in phyloseq v. 1.22.2 [32] for both fungal and prokaryotic barcodes.
242 After subsetting by crop and location, richness and evenness were estimated from
243 rarefied datasets of the raw sequence counts using vegan. DESeq2 v. 1.18.1 [33] was used to
244 produce variance stabilized datasets [34]. Bray-Curtis dissimilarity for each sample was used for
245 PCA. We used PERMANOVA to determine significance of main effects and interactions
246 between the following factors: farming system, soil zone, glyphosate treatment, sampling date
247 and year. The Farming System factor had 4 categories for Beltsville (CT, NT, Org3 and Org6)
248 and 2 for Stoneville (NT_none, NT_15yr). All other factors had two categories at both locations:
249 Soil Zone (bulk and rhizosphere); Year (2013, 2014); glyphosate treatment (spray, no spray);
250 sampling date (PRE glyphosate application, and Post glyphosate application). A repeated
251 measures model based on the plot ID was used.
252 The effect of glyphosate treatment on microbial communities was tested with the
253 Wilcoxon signed-rank test of differences between dates as implemented in the longitudinal plug-

254 in for Qiime2 [35]. The test was applied separately for three measures of richness: observed,
255 Shannon's and Simpson's.
256 *Differentially abundant taxa.* Tests for differentially abundant taxa in response to
257 glyphosate treatment were conducted in DESeq2 using likelihood ratio tests after subsetting
258 fungal and prokaryotic data by location, crop and management type. The test compared a full
259 model including group, sampling date terms, as well as an interaction term, where group is
260 defined as the combination of management type and treatment (e.g. Org3_spray) and sampling
261 date corresponds to PRE and POST glyphosate application. The full model was compared to a
262 reduced model lacking the interaction term. Thus significant interaction terms would indicate
263 sampling date and glyphosate application interacted to be important predictors of microbial
264 abundance. This was tested for every fungal and bacterial taxon identified. Datasets with
265 untransformed counts were used as the starting data, which were then variance-stabilized
266 during testing.
267 **Results**
268 From the sequencing analysis a total of 68,964 unique fungal and 72,454 unique
269 prokaryotic sequence variants were identified across all samples. Beltsville and Stoneville
270 shared 13,964 bacterial and 5,740 fungal taxa. Stoneville featured 62,985 and 29,780 bacterial
271 and fungal taxa, respectively. Beltsville featured 41,538 and 44,924 unique bacterial and fungal
272 taxa. Fungal richness was higher for all Beltsville farming systems, compared with Stoneville,
273 with the exception of the Shannon's and Simpson's diversity metrics for Org_3 (Figure 1A).
274 Conversely, prokaryotic diversity was greater for Stoneville in all measures (Figure 1B).
275 Principal component analysis showed that Beltsville and Stoneville communities were
276 distinct (Figure 1C & D). Permanova analysis of relative abundance for fungi and prokaryotes
277 revealed that site was the most significant factor accounting for Bray-Curtis dissimilarity
278 distances in fungi and prokaryotes (p = 0.001 in both cases. Fungal R^2 = 0.19, Prokaryote R^2 =
279 0.16 ; supplemental data). Differences between the Stoneville and Beltsville microbial

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

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

299 Farming system was also a driver of prokaryote community structure in Beltsville
300 (Permanova; corn: p = 0.001, R^2 = 0.096; soybean; p = 0.001, R^2 = 0.09) and Stoneville
301 (Permanova; corn: p = 0.001, R^2 = 0.21; soybean; p = 0.001, R^2 = 0.16).Farming explained less
302 variation in Beltsville prokaryotic communities (Figure 2), than in Stoneville (Figure 3). The year
303 term explained a lesser amount of variance for Beltsville (corn: p = 0.001, R^2 = 0.096; soybean;
304 p = 0.001, R^2 = 0.086) and Stoneville (corn: p = 0.001, R^2 = 0.051; soybean; p = 0.001, R^2 =
305 0.069). The interaction between glyphosate with sampling date was not significant for either

306 crop (Supplemental data). Likelihood ratio tests of taxon abundance in DESeq2 also confirmed
307 no glyphosate treatment; the sampling date-glyphosate treatment interaction did not significantly
308 increase the explanatory power of the model for any taxon (supplementary data), regardless of
309 crop or farming system (supplementary data).

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

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

329 There was no significant difference in corn yield among systems or among glyphosate
330 application treatments for either 2013 or 2014 (Table 1). Corn yields were not significantly
331 different from the county averages for all systems with a mean among systems of 9339.4 kg/ha.

332 In 2013 an error occurred while using the small plot combine and beans harvested from different
333 microplots were mixed rendering the data unusable. In 2014 soybean yields were similar to the
334 county averages with a mean of 2326.5 kg/ha. There was no significant difference in yield
335 across farming systems, and no effect of glyphosate treatment on yield (Table 1)
336
337 **Discussion**
338 The structure of prokaryote and fungal communities among cropping systems and
339 between sampling dates were not driven by the application of glyphosate. Instead, tillage and
340 carbon inputs associated with different management practices appear to be the primary drivers
341 of soil microbiome structure. Case in point, even though the Beltsville site had a common history
342 of no-till management prior to 1996, the microbial communities are easily differentiated by the
343 current management regimens. Differences in management have effects that extend beyond
344 microbial taxa to include nematodes [36], as well as soil organic matter and phosphorous
345 concentrations, greenhouse gas emissions and total energetic costs of the farming system [23,
346 37, 38].
347 The absence of glyphosate effects in naïve soil communities suggests that typical
348 application rates of glyphosate do not alter the overall microbial community. Existing literature
349 suggests most microbial communities are susceptible to disturbance, although bias against
350 reporting of no treatment could affect this view [39]. Understanding the factors contributing to
351 resistance of microbes in agroecosystems remains an important goal [40]. In the current study
352 resilience to glyphosate spray could be linked to several factors. Some bacterial and fungal
353 species are known to metabolize glyphosate, and the presence of these organisms may protect
354 susceptible species [41, 42]. Studies reporting effects of glyphosate on soil microbes often use
355 higher concentrations of the herbicide than the approved rate, which may overwhelm buffering
356 by resistant members. Concentration dependent effects of glyphosate on soil microbial

357 respiration and biomass have been reported and are in keeping with other agrochemicals,
358 showing only transient effects at recommended application rates [43].
359 Greenhouse studies with GR wheat conducted in the Pacific Northwest found only minor
360 effects of glyphosate on microbial communities, and determined location was a major driver of
361 soil microbial community structure [19, 20]. While these studies did detect effects of glyphosate
362 on the prevalence of a few microbial taxa, they applied glyphosate at twice the recommended
363 rate, increasing the likelihood that the microbial community experienced a significant effect.
364 These methodological differences may account for the detection of an effect on the abundance
365 of some taxa after glyphosate exposure where none was detected here, and ultimately increase
366 confidence in our finding that glyphosate has minimal effect on the microbial community when
367 applied at the recommended rate.
368 The Beltsville and Stoneville sites differ in soil chemistry and physical characteristics
369 (OM, pH). Soil microbial communities in these soils also differ considerably between sites
370 (Figure 1), with Beltsville having higher overall fungal richness and Stoneville having higher
371 prokaryotic richness (Figure 1). The higher richness of fungi and prokaryotes in Beltsville NT
372 plots relative to the other Beltsville management types receiving tillage is consistent with
373 previous studies, and may be due to the spatial heterogeneity that develops over time in the
374 absence of tillage [44]. However, in spite of differences in microbial communities between sites
375 and among management histories, fungal and prokaryotic richness were unaffected by
376 applications of glyphosate in all management and crop treatments.
377 Community richness changed across the growing season regardless of glyphosate
378 concentration (Figure 4). These results are similar to those of Hart et al. [45] in which the GR
379 corn and its genetically close isoline were grown for one season in Canada and the microbial
380 community compared by TRFLP with and without glyphosate application, although this study
381 could not have tested the long term legacy of glyphosate application. This study also found that

382 seasonality was a significant controling factor in microbial community structure with and without
383 glyphosate under field conditions.

384 Previous culture-based work has found that *Fusarium* abundance increases and
385 *Pseudomonas* abundance decreases under glyphosate treatment [11]. In those studies,
386 *Fusarium* were presumed to be pathogenic while *Pseudomonas* were presumed to be
387 symbionts. However, our metabarcoding and culture data failed to detect an effect of glyphosate
388 on the abundance of any *Fusarium* or *Pseudomonas* spp. And while both barcoding and culture
389 surveys detected other pathogens, none responded to glyphosate (Supplementary Data). Our
390 results are consistent with previous metabarcoding studies [19, 20].

391 It is important to note that the ITS and 16s gene loci fail to resolve diversity at an
392 adequate level to differentiate pathogenic genotypes from closely related non-pathogenic
393 genotypes [46, 47]. For example, several species of *Metarhizium* known to occur at this site [48]
394 were not represented in the samples from this study. It is possible (indeed likely) that
395 pathogenic strains were missed in this study. However, even if they are not identified to the
396 strain level, pathogenic species contribute to the relative abundance of their constituent OTU,
397 and we did not detect any change in total numbers of *Fusarium* spp. OTUs associating with
398 crops due to glyphosate application. This holds true for other genera of pathogenic fungi such
399 as *Alterneria* spp. and *Macrophomina* spp. (supplementary data). It should also be noted that
400 while *Pseudomonas* spp. are often taken to be inherently beneficial, there are at least a few
401 confirmed pathogens [47]; and the type of beneficial function may differ substantially across
402 strains. Regardless, as with fungi, no *Pseudomonas* spp. changed in prevalence as a result of
403 glyphosate treatment.

404 We also found no reductions in yield by glyphosate application on GR corn or GR
405 soybean in fields with a long history of glyphosate use or with no history of glyphosate use [49,
406 50]. In a similar study with GR sweet corn, there was even a slight increase in yield associated
407 with glyphosate application [51], which could have been due to hormesis, a common

408 phenomenon with non-phytotoxic doses of glyphosate [52]. Lack of effects on yields are
409 consistent with no substantial detrimental effects on rhizosphere microbes.
410 Although glyphosate is widely used across the globe, relatively few studies have
411 investigated the effect of this herbicide on soil microbial communities in cropping systems with
412 and without a legacy of glyphosate application. This work provides an important contribution into
413 determining the effect of glyphosate on bacterial and fungal communities found in soils. No
414 changes due to glyphosate, coupled with a trend towards higher species richness in no-till plots
415 suggests this widely employed management practice is not at risk of altering soil microbial
416 communities in a negative manner. Whether this biological component of no-till systems
417 translates to increases in ecosystem function supportive of crop productivity remains to be fully
418 elucidated.

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421 identification.

422 **Conflict of interest**
423 The authors declare they have no conflict of interest.
424

425

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559
560

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

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 Round up ready 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.

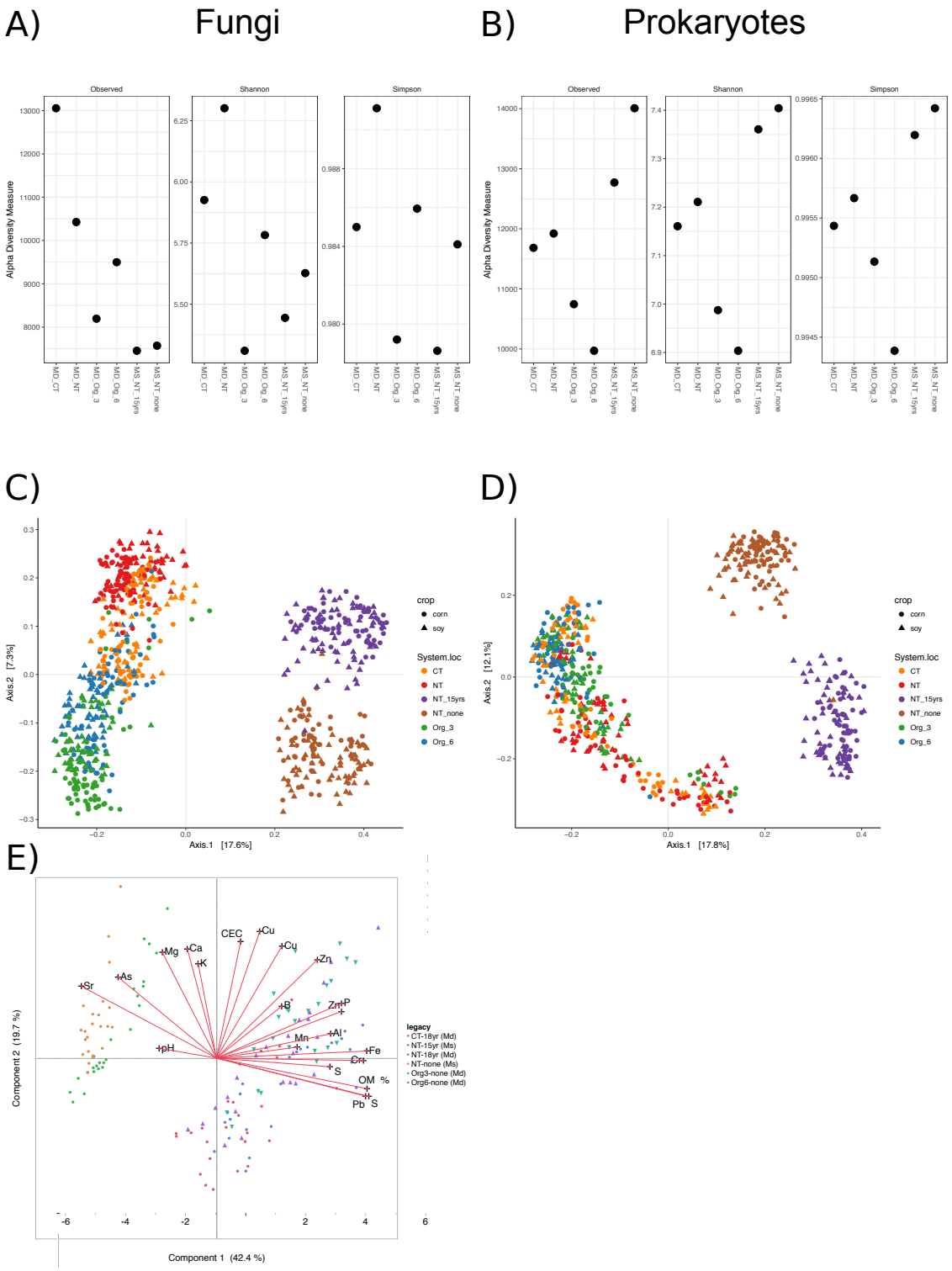
A)
Fungi
B)
Prokaryotes
Observed
Shannon
Simpson
Alpha Diversity Measure
MD_CT
MD_NT
MD_Org_3
MD_Org_6
MS_NT_15yrs
MS_NT_none
C)
D)
crop
corn
soy
System.loc
CT
NT
NT_15yrs
NT_none
Org_3
Org_6
Axis.1 [17.6%]
Axis.2 [7.3%]
Axis.1 [17.8%]
Axis.2 [12.1%]
E)
CEC
Cu
Mg
Ca
K
As
Sr
Zn
B
Zn
P
Al
Mn
Fe
pH
Cr
S
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

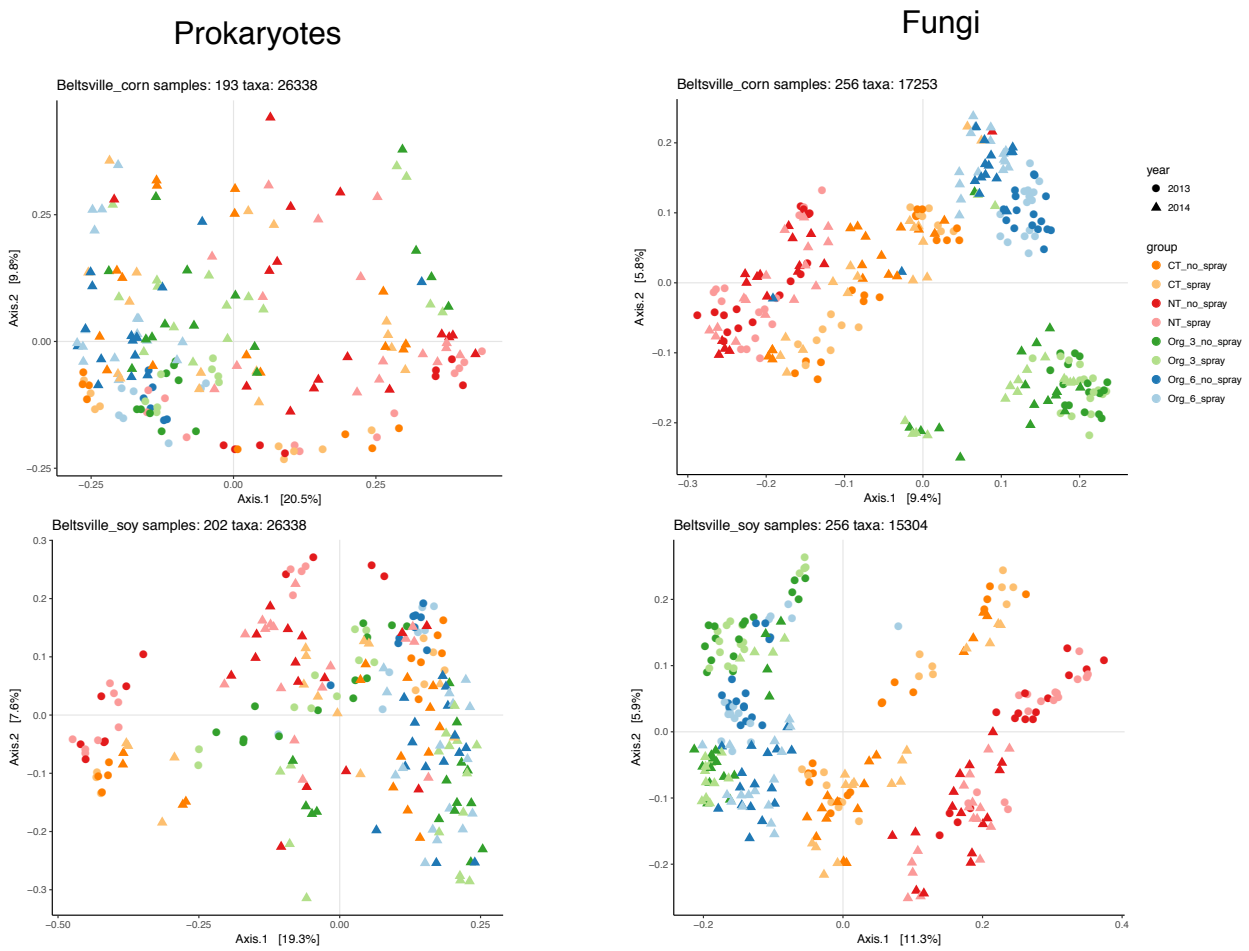
Prokaryotes
Fungi
Beltsville_corn samples: 193 taxa: 26338
Axis.1 [20.5%]
Axis.2 [9.8%]
Beltsville_corn samples: 256 taxa: 17253
Axis.1 [9.4%]
Axis.2 [5.8%]
Beltsville_soy samples: 202 taxa: 26338
Axis.1 [19.3%]
Axis.2 [7.6%]
Beltsville_soy samples: 256 taxa: 15304
Axis.1 [11.3%]
Axis.2 [5.9%]
year
2013
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

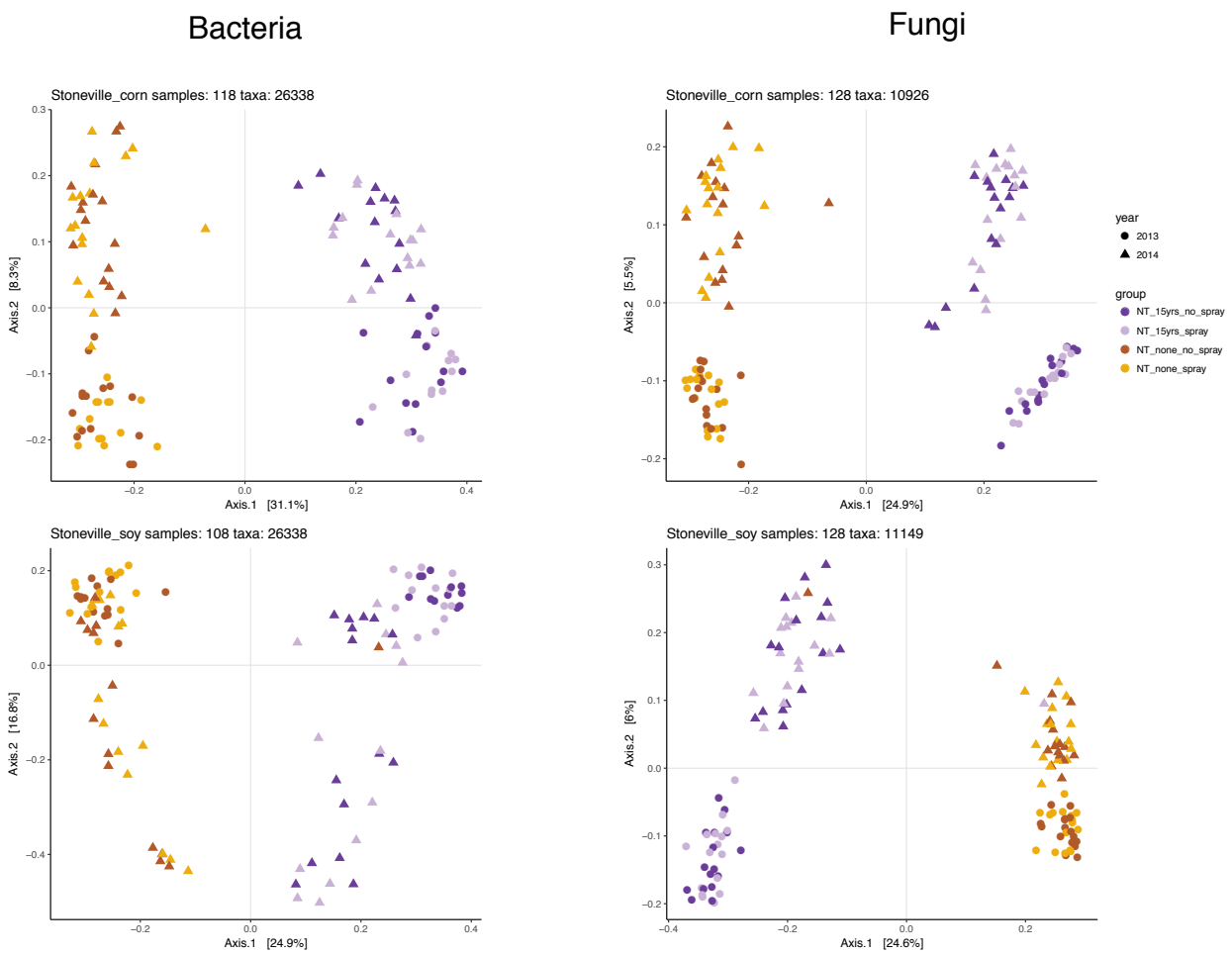
Bacteria
Fungi
Stoneville_corn samples: 118 taxa: 26338
Axis.2 [8.3%]
Axis.1 [31.1%]
Stoneville_corn samples: 128 taxa: 10926
Axis.2 [5.5%]
Axis.1 [24.9%]
Stoneville_soy samples: 108 taxa: 26338
Axis.2 [16.8%]
Axis.1 [24.9%]
Stoneville_soy samples: 128 taxa: 11149
Axis.2 [6%]
Axis.1 [24.6%]
year
2013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

Figure 3

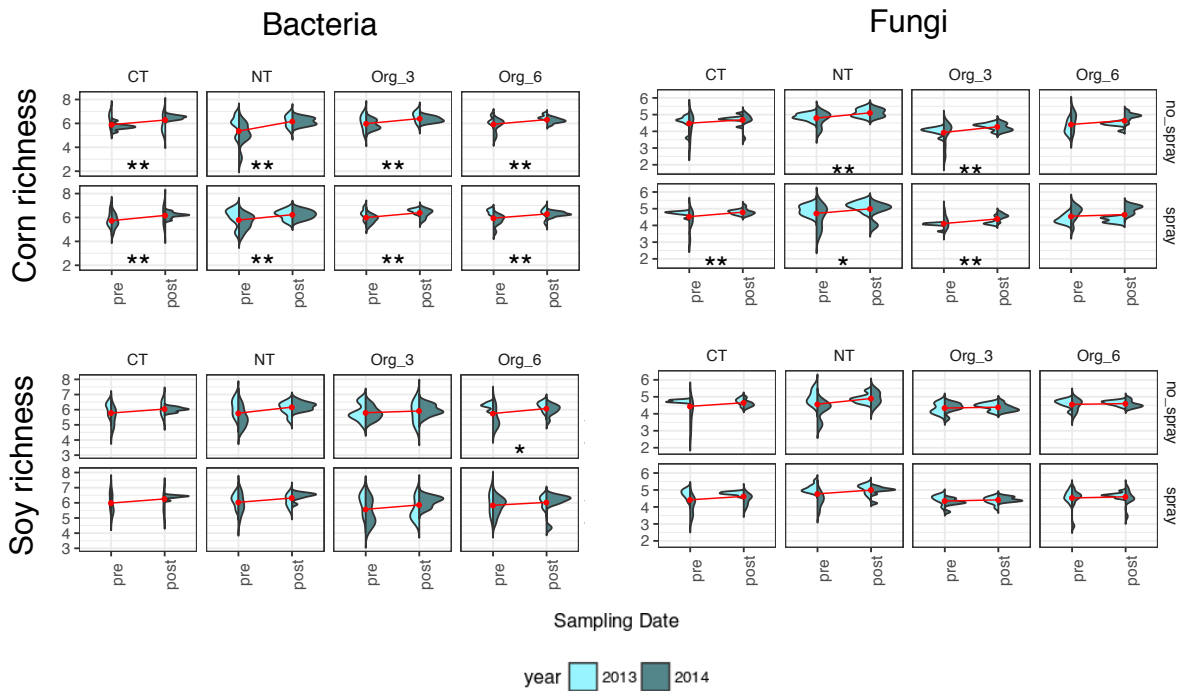
Bacteria
Fungi
Corn richness
Soy richness
CT
NT
Org_3
Org_6
no_spray
spray
pre
post
Sampling Date
year
2013
2014

Figure 4

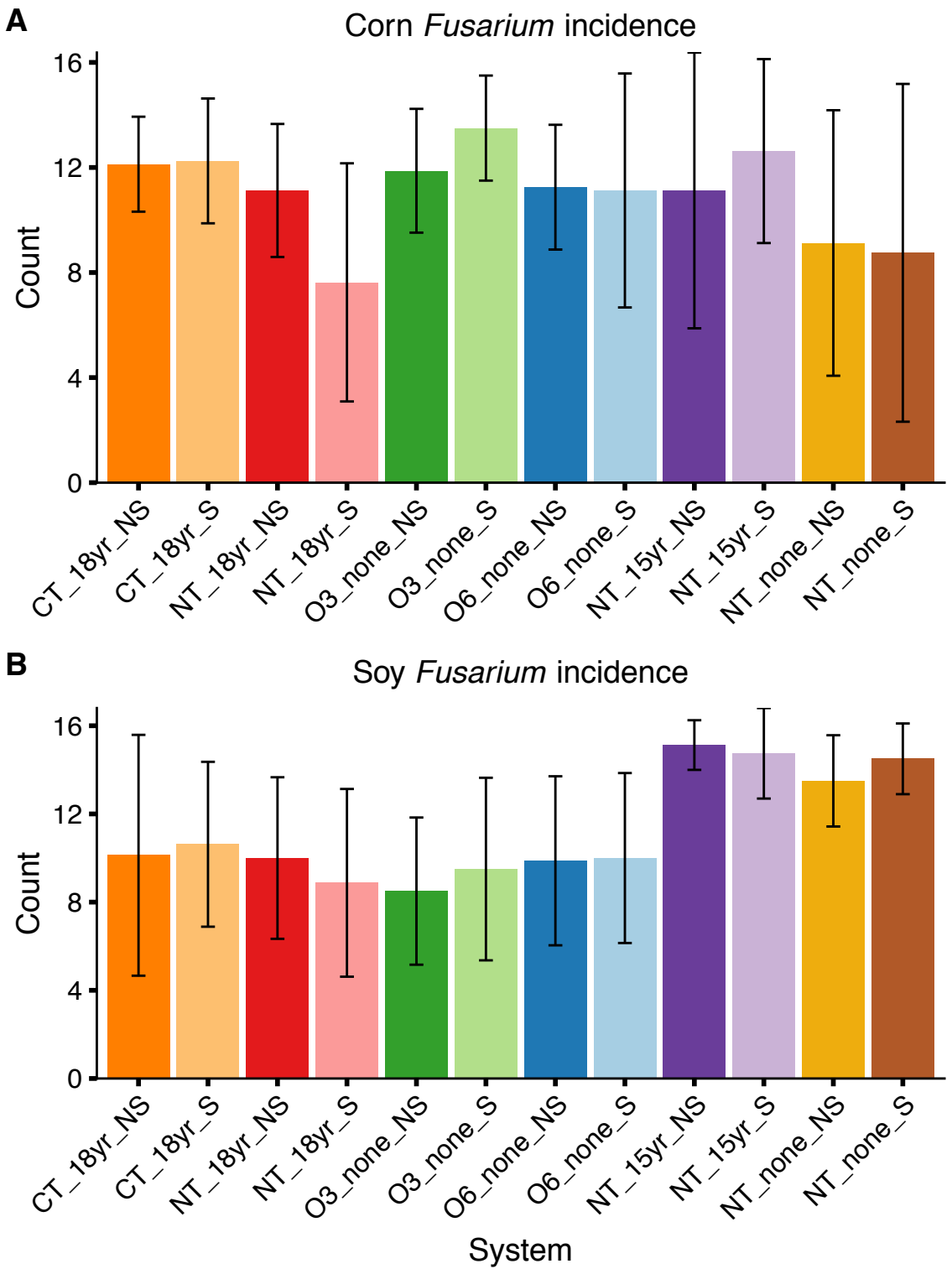
A
Corn *Fusarium* incidence
Count
16
12
8
4
0
CT_18yr_NS
CT_18yr_S
NT_18yr_NS
NT_18yr_S
O3_none_NS
O3_none_S
O6_none_NS
O6_none_S
NT_15yr_NS
NT_15yr_S
NT_none_NS
NT_none_S
B
Soy *Fusarium* incidence
Count
16
12
8
4
0
CT_18yr_NS
CT_18yr_S
NT_18yr_NS
NT_18yr_S
O3_none_NS
O3_none_S
O6_none_NS
O6_none_S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

Figure 5