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Soil Microbial Communities in Diverse Agroecosystems Exposed to the Herbicide Glyphosate

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ABSTRACT Despite glyphosate's wide use for weed control in agriculture, questions remain about the herbicide's effect on soil microbial communities. The existing scientific literature contains conflicting results, from no observable effect of glyphosate to the enrichment of agricultural pathogens such as *Fusarium* spp. We conducted a comprehensive field-based study to compare the microbial communities on the roots of plants that received a foliar application of glyphosate to adjacent plants that did not. The 2-year study was conducted in Beltsville, MD, and Stoneville, MS, with corn and soybean crops grown in a variety of organic and conventional farming systems. By sequencing environmental metabarcode amplicons, the prokaryotic and fungal communities were described, along with chemical and physical properties of the soil. Sections of corn and soybean roots were plated to screen for the presence of plant pathogens. Geography, farming system, and season were significant factors determining the composition of fungal and prokaryotic communities. Plots treated with glyphosate did not differ from untreated plots in overall microbial community composition after controlling for other factors. We did not detect an effect of glyphosate treatment on the relative abundance of organisms such as *Fusarium* spp.

IMPORTANCE Increasing the efficiency of food production systems while reducing negative environmental effects remains a key societal challenge to successfully meet the needs of a growing global population. The herbicide glyphosate has become a nearly ubiquitous component of agricultural production across the globe, enabling an increasing adoption of no-till agriculture. Despite this widespread use, there remains considerable debate on the consequences of glyphosate exposure. In this paper, we examine the effect of glyphosate on soil microbial communities associated with the roots of glyphosate-resistant crops. Using metabarcoding techniques, we evaluated prokaryotic and fungal communities from agricultural soil samples ($n = 768$). No effects of glyphosate were found on soil microbial communities associated with glyphosate-resistant corn and soybean varieties across diverse farming systems.

KEYWORDS agriculture, agroecology, corn, disease ecology, glyphosate, metabarcode, microbiome, organic, soybean

Microbes associated with agricultural crops affect multiple dimensions of plant health. They can play important roles related to plant physiology, such as nutrient acquisition (1, 2) and hormone modulation (3), in addition to aiding the defense against biotic stressors (4) or acting as important pathogens. Despite this importance to plant health, it is only recently that managing microbial diversity has come to be viewed as

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a realistic possibility for the sustainable increase in crop productivity needed to meet food demand in the face of human population growth and climate change (5–7). The intensification of modern agriculture has been driven by the use of pesticides, fertilizers, and other amendments known to affect soil microbial communities (8). However, many studies lack the spatial and temporal replication necessary for statistical rigor (9). A better understanding of how farming systems (including crops) and geography interact to shape microbial communities is needed in order to leverage agricultural microbiomes for food security (10).

The introduction of genetically modified glyphosate-resistant (GR) crops has transformed agroecosystems across much of the globe by increasing the adoption of no-tillage and reduced-tillage agriculture where weeds are controlled chemically (11, 12). No-till farming systems improve soil structure and nutrient retention by reducing erosion while also reducing expenses and fossil fuel use associated with machine operation. Microbial communities in no-till soils are generally more diverse than those in systems receiving tillage due to the increase in niche heterogeneity (13, 14).

Glyphosate interrupts the shikimate biosynthesis pathway (15), 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) with respect to phosphoenolpyruvate, and it is lethal to most species of plants and a large proportion of fungi (16). However, some microbes are resistant to glyphosate due to the rapid metabolism of glyphosate or to a GR form of the gene encoding EPSPS (16, 17). Once this biosynthetic pathway is blocked, plants die due to metabolic disruption. Even at sublethal application rates, glyphosate can weaken a plant's pathogen defenses enough that pathogens are able to infect and kill the plant (18, 19). In the absence of a pathogen, the plant may have a stunted appearance for a few weeks but then recover.

Glyphosate is a foliar-applied herbicide that rapidly translocates from the foliage to the rest of the plant, including the roots (20). Plants can exude glyphosate from their roots within 24 h of foliar application (21, 22). Glyphosate strongly binds to some soil components, being nearly immobilized in most soil types (23). Its tight binding to soil contributes to its weak phytotoxicity to plants as a soil-applied herbicide. Episodic root 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. However, changes in the microbial community are difficult to detect given the concurrent effects of seasonality, changing crop species, and soil type.

While there are clear benefits to no-till agriculture, reports differ on the effect of glyphosate on microbial diversity. Concerns have been raised about increased pathogen loads and suppression of beneficial organisms associated with glyphosate use (24, 25). There are several mechanisms by which glyphosate could enrich the soil for plant pathogens, as follows: (i) pathogens could attack glyphosate-susceptible weeds that succumb to the herbicide, the dying biomass of which then acts as refugium for subsequent crop infestation (green bridge); (ii) pathogens could gain a "foothold" in a glyphosate-resistant plant due to reduced immune response from alterations in the shikimate pathway, resulting in a nonlethal infection while allowing the pathogen to propagate; and (iii) the removal of susceptible microbial taxa could also result in reduced competition for root niche space, allowing pathogens access to plant tissues. A review of all GR crops by Hammerschmidt (19) determined that there is no conclusive evidence that glyphosate increases the susceptibility of GR crops to disease. Another review (26) challenges this assessment. Several studies have observed that GR beets and soybean have increased susceptibility to pathogens when glyphosate is applied at recommended rates (26–28). One study found no effect of glyphosate on disease induction in GR beets until normal field application rates were exceeded by 1 order of magnitude (29). However, other studies with GR crops have found no influence of glyphosate on disease (30) and even some instances of fungicidal activity of glyphosate against some plant pathogens, especially rusts (reviewed by Duke [31]).

Two key studies have supported the glyphosate-pathogen-enrichment hypothesis, finding over long study periods that glyphosate repeatedly increases the rate of colonization of crops by *Fusarium* spp. (presumed to be pathogenic strains) while decreasing the abundance of fluorescent *Pseudomonas* bacteria (taken as putative beneficial organisms) in the soil (24, 28). These studies are often cited as conclusive evidence that long-term use of glyphosate increases the pathogen load and decreases the abundance of growth-promoting bacteria in soils. Both studies applied culture-based methodology to quantify these microbial groups, with molecular analysis of the ribosomal internal transcribed spacer region (ITS) for fungi. Studies using culture-free methodology to characterize microbial communities have failed to detect substantial glyphosate effects on pathogen abundance (32, 33). Farming systems, soil factors, crop varieties, and glyphosate use history can all impact the behavior of glyphosate and its interaction with the crop and soil microbiomes (34) and must be included in the experimental design.

We conducted two field-scale studies to determine the effects of glyphosate on the soil microbiome and plant health for GR corn and soybean varieties, testing the hypothesis that glyphosate changes the composition of the soil microbiome under different soil types, crops, sampling time points, and farming systems. Furthermore, we tested the hypothesis that *Fusarium* sp. sequence abundance or culturable numbers would increase due to glyphosate treatment. Our study included six farming systems studied across 2 years, representing diverse agricultural practices as implemented on working farms. Our study targeted both naive soil microbiomes that had not been exposed to glyphosate and those exposed to glyphosate annually. High-throughput sequencing was used to generate bacterial and archaeal 16S rRNA gene profiles and fungal nuclear ribosomal ITS profiles.

RESULTS

Summary of fungal and prokaryotic diversity across all sites. From sequencing analysis, a total of 68,964 unique fungal and 72,454 unique prokaryotic amplicon sequence variants (ASVs) were identified across all samples. Beltsville, MD, and Stoneville, MS, shared 13,964 prokaryotic and 5,740 fungal taxa. Stoneville featured 62,985 and 29,780 prokaryotic and fungal ASVs, respectively. Beltsville featured 41,538 and 44,924 unique prokaryotic and fungal ASVs, respectively. Fungal diversity was higher for Beltsville than for Stoneville, except for the Shannon's and Simpson's diversity metrics for Org3 (see description of field rotations in Materials and Methods) (Fig. 1A). Conversely, prokaryotic diversity was greater in Stoneville than in Beltsville in all measures (Fig. 1B).

Detrended correspondence analysis showed that the Beltsville and Stoneville communities were distinct (Fig. 1C and D). Permutational multivariate analysis of variance (PERMANOVA) of relative abundance for fungi and prokaryotes revealed that location was the most significant factor accounting for differences in fungal and prokaryotic soil communities ($P = 0.001$ in both cases; fungal $R^2 = 0.19$, prokaryote $R^2 = 0.16$; see Table S1 in the supplemental material). Differences between the Stoneville and Beltsville microbial communities were driven by differences in edaphic factors. Soil chemical characteristics differed between the two locations (canonical discrimination analysis, $P < 0.001$, $R^2 = 0.99$) and among farming systems (canonical discrimination analysis, $P < 0.001$, $R^2 = 0.99$). The soil in Stoneville was significantly higher in pH and the cations As and Sr (analysis of variance [ANOVA], $P < 0.001$), whereas Beltsville soil contained significantly more P, Pb, S, Fe, and organic matter (OM) (ANOVA, $P < 0.001$). Nonmetric multidimensional scaling (NMDS) ordinations of Bray-Curtis dissimilarity for soil chemistry between locations and farming systems are presented in Fig. 2. To increase power to detect the local effects of glyphosate treatment, we analyzed crop (corn versus soybean) separately within each location (Beltsville versus Stoneville).

Fungal community structure and response to glyphosate. Farming system was the largest driver of fungal community structure regardless of crop (Fig. 3) in both Beltsville (PERMANOVA; corn, $P = 0.001$, $R^2 = 0.16$; soybean, $P = 0.001$, $R^2 = 0.16$) and Stoneville (PERMANOVA; corn, $P = 0.001$, $R^2 = 0.24$; soybean, $P = 0.001$, $R^2 = 0.23$). The

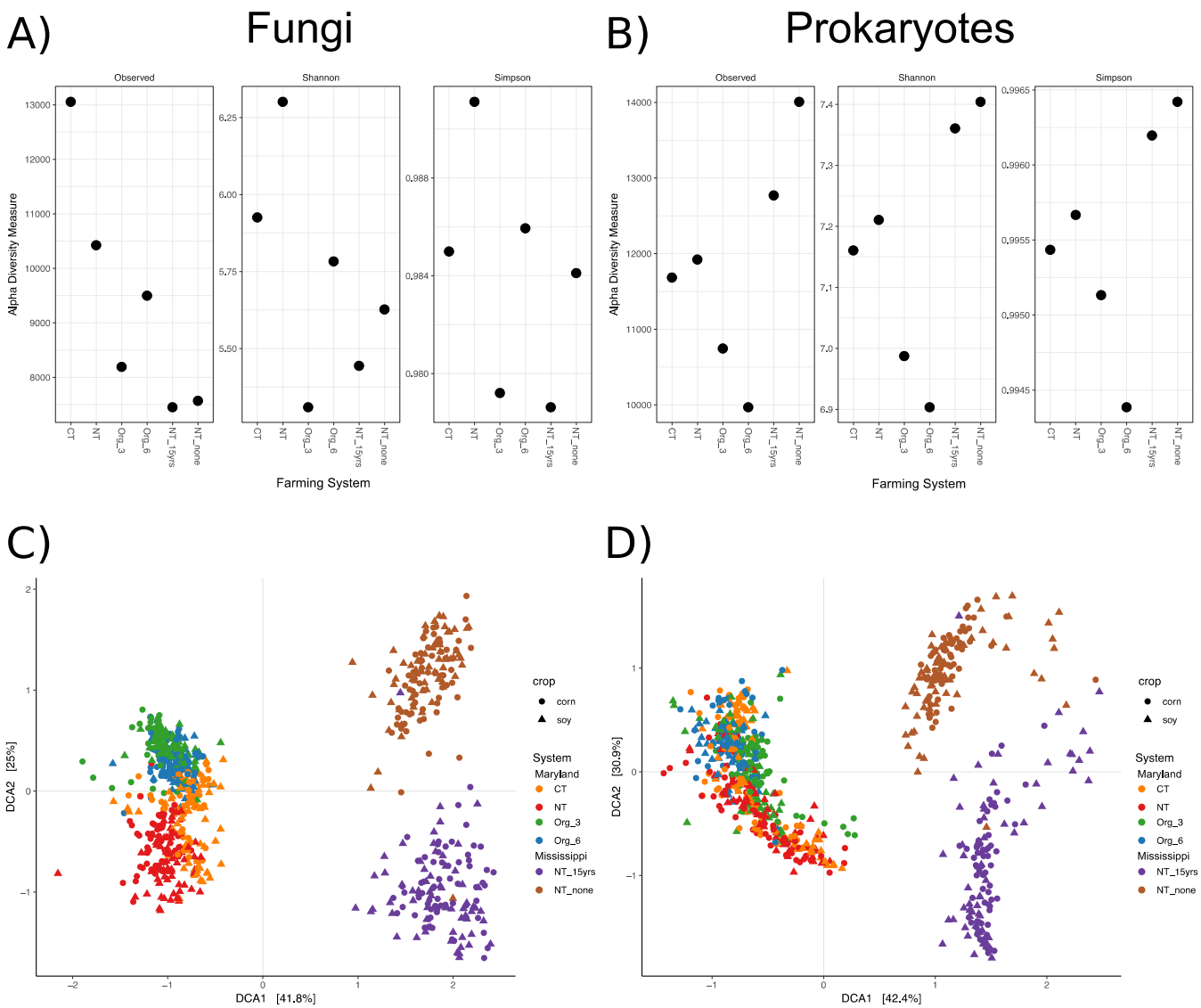

FIG 1 Summary of species diversity and bacterial and fungal community structures for all soil samples from Beltsville, MD, and Stoneville, MS ($n = 768$). (A) Species diversity estimates for fungi across all farming systems. (B) Species diversity for prokaryotes across all farming systems. (C) Detrended correspondence analysis (DCA) of Bray-Curtis dissimilarity for fungal communities from all samples after relative abundance transformation of total counts. (D) DCA of Bray-Curtis dissimilarity for prokaryotic communities from all samples after relative abundance transformation of total counts.

year of sampling was also significant but explained less variance than did the farming system in both Beltsville (corn, $P = 0.001$, $R^2 = 0.046$; soybean, $P = 0.001$, $R^2 = 0.043$) and Stoneville (corn, $P = 0.001$, $R^2 = 0.051$; soybean, $P = 0.001$, $R^2 = 0.052$). The taxonomic identity of fungal diversity is summarized at the level of order in Fig. 4 (see also Fig. S1 and S5 in the supplemental material). Differences among systems are spread along axis 1 of canonical correspondence analysis (CCA) plots, and differences related to year are reflected in the spread along axis 2 in Fig. 3. No significant interaction was noted between sampling date and glyphosate treatment ($P = 0.488$ and 0.296 for corn and soybean, respectively). Rhizosphere partitions (near and far) were also not significantly different (Table S1) for any crop or location. Likelihood ratio tests of taxon abundance in DESeq2 also found no significant increase in the explanatory power of a model containing the sampling date-glyphosate treatment interaction for any taxon, regardless of crop or farming system (Tables S4 and S5).

Prokaryotic community structure and response to glyphosate. Farming system was also significant for prokaryote community structure (Fig. 5) in Beltsville (PER-

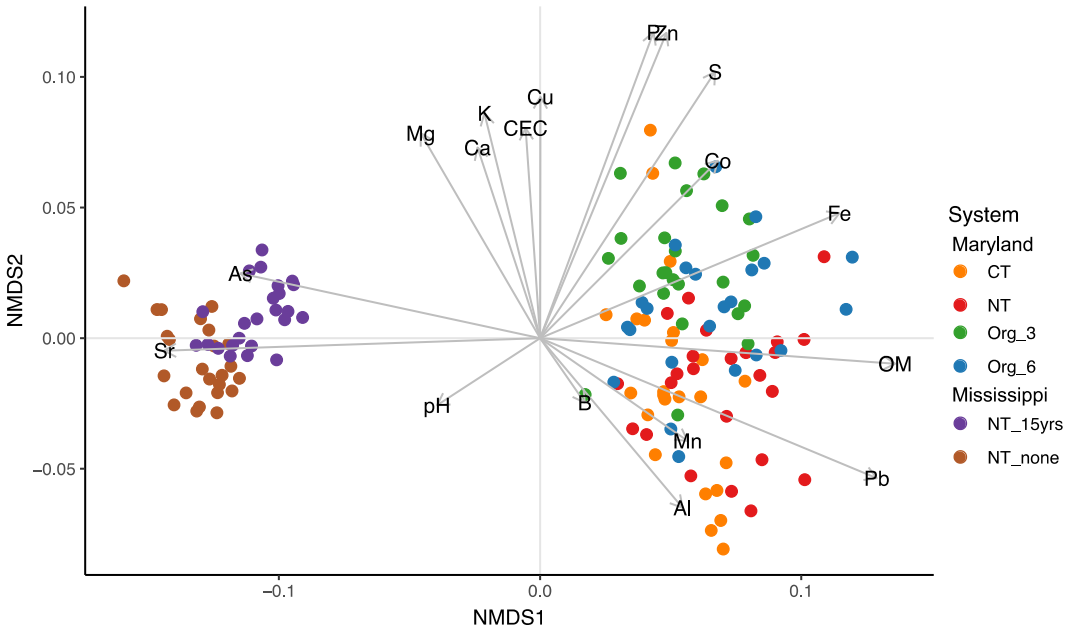

FIG 2 Nonmetric multidimensional scaling ordination of Bray-Curtis dissimilarity for soil chemistry of sites examined in this study. Arrows indicate vectors of the various components of soil composition.

MANOVA; corn, $P = 0.001$, $R^2 = 0.096$; soybean, $P = 0.001$, $R^2 = 0.09$) and Stoneville (PERMANOVA; corn, $P = 0.001$, $R^2 = 0.21$; soybean, $P = 0.001$, $R^2 = 0.16$). The year term explained a smaller amount of variance than did farming system for Beltsville (corn, $P = 0.001$, $R^2 = 0.096$; soybean, $P = 0.001$, $R^2 = 0.086$) and Stoneville (corn, $P = 0.001$, $R^2 = 0.051$; soybean, $P = 0.001$, $R^2 = 0.069$). Differences among systems are spread along axis 1 of CCA plots, and differences related to year are reflected in spread along axis 2 of Fig. 5. Taxonomic identity of prokaryotic diversity is summarized at the level of order in Fig. 6, S6, and S10. The interaction between glyphosate and sampling date was not significant for either crop (Table S1). Likelihood ratio tests of taxon abundance in DESeq2 indicate that the sampling date-glyphosate treatment interaction did not significantly increase the explanatory power of the model for any taxon, regardless of crop or farming system (Tables S6 and S7).

Community richness differences between pre- and postspray samples. Wilcoxon signed-rank tests showed several instances where species diversity differed significantly between the prespray and postspray sampling dates (Fig. 7 and Tables S2 and S3); however, differences were observed in both spray and no-spray treatments for most crop farming system combinations, indicating that this is a seasonality effect and not due to glyphosate exposure. In Beltsville, corn and soybean differed in their response over the two dates. Prokaryote diversity for corn in every Beltsville farming system was significantly different between the two dates. This trend was also observed, but to a lesser degree, in fungal communities. Half of the treatments differed significantly for both spray and no-spray treatments. Fungal communities did not differ seasonally in the Beltsville soybean plots, and fungal species diversity was unaffected by sampling date for both corn and soybean in the Stoneville samples.

Quantification of *Fusarium* CFU. The root endophyte screening required analysis of over 6,100 root segments and identified over 2,400 fungal colonies. Significantly more CFU were observed in 2013 than in 2014 at the Beltsville location ($P < 0.0003$), but no difference in the number of CFU was observed between years at the Stoneville location. A total of 384 of the typical morphotypes were ITS amplicon sequenced, resulting in the following 8 identified dominant taxa: *Fusarium*, *Macrophomina*, *Alternaria*, *Cladosporium*, *Penicillium*, *Zygomycota*, *Trichoderma*, and *Epicoccum*. There are no significant differences in *Fusarium* CFU observed among the glyphosate spray and

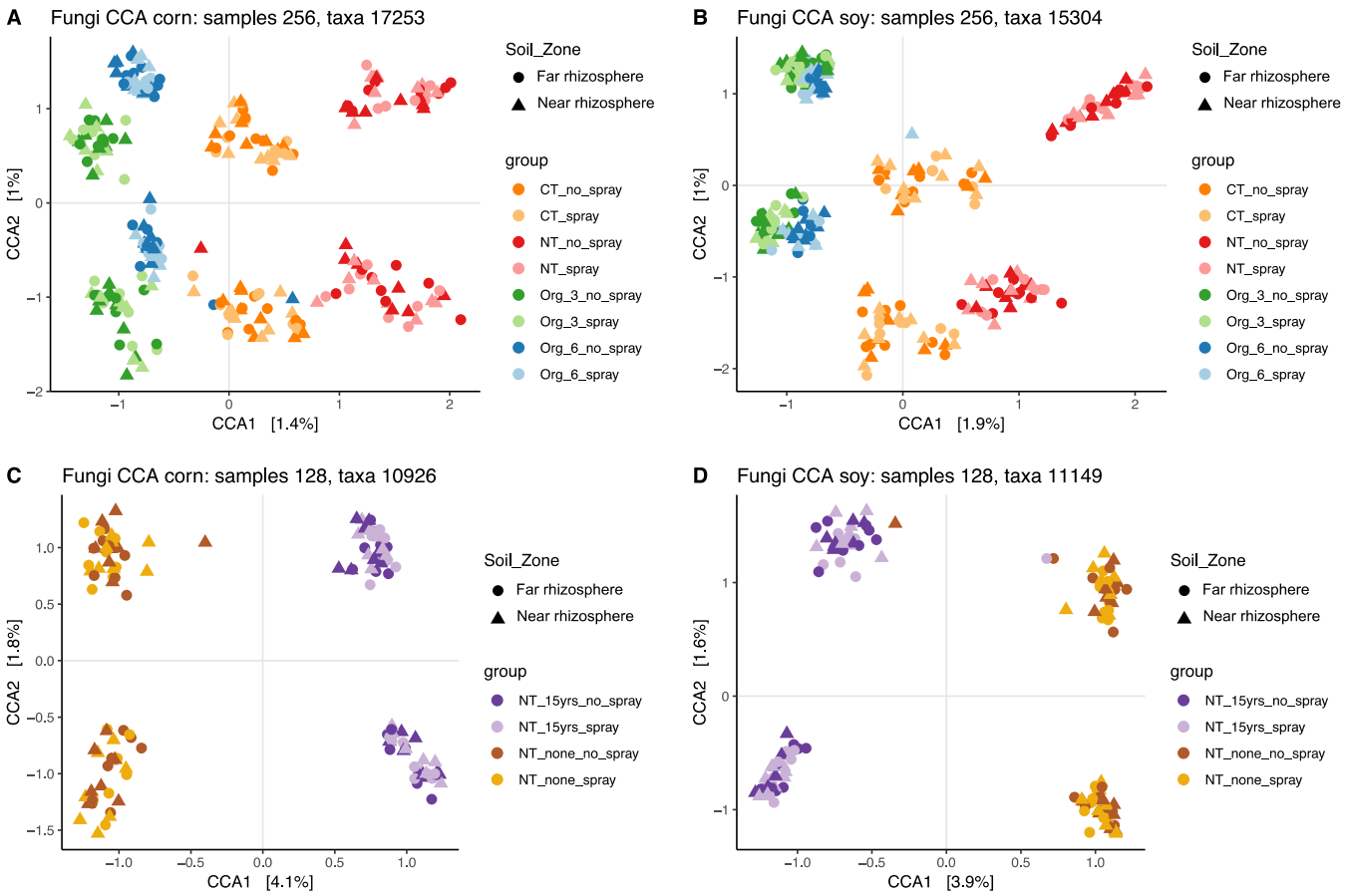

FIG 3 Canonical correspondence analyses (CCA) of soil fungal communities in Beltsville, MD, and Stoneville, MS, partitioned by crop. Data have been variance stabilized with negative binomial transformation in DESeq2. (A) Beltsville corn plots. (B) Beltsville soybean plots. (C) Stoneville corn plots. (D) Stoneville soybean plots.

no-spray treatments for corn or soybean (ANOVA, $P < 0.07$). Although the P value is close to significance, the variance around the means shows no detectable trend in the data (Fig. 8).

Corn and soybean yields. There were no significant differences in corn yield with systems or among glyphosate application treatments for either 2013 or 2014 (Table 1). The corn and soybean yields in this study have been previously published (35, 36). Corn yields were not significantly different from the MD county averages for all systems, with a mean among systems of 9,339 kg ha^{-1}. 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 2,327 kg ha^{-1}. There was no significant difference in yield across farming systems and no effect of glyphosate treatment on yield (Table 1).

DISCUSSION

The structures of prokaryotic and fungal communities among farming systems and between sampling dates were not driven by glyphosate use. Instead, tillage and other farming system differences appear to be the primary drivers of soil microbiome structure (Fig. 1C and D, 3, and 5). For instance, even though all Beltsville fields were under no-till management until 1996, differences in management since then are significant predictors of the current microbial community structure. Organic systems showed an increase in fungi of the order Pezizales (Fig. 4A and B), possibly a response of saprobic taxa to the addition of poultry litter in these plots. Land use history in Stoneville differed significantly between systems, with one system being under no-till

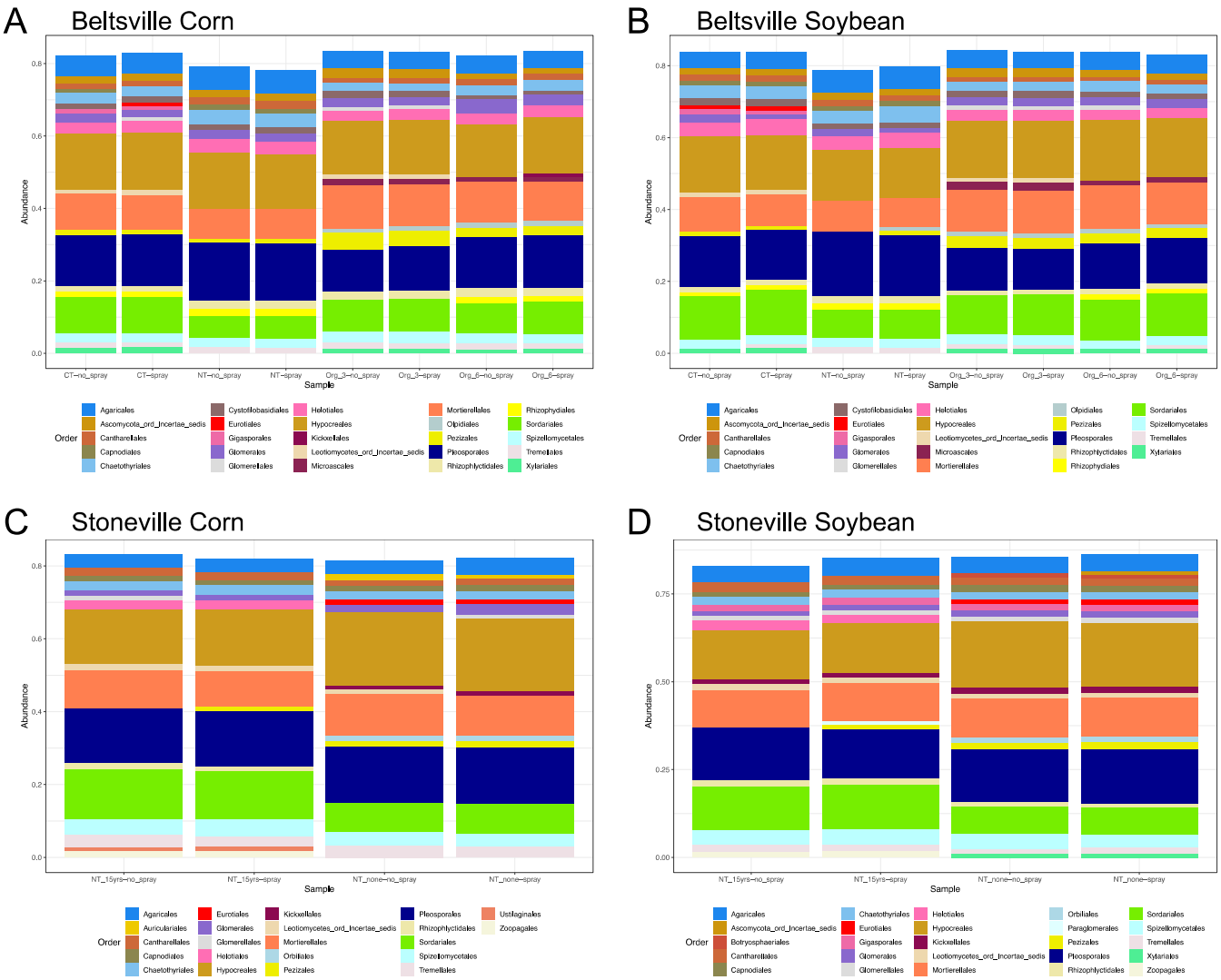

FIG 4 Proportional abundances of soil fungal communities in Beltsville, MD and Stoneville, MS partitioned by crop from data in Fig. 3. Taxa with abundance less than 1% excluded. Plots with all taxa included can be found in Fig. S1 in the supplemental material and 5. (A) Beltsville corn plots. (B) Beltsville soybean plots. (C) Stoneville corn plots. (D) Stoneville soybean plots.

agricultural management with a 15-year history of glyphosate application and the other being a cogongrass monoculture with no history of glyphosate exposure. Differences between fungal communities in the Stoneville plots appear to be due to shifting proportions, with few orders over 5% abundance present in one system while absent in the other (Fig. 4C and D).

Farming management legacy shifted the prokaryotic communities among systems in both Maryland and Mississippi. A history of no-till management appeared to change the structure of the microbial community relative to the conventional till and organic treatments. For example, acidobacteria were detected, although at low relative levels, in the highest proportions in the no-till systems with at least a 15-year history. Acidobacteria are positively responsive to nitrate in the soil and have been shown to produce the plant growth hormone indole acetic acid (IAA), which can promote plant root growth (37). In addition, *Chloroflexi* tended to be at lower relative abundance in the no-till systems in Maryland. Ishaq et al. (38) found the *Chloroflexi* to be one of the most responsive taxa to changes in farming systems. A not-well-known taxon, the *Ktedonobacteria*, also showed differential response to the system-level treatments. Members of this group appear to be sensitive to pH, occupy a diverse array of

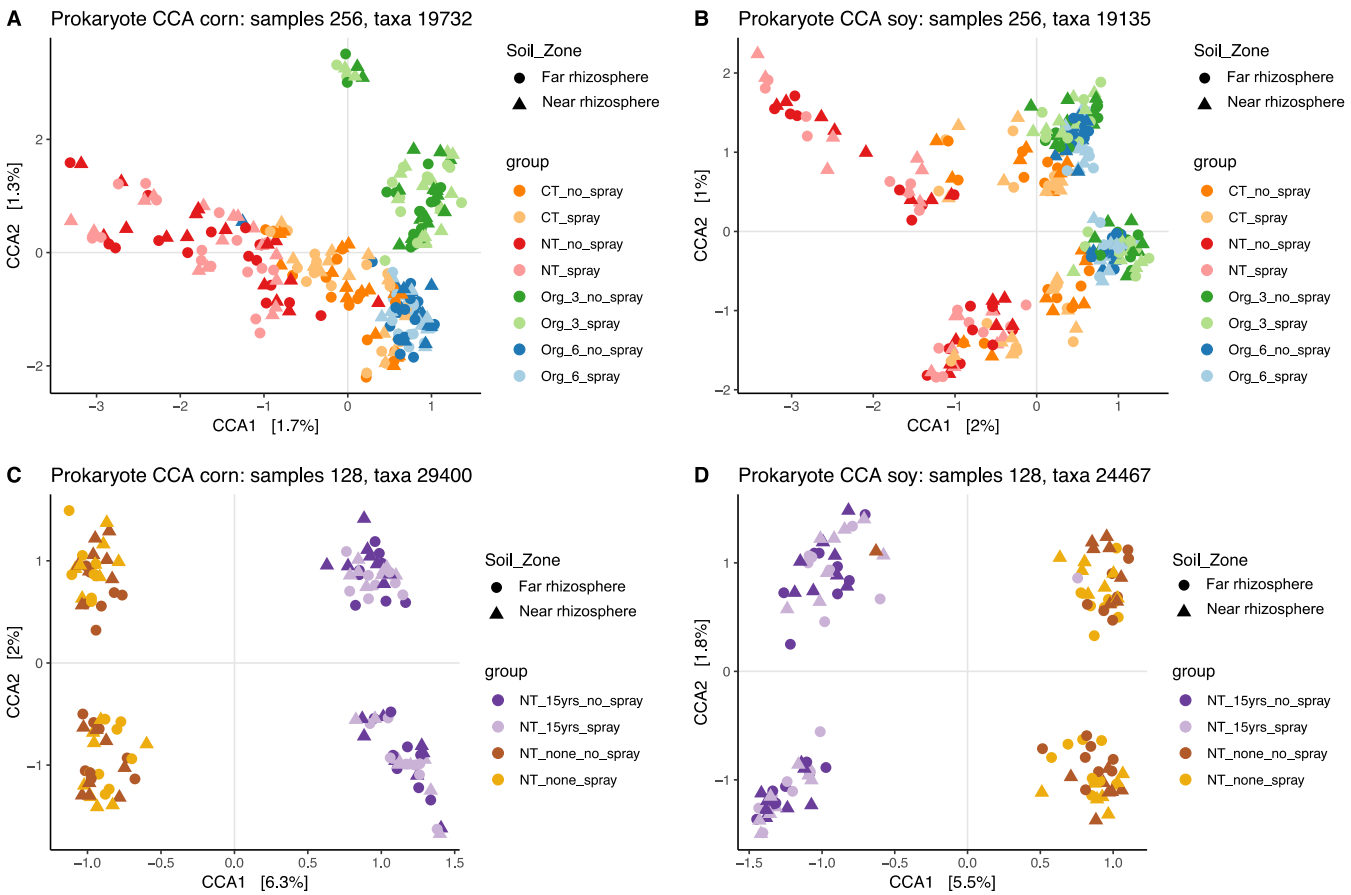

FIG 5 Canonical correspondence analyses (CCA) of soil prokaryotic communities in Beltsville, MD, and Stoneville, MS, partitioned by crop. Data have been variance stabilized with negative binomial transformation in DESeq2. (A) Beltsville corn plots. (B) Beltsville soybean plots. (C) Stoneville corn plots. (D) Stoneville soybean plots.

environments, and are genetically similar to the *Chloroflexi* (39). The results for the prokaryotic and fungal taxa investigated in this study are consistent with farming system differences observed in other dimensions of soil ecosystems in Beltsville, including soil nematode communities (40), soil OM and P concentrations, greenhouse gas emissions, and total energetic costs of the farming system (41–43).

The absence of glyphosate effects in previously naive soil communities suggests that typical application rates of glyphosate do not alter the overall microbial community at the resolution of taxa recovered in our study. Existing literature suggests most microbial communities are susceptible to disturbance, although bias against reporting of no treatment effects could affect this view (44). In the current study resilience to glyphosate could be linked to several factors. Some prokaryotic and fungal species are known to metabolize glyphosate, and the presence of these organisms may protect susceptible species (16, 17, 45, 46). Studies reporting effects of glyphosate on soil microbes often use higher concentrations of the herbicide than the approved rate, which may overwhelm buffering by resistant members. Glyphosate is strongly bound to soil components (17, 23), but how this affects its bioavailability to soil microbes is unknown. Nevertheless, its half-life in temperate climate soils averages about 30 days (47). Concentration-dependent effects of glyphosate on soil microbial respiration and biomass have been reported and are consistent with reports on other agrochemicals, showing only transient effects at the recommended application rates (48).

Greenhouse studies with GR wheat grown in soils from throughout the Pacific Northwest found only minor effects of glyphosate on microbial communities, and the determined location was a major driver of soil microbial community structure (32, 33).

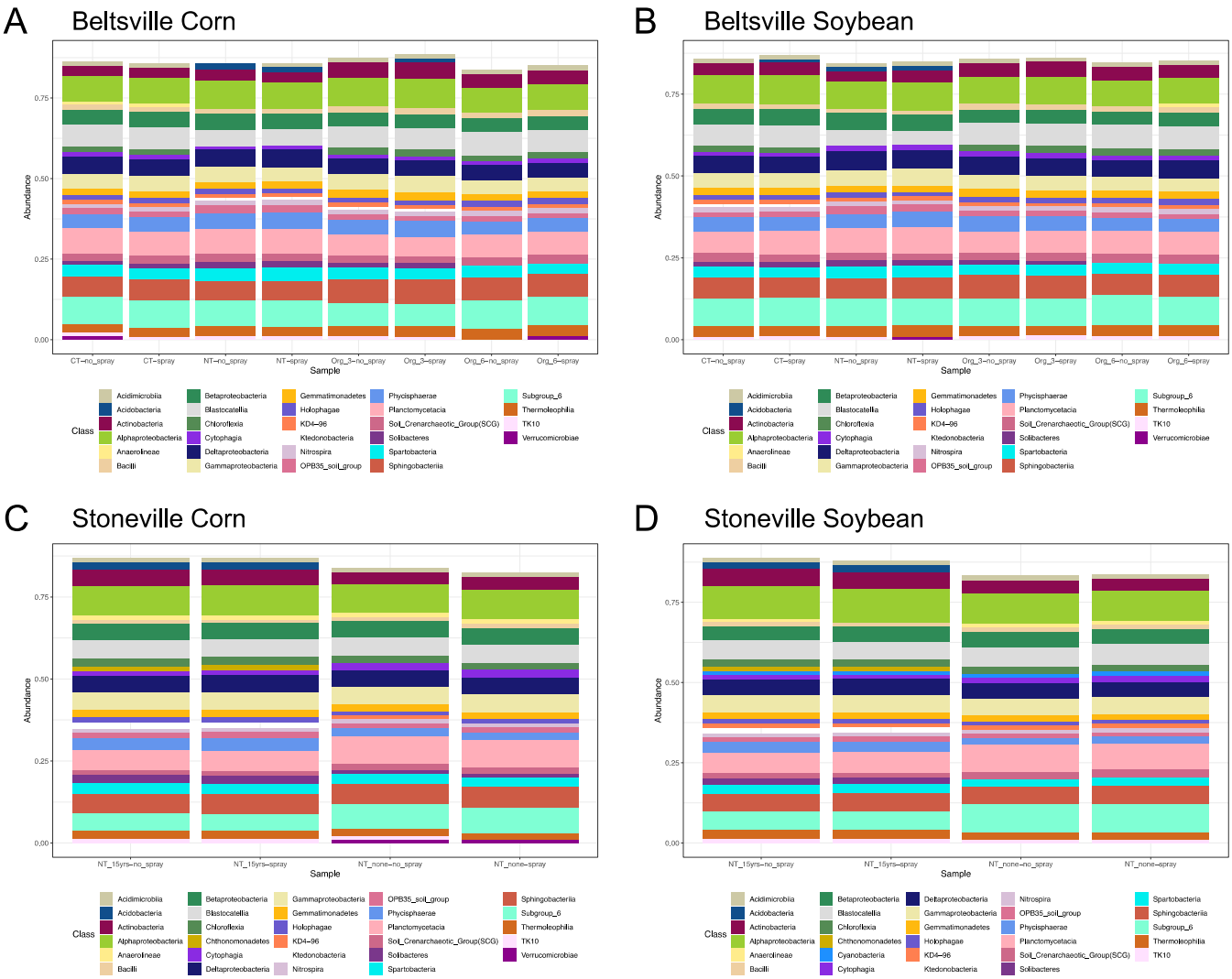

FIG 6 Proportional abundances of soil prokaryotic communities in Beltsville, MD, and Stoneville, MS partitioned by crop from data in Fig. 5. Bars do not sum to 1 because taxa with abundances of less than 1% are excluded, which may cause the appearance of taxa missing among treatments. Plots with all taxa included can be found in Fig. S6 and S10. (A) Beltsville corn plots. (B) Beltsville soybean plots. (C) Stoneville corn plots. (D) Stoneville soybean plots.

While these studies did detect slight effects of glyphosate on microbial communities, glyphosate was applied at twice the recommended rate, increasing the likelihood that the microbial community experienced a detectable effect. This methodological difference may account for detecting an effect on the abundance of some taxa after glyphosate exposure for wheat grown in the greenhouse, while none was detected in our study. This increases confidence in our finding that glyphosate has a minimal effect on the microbial community when applied at the recommended rate.

Community diversity changed across the growing season regardless of glyphosate application (Fig. 7). These results are similar to those of Hart et al. (49) who grew GR corn and its genetically close isoline with and without glyphosate application for one season in Canada. Using terminal restriction fragment length polymorphism (TRFLP) to compare microbial communities, they also showed changes in microbial community diversity over time but not in relation to glyphosate.

Previous culture-based work found that *Fusarium* abundance increased and *Pseudomonas* abundance decreased with glyphosate use (24). In those studies, *Fusarium* spp. were presumed to be pathogenic, while *Pseudomonas* spp. were presumed to be symbionts. Our metabarcoding failed to detect any effect of glyphosate on the abundance of any *Fusarium* or *Pseudomonas* sp. (see the supplemental material).

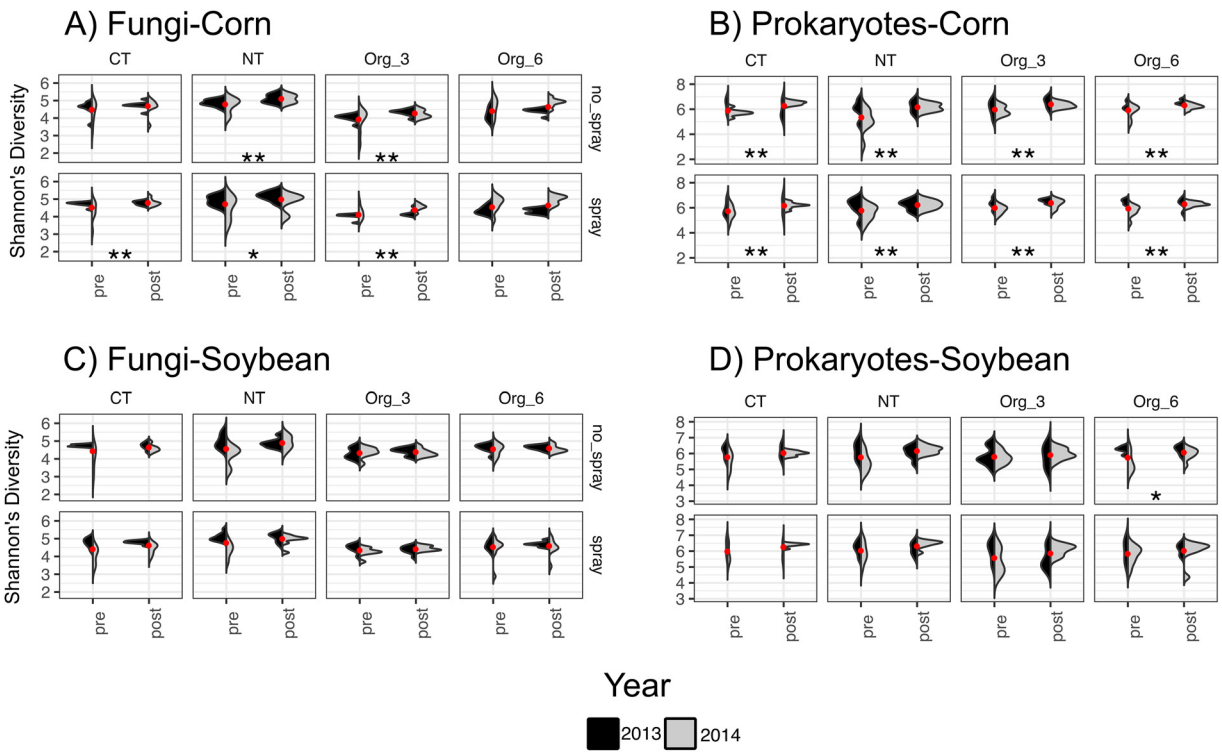

FIG 7 Change in Shannon's diversity of rarefied data across sampling dates in no-spray and spray treatments. The asterisks on each plot are for raw (*) and false-discovery rate corrected (**) *P* values less than 0.05 from Wilcoxon signed-rank test of differences between dates. Years are pooled although graphed separately. Red points represent mean diversity. (A) Fungal Shannon's diversity in corn. (B) Prokaryotic Shannon's diversity in corn. (C) Fungal Shannon's diversity in soybean. (D) Prokaryotic Shannon's diversity in soybean.

It is important to note that the ITS and 16S rRNA genes fail to resolve species-level classifications for certain groups (50, 51). For example, the ITS is known to have limited ability to discriminate between species of *Metarhizium* relative to other available markers (52). Several species of *Metarhizium* known to occur at the Maryland location (53) were not represented in the samples from this study. Most likely, plant pathogens were missed in this study. However, pathogenic species contribute to the relative abundance of their constituent OTU, and we did not detect any change in the relative abundances of *Fusarium* sp., *Alternaria* sp., or *Macrophomina* sp. OTUs increasing due to glyphosate application (see the supplemental material). Although *Pseudomonas* spp. are often taken to be inherently beneficial, there are at least a few confirmed pathogens (51), and the type of beneficial function may differ substantially across strains. Regardless, as with fungi, no *Pseudomonas* spp. changed in prevalence as a result of glyphosate treatment.

We also found no reductions in yield by glyphosate application on GR corn or GR soybean in fields with a long history of glyphosate use or with no history of glyphosate use (Table 1). In a similar study with GR sweet corn, there was even a slight increase in yield associated with glyphosate application (54). This could have been due to hormesis, where nonphytotoxic doses of glyphosate stimulate plant growth (55). The lack of effects on yields are consistent with no substantial detrimental effects on rhizosphere microbes.

Glyphosate is the most widely used herbicide globally, and GR crops are the most widely used transgenic crops (11). In the United States, more than 90% of cultivated farmlands in soybean, cotton, sugar beet, and maize are planted in GR cultivars (56). In 2014, GR crops received 88% of the glyphosate used in U.S. agriculture. The adoption of GR soybeans and the associated heavy use of glyphosate in Argentina and Brazil have followed a trend similar to that in the United States (11). Although yields of corn and soybean in the United States continue to increase at about the same rates as before

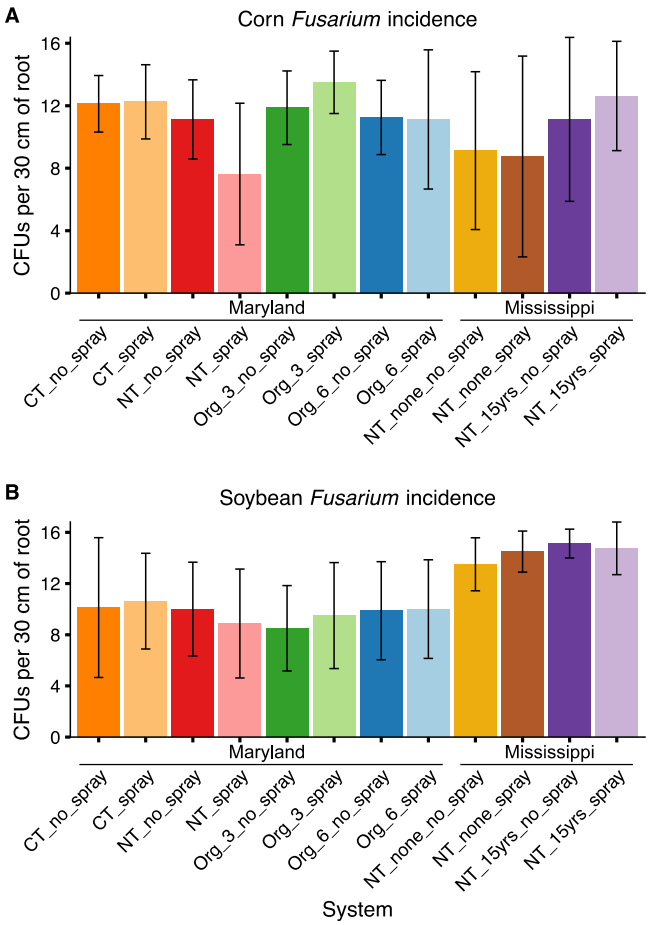

FIG 8 Abundance of *Fusarium* isolates ± standard deviation. (A) Corn roots. (B) Soybean roots. Mississippi and Maryland sites analyzed with ANOVA separately. Colors follow those used in Fig. 3 and 4.

GR crops were introduced (57), a significant amount of literature suggests that glyphosate should compromise GR crops by negatively altering soil microbe populations (see, e.g., references 24–26). However, many of the studies supporting this view have not been conducted in realistic farming situations. For example, one of the studies cited as evidence of strong glyphosate effects on plant-associated microbes was done in a greenhouse on hydroponically grown plants (58). Relatively few studies have investigated the effect of glyphosate on soil microbial communities in farming systems with and without a legacy of glyphosate application. The work described in the current paper provides an important contribution to determining the effect of glyphosate on soil prokaryotic and fungal communities, as it is a well-replicated (in time and space) study at two geographically separated sites in realistic farming systems with well-documented glyphosate use histories. The fact that there are no changes due to glyphosate, coupled with a trend toward higher species diversity in no-till plots, suggests that this widely employed management practice is not at risk of altering soil microbial communities in a negative manner.

MATERIALS AND METHODS

Field conditions and experimental design. The study was conducted in 2013 and 2014 at two U.S. Department of Agriculture, Agricultural Research Service (USDA-ARS) locations, the Sustainable Agricultural Systems Laboratory in Beltsville, MD, and the Crop Production Systems Research Unit in Stoneville, MS (Table 2).

The Beltsville location is managed as part of a USDA Long-Term Agroecological Research site that includes typical farming systems of the mid-Atlantic region described previously (41, 59). We conducted the study in two conventional farming systems, including one using a chisel plow for primary tillage (CT), and one under no-tillage (NT) management. These two systems rely on mineral fertilizers, herbicides, and other pesticides as

TABLE 1 Yield of Maryland corn and soybean for glyphosate-treated or untreated plots in chisel till, no-till, organic 3-year rotation, or organic 6-year rotations[a]

Yr	Crop	Management philosophy	Primary tillage	Yield in glyphosate treatment (kg ha^{-1})[b]	
				Spray	No spray
2013	Corn	Conventional	Chisel till	8,848	9,536
		Conventional	No till	9,141	9,780
		Organic 3-yr	Moldboard plow	7,634	9,210
		Organic 6-yr	Moldboard plow	8,510	8,832
	Soybean	Conventional	Chisel till	–	–
		Conventional	No till	–	–
		Organic 3-yr	Moldboard plow	–	–
		Organic 6-yr	Moldboard plow	–	–
2014	Corn	Conventional	Chisel till	8,967	9,798
		Conventional	No till	11,123	10,757
		Organic 3-yr	Moldboard plow	9,497	10,225
		Organic 6-yr	Moldboard plow	8,185	7,627
	Soybean	Conventional	Chisel till	2,160	2,438
		Conventional	No till	2,016	2,264
		Organic 3-yr	Moldboard plow	2,907	2,315
		Organic 6-yr	Moldboard plow	2,733	2,290

[a]Comparison of means was calculated within each system for the glyphosate-resistant genotype either treated with glyphosate or not.
[b]No significant difference was found between glyphosate-treated or untreated plots within each system in 2014. –, in 2013, an error in plot harvesting resulted in mixing of treated and untreated plots, therefore making the yield data unusable.

needed to manage a corn (*Zea mays*)-rye (*Secale cereale*)-soybean (*Glycine max*)-winter wheat (*Triticum aestivum*)/soybean rotation. Additionally, two organic systems were used at this site. One organic system is a 3-year corn-rye cover crop-soybean-winter wheat/hairy vetch (*Vicia villosa*) rotation (Org3). The second is a 6-year crop rotation (Org6) in which alfalfa (*Medicago sativa*), a perennial crop in place for 3 years, replaces the vetch present in Org3. The organic systems rely on legumes, poultry litter, and K_2SO_4 to supply crop nutrients in accordance to soil test results and local regulations. A moldboard and/or chisel plow is used for primary tillage, and weed control included the use of a rotary hoe and between-row cultivation after corn and soybeans were planted in the organic systems.

In Stoneville, the experiment is composed of two farming systems established in two adjacent fields, one with a 15-year legacy of glyphosate use (NT_15yrs) and the other with no glyphosate history (NT_none). There were four replicates delineated within each field for each farming system. 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*) 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. During the experiment, each field (NT_15yrs or NT_none) was split in half, with one half planted to the corn and one half planted to the soybean phase of the experiment. The following year, portions of the field that had been planted to corn were planted to soybean and vice versa.

The experiment was conducted during both the corn and soybean phases of crop rotations at both locations. At each location within each farming system and crop combination, the following glyphosate treatments were established: a GR cultivar with no glyphosate applied, and the same GR cultivar with glyphosate applied at 0.87 kg ha^{-1} twice at 4 weeks after planting. There were two sampling events for each experimental unit. Soil and root samples were taken at the "prespray" time point, which was at growth stage V4. The next day, glyphosate was applied to the plots scheduled to receive glyphosate. Approximately 20 days later, a "postspray" sample was taken in each experimental unit. Experimental units at all locations were four rows 4.6 m wide and 6.1 m long. Soybean cultivar USG Allen (GR) was planted at 526,400 seeds ha^{-1}, and the corn cultivar DKC 65-17 RR2 (GR) was planted at 67,600 seeds ha^{-1}. In Beltsville, the corn or soybean plots are each a phase of the main plot rotation which is a farming

TABLE 2 Description of farming systems represented in field experiments in Beltsville, MD, and Stoneville, MS, in 2013 and 2014[a]

Location	Management philosophy	Primary tillage	Glyphosate history	Farming system name
Beltsville	Conventional	Chisel till	Yes	CT
	Conventional	No till	Yes	NT
	Organic, 3-yr rotation	Moldboard plow	No	Org_3
	Organic, 6-yr rotation	Moldboard plow	No	Org_6
Stoneville	Conventional	No till	Yes	NT_15yrs
	Conventional	No till	No	NT_None

[a]Each farming system was represented by glyphosate-resistant (GR) corn and soybean, and each was not treated or treated with glyphosate at 0.87 kg ha^{-1}.

system (NT, CT, Org3, or Org6); thus, each phase of the rotation is considered a split-plot of the main plot. At both locations, four replicates of each factor and level were established. All plots were kept weed free by hand hoeing as needed.

In October of each year at both locations, corn was harvested with an Almaco small-plot combine (Nevada, IA); grain yield was calculated at 15.5% moisture from the two center rows of the 6.1-m plots. In both years at the Stoneville site, soybean was harvested with an Almaco small-plot combine. In Beltsville in 2013, soybean was harvested with an Almaco small-plot combine, and in 2014, soybean was 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 silt loam Ultisols consisting primarily of Christiana, Keyport, Matapeake, and Mattapex soil map units. The Stoneville soils were a silt loam typic Alfisol 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) sampled in a semirandom 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, 1 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) content, cation exchange capacity (CEC), and P, K, Mg, Ca, S, B, Zn, Mn, Fe, Cu, As, Al, Ba, Cd, Co, Cr, Ni, Pb, Se, and Sr content. 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 and Ketterings (60). Mehlich 3 extractions were conducted to obtain soil Ca, Mg, and K; all other metals are expressed as total sorbed element using the EPA 3050 method (61).

Rhizosphere soil and root sampling. At the V3 to V4 crop growth stage (4 to 6 weeks after planting) and 1 day prior to glyphosate application (prespray), six plants and root-associated soil were excavated from each plot by removing soil monoliths with a 30-cm diameter (crop stem at center) and 15 cm deep using surface-sterilized sharpshooter shovels. Monoliths were placed on a sieve, and soil around the root ball was gently removed by shaking and passed through a 2-mm sieve, here called "far rhizosphere soil." Soil adhering to roots after this procedure was brushed onto a 2-mm sieve using a camel hair brush, here called "near rhizosphere soil." Roots were brushed thoroughly without compromising the integrity of the root surface. Rhizosphere samples from the six plants were pooled and 5 g added to a 15-ml Falcon tube containing 10 ml of Mo Bio LifeGuard nucleic acid preservation solution. The contents of the tubes were mixed and frozen at −80°C. Plants were placed at 4°C until further processing.

Identification of endophytes from roots. Two-centimeter sections of root were cut at random 16 times from each of six fresh root systems for each treatment. The total wet weight of the 16 sections was recorded. Sections were surface sterilized for 2 min in 1.25% sodium hypochlorite, followed by three rinses in sterile distilled water. Sections were blotted dry on sterile paper towels, and eight root sections were placed on a plate containing Komada's medium (62). Plated roots were incubated in ambient light at room temperature until colonies emerged. Fungal mycelia and spores from emerging colonies were sampled and examined on a Nikon E60 microscope and identified to the genus level, or to broader morphological groups, based on taxonomic features. Colonies of typical morphology were plated onto minimal medium to induce sporulation for further identification. PCR screens for ITS, followed by cloning and sequencing, were conducted on over 384 colonies of typical morphology to validate microscopic identification. The methods followed those described in reference 63. Sequences were quality checked and aligned using the DNAStar suite software (DNAStar, Madison, WI, USA) and identified using the Basic Local Alignment Search Tool and GenBank nucleotide data bank from the National Center for Biotechnology Information in Bethesda, MD (https://www.ncbi.nlm.nih.gov/).

Illumina sequencing library preparation from rhizosphere soils. Rhizosphere and bulk soils preserved in LifeGuard at −80°C were thawed, and 800 μl of each slurry was processed using a PowerSoil-htp 96-well soil DNA isolation kit (Mo Bio Laboratories, Inc., Solana Beach, CA), according to the manufacturer's recommendations. DNA was quantified and quality verified using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Pittsburgh, PA). Bacterial 16S rRNA gene amplicons were generated with the primers f515 and r806 (64). Fungal ITS amplicons were generated with the primers ITS1 and ITS2 (65). 16S rRNA gene and ITS metabarcode sequencing was conducted according to the Illumina protocol library preparation manual (part number 1504423 rev. B; Illumina, Inc.). Five microliters of cleaned adapter amplicon product for each sample was used for index PCR using the Nextera XT index kit (part no. FC-131-1002; 16S metagenomic sequencing library preparation manual part no. 1504423 rev. B; Illumina, Inc.). Index PCR products were cleaned according to the Illumina protocol (16S metagenomic sequencing library preparation manual part no. 1504423 rev. B; Illumina, Inc.), and 2-μl aliquots per sample from each 96-well PCR plate were pooled for the final Illumina library. For analysis, 100 μl of 10 nM solutions of each library pool were frozen and shipped on dry ice for analysis on an Illumina MiSeq system at the Center for Genome Research and Bioinformatics (CGRB), Oregon State University, Corvallis, OR. For Beltsville, a total of 512 samples were sequenced. For Stoneville, 256 samples were sequenced.

Bioinformatics and statistical analysis. (i) Sequence filtering and trimming. Reads were returned from the CGRB after initial quality control with standard Illumina workflows, including quality filtering and adapter trimming. The scripts used in subsequent steps can be found at https://github.com/rmkepler/FSP_script_repository. Prior to joining paired ends and taxonomy assignment, forward and reverse primers were removed and sequences quality trimmed (-q 22) at the 3′ end using Cutadapt (version 1.8.3) (66). Reads lacking primer sequences or shorter than 75 bp before trimming were discarded.

(ii) Assembly and taxonomy assignment. The R package Dada2 (67) was used for paired-end assembly and taxonomy assignment. The command "filterandtrim" was used to remove sequences with an expected error rate of >2 and any sequences containing "N" values (unreadable bases). Error rates

were estimated for forward and reverse reads. The filtered reads were then dereplicated with the "derepFastq" command. Dereplicated sequences were denoised with the "dada" command, and then paired ends were merged. Chimeric sequences were removed with the command "removeBimeraDenovo." Taxonomy was assigned to the chimera-free table of sequences with the dada2 implementation of the RDP Classifier (68). The UNITE database (v. 7.2) (69) was used as the reference for identification of fungal ITS sequence variants, and SILVA (release 132) (70) was used for prokaryotes.

(iii) Community analysis. We transformed 16S rRNA gene and ITS community count data into relative abundances and then calculated Bray-Curtis dissimilarity. Detrended correspondence analysis (DCA) was applied to the Bray-Curtis dissimilarity matrix using the VEGAN package v. 2.4 (71) as implemented in phyloseq v. 1.22.2 (72) for both fungal and prokaryotic barcodes. PERMANOVA was used to assess the significance of the crop and location factors.

After subsetting by crop and location, richness and evenness were estimated from rarefied data sets of the raw sequence counts using VEGAN. DESeq2 v. 1.18.1 (73) was used to produce variance stabilized data sets (74) from unrarefied counts. Ordinations were produced using canonical correspondence analysis (CCA) and a model of the form "$\sim$ system + year" with VEGAN. We used PERMANOVA to determine significance of main effects and interactions between the following factors: farming system, soil zone, glyphosate treatment, sampling date, and year. The farming system factor had 4 categories for Beltsville (CT, NT, Org3, and Org6) and 2 for Stoneville (NT_none and NT_15yr). All other factors had two categories at both locations, as follows: soil zone (bulk and rhizosphere), year (2013 and 2014), glyphosate treatment (spray and no spray), and sampling date (prespray glyphosate application and postspray glyphosate application). A repeated-measures model based on the plot identification (ID) was used.

The effect of glyphosate treatment on microbial communities was tested with the Wilcoxon signed-rank test of differences between dates as implemented in the longitudinal plug-in for Qiime2 (75). The test was applied separately for three measures of diversity for variance-stabilized data as determined with VEGAN, observed, Shannon's index, and Simpson's index.

(iv) Differentially abundant taxa. Tests for differentially abundant taxa in response to glyphosate treatment were conducted in DESeq2 using likelihood ratio tests after subsetting fungal and prokaryotic data by location, crop, and farming system. The test compared a full model including group, sampling date terms, and an interaction term, where group is defined as the combination of farming system and glyphosate treatment (e.g., Org3_spray) and sampling date corresponds to prespray and postspray sampling events. The full model was compared to a reduced model lacking the interaction term. Thus, taxa with significant *P* values indicate that sampling date and glyphosate application interacted to be important predictors of their abundance. This was tested for every fungal and prokaryotic taxon identified. Data sets with untransformed counts were used as the starting data, which were then variance stabilized during testing.

Differences in soil chemistry were assessed by canonical discriminant analysis using the candisc v. 0.8.0 package in R. Differences in soil chemistry were visualized with nonmetric multidimensional scaling ordination of Bray-Curtis dissimilarity and vectors plotted for the various chemical constituents using the metaMDS and envfit functions of VEGAN, respectively.

Data availability. Data are accessible under NCBI BioProject number PRJNA548504.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 1 MB.

SUPPLEMENTAL FILE 2, XLSX file, 6.3 MB.

SUPPLEMENTAL FILE 3, XLSX file, 3.1 MB.

SUPPLEMENTAL FILE 4, XLSX file, 8.3 MB.

SUPPLEMENTAL FILE 5, XLSX file, 8.7 MB.

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We declare no conflicts of interest.

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