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# Glyphosate Applications, Glyphosate Resistant Corn, and Tillage on Nitrification Rates and Distribution of Nitrifying Microbial Communities

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**We tested the null hypothesis that nitrification and the distribution of archaeal and bacterial nitrifying communities would not be impacted by long-term applications of the herbicide, glyphosate, to glyphosate-resistant (GR) and non-glyphosate-resistant corn (nonGR) under conventional tillage (CT) and reduced tillage (RT) systems. Bulk and rhizosphere soil nitrification potential rates and quantification of *amo*A genes of archaeal (AOA) and bacterial (AOB) communities were undertaken. In 2013, the nitrification rate of nonGR corn with no glyphosate application treatment associated with bulk and rhizosphere soils under RT displayed greater ($P < 0.05$) nitrification rates than GR corn with glyphosate applications. In 2014, the nitrification rate of the rhizosphere soil under RT in GR corn with no glyphosate application treatment was greater ($P < 0.05$) than other rhizosphere soil treatments. Ratios between archaeal and bacterial *amo*A genes indicated distinct dominance of AOA communities in the rhizosphere soil. The ratios between AOA and AOB *amo*A genes in bulk soil were more balanced. Regression analyses indicated more significant correlations between nitrification potential determinations and bulk soil nutrients ratios, nutrient acquisition ratios, and microbial communities than between analogous rhizosphere soil characteristics. Compared to non-glyphosate treatments, glyphosate applications appeared to disrupt rhizosphere nutrient, exoenzymatic, and microbial relationships. Glyphosate applications had an inconsistent inhibitory effect on the nitrification process. In contrast to bulk soil, rhizosphere soil nitrifying communities were most likely in competition with corn roots for ammonia-N, and were thus dominated by more oligotrophic archaeal nitrifiers compared to bacterial nitrifiers.**

**Abbreviations:** AOA, archaeal ammonia oxidizing; AOB, bacterial ammonia oxidizing; AP, phosphatase; BG, β-glucosidase; CT, conventional tillage; FDA, fluorescein diacetate; GR, glyphosate-resistant; LAP, leucine aminopeptidase; NAG, β-N-acetylglucoaminidase; RT, reduced tillage.

## Core Ideas

- **The herbicide glyphosate can affect nitrification rates.**
- **Archaeal nitrifiers were dominant in the rhizosphere.**
- **In bulk soil, bacterial and archaeal nitrifiers were evenly distributed.**

Modern cropping systems in the mid-southern United States are dominated by glyphosate resistant (GR) crops. In 2016, 89% of corn, 94% of soybean, and 89% of cotton acreage was planted with GR cultivars (USDA–NASS, 2016). The availability of GR crops along with strategic applications of the non-selective herbicide glyphosate [N-(phosphomonomethyl) glycine] for controlling weeds has facilitated the adoption of conservation tillage (Locke et al., 2008). In 2008, about 40% of corn production in the United States was under some form of conservation tillage (Conservation Technology Information Center, 2016). Wide spread adoption of GR crops has increased glyphosate use and public concern. Some studies suggest that glyphosate may result in shifts in soil microorganisms, with beneficial organisms decreasing and non-beneficial organisms increasing (Kremer and Means, 2009; Zobiole et al., 2011).

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Glyphosate is a xenobiotic compound with a half-life in soil ranging between 1.7 and 142 d (Coupe et al., 2011). The sorption coefficient ($K_f$) of glyphosate ranges between 0.6 and 215, and contradicts the assertion that it always adsorbs readily to soil (Locke et al., 2008). When applied to crop foliage, glyphosate is translocated into metabolic sinks including roots and may be released into the rhizosphere. Glyphosate from corn root exudates can diffuse into rhizosphere soil and be hydrologically transported into bulk soil (Coupland and Caseley, 1979). Detection of glyphosate and its metabolite, aminomethylphosphonic acid (AMPA), in soil water implies these compounds interact with rhizosphere and bulk soil microbial communities and may affect their metabolic processes, as well as their exoenzyme activities that initiate organic matter mineralization (Jenkins et al., 2017). Sannino and Gianfreda (2001), for example, reported that glyphosate inhibited phosphatase activity of various Italian soils. Martens and Bremner (1993) demonstrated inhibitory effects of glyphosate on nitrification of urea N in two out of four Iowa soils. Carlisle and Trevors (1986) reported the potential of glyphosate to inhibit nitrification. In contrast, Stratton and Stewart (1991) reported results of an eight month field study showing glyphosate had no effect on nitrification in a conifer forest. A recent comprehensive review on glyphosate effects on crop rhizosphere microbiota concluded that most of the available data support the view that neither the GR transgenes nor glyphosate use in GR crops increases crop disease (Duke et al., 2012). We present experiments designed to determine the effects of glyphosate on soil nitrification and nitrifying microbial communities in fields of GR corn in mid-southern United States.

Nitrification, a fundamental process in the global nitrogen cycle, is the microbial mediated transformation (oxidation) of ammonia to nitrite, and nitrite to nitrate (Kowalchuk and Stephen, 2001). Until Leininger et al. (2006) demonstrated that members of the crenarchaeota are a significant component of the soil nitrifying community, the first step in the nitrification process, ammonia to nitrite, was thought to be undertaken solely by autotrophic ammonia-oxidizing $\beta$- and $\gamma$-proteobacteria (Kowalchuk and Stephen, 2001). Recently, van Kessel et al. (2015) and Daims et al. (2015) reported on the isolation and characterization of a member of the genus *Nitrospira* that oxidizes ammonia to nitrite and nitrite to nitrate to complete the nitrification cycle. The focus of our research was on the microbial communities that initiate the nitrification process, the oxidation of ammonia to nitrite.

The observation that glyphosate appeared to inhibit nitrification implies that it can have a direct impact on microbial communities involved in initial oxidation of ammonia to nitrite. Repeated applications of other herbicides to fields appear to have the potential to impact both archaeal ammonia oxidizing (AOA) and bacterial ammonia oxidizing (AOB) communities. Hernandez et al. (2011) reported results of a microcosm study that demonstrated inhibitory effects of the herbicide simazine on nitrification in a soil amended with ammonium, and appeared to affect the AOB community, but not the AOA community. Results of another microcosm study indicated that the herbicide chlorimuron-ethyl inhibited growth of both AOA and AOB communities (Tan et al., 2013). Li et al. (2015) reported results of a long-term field study indicating that reduced tillage (RT) compared to conventional tillage (CT) decreased rates of nitrification without affecting the concentrations of AOA and AOB communities. Segal et al. (2017) reported results of a long-term, continuous maize study indicating that disk tillage decreased by 10-fold the AOB community but had no effect on the AOA community. Results of a 30-yr crop rotation and tillage study indicated that no-till supported greater abundances of archaea and AOA communities than conventional tillage (Munroe et al., 2016).

The objective of this study was to test the hypothesis that routine applications of glyphosate on GR and nonGR corn (isogenic cultivars) under CT and RT management practices would affect bulk and rhizosphere soil nitrification rates or distribution of ammonia oxidizing archaeal (AOA) and bacterial (AOB) communities and their relation to total archaeal and bacterial 16S rRNA gene copy numbers. As a sub-objective, we determined correlation coefficients between nitrification potential activities, and activities of four exoenzymes involved in the initial steps of organic matter mineralization, nutrient acquisition ratios (Sinsabaugh et al., 2009), total microbial activity as measured by fluorescein diacetate (FDA) activity, gene copies and ratios of AOA and AOB nitrifying communities, and gene copies and ratios of archaeal and bacterial 16S rRNA gene copies.

## MATERIALS AND METHODS

### Experimental Design

Our study was established on the USDA–ARS Crop Production Systems Research Unit Farm, Stoneville, MS in the fall of 2007 as described by Jenkins et al. (2017). In brief, the experimental design consisted of a split-split plot with four replications: the main treatments were conventional tillage (CT) and reduced tillage (RT); the split treatment was isogenic cultivar (glyphosate-resistant [GR] and non-glyphosate-resistant [nonGR]), and the split-split treatment was with and without glyphosate application.

### Field Plot Establishment

Field plot preparation was initiated in 2007, and consisted of disking, subsoiling, and disking. Conventional tillage plots were disked each fall after corn harvest from 2007 to 2014. Reduced tillage plots received no tillage operations after their establishment in the fall of 2007. Each year a regime of herbicide applications was applied to each plot that consisted of (i) burning down of all plots with herbicides 2,4-D (1.1 kg a.i. $ha^{-1}$) in February, and (ii) paraquat (1.1 kg a.i. $ha^{-1}$) before planting. Pre-emergence herbicides atrazine (1.7 kg a.i. $ha^{-1}$) and metolachlor (1.7 kg a.i. $ha^{-1}$) were applied right after corn planting. Glyphosate, Roundup Weathermax formulation (Monsanto Agricultural Co., St. Louis, MO), was applied as recommended at 0.84 kg a.i. $ha^{-1}$ once at early and once at late crop development. There was a no glyphosate control. For each year of this field experiment the first glyphosate application occurred within a month after planting the corn and was applied over-the-top. The second application

was applied 2 to 4 wk after the initial glyphosate application and was directed to the base of the corn. In nonGR corn plots glyphosate applications corresponded with applications to the GR treatments and were made with a hooded sprayer. In the third week of May, Halosufuron (0.07 kg $ha^{-1}$) was applied on all plots to manage yellow nutsedge. To all plots 225 kg N $ha^{-1}$ (in the form of a mixture of liquid urea and ammonium nitrate) was applied 3 wk after planting. Eight rows spaced 102 cm apart and 32 m long made up each plot. To establish a baseline with no transgenic influence, non-glyphosate-resistant soybean [*Glycine max* (L.) Merr.] was planted in 2007. In 2008 and each year to 2012 commercial GR and nonGR corn hybrids were planted in respective plots the first 5 yr of the study. Commercially available isogenic cultivars, glyphosate-resistant DeKalb DKC65-17 (RR2) and non-glyphosate-resistant DeKalb DKC65-18 (conventional) hybrids, were planted on 20 Mar. 2013, and 20 Mar. 2014.

At the corn R2 growth stage (27 and 42 d, respectively, after the last glyphosate application) in 2013 and 2014 bulk soil and rhizosphere soil samples were taken from the field plots as described below. Sampling bulk and rhizosphere soils at this phenological stage was undertaken because it concurred with optimal time to sample corn leaf nutrient status (Jones and Hicks, 1973). Thus we assessed the effects of 6 and 7 yr that transgene corn and glyphosate applications within CT and RT systems were on soil ecology and chemical parameters.

## Soil Sampling and Processing

### Bulk Soil

With an ethanol-flame sterilized probe five 10 cm-depth soil samples were taken at random from each plot. They were composited and mixed in a sterile ziplock bag. The bags of soil were stored at 4°C until analysis for enzyme activities, soil moisture, extractable P, total C, and total N, and nitrification potential determinations. Tared soil samples were dried at 105°C for gravimetric moister determinations. For DNA extraction subsamples were stored at −80°C. All the above determinations were based on a dry soil basis.

### Rhizosphere Soil

With a shovel that was flame sterilized with ethanol seven root balls were removed from each field plot; each root ball was hit against the shovel to remove loose soil. Seven root balls per plot were placed in paper bags and transited on ice to the laboratory for processing (Supplemental Fig. S1). To collect rhizosphere soil from root balls, each one of the seven per plot were placed in a sterile metal bucket with 4 L of sterile, deionized water and agitated by hand. The rhizosphere soil slurry was then poured through a flame sterilized stainless steel funnel and through a flame sterilized 2-mm sieve into an autoclaved 10-L glass jar. The soil slurry was suspended by swirling before being poured through a small surface sterilized funnel into six sterile 250-mL centrifuge bottles, and were centrifuged at 10,000 *g* for 15 min. Each pellet was resuspended in a minimal volume of supernatant and then aseptically transferred to sterile Falcon tubes. Soil slurries were stored at 4°C before determinations of exoenzyme activities, nitrification potentials, and chemical analyses. An aliquot of slurry was dried at 105°C for dry-weight determination. These data were used to calculate gene copies per gram of rhizosphere soil. A subsample was stored at −80°C for DNA extraction.

## Exoenzyme and Total Microbial Community Determinations

Enzyme activities of phosphatase (AP), β-glucosidase (BG), β-N-acetylglucoaminidase (NAG), and leucine aminopeptidase (LAP) were estimated using ρ-nitrophenyl (pNP) linked substrates [(pNP-phosphate, pNP-β-glucopyranoside, pNP-β-N-acetylglucosaminide, and leucine p-nitroanilide, respectively (Sigma Aldrich, St Louis, MO)] as previously described by Jackson et al. (2013). An estimate of overall microbial activity was made using FDA hydrolysis (Green et al., 2006). All enzyme assays were completed within 7 d of sample processing. Detailed descriptions of these assays are described in Jenkins et al. (2017), and are described in detail in the Supplemental Material.

## Nitrification Potential Determinations

In 2013 and 2014, nitrification potential was determined using the protocol described by Hart et al. (1994). In brief, bulk and rhizosphere soil samples in 15-g quantities were transferred to 250 mL Erlenmeyer flasks and 100 mL of buffered ammonium sulfate (0.2 M $KH_2PO_4$, 0.2 M $K_2HPO_4$, 50 mM $(NH_4)_2SO_4$) was added. Flasks were covered with parafilm in which a hole was punched for air exchange. Flasks were incubated at 28°C in an orbital shaker at 180 rpm. Just before 10 mL of sample was removed after 2, 5, 19, and 24 h, each flask was swirled to maintain a constant soil/solution ratio over the incubation period. The 10-mL aliquots of sample were transferred to 50 mL centrifuge tubes and centrifuged at 8000 *g* for 15 min at 4°C. The supernatant was filtered through syringe filters and transferred to 15 mL Falcon tubes and frozen until analyzed for nitrate. In 2013 analyses were performed with an automated cadmium reduction method (Quick Chem Method 10-107-04-1-C) using Lachat (Lachat Instruments, Hach Company, Loveland, CO). For logistical reasons, in 2014 the Low Range Microplate Nitrate Test Kit (Nitrate Elimination Co., Inc., Lake Linden, MI) was used instead of the cadmium reduction method on incubated samples as described above. This protocol can be accessed at http://nitrate.com/store/index.php/nitrate/laboratory-test-kits/microplate-format-nitrate-test-kits-com. Nitrate concentrations were plotted against elapsed time and the slope of the linear regression determined the rate of potential nitrification.

## Quantification of Archaeal and Bacterial 16S rRNA and *amo*A Genes

Total genomic DNA was extracted from bulk and rhizosphere soil samples with Mo Bio Power Soil DNA Isolation kit (Mo Bio Laboratories, Inc.). Extracted DNA was quantified with a Nanodrop 2000 Spectrophotomer (Thermo Fisher Scientific, Wilmington, DE, USA), and was freeze-dried for storage and accurate weighing. Extraction of DNA and qPCR were performed according to Minimum Information Publication of Quantitative

Real-Time PCR Experiments (MIQE) guidelines (Bustin et al., 2009). Total archaeal and bacterial community densities were determined in triplicate by real-time TaqMan quantitative polymerase chain reaction (qPCR) using primers and probes specific for archaeal and bacterial 16S rRNA genes (Takai and Horikoshi, 2000; Nadkarni et al., 2002). The archaeal ammonia monooxygenase gene (*amo*A) was quantified with primers developed by Francis et al. (2005) and Power SYBR Green Master Mix (Applied Biosystems) (Mao et al., 2013). The bacterial *amo*A gene was quantified with primers developed by Rotthauwe et al. (1997) and Power SYBR Green Master Mix (Applied Biosystems) (Mao et al., 2013) (Supplemental Table S1). Sample derived standard curves for these genes were developed as described by Yu et al. (2005) and Mao et al. (2013). All real-time qPCR determinations were performed on an ABI Prism 7000 Sequence Detection System. Standard curves were quantified with Qubit (Life Technologies Corporation, Carlsbad, CA). Real-time qPCR determinations of archaeal and bacterial 16S rRNA, and archaeal and bacterial *amo*A genes were performed on ABI Prism 7000 Sequence Detection System; their respective mean efficiencies were 1.88, $R^2 = 0.997$, 1.92, $R^2 = 0.995$, 1.84, $R^2 = 0.996$, 1.89, $R^2 = 0.995$ ($n = 8$).

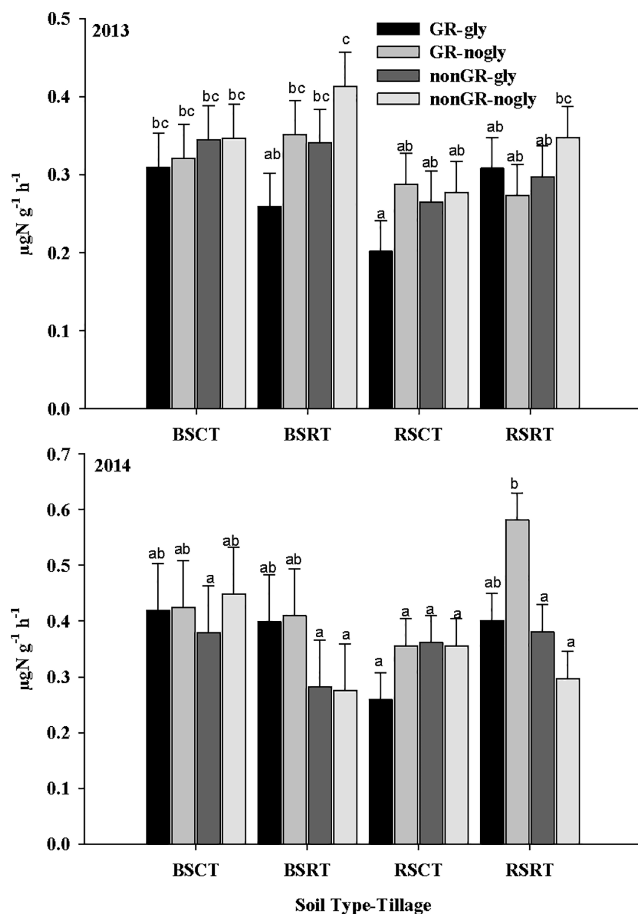


**Fig. 1. Mean nitrification potential rates determined for bulk soil (BS), and rhizosphere soil (RS), under conventional tillage (CT), and reduced tillage (RT) with glyphosate-resistant (GR) and non-glyphosate resistant (nonGR) corn with and without glyphosate applications in 2013 and 2014. Different letters indicate mean differences between BS and RS treatments at $P < 0.05$.**

## Carbon, Nitrogen, and Phosphorus Analyses

For analyses of bulk and rhizosphere soil C and N content, duplicate 1.0-mL aliquots of slurries from the enzyme analyses were analyzed on a vario Max CNS analyzer (Elementar Americas, Inc., NJ). Mehlich 3 extraction procedure was undertaken for determining soil P concentrations (Mehlich, 1984; Sikora et al., 2005). Data from these analyses were presented in Jenkins et al. (2017), and were used in regression analyses as described below.

## Data Analysis

Similar to Jenkins et al. (2017) differences in mean nutrient C/N, C/P, and N/P ratios; BG, AP, LAP, and NAG enzyme activities; nutrient acquisition ratios: BG/AP, BG/(LAP+NAG), and (LAP+NAG)/AP), respectively (Sinsabaugh et al., 2009); copies of archaeal and bacterial *amo*A genes; and archaeal and bacterial 16S rRNA genes were determined with mixed models of Proc Mixed of SAS (SAS, 2004) with replicate ratios as random variables and tillage and treatments as fixed variables. Data on nitrification potential, archaeal and bacterial 16S rRNA and AOA*amo*A/AOB*amo*A ratios were log-normal transformed for analysis. Regression analyses on natural log transformed data ($P < 0.05$) were determined with the linear regression application of SAS (Littell et al., 2006).

# RESULTS

## Nitrification Potential Rates

In 2013, the rate of potential nitrification activity of the control treatment (nonGR corn with no glyphosate) associated with bulk soil in RT and rhizosphere soil in RT treatments were greater than the bulk soil in RT for GR corn with glyphosate and rhizosphere soil in CT for GR corn with glyphosate, respectively (Fig. 1). The difference between these treatments appeared to indicate the occurrence of a glyphosate effect and appeared to demonstrate a potential inhibitory effect of glyphosate on the nitrification process. This pattern, however, was not observed in 2014; instead, the GR corn with no glyphosate treatment associated with rhizosphere soil in RT had a greater potential nitrification activity rate than the other rhizosphere soil treatments. Although appearing anomalous, because it was a non-glyphosate treatment with the greatest rate, it appeared to add to the evidence that glyphosate may inhibit the microbe-mediated nitrification process.

## Archaeal and Bacterial 16S rRNA Copies

Treatment differences between archaeal 16S rRNA gene copies were observed both sampling years (Supplemental Table S2). In 2013, the greatest number of gene copies was associated with the bulk soil in RT and rhizosphere soil in RT for nonGR corn without glyphosate; whereas, in 2014 the only difference in archaeal 16S rRNA gene copies was observed for the rhizosphere soil in RT for GR corn without glyphosate application. The overall mean of archaeal 16S rRNA gene copies for bulk soil treatments (at 7.58 $\log_{10}$ copies $g^{-1}$ dry soil) in 2013 was significantly greater than the overall mean copies associated with rhizosphere soil treatments (at 7.47 $\log_{10}$ copies $g^{-1}$ dry soil) ($P = 0.005$). In contrast no dif-

ferences in overall archaeal 16S rRNA gene copies were observed between bulk soil and rhizosphere soil treatments in 2014.

Significant differences in bacterial 16S rRNA gene copies between treatments were observed in 2013 (Supplemental Table S2). Glyphosate treatments, bulk soil in RT, rhizosphere soil in CT, and rhizosphere soil in RT, had significantly fewer bacterial 16S rRNA gene copies. A similar pattern was not observed in 2014. In contrast to archaea, no differences in overall mean copies of the bacterial 16S rRNA gene between bulk soil and rhizosphere soil were observed in 2013; in 2014 overall mean of bacterial 16S rRNA gene copies for bulk soil treatments (8.91 $\log_{10}$ copies $g^{-1}$ dry soil) was significantly greater ($P = 0.047$) than the overall mean of rhizosphere soil treatments (8.88 $\log_{10}$ copies $g^{-1}$ dry soil).

## Archaeal 16S rRNA/Bacterial 16S rRNA Ratios

Treatment differences between bulk soil Archaeal 16S rRNA/Bacterial 16S rRNA ratios in 2013 were observed (Fig. 2). In contrast, no differences were observed in 2013 between rhizosphere soil treatments. In 2014 differences between bulk soil treatments were observed; the Archaeal 16S rRNA/Bacterial 16S rRNA ratio for bulk soil in RT for nonGR corn with glyphosate was greater than the bulk soil in CT for GR corn with glyphosate, bulk soil in RT for GR corn with glyphosate, and nonGR corn without glyphosate application. As in 2013 no differences between rhizosphere soil treatments in 2014 were observed. The overall mean for the Archaeal 16S rRNA/Bacterial 16S rRNA ratio of 0.0585 for bulk soil treatments in 2013 was greater ($P < 0.003$) than the overall mean ratio of 0.0395 associated with rhizosphere soil treatments. In 2014, no differences between the overall means of bulk soil and rhizosphere soil treatments were observed.

## Archaeal and Bacterial *amo*A Copies

Mean copy numbers of AOA and AOB *amo*A genes associated with bulk soil and rhizosphere soil did not display a pattern that would indicate a particular treatment effect (Supplemental Table S3). The overall mean of AOA *amo*A gene copies for bulk soil in 2013 was 6.99 $\log_{10}$ copies $g^{-1}$ dry soil, and was greater ($P < 0.0023$) than the overall mean for AOB *amo*A gene copies at 6.79 $\log_{10}$ copies $g^{-1}$ dry soil. For rhizosphere soil in 2013 the overall mean *amo*A gene number for AOA was 7.23 $\log_{10}$ copies $g^{-1}$ dry soil and was greater ($P < 0.0001$) than the overall mean for AOB *amo*A gene copies at 6.47 $\log_{10}$ copies $g^{-1}$ dry soil. In 2014 the overall mean AOA *amo*A gene copy number for bulk soil was 6.72 $\log_{10}$ copies $g^{-1}$ dry soil and was not statistically different from the overall mean of bulk soil AOB *amo*A gene copy number of 6.81 $\log_{10}$ copies $g^{-1}$ dry soil. In contrast, the mean rhizosphere soil *amo*A gene copy number for AOA in 2014 at 7.14 $\log_{10}$ copies $g^{-1}$ dry soil was greater ($P < 0.0001$) than the rhizosphere soil *amo*A gene copy number for AOB at 6.42 $\log_{10}$ copies $g^{-1}$ dry soil. Thus, except for the similar bulk soil *amo*A gene copy numbers between AOA and AOB nitrifying communities in 2014, AOA *amo*A gene copy numbers tended to be greater than AOB *amo*A gene copy numbers for both bulk soil and rhizosphere soil environments. In 2013 and 2014 bulk soil AOA nitrifying communities were less than rhizosphere soil AOA nitrifying communities ($P < 0.0001$), and conversely, bulk soil AOB nitrifying communities were greater than rhizosphere soil AOB nitrifying communities ($P < 0.0001$).

## AOA*amo*A/Archaeal 16S rRNA and AOB*amo*A/Bacterial 16S rRNA Ratios

AOA*amo*A/Archaeal 16S rRNA ratios varied between treatments and soil types in 2013 but without a pattern indicative of any particular treatment effect (Fig. 3). The overall mean ratio for rhizosphere soil treatments at 0.597 was greater ($P < 0.0001$) than the overall mean ratio for bulk soil treatments at 0.288. In 2014 no treatment differences were observed for bulk soil and only one rhizosphere soil treatment, nonGR corn with glyphosate, had a greater ratio. Following the pattern observed in 2013, in 2014 the overall mean ratio for rhizosphere soil treatments at 0.463 was greater ($P < 0.007$) than the overall mean ratio for bulk soil treatments at 0.215.

Differences in AOB*amo*A/Bacterial 16S rRNA ratios were observed in 2013 between bulk soil treatments, notably between CT and RT treatments with the bulk soil in RT for GR corn with glyphosate ratio appearing to be anomalous with the greatest ratio

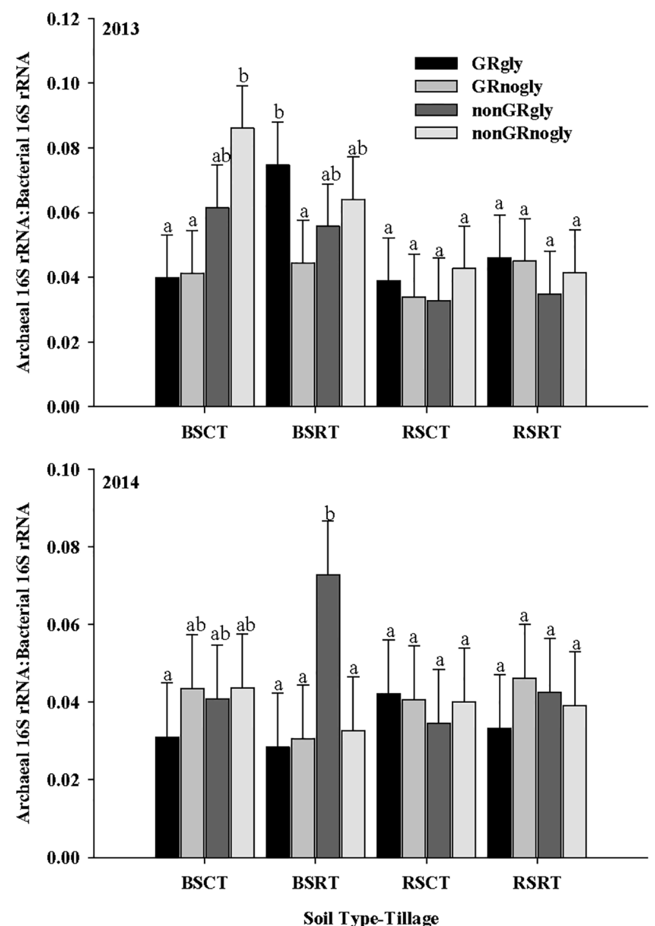


**Fig. 2. Mean Archaeal 16S rRNA:Bacterial 16DrRNA ratios for bulk soil (BS) and rhizosphere soil (RS) under conventional tillage (CT) and reduced tillage (RT) with glyphosate-resistant (GR) and non-glyphosate resistant (nonGR) corn with and without glyphosate applications in 2013 and 2014. Different letters indicate mean differences between BS and RS treatments at $P < 0.05$.**

(Fig. 4). In contrast, no differences in AOB*amo*A/Bacterial 16S rRNA ratios were observed for rhizosphere soil. In 2014 the bulk soil in CT for nonGR corn without glyphosate and bulk soil in RT for GR corn without glyphosate had greater AOB*amo*A/Bacterial 16S rRNA ratios than other treatments. No differences were observed in AOB*amo*A/Bacterial 16S rRNA ratios for rhizosphere soil treatments. In contrast to the archaeal community, the overall mean AOB*amo*A/Bacterial 16S rRNA ratio for bulk soil treatments at 0.011 was greater ($P < 0.0001$) than the overall mean for rhizosphere soil treatments at 0.001 in 2013; and in 2014, the overall mean ratio for bulk soil treatments at 0.010 was greater ($P < 0.001$) than the overall mean ratio for rhizosphere soil treatments at 0.003.

## Ratio of AOA *amo*A to AOB *amo*A

Although the overall mean copy numbers for the *amo*A gene were greater for the AOA than the AOB nitrifying communities, differences appeared between AOA and AOB *amo*A gene ratios (Fig. 5). The AOA/AOB *amo*A gene ratios for rhizosphere soil ranged between 3.78 and 9.90 in 2013, and between 3.54 and 11.18 in 2014, and reflected an overall AOA dominance of nitrifying communities in the rhizosphere soil environment. Compared to other treatments within rhizosphere soil and both the CT and RT treatments, significantly greater AOA/AOB *amo*A ratios were associated with the control treatment (nonGR corn without glyphosate) in both 2013 and 2014.

In bulk soil environments AOB nitrifying communities appeared to dominate several treatments with ratios ranging between 0.45 and 3.68 in 2013, and between 0.23 and 2.32 in 2014. Differences in AOA/AOB *amo*A ratios between treatments and between bulk soil and rhizosphere soil, were observed both sampling years; however, unlike the treatments associated with RT, no pattern of treatment differences for CT were observed. The archaeal nitrifying communities dominated rhizosphere soil with overall mean AOA/AOB *amo*A ratios of 6.87 in 2013 and 6.83 in 2014 compared to overall mean ratios of 2.27 in 2013 and 1.17 in 2014 for the bulk soil nitrifying communities. The bulk soil nitrifying communities appeared to have relatively balanced AOA/AOB *amo*A ratios both sampling years.

## Correlation and Regression Analysis

The fundamental difference between the two isogenic corn cultivars was the insertion of the glyphosate resistance determinant. No definitive differences in nitrification potential activity between the two cultivars were observed. We, therefore, have focused on

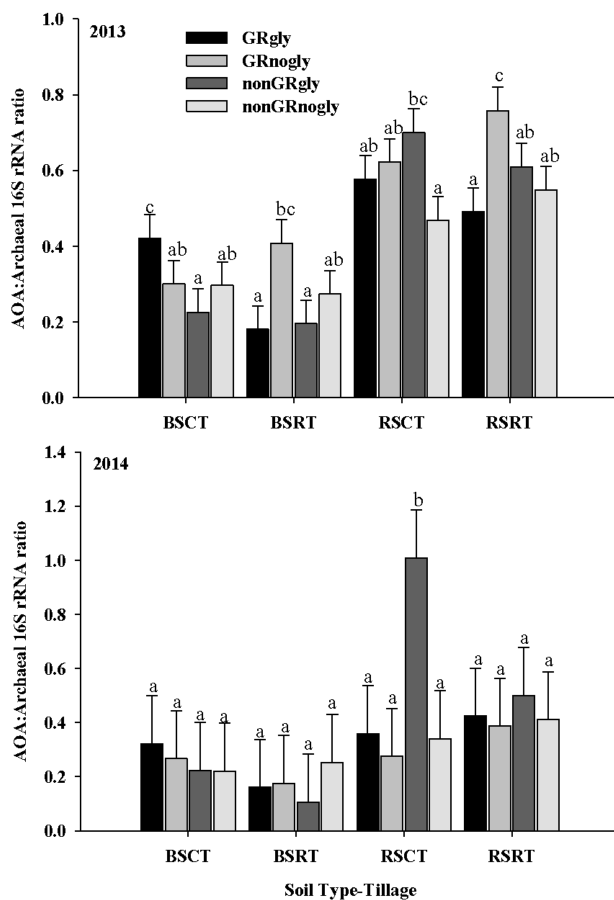


Fig. 3. Mean AOA *amo*A:Archaeal 16S rRNA ratios for bulk soil (BS) and rhizosphere soil (RS) under conventional tillage (CT) and reduced tillage (RT) with glyphosate-resistant (GR) and non-glyphosate resistant (nonGR) corn with and without glyphosate applications in 2013 and 2014. Different letters indicate mean differences between BS and RS treatments at $P < 0.05$.

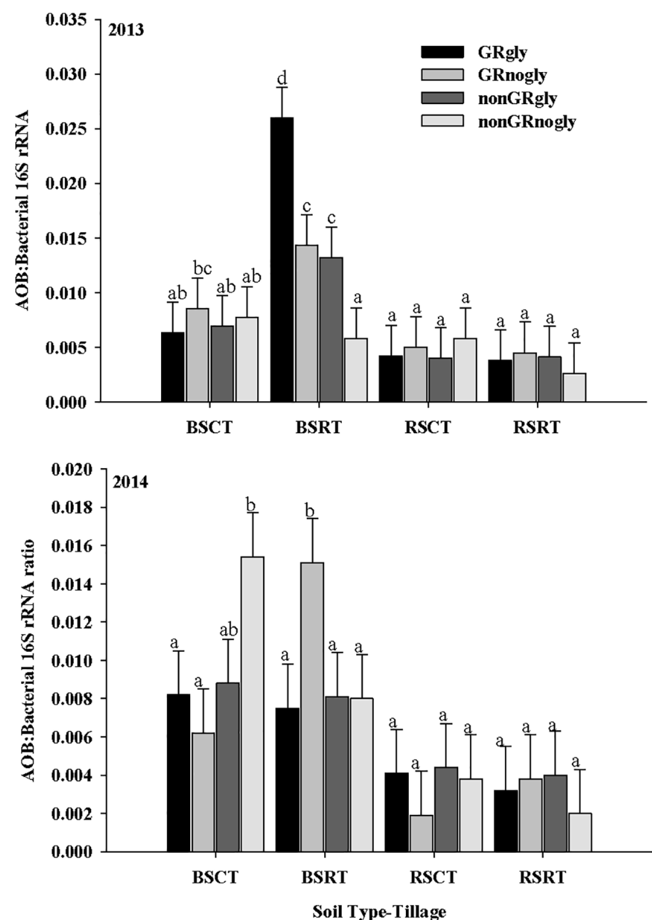


Fig. 4. Mean AOB *amo*A:Bacterial 16S rRNA ratios for bulk soil (BS) and rhizosphere soil (RS) under conventional tillage (CT) and reduced tillage (RT) with glyphosate-resistant (GR) and non-glyphosate resistant (nonGR) corn with and without glyphosate applications in 2013 and 2014. Different letters indicate mean differences between BS and RS treatments at $P < 0.05$.

the impact of glyphosate applications as the potential determining factor on the relationships between nitrification potential activity and soil macronutrient ratios, nutrient acquisition ratios, archaeal and bacterial nitrifier ratios, total microbial activity as measured by FDA activity, AOA*amo*A and AOB*amo*A gene copies, and archaeal and bacterial 16S rRNA gene copies and their ratios. Thus, to discern significant connections between them, regression analyses on these relationships were undertaken (Table 1).

In 2013 significant coefficients of determination were observed between nitrification potential rates and C/N, BG/AP, AOA/AOB ratios, FDA activities, and AOA*amo*A, Arch16S, Bac16S gene copies associated with bulk soil with glyphosate treatments. In comparison, bulk soil without glyphosate treatment correlations were observed between nitrification potential and C/N, and (LAP+NAG)/AP ratios, and Bac16S copies; shared correlations were between C to N ratios and Bac16S gene copies. In contrast to bulk soil coefficients of determination, more significant correlations were observed for the rhizosphere soil without glyphosate treatments than for rhizosphere soil with glyphosate treatments. The rhizosphere soil without glyphosate correlations between nitrification potential and C/N, AOA/AOB ratios, FDA activity, and Bac16S gene copies were shared with bulk soil with glyphosate treatments; the singular correlation between nitrification potential and N/P ratio appears to be anomalous. The correlation between nitrification potential and AOB*amo*A gene copies was the only correlation observed for rhizosphere soil with glyphosate treatments in 2013.

As in 2013, more significant correlations between nitrification potential and nutrient, enzymatic, and genetic variables were observed for bulk soil with glyphosate treatments than for bulk soil without glyphosate treatments. As in 2013, correlations between nitrification potential and BG/AP nutrient acquisition ratio, and Arch16S and Bac16S gene copies were observed in 2014. The 2014 correlations between nitrification potential and BG and AP exoenzymatic activities, and (LAP+NAG)/AP nutrient acquisition ratios were not observed in 2013. No correlations were observed between nitrification potential from rhizosphere soil with glyphosate treatments in 2014, and only two correlations were observed for rhizosphere soil without glyphosate treatments; these were between nitrification potential and AOB*amo*A and Bac16S gene copies. Some shared correlations were observed both sampling years for bulk soil treatments. Fewer relationships were observed for rhizosphere soil treatments.

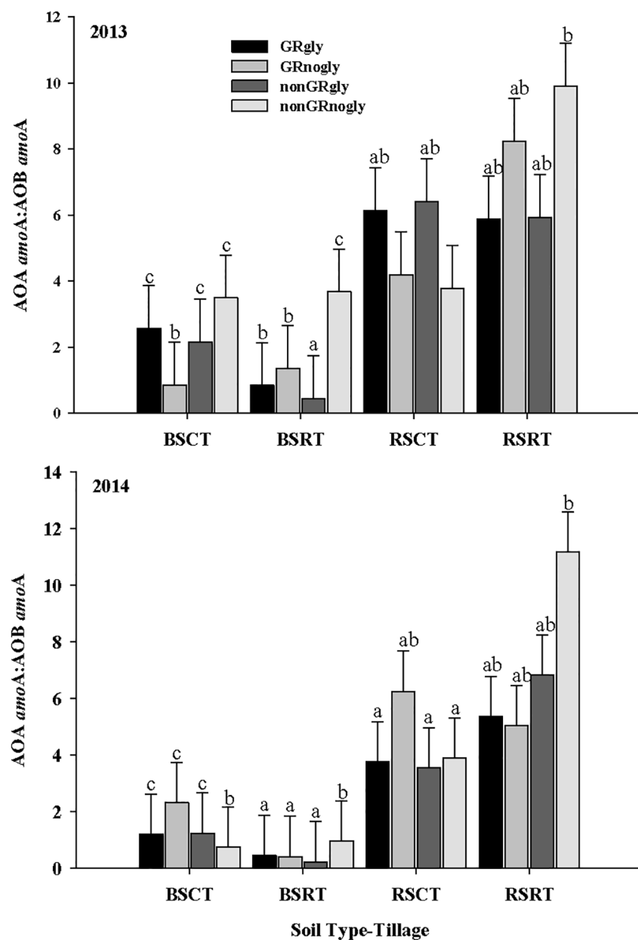


**Fig. 5. Mean AOA*amo*A:AOB*amo*A ratios for bulk soil (BS) and rhizosphere soil (RS) under conventional tillage (CT) and reduced tillage (RT) with glyphosate-resistant (GR) and non-glyphosate resistant (nonGR) corn with and without glyphosate applications in 2013 and 2014. Different letters indicate mean differences between BS and RS treatments at $P < 0.05$.**

## DISCUSSION

Microbial transformation of ammonia to nitrate in soil is initiated by autochthonous archaeal and bacterial communities (Leininger et al., 2006). The greater potential nitrification activities were, on the whole, associated with no glyphosate treatments, indicating glyphosate applications appeared to inhibit the nitrification process in both bulk soil and rhizosphere soil under both tillage practices (Fig. 1). This observation appears to corroborate other studies indicating that glyphosate applications can inhibit nitrification in agricultural soils (Martens and Bremner, 1993; Carlisle and Trevors, 1986).

Indications that glyphosate applications may inhibit nitrification imply that glyphosate appears to affect both bulk soil and rhizosphere soil microbial communities. Coupland and Caseley (1979) demonstrated that glyphosate can be released by plant roots into the ambient soil environment where it can interact with the soil microbiota. Archaeal 16S rRNA data indicated greater concentrations of archaea were associated with treatments where glyphosate was not applied (Supplemental Table S2), thus indicating a potential inhibiting interaction with glyphosate. Although glyphosate applications appeared to affect the archaeal bulk soil and rhizosphere soil microbial communities, no parallel pattern was observed for the archaeal copies of *amo*A genes (Supplemental Table S3). AOA*amo*A gene copies were greater than AOB*amo*A gene copies in 2013, and appeared to follow a consensus pattern reported by Erguder et al. (2009). In contrast to differences observed between archaeal 16S rRNA distributions as affected by glyphosate applications, differences in distributions of AOA*amo*A and AOB*amo*A gene copies were associated with soil type (Supplemental Table S3). Mean rhizosphere soil gene copies of AOA*amo*A were greater than mean bulk soil copies; the

**Table 1. Coefficients of determination ($R^2$) between nitrification potential activity rates and soil macronutrient ratios (C/N, C/P, NP), exoenzyme activities (phosphatase [AP], β-glucosidase [BG], leucine aminopeptidase and β-N-acetylglucoaminidase [LAP+NAG]), nutrient acquisition ratios (BG/AP, BG/[LAP+NAG], [LAP+NAG]/AP), total microbial activity (FDA), AOA/AOB *amo*A ratios (AOA/AOB), AOA and AOB *amo*A gene copies, and archaeal and bacterial 16S rRNA (Arch 16S and Bac 16S) gene copies associated with bulk and rhizosphere soils receiving glyphosate (Gly) and not receiving glyphosate (No gly) applications.**

| | | Bulk soil | | Rhizosphere soil | |
|---|---|---|---|---|---|
| | | $R^2$, Pr > F | | | |
| Year | Nitrification vs | Gly | No gly | Gly | No gly |
| 2013 | C/N | 0.436, 0.005* | 0.310, 0.025* | 0.006, 0.784 | 0.270, 0.039* |
| | C/P | 0.059, 0.363 | 0.004, 0.805 | 0.033, 0.498 | 0.179, 0.102 |
| | N/P | 0.091, 0.256 | 0.020, 0.554 | 0.037, 0.476 | 0.257, 0.045* |
| | BG | 0.028, 0.537 | 0.198, 0.084 | 0.022, 0.583 | 0.036, 0.483 |
| | AP | 0.211, 0.073 | 0.019, 0.610 | 0.043, 0.442 | 0.184, 0.098 |
| | LAP+NAG | 0.013, 0.679 | 0.196, 0.086 | 0.004, 0.814 | 0.002, 0.868 |
| | BG/AP | 0.248, 0.050* | 0.0002, 0.955 | 0.011, 0.699 | 0.161, 0.123 |
| | BG/(LAP+NAG) | 0.003, 0.834 | 0.227, 0.062 | 0.059, 0.366 | 0.044, 0.436 |
| | (LAP+NAG)/AP | 0.175, 0.107 | 0.462, 0.004* | 0.070, 0.321 | 0.213, 0.072 |
| | FDA | 0.298, 0.029* | 0.004, 0.815 | 0.003, 0.843 | 0.298, 0.029* |
| | AOA/AOB | 0.268, 0.040* | 0.001, 0.902 | 0.050, 0.404 | 0.214, 0.071* |
| | AOA *amo*A | 0.372, 0.012* | 0.044, 0.434 | 0.015, 0.647 | 0.126, 0.178 |
| | AOB *amo*A | 0.011, 0.703 | 0.019, 0.611 | 0.250, 0.049* | 0.0002, 0.963 |
| | Arch 16S | 0.395, 0.009* | 0.220, 0.067 | 0.075, 0.305 | 0.183, 0.098 |
| | Bac 16S | 0.243, 0.052* | 0.467, 0.004* | 0.078, 0.295 | 0.350, 0.016* |
| | Arch16S/Bac16S | 0.041, 0.453 | 0.005, 0.795 | 0.012,0.666 | 0.0001, 0.968 |
| 2014 | C/N | 0.220, 0.067 | 0.176, 0.106 | 0.015, 0.651 | 0.159, 0.127 |
| | C/P | 0.042, 0.449 | 0.035, 0.487 | 0.070, 0.322 | 0.047, 0.422 |
| | N/P | 0.094, 0.236 | 0.055, 0.380 | 0.071, 0.320 | 0.064, 0.343 |
| | BG | 0.285, 0.033* | 0.266, 0.041* | 0.022, 0.595 | 0.069, 0.344 |
| | AP | 0.398, 0.009* | 0.131, 0.169 | 0.006, 0.771 | 0.005, 0.801 |
| | LAP+NAG | 0.095, 0.245 | 0.148, 0.141 | 0.136, 0.176 | 0.017, 0.627 |
| | BG/AP | 0.593, 0.0005* | 0.462, 0.004* | 0.030, 0.534 | 0.048, 0.434 |
| | BG/(LAP+NAG) | 0.008, 0.743 | 0.0006, 0.929 | 0.0000, 0.987 | 0.057, 0.393 |
| | (LAP+NAG)/AP | 0.463, 0.004* | 0.293, 0.031* | 0.027, 0.559 | 0.020, 0.601 |
| | FDA | 0.002, 0.864 | 0.142, 0.150 | 0.001, 0.895 | 0.001, 0.904 |
| | AOA/AOB | 0.012, 0.690 | 0.180, 0.102 | 0.002, 0.868 | 0.080, 0.287 |
| | AOA *amo*A | 0.021, 0.589 | 0.306, 0.026* | 0.045, 0.432 | 0.207, 0.077 |
| | AOB *amo*A | 0.126, 0.178 | 0.021, 0.596 | 0.036, 0.482 | 0.405, 0.005* |
| | Arch 16S | 0.334, 0.019* | 0.103, 0.225 | 0.020, 0.597 | 0.222, 0.065 |
| | Bac 16S | 0.307, 0.026* | 0.051, 0.403 | 0.020, 0.597 | 0.287, 0.032* |
| | Arch16S/Bac16S | 0.043, 0.440 | 0.045, 0.428 | 0.150, 0.138 | 0.056, 0.318 |

* Significant at $P \leq 0.05$.

converse of this difference was observed for the distribution of AOB*amo*A copies with greater mean bulk soil AOB*amo*A gene copies than mean rhizosphere soil copies. This variation between the distribution of AOA and AOB *amo*A gene copies between bulk soil and rhizosphere soil appears to indicate a fundamental difference between the communities and microbial ecology of the two soil environments. This difference is underscored by the differences in AOA/AOB *amo*A gene copy ratios between bulk soil and rhizosphere soil environments (Fig. 5). AOA*amo*A gene copies dominated AOB*amo*A gene copies in rhizosphere soil. A more equitable distribution of these two *amo*A genes was observed for bulk soil. Chen et al. (2008) reported a similar distribution pattern between bulk soil and rhizosphere soil for several rice paddy soils in China. In contrast to AOB, AOA communities favor oligotrophic, low ammonia environments (Valentine, 2007; Glaser et al., 2010; Martens-Habbena et al., 2015; Thion et al., 2016). In this study, measurements of *amo*A and 16S rRNA gene copies were taken at the $V_2$ stage of corn development when N uptake by corn roots is at a high rate. Thus it appears that the competition between corn roots and the rhizosphere soil AOA and AOB communities for ammonia favors the more oligotrophic AOA community (Thion et al., 2016; Stahl and de la Torre, 2012).

Differences in AOA*amo*A/Archaeal 16S rRNA and AOB*amo*A/Bacterial 16S rRNA ratios appear to mirror the AOA/AOB *amo*A ratios associated with bulk soil and rhizosphere soil environments. In both sampling years bulk soil AOA*amo*A/Archaeal 16S rRNA ratios were significantly less than the ratios associated with rhizosphere soil indicating a greater proportion of total archaeal community in rhizosphere soil appeared to be involved in the nitrification process compared to the bulk soil archaeal communities. The converse of this pattern was observed for the bacterial AOB*amo*A/Bacterial 16S rRNA ratios with greater ratios associated with bulk soil than rhizosphere soil both sampling years. This apparent alternating dynamic between archaeal and bacterial bulk soil and rhizosphere soil communities as affected by the differences in available ammonia supports previous observations. The AOA and AOB communities prefer different soil N conditions with AOA preferring low ammonia conditions and AOB preferring high ammonia conditions (Erguder et al., 2009; Valentine, 2007; Di et al., 2010).

Ratios between total archaeal and bacterial communities as measured by 16S rRNA gene copies appeared to be dynamic, with apparent treatment differences for bulk soil but not rhizosphere soil communities both sampling years. The overall mean Archaeal 16S rRNA/Bacterial 16S rRNA ratio for bulk soil in 2013 was greater than the overall mean for rhizosphere soil, indicating a greater proportion of archaea associated with bulk soil than rhizosphere soil. This result was not observed in 2014, further indicating the dynamic of the two microbial communities within bulk soil and rhizosphere soil environments.

Regression analyses of nitrification potential activity rates and soil nutrient, enzymatic, and microbial measurements associated with bulk soil, rhizosphere soil, glyphosate and no glyphosate treatments (Table 1) indicated several more significant relationships for bulk soil treatments than rhizosphere soil treatments. Five significant bulk soil $R^2$ values appeared in both 2013 and 2014. These were the nutrient acquisition ratios for C/P and N/P, the copy numbers of AOA *amo*A genes, Arch16S rRNA, and Bac16S rRNA genes. Whereas we have observed a correlation be-

tween nitrification activity and AOA*amo*A gene copies, Di et al. (2010) reported correlations between AOB*amo*A gene copies and nitrification activity, but not between AOA*amo*A gene copies and nitrification activities. Along with the apparent disconnected significant $R^2$ values observed for bulk soil, fewer significant $R^2$ values were observed within rhizosphere soil in 2013 and 2014. The difference in number of significant correlations between bulk soil and rhizosphere soil may be attributed to the various physiological activities of corn roots and competition between rhizosphere microbial community and corn roots for nutrients. Altogether, results for both bulk soil and rhizosphere soil appear to reflect complexities and dynamics of soil-plant ecological relationships.

Observations presented in this study were made at a particular time in the phenological development of the corn crop. At this stage of development glyphosate applications appeared to have an inconsistent inhibiting effect on nitrification under field conditions. This apparent inhibition of the nitrification process was underscored by the observation that there were interactions that indicated this was a variable effect. Both archaeal and bacterial communities associated with no glyphosate treatments were greater than treatments receiving glyphosate. The apparent inhibiting effect of glyphosate on the nitrification process and total microbial concentrations, however, did not appear to affect the distribution of the nitrifying components of the two microbial communities. Whereas glyphosate applications affected the total microbial distribution, soil type appeared to affect the distribution of the two nitrifying communities. The archaeal community dominated rhizosphere soil where there was competition with corn roots for ammonia-nitrogen; the bacterial nitrifiers were more prominent in bulk soil. Results of this study appear to reflect the dynamics of the nitrifying community under agronomic conditions.

## SUPPLEMENTAL MATERIAL

A supplemental document is available with the online version of this article. Supplemental Fig. S1 is a diagram illustrating the preparation of soil slurry samples for analyses. Supplemental Methods describe exoenzyme and total microbial community activity determinations. Supplemental Table S1 contains qPCR primers and thermocycler conditions with references. Supplemental Table S2 contains the concentrations of archaeal and bacterial 16S rRNA genes in bulk and rhizosphere soil under conventional tillage and reduced tillage associated with the four experimental practices. Supplemental Table S3 contains the concentrations of archaeal *amo*A and bacterial *amo*A in bulk and rhizosphere soil under conventional tillage and reduced tillage associated with the four experimental treatments.

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