Science of the Total Environment 720 (2020) 137514

Contents lists available at ScienceDirect

Science of the Total Environment

journal homepage: www.elsevier.com/locate/scitotenv

Acidification in corn monocultures favor fungi, ammonia oxidizing bacteria, and nirK-denitrifier groups

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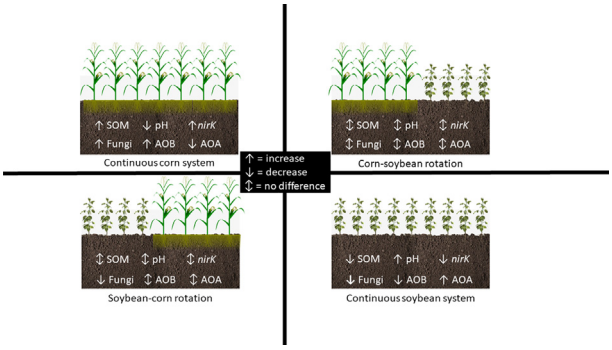
HIGHLIGHTS

- Fungal abundance increased under continuous corn compared to soybean monocultures.
- Monocropping of corn lowered soil pH.
- Abundance of bacterial *amoA* nitrifiers was highest within corn monocultures.
- Denitrifiers carrying *nirK* genes were highest within continuous corn monocultures.
- Observed changes have direct implications on the potential for N_2O emissions.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 17 January 2020
Received in revised form 21 February 2020
Accepted 21 February 2020
Available online 29 February 2020

Editor: Frederic Coulon

Keywords:
Microbial N cycle
Soil health
Soil degradation
Nitrogen fixation
Nitrification
Denitrification
Crop rotation
Tillage

ABSTRACT

Agricultural practices of no-till and crop rotations are critical to counteract the detrimental effects of monocultures and tillage operations on ecosystem services related to soil health such as microbial N cycling. The present study explored the main steps of the microbial N cycle, using targeted gene abundance as a proxy, and concerning soil properties, following 19 and 20 years of crop monocultures and rotations of corn (*Zea mays* L.), and soybean [*Glycine* max (L.) Merr.], either under no-till or chisel tillage. Real-time quantitative polymerase chain reaction (qPCR) was implemented to estimate phylogenetic groups and functional genes related to the microbial N cycle: *nifH* (N_2 fixation), *amoA* (nitrification) and *nirK*, *nirS*, and *nosZ* (denitrification). Our results indicate that long-term crop rotation and tillage decisions affect soil health as it relates to soil properties and microbial parameters. No-till management increased soil organic matter (SOM), decreased soil pH, and increased copy numbers of AOB (ammonia oxidizing bacteria). Crop rotations with more corn increased SOM, reduced soil pH, reduced AOA (ammonia oxidizing archaea) copy numbers, and increased AOB and fungal ITS copy numbers. *NirK* denitrifier groups were also enhanced under continuous corn. Altogether, the more corn years included in a crop rotation multiplies the amount of N needed to sustain yield levels, thereby intensifying the N cycle in these systems, potentially leading to acidification, enhanced bacterial nitrification, and creating an environment primed for N losses and increased N_2O emissions.

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https://doi.org/10.1016/j.scitotenv.2020.137514

1. Introduction

Agricultural management decisions fundamentally affect the soil microbial community, and as a result, soil health. Soil health is directly associated with the health of humans, animals, and ecosystems through plant growth. Thus, global food and nutritional security are correlated to the supply of soil macro- and micronutrients (Lal, 2016). Unfortunately, each year valuable cropland is lost to erosion, flooding, mining, urban development, and overuse (FAO, 2015), thus contributing to the degradation of soil health. Fundamental management decisions regarding crop rotation and tillage influence and alter the soil environment, leading to changes in the microbial community structure and essential functions. Essential soil functions include soil organic matter (SOM) dynamics, residue breakdown, formation and stabilization of soil aggregates, aeration and gaseous exchanges, retention and cycling of nutrients and water, and maintenance of biodiversity (Lal, 2016; Schimel and Schaeffer, 2012). Crop rotation has been documented as one management practice used to increase and stabilize yields, while potentially lowering fertilization inputs (Behnke et al., 2018b; Gentry et al., 2013). Another common management practice used to improve yields in systems of high organic matter is tillage (Behnke et al., 2018b; Behnke and Villamil, 2019). However, the ubiquitous practice used throughout the world to enhance crop yields is N fertilization, which directly alters potential N acquisition and transformation by soil microbial communities (Huang et al., 2019).

In order to understand the changes brought about by agronomic management on the soil metagenome, quantifying changes in target phylogenetic and functional genes relating to the microbial N cycle is essential. The availability of this information will provide valuable insight as to the genetic potential of soil microbes to support biological N fixation, nitrification, and denitrification under different management practices (de Vries et al., 2015; Levy-Booth et al., 2014). Numerous marker genes are used to quantify changes to critical steps of the microbial N cycle. The *nifH* gene, which encodes the enzyme nitrogenase reductase, is used as an indirect measure of biological N fixation. Nitrification potential relates to the number of *amoA* genes, which encodes the enzyme ammonia monooxygenase. Likewise, denitrification potential is regulated by a series of different marker genes: *nirK* and *nirS*, which encodes the enzyme for different nitrite reductases; and *nosZ*, which encode enzymes for nitrous oxide reductase (Jetten, 2008; Levy-Booth et al., 2014).

Fertilization has been shown to exacerbate each step of the microbial N cycle by flooding the system with synthetic N (Huang et al., 2019; Ouyang et al., 2018). Nitrification increases substantially, leading to greater losses to the environment through leaching and denitrification. Similarly, biological N fixation is reduced due to an abundance of easily accessible N in the system. Following 25 years of inorganic N fertilization in southern China, Wang et al. (2017) found that the addition of N fertilizer reduced *nifH* copy numbers; however, the use of lime (P + K) fertilizer (no N) increased *nifH* copy numbers. Carey et al. (2016) observed that additions of N increased both AOA (ammonia oxidizing archaea) and AOB (ammonia oxidizing bacteria) abundances, with AOB showing an order of magnitude larger increases. These authors found that the increase in AOB populations was correlated to increases in nitrification potential with the largest increases coming from unmanaged soils compared to agroecosystems. In a controlled microcosm study using agricultural soils in Scotland, Hink et al. (2018), found that AOB was dominant in soils with greater concentrations of NH_4^+ and AOA was dominant in soils with low NH_4^+.

Ouyang et al. (2018), conducted a meta-analysis on 47 field studies from a wide range of agroecosystems from across the world and observed that N fertilization had no effect on the abundance of *nifH* but increased the abundance of *amoA* from both archaea and bacteria, as well as the abundance of denitrifier groups (carrying either *nirK*, *nirS*, or *nosZ* genes). The authors also found that crop rotation and soil pH were important factors in N cycling. For example, crop rotation increased copy numbers of AOB genes compared to monocultures, while no differences were observed in AOA. On the other hand, soil pH greater than six was found to increase levels of both AOA and AOB. Furthermore, denitrification gene counts (*nirK*, *nirS*, *and nosZ*) were higher under crop rotations compared to monocultures (Ouyang et al., 2018). Research from continuous cropping of peanuts (*Arachis hypogaea* L.), Zhang et al. (2019) concluded that implementing a rotation of corn (*Zea mays* L.) and soybean [*Glycine* max (L.) Merr.] compared to a monoculture of either improves soil nutrient content by influencing the composition of the bacterial community. In corn-soybean systems in Indiana, Smith et al. (2016) found that crop type and tillage had little to no effect on the quantity and diversity of species, changes were only evident in the species composition. The authors reported a greater effect of tillage compared to the crop planted, with tillage affecting soil properties more than the composition of the microbial communities. The authors concluded that altering nutrient levels through tillage systems leads to changes in microbial community that cycle these nutrients.

Tillage affects soil organisms in a number of ways: mechanically harming or killing them, destroying soil aggregates, reducing soil moisture, and raising soil temperatures (Beare et al., 1994; Carter, 1992; Martens, 2000). Furthermore, tillage has been widely reported to increase the mineralization of SOM and N, which can lead to a reduction in soil fertility and crop production (Cheneby et al., 2009; Zuber et al., 2015; Zuber et al., 2018). In a review conducted by Balesdent et al. (2000), SOM increases under no-till (NT) were attributed to a slower decomposition rate of crop residues compared to conventional tillage. In a global meta-analysis conducted by Zuber and Villamil (2016) NT was found to increase microbial biomass C and N compared to intensive tillage regimes, however, chisel tillage was not different from NT. In another meta-analysis that focused on the effect of management practices on SOM and microbial diversity, de Graaff et al. (2019) found that tillage decreased bacterial and faunal biodiversity, yet it did not affect soil fungi, including arbuscular mycorrhizal fungi (AMF).

While the functions of the soil microbiome regarding N cycling are relatively known and documented (Hirsch and Mauchline, 2015; Huang et al., 2019), numerous knowledge gaps exist about how the soil microbiome relates to typical agricultural management practices such as rotation and tillage, especially from long-term, replicated, stable cropping systems. Previous studies regarding AOA and AOB from agroecosystems have focused primarily on fertilizer amendments, yet little is known about the combined effects of crop rotation and tillage practices (Munroe et al., 2016). Moreover, the effects of tillage on *amoA* abundance are contrasting in their results (Munroe et al., 2016; Segal et al., 2017), or are even different within one study from different ecosystems (Phillips et al., 2000). Of the 157 total observations used in the meta-analysis by Ouyang et al. (2018), only 24 were from corn or soybean cropping systems; and of these 24 studies, only 15 used inorganic N fertilizer (along with P and K fertilization). Likewise, only four studies included in the meta-analyses of Ouyang et al. (2018) presented data from no-till practices, none of them related to corn or soybean agroecosystems.

No published studies to date have investigated the response to long-term use of rotations and tillage practices in a monoculture or rotated corn-soybean crops by quantifying all critical functional genes involved in the microbial N cycle simultaneously. Our main goal was to fill that information gap to increase our understanding of microbial N processes concerning soil health within an agroecosystem of critical relevance for U.S. agriculture, following 20 years of rotation and tillage treatments in monocultures of corn, soybean, as well as their corn-soybean rotation. We hypothesized that continuous corn and soybean systems will show the most contrast on the abundance of functional genes, whereas the corn-soybean phases of the corn-soybean rotation will show intermediate changes. The detected changes on copy numbers will be closely associated to the number of corn crops in the system due to the required N fertilizer additions to maintain yield levels. We hypothesized that compared to NT, tillage will decrease the number of copies of functional

genes associated with the N cycle caused by a reduction of SOM, physical destruction of microbial biomass, enhanced soil aeration, and desiccation of the topsoil layer. The availability of this information is essential to furthering our knowledge of how agronomic practices affect the soil microbial community and overall soil health.

2. Materials and methods

2.1. Experimental site description

The experimental site was initiated in 1996 at the Northwestern Illinois Agricultural Research and Demonstration Center (40°55′50″ N, 90°43′38″ W), approximately 8 km northwest of Monmouth, Illinois. The mean annual precipitation at Monmouth is 914 mm with a mean annual temperature of 10.6 °C (Illinois Climate Network, 2019). Research plots were located on Sable silty clay loam (fine-silty, mixed, mesic Typic Endoaquoll), and on Muscatune silt loam (fine-silty, mixed, superactive, mesic Aquic Argiudoll), with about 10% of the study area on Osco silt loam (fine-silty, mixed, mesic Typic Argiudoll). These prime agricultural soil series are dark-colored, very deep soils on nearly flat topography, with low to moderate permeability, and low surface runoff potential (Soil Survey Staff, 2019). Soils in these series developed under prairie vegetation in a layer of loess 2–3 m thick, over glacial till (Soil Survey Staff, 2019).

2.2. Treatments and cultural practices

Research plots were set up to study the effects of rotation and tillage practices on crop yields when the crop is in a monoculture (CCC, continuous corn; SSS, continuous soybean) or a short rotation with each other with each phase present every year (Cs, Sc). The experimental layout was a split-plot arrangement of rotation (CCC, Cs, Sc, SSS) as main plots, and tillage options (T, chisel tilled; NT, no-till) as the subplot treatment, in a randomized complete block design with three replications. The main plot of Cs is the corn phase of the corn-soybean rotation with corn as the crop harvested before soil sampling; the main plot of Sc is the soybean phase of the-corn-soybean rotation with soybean as the crop harvested before sampling. The main plots were 22 m long by 12 m wide. The subplots were 22 m long by 6 m wide. Conventional tillage consisted of primary tillage with a chisel plow 20- to 25-cm deep in the fall after harvest, and secondary tillage with a field cultivator before planting in the spring. Corn and soybeans were planted in April, May, or June each year in 76- and 38-cm wide row-to-row spans, respectively. Corn was planted at 75,000 to 85,000 seeds ha^{-1}, and soybean at 340,000 to 350,000 seeds ha^{-1}. Fertilizer and pest management decisions were based on best management practices for the site according to the Illinois Agronomy Handbook (Nafziger, 2009). Nitrogen fertilization of corn occurred in the spring at or before planting as knife-injected incorporated urea ammonium nitrate liquid solution (UAN 28%) at a rate of 246 kg N ha^{-1} and 224 kg N ha^{-1} for continuous and rotated corn, respectively. No N fertilizer was used in soybeans. P fertilizer at a rate of 225 kg ha^{-1} (as diammonium phosphate, DAP: 18–46-0), and lime at a rate of 4.5 Mg ha^{-1}, were last applied to the entire experimental area in the fall of 2013 based on soil test results and did not differ based on crop rotation or tillage options.

2.3. Soil sampling and determinations

Soil samples were collected following cash crop harvest in October 2015 and October 2016, after 19 and 20 years respectively, of the start of the experiment. We used an Eijkelkamp grass plot sampler (Eijkelkamp North America http://www.eijkelkamp-usa.com) to take three composited soil subsamples per plot to a depth of 10 cm. Each composited subsample was taken walking the plot in a zig-zag pattern and contained about 10 pushes of the soil sampler probe, some 500 g each. For soil DNA analyses, subsamples were immediately preserved on ice, and frozen to −20 °C upon arrival to our lab facilities. After determining gravimetric water content for each soil sample, field moist soil was analyzed for available N (NO_3^- and NH_4^+ in mg kg^{-1}) using KCl extraction (1:5 ratio of soil to solution) followed by flow injection analysis with a SmartChem 200 (Westco Scientific Instruments, Inc., Danbury, CN, USA). Remaining soil was sent to a commercial laboratory (Brookside Laboratories, Inc., New Bremen, OH), that deploys standard procedures recommended for the U.S. North Central Region (https://www.blinc.com/resources/testing-methods). Determination of soil organic matter (SOM, %) was thus conducted by loss on ignition; soil pH (1:1 soil:water) via potentiometry; available phosphorus (P, mg kg^{-1}) with Bray I extraction; macronutrients (K, Ca, Mg, S) and micronutrients (B, Fe, Mn, Cu, Zn) as well as Na and Al levels, via Mehlich III extraction (expressed in mg kg^{-1}); and cation exchange capacity (CEC, cmol kg^{-1}) by the summation method of exchangeable cations (Ca, Mg, K, Na, and H).

2.4. Soil DNA extraction and real-time quantitative PCR analysis

Guidelines and protocols for soil DNA extraction and quantitative PCR analyses are thoroughly detailed in Huang et al. (2019). Briefly, soil DNA was extracted using Power Soil DNA isolation kits (MoBio Inc., Carlsbad, CA), following the manufacturer's instructions. Extracted DNA purity and quantity were checked with a Nanodrop1000 Spectrophotometer (Thermo Fisher Scientific, USA) and gel electrophoresis. Soil DNA concentrations were between 10 and 40 ng/μl with A260/A280 values ranged 1.7–2.0. Quantification of abundance for nine marker genes (fungal ITS, bacterial 16S, Archaeal 16S, AOA, AOB, *nifH*, *nirK*, *nirS*, and *nosZ*) was obtained via high-throughput qPCR at the Roy Carver Biotechnology Center for Functional Genomics lab at the University of Illinois at Urbana-Champaign, using known primers published in previous studies (Huang et al., 2019, Supplementary information) in a Fluidigm BioMark HD™ System. DNA standard curves for each target gene were prepared using a dilution series (representing 10^2–10^8 copies each target gene/μl) of fluidigm-prepared amplicons from 225 pooled soil samples as detailed in Huang et al. (2019). Standards, samples, and controls were run in six replicates on six plates. Melting curve analyses after the final qPCR cycle allowed monitoring amplicon specificity. Under the specified qPCR conditions, the amplification efficiencies were within 86%–96% for all the primers. Copy numbers of target genes were calculated from standard curves of known concentrations using the comparative Ct ($2^{-\Delta\Delta Ct}$) method (Livak and Schmittgen, 2001).

2.5. Statistical analyses

The statistical methodology deployed for this study followed the general steps thoroughly explained in Huang et al. (2019). Briefly, we deployed principal component analyses (PCA) as a data reduction technique in SAS software version 9.4 (SAS Institute, Cary, NC, USA) to avoid problems of multicollinearity by compiling the soil variables information into a new smaller set of uncorrelated variables. Table S1 includes the mean and standard errors for each of these soil variables measured under the different rotation|phases and tillage options studied. Thus, using the FACTOR procedure with priors = 1 option, we extracted PC scores with eigenvalues ≥1 (Table 1). Soil variable loadings greater than |0.5| were considered in the interpretation of each PC. Linear mixed models were then fit to each of the PCs extracted using GLIMMIX procedure to evaluate the effect of rotation|phase, tillage, and their interaction effects on soil quality parameters now summarized within our PCs. Blocks and years were considered random effects, and rotation, tillage, and their interaction, as fixed effects. Similarly, linear mixed models were fit to each of the soil microbial parameters, e.g. the quantification of phylogenetic genes of Bacteria, Fungi and, Archaea, and functional genes involved in the N cycle (*nifH*, *amoA* of AOB and AOA, *nirK*, *nirS*, and *nosZ* -expressed as copies per microgram of DNA).

Table 1
Principal component analysis of soil variables (17) for 0–10 cm soil depth with eigenvalues and cumulative proportion of the data set variability explained by the five principal components (PC) extracted with eigenvalues >1. Component correlation scores (eigenvectors) with loadings greater than |0.5| are bolded. Probability values for the analysis of variance (ANOVA) and degrees of freedom (df) available for the effects of rotation|phase (Rotation), tillage (Tillage), and their interaction are shown for each extracted PCs.

		PC1	PC2	PC3	PC4	PC5
Eigenvalue		4.80	4.07	1.87	1.32	1.20
Cum. proportion		0.28	0.52	0.63	0.71	0.78
Soil variable		**Component correlation scores**				
pH		**0.76**	**−0.60**	−0.06	0.02	0.09
CEC		−0.11	**0.95**	−0.04	−0.08	−0.11
SOM		−0.23	**0.59**	**−0.61**	−0.01	0.21
NO_3^-		0.27	0.16	**0.66**	−0.40	−0.17
NH_4^+		−0.38	0.15	−0.26	**0.65**	0.08
P		0.31	0.41	0.25	0.39	0.35
S		−0.32	0.12	0.13	**−0.55**	**0.55**
K		**−0.55**	0.25	0.34	0.04	0.23
Na		**0.55**	**0.71**	−0.14	−0.07	−0.16
Ca		**0.87**	0.35	−0.06	0.12	−0.14
Mg		−0.05	0.28	0.39	0.10	**−0.58**
Al		**−0.74**	0.08	**0.49**	0.29	−0.06
Fe		**−0.49**	**0.77**	0.20	0.10	0.01
B		**0.70**	0.09	0.28	0.15	0.33
Zn		0.44	**0.71**	0.18	−0.06	0.29
Cu		**0.77**	0.45	−0.17	−0.02	−0.11
Mn		**0.56**	−0.37	0.43	0.36	0.20
Factor	**df**	**Probability values**				
Rotation	3	*0.0061*	*0.0175*	*0.0638*	*0.0005*	*0.0574*
Tillage	1	*0.0012*	*0.0006*	*<0.0001*	0.5677	*0.0367*
Rotation × Tillage	3	0.8511	0.8368	0.2230	0.7103	0.6221

Statistically significant effects are italicized.

Table S2 includes the mean and standard errors for each of these soil microbial parameters determined under the different rotation|phases and tillage options. Since the model residuals of each of these soil microbial parameters were not normally distributed, we used a lognormal distribution link function (dist = logn) with a Kenward-Rogers adjustment to the degrees of freedom (ddfm = kr) in the model statement to account for model complexity (Gbur et al., 2012). When appropriate, lsmeans were separated using the lines option of the lsmeans statement, setting the probability of Type I error (α) at 0.10. To assess relationships among the soil properties and microbial parameters, we used Pearson's correlation coefficients obtained with the CORR procedure. All plots were created within the R environment, version 3.6.1 (R Core Team, 2018), deploying package ggplot2 (Wickham, 2016).

3. Results

3.1. Rotation and tillage effects on soil properties

To characterize the microbial environment, our soil variables dataset included 17 soil properties of pH, CEC, SOM, and macronutrients of available N (NO_3^- and NH_4^+), available P, exchangeable K, S, Ca, and Mg, along with Na and Al levels, and micronutrients of Fe, B, Zn, Cu, and Mn. Thus, the PCA analyses conducted on soil properties rendered a set of five uncorrelated PCs (PC1 to PC5) with eigenvalues >1 that together explained 78% of the variability of the soil data set (Table 1). PC1 had the largest eigenvalue (4.8) and explained 28% of the soil variability; its eigenvector included positive loadings (>0.50) for pH, Na, Ca, B, Cu and Mn, as well as negative loadings for K, Al, and Fe. This component reflects how K, Al, and Fe levels drop and exchange sites became enriched with Ca and micronutrients as the pH of the soil increases. PC2 showed an eigenvalue of 4.07 and explained an additional 24% of the soil variability with positive loadings for CEC, SOM, Fe, and Zn, and negative loadings for pH. This component then showed how the pH of these soils seemed to be inversely related to CEC, SOM, Fe, Zn, and levels. The eigenvalue for PC3 was 1.87 with an additional 11% explained, showing positive loadings for NO_3^-, and Al, and negative loadings for SOM. The eigenvalue of PC4 and PC5 were 1.32 and 1.20, each explaining an additional 8% and 7% of the variability, respectively. PC4 and PC5 both reflect a specific contrast between nutrients in the soils. While PC4 showed a dominant positive loading of NH_4^+ and a negative loading for S, PC5 showed dominant positive loading for S and a negative loading for Mg (Table 1). The five PCs were then used as independent variables in follow-up analyses of variance (ANOVA) testing the effect of rotation|phase, tillage, and their interaction (Rotation × Tillage) on the soil quality parameters represented by each PC. The probability values and degrees of freedom associated with these analyses are shown in the lower portion of Table 1 for each extracted PC. Results showed that the interaction effect (Rotation × Tillage, Table 1) was not statistically significant for any of the PCs evaluated. The lack of interaction effect indicated that the soil variables represented by those PCs responded similarly to the rotation and tillage treatments. All PCs showed statistically significant effects of the rotation, though for PC3 and PC5 these were marginally significant ($p < 0.0638$, and $p < 0.0574$ respectively). Likewise, most PCs but PC4 ($p < 0.5677$), showed statistically significant effects of tillage practices (Table 1). Specifically, more soybean in the rotation increased PC1 values (Table 2), and accordingly, soil pH increased from 6.1 under CCC to 7.1 under SSS (Table S2). Lower levels of K, Al, and Fe accompanied this increase in soil pH with an increasing number of soybean crops. PC1 values measured for the corn phase of the corn-soybean rotations (Cs), were intermediate between those measured under CCC and the Sc values, which in turn were intermediate between Cs and the SSS PC1 scores (Table 2). Higher PC1 scores were also recorded under chisel tillage compared to NT, but the effect on nutrients was less pronounced (Tables 2 and S2). On the other hand, PC2 scores (dominated by CEC, SOM, Fe, and Zn along with pH) showed a statistically significant main effect of rotation ($p = 0.0061$, Table 1) and of tillage as well ($p = 0.0012$). Thus, for PC2, continuous corn (CCC) had higher PC2 estimates (lower pH and higher CEC, SOM, Fe Zn and Na values) than continuous soybean (SSS) and the corn phase of the corn-soybean rotation (Cs); whereas the soybean phase (Sc) showed intermediate values (Table 2). On average, CEC went from 25.2 cmol kg^{-1} under CCC to 20.5 cmol kg^{-1} under SSS; SOM went from 5.1% under CCC to 4.3 under SSS (Table S2). Long-term tillage practices decreased PC2 scores, rising pH and lowering associated levels of CEC and SOM when compared to NT (Table 2). A slightly significant trend was determined for the main effect of rotation on PC3 scores ($p < 0.0638$); PC3, in this case, contrasted both phases of the corn-soybean rotations on their levels of SOM and NO_3^- in the soils with the monocultures having intermediate scores. Both Cs and Sc showed a trend to higher NO_3^- levels that their respective monocultures, yet the Sc phase also showed higher SOM than any rotation|phase (Table S1). Tillage, on the other hand, showed higher PC3 scores than NT, reflecting lower levels of SOM yet higher NO_3^- under chisel tillage (Tables 2 and S1). Lower levels of S in the soil under SSS drives the difference in the comparison of PC4 scores from SSS with the CCC and both corn-

Table 2
Results of the mean separation procedures for the principal components (PCs) that showed a statistically significant response to the main effect of rotation, and of tillage. Within a column and for each factor, means followed by the same letter are not statistically different (α = 0.05).

Factor[§]		PC1		PC2		PC3		PC4		PC5	
Rotation	Tillage										
CCC		−0.63	c	0.64	a	0.03	ab	−0.54	b	0.65	a
Cs		−0.29	bc	−0.28	b	0.30	a	−0.14	b	−0.52	b
Sc		0.21	ab	0.16	ab	−0.39	b	−0.12	b	−0.40	b
SSS		0.72	a	−0.51	b	0.06	ab	0.79	a	0.26	ab
	NT	−0.23	B	0.25	A	−0.26	B	−0.05		0.21	A
	Tillage	0.23	A	−0.25	B	0.26	A	0.05		−0.21	B

[§] Rotation: CCC, continuous corn; SSS, continuous soybean; Cs and Sc represent the corn and soybean phases of the corn soybean rotation, respectively. NT, no-till; Tillage, chisel till.

soybean phases of the CS rotation (Table 2). Lastly, for PC5, higher S under CCC and higher Mg with soybean in the rotations drive the differences recorded among the monocultures and the corn and soybean phases of the CS rotation (Tables 2 and S1). Soils under tillage had higher Mg levels than soils in NT reflected by higher PC5 scores for NT in Table 2.

3.2. Rotation and tillage effects on the abundance of microbial communities and functional genes

The averaged copy number of bacterial *16S* rRNA ranged from 1.5×10^8 to 1.9×10^8 copies/μg DNA, while the copy number of fungi *ITS* region in the soil samples collected from our study ranged from 1.3×10^7 to 3.4×10^7 copies/μg DNA on average (Table S2). The copy number of archaeal *16S* rRNA ranged from 8.0×10^6 to 1.1×10^7 copies/μg DNA on average. Increasing the number of soybean crop years significantly decreased the abundance of the fungal *ITS* region in the soil regardless of tillage options, yet no statistically significant differences in bacterial and archaeal *16S* gene abundance were detected within crop rotations or tillage options (Fig. 1).

The copy number of *nifH* in the soil samples collected from our study ranged from 2.5×10^5 to 3.6×10^5 copies/μg DNA on average (Table S2). The estimated abundance of *nifH* genes was not statistically affected by rotation ($p = 0.8903$), and tillage ($p = 0.6648$) treatments, or their interaction ($p = 0.5871$).

The copy number of *amoA* genes (AOB and AOA) ranged from 2.8×10^6 to 2.4×10^7 copies/μg DNA on average (Table S2). The copy number of amoA of AOB showed statistically significant effects of the interaction of rotation × tillage treatments ($p < 0.0281$). This interaction was driven by a differential response of AOB to NT management within the monocultures, with higher amoA of AOB copy numbers under CCC compared to SSS (Fig. 2). A significant statistical effect of rotation was detected for AOA ($p < 0.0001$), with CCC showing the lowest copy numbers and SSS the highest, whereas the corn and soybean phases of the CS rotation showed intermediate values (Fig. 3A).

The averaged copy number of *nirK*, *nirS*, and *nosZ* ranged from 3.4×10^6 to 6.2×10^6, 9.5×10^4 to 2.7×10^5, and 2.5×10^6 to 2.9×10^6 copies/μg DNA, respectively (Table S2). A statistically significant effect of rotation was determined for *nirK* denitrifiers ($p = 0.0337$), showing a reduction in their copy numbers SSS when compared to CCC (Fig. 3B). No statistical differences were detected for the *nirS* and *nosZ* denitrifying groups.

3.3. Correlation among soil microbial parameters and soil properties

The matrix of Pearson's correlation coefficients among all five PCs and the soil microbial parameters is shown in Table 3. Most statistically significant correlations found fell within the 'weak' (|0.2–0.4|), or 'moderate' (|0.4–0.6|), association ranges (Cohen, 1988). Fungal ITS copy numbers were positively and moderately associated with PC3 values ($r = 0.52$, $p < 0.0001$), and weakly and negatively associated to PC1 ($r = -0.25$, $p = 0.05$) and PC4 ($r = -0.28$, $p = 0.02$) scores. Bacterial *16S* rRNA counts (bacteria Table 3), were weakly and positively associated with PC3 scores ($r = 0.31$, $p = 0.01$), and weakly yet negatively related to both PC4 ($r = -0.33$, $p = 0.01$) and PC5 ($r = -0.24$, $p = 0.05$). Archea*16S* copy numbers (Arch Table 3) were weakly and positively associated to PC1 ($r = 0.30$, $p = 0.01$) yet negatively related to PC4 ($r = -0.30$, $p = 0.02$) and PC5 ($r = -0.25$, $p = 0.04$). Likewise, *nifH* counts were weakly and positively associated with PC1 ($r = 0.32$, $p = 0.01$) and negatively correlated with PC4 ($r = -0.33$, $p = 0.01$). Estimates of AOB abundance were weakly and positively associated with PC2 ($r = 0.27$, $p = 0.03$) and moderately correlated to PC3 scores ($r = 0.42$ $p = 0.0005$) while showing a negative moderate association to PC4 ($r = -0.45$, $p = 0.0002$). In addition, the denitrifier groups carrying

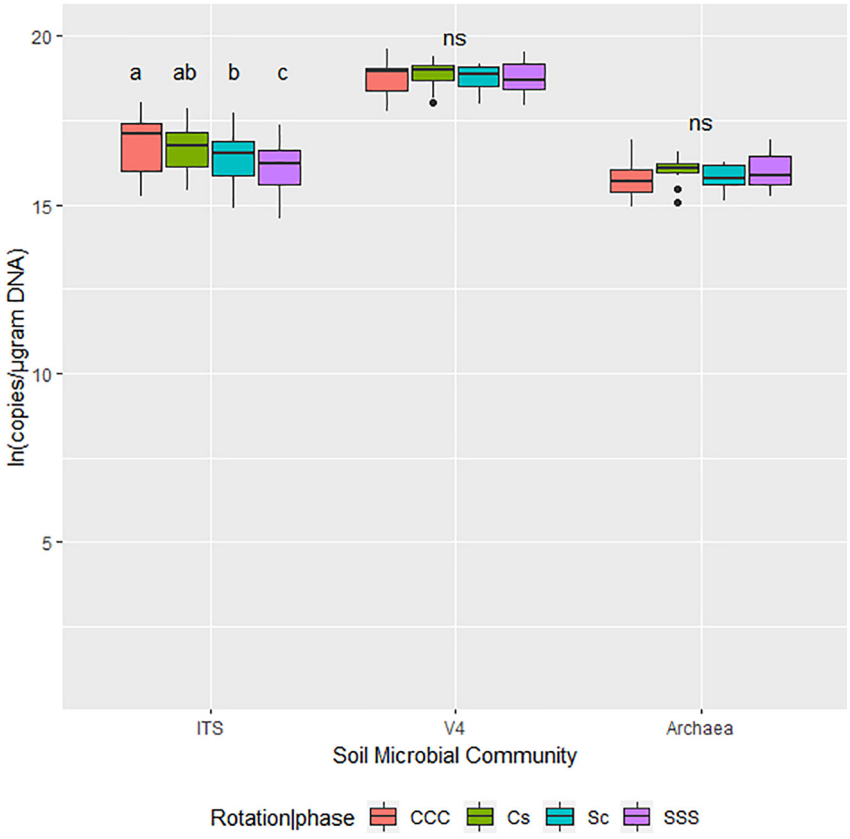

Fig. 1. Distribution of gene copy numbers of fungal (ITS), bacterial (Bacteria), and archaeal (Archaea) groups in the soils following long-term rotation treatments. Within each group, boxplots with the same letter are not significantly different; likewise, "ns" indicates no statistically significant difference within a group ($\alpha = 0.05$).

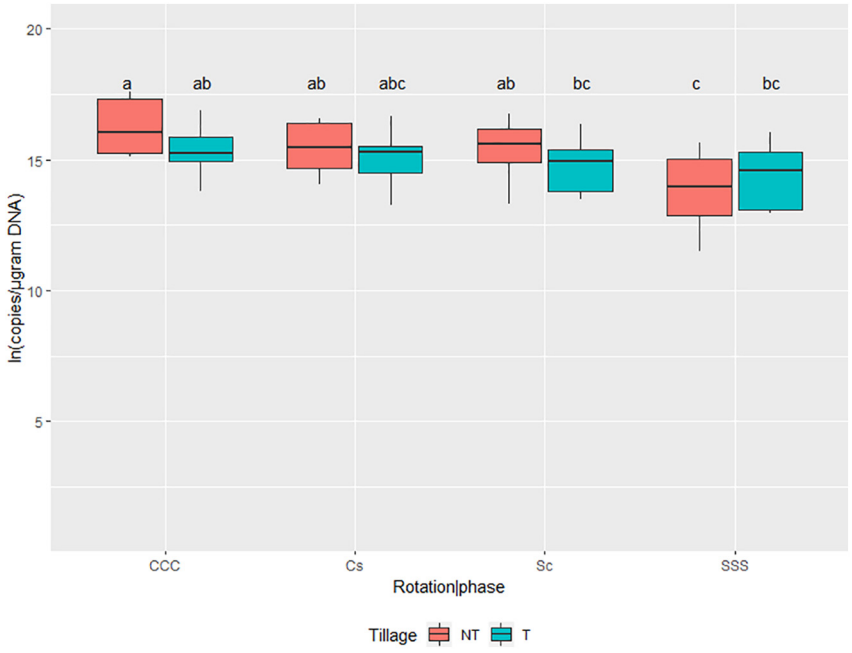

Fig. 2. Distribution of gene copy numbers of bacterial amoA (AOB) functional genes in the soils following long-term management practices of rotation and tillage treatments. Boxplots with the same letter are not significantly different ($\alpha = 0.05$).

nirK, *nirS*, or *nosZ* genes showed a positive association with PC3 scores, yet while the relation with *nirK* was moderate ($r = 0.43$, $p = 0.0004$), *nirS* and *nosZ* showed a weak relationship with PC3 scores (*nirS* $r = 0.30$, $p = 0.01$; *nosZ* $r = 0.29$, $p = 0.02$). In addition, denitrifiers had a weak (*nirK* and *nosZ*), or moderate (*nirS*) negative association to PC4 (*nirK* $r = -0.29$, $p = 0.02$; *nirS* $r = -0.41$, $p = 0.0007$; *nosZ* $r = -0.36$, $p = 0.003$).

All PCs showed a response to the main effects of rotation and tillage practices with the exemption of PC4 which showed only rotation effects (Tables 1 and 2). PC1 and PC2 both reflect the effect of changes in soil pH and the correlation analyses allow us to see the differential effect of this soil parameter on the communities of fungal ITS and archaea (Table 3) as well as on the archaeal nitrifiers carrying *amoA* genes (AOA). With the clear exception of *nifH*, and archaea *16S* rRNA, most targeted genes correlated relatively more strongly with PC3 (positive associations) and PC4 (negative relations) scores than with the other PCs.

Correlations among soil microbial parameters (Table 3) fell within the strong (|0.6–0.8|) and very strong (>|0.8|) categories. Among the latter set, the association between *nifH* and bacterial 16S rRNA and Archaeal *16S* rRNA counts were both statistically significant ($p < 0.0001$) and had coefficients of $r = 0.87$ and $r = 0.80$, respectively. Based on those associations, we can say that 76% and 64% of the variation observed in *nifH* counts could be attributed to the presence of bacteria and archaea groups, respectively. In addition, *amoA* of AOB was strongly associated with fungal ITS counts ($r = 0.83$, $p < 0.0001$). *NirK* showed strong associations to both ITS ($r = 0.94$, $p < 0.0001$) and bacterial *16S* rRNA counts ($r = 0.82$, $p < 0.0001$). *NosZ* on the other hand, was strongly correlated to bacterial 16S rRNA abundance ($r = 0.87$, $p < 0.0001$), *nifH* ($r = 0.83$, $p < 0.0001$), and *nirK* ($r = 0.88$, $p < 0.0001$) as well (Table 3).

4. Discussion

The results from this long-term study indicate that crop rotation and tillage significantly alter soil properties in different ways. An inverse relationship between soil pH and SOM was observed for the chisel tillage treatment with NT having greater amounts of SOM compared to chisel tillage and chisel tillage having a greater pH compared to NT. In a NT system, residues accumulate on the soil surface and break down slower compared to a tilled system, leading to increases in SOM (Varvel and Wilhelm, 2010; Zuber et al., 2017). From our PCA we can see that chisel tillage increased levels of NO_3^- compared to NT (10.1 vs 8.1 mg kg^{-1}, respectively), likely related to increased microbial access to those residues, resulting in an increased rate of decomposition and mineralization (Rice et al., 1986; Zuber et al., 2017), confirmed by the reduced SOM values in the chisel tillage treatments. Moreover in NT systems, fertilizer N is not incorporated into the soil profile as happens in tilled systems, so acidification and a reduction in soil pH at the surface is commonly observed (Crozier et al., 2008; Zuber et al., 2017). Thus, surface levels of NH_4^+ were greater under NT compared to chisel tillage (3.5 vs 2.9 mg kg^{-1}, respectively; Table S1), corresponding to the lower pH values in NT, which then lead to an increase of AOB copy numbers compared to AOA, which prefers low concentrations of NH_4^+ (Lehtovirta-Morley, 2018; Martens-Habbena et al., 2009). This phenomenon was influential in the observed interaction between rotation and tillage for copy numbers of AOB, with chisel tillage raising the pH and reducing AOB copy numbers in three of the four rotations (Fig. 2). An inverse relationship between AOB and soil pH due to increases in N fertilization was observed in Huang et al. (2019). Likewise, our results agreed with Segal et al. (2017) and de Graaff et al. (2019) as those authors also found that AOB copy numbers were reduced by tillage compared to NT.

From our PCA we can see that SOM has an inconsistent relationship to AOB copy numbers (PC2, PC3, and PC4 for rotation) showing both a positive and negative correlation. Thus, due to a stronger, unwavering relationship with pH, we can conclude that in our study AOB copy numbers are inversely related to pH, with chisel tillage having a greater pH in each rotation × tillage combination (Table S1), corresponding to lower AOB copy numbers. Other studies from inorganic fertilized systems have detected this inverse relationship as well (Carey et al., 2016; Huang et al., 2019). However, the importance of SOM should not overlooked, lower SOM amounts are commonly observed under chisel tillage compared to NT and corroborated from each rotation × tillage combination (Table S1). In a meta-analysis conducted by de Graaff et al. (2019) looking at agriculture and soil biodiversity, the authors found that N fertilization for periods of longer than five years can lead to an increase in SOM due to greater biomass returns. The increase in SOM provides resources for soil microorganisms in addition to

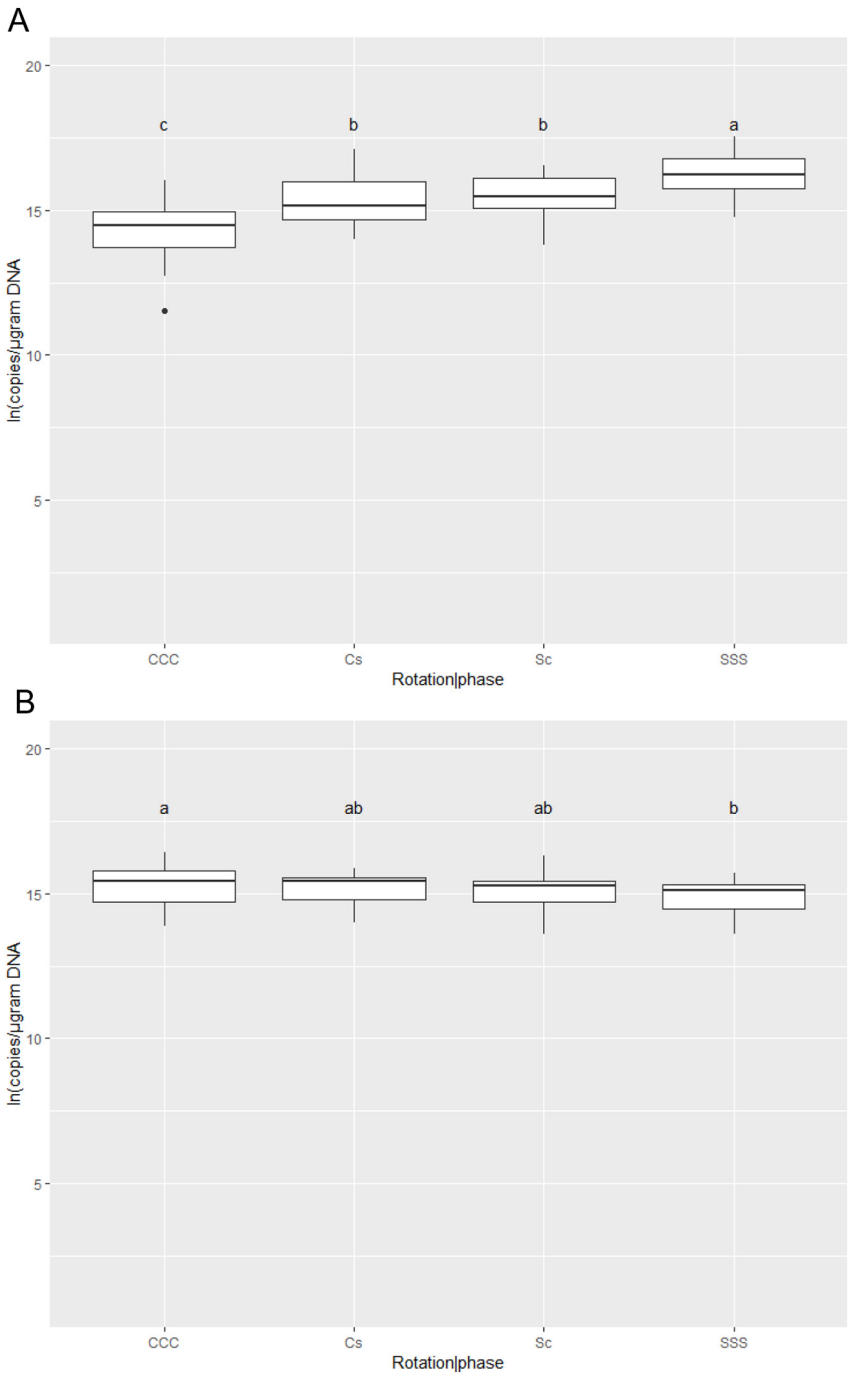

Fig. 3. A. (Top panel) Distribution of gene copy number of archaeal *amoA* (AOA) in the soils following long-term rotation treatments. Boxplots with the same letter are not significantly different ($\alpha = 0.05$). B. (Lower panel) Distribution of gene copy number of *nirK* in the soils following long-term rotation treatments. Boxplots with the same letter are not significantly different ($\alpha = 0.05$).

improving soil physical properties like structure (Whalen and Chang, 2002) and hydrological properties like aeration, water holding capacity, and drainage (Miller et al., 2002). Therefore, agricultural practices that increase SOM are likely to retain or increase soil bacterial diversity (de Graaff et al., 2019).

Our PCA detected a significant effect due to crop rotation for several of our response variables, including fungal ITS, AOA, AOB, and *nirK*. The inclusion of a crop rotation compared to monoculture improved soil quality by raising the soil pH compared to CCC. The rotated phases of corn and soybean showed an increase in SOM compared to SSS, but lower values than CCC. Soil pH and SOM both affect the availability of macro- and micronutrients (Zuber et al., 2017). The lower pH in the CCC rotation is likely due to increased fertilizer application compared to the rotated corn, and lack of fertilizer in the soybean plots; other studies corroborate these findings (Behnke et al., 2018a; Segal et al., 2017; Zuber et al., 2017). Rotated or monocultures of soybeans return substantially less residue to the soil compared to corn crops and are not fertilized, leading to our observed inverse relationship between pH and CEC, similar to that described for SOM, and evident in their positive relationship in PC2 (Table 1). Traditionally, SOM and CEC are positively related as SOM (and clay minerals) is one of the sources of negatively charged binding sites for the elements included in CEC (Parfitt et al., 1995). As pH decreases, Al^{3+} and Fe^{3+} increase and begin to displace important macro- and micronutrients. In our study,

Table 3
Matrix of Pearson' correlation coefficients among soil properties (PCs) and soil microbial parameters describing the abundance of fungi (copy numbers of ITS region) and prokaryotes (copy numbers of bacteria 16S, Bact; and archaea 16S, Arch), and functional genes of the microbial N cycle (*nifH*, AOB, AOA, *nirK*, *nirS*, and *nosZ*). All soil microbial parameters included were expressed as ln(copy numbers/μgram DNA). Bolded correlation coefficients indicates statistical significance at $\alpha = 0.05$.

	PC1	PC2	PC3	PC4	PC5	ITS	Bact	Arch	nifH	AOB	AOA	nirK	nirS	nosZ
PC1	1													
PC2	0	1												
PC3	0	0	1											
PC4	0	0	0	1										
PC5	0	0	0	0	1									
ITS	**−0.25**	0.18	**0.52**	**−0.28**	−0.11	1								
Bact	0.11	−0.04	**0.31**	**−0.33**	**−0.24**	**0.75**	1							
Arch	**0.30**	−0.23	0.16	**−0.30**	**−0.25**	**0.44**	**0.89**	1						
nifH	**0.32**	0.05	0.22	**−0.33**	−0.22	**0.60**	**0.87**	**0.80**	1					
AOB	−0.24	**0.27**	**0.42**	**−0.45**	−0.08	**0.83**	**0.61**	**0.35**	**0.45**	1				
AOA	**0.50**	**−0.30**	0.15	0.05	−0.18	0.22	**0.47**	**0.55**	**0.51**	0.11	1			
nirK	−0.16	0.21	**0.43**	**−0.29**	−0.14	**0.94**	**0.82**	**0.52**	**0.73**	**0.81**	**0.27**	1		
nirS	0.15	0.16	**0.30**	**−0.41**	−0.21	**0.69**	**0.72**	**0.52**	**0.73**	**0.64**	**0.42**	**0.78**	1	
nosZ	0.15	−0.03	**0.29**	**−0.36**	−0.10	**0.78**	**0.87**	**0.70**	**0.83**	**0.64**	**0.50**	**0.88**	**0.74**	1

the CCC rotation showed the lowest pH and the highest concentrations of Al^{3+} and Fe^{3+}. Globally, soil pH reductions from agricultural soils are frequently observed and are a significant issue for crop production, adding cost to growers to raise the pH to maintain or improve yields (Tian and Niu, 2015; Wang et al., 2018).

The reduction of soil pH associated with more corn (and more N fertilization) years, caused an increase in fungal ITS abundance, as evident in Table 3 and Fig. 1, in which the CCC rotation had significantly greater ITS copies compared to the rotated crops or the SSS. Fisher et al. (2017) also observed increases in fungal ITS gene copy numbers at lower pH; the authors attributed this effect to decreasing bacterial communities, thereby removing the competitive constraint and allowing fungal communities to flourish. Other studies have detected similar findings (Huang et al., 2019; Rousk et al., 2009; Wang et al., 2019). We also determined a positive correlation between soil pH and Archaea*16S rRNA*, AOA, and *nifH* copy numbers (Table 3). In our study, AOA copy numbers were highest in the SSS (with no inorganic N fertilization, highest pH), intermediate in the Cs and Sc, and lowest in the CCC (highest levels of inorganic N fertilization, lowest pH) (Fig. 3 A). From the PCA we see that pH is positively related to AOA copy numbers, meaning that the crop rotations with a lower pH correlate with lower AOA copy numbers (Table 3). Similarly, in the meta-analysis by Ouyang et al. (2018), the authors found that pH was significant and positively correlated to AOA copy numbers. However, in a meta-analysis by Carey et al. (2016), the authors found AOA was not influenced by pH, though AOB was. Both meta-analyses conclude that in systems with N applications, AOB was more responsive to inputs and management compared to AOA. In a similar study of monoculture versus rotated corn, Munroe et al. (2016) also concluded that management history may have caused AOB to be elevated due to inorganic N fertilization and regular disturbance. As inorganic N is added to the system in increasing amounts as is the case in our study (CCC receives more fertilizer N compared to CS), AOB levels were observed to increase (Fig. 2). Increases in bacterial nitrification have been shown to also enhance acidification, NO_3^-, and N_2O production (Ai et al., 2013; Hink et al., 2018; Hink et al., 2017). Conversely, the AOB copy numbers were highest in the CCC-NT and lowest in the SSS-NT (Fig. 2). Our results are thus consistent with several studies showing that AOB is the dominant group responsible for ammonia oxidation (nitrification) within inorganic N fertilizer systems (Carey et al., 2016; Zabaloy et al., 2017; Huang et al., 2019; Ouyang et al., 2018).

From our results, we measured a positive relationship between AOB and denitrification (*nirK*, *nirS*, and *nosZ*) (Table 3). The PCA also detected a positive relationship between those denitrifiers and NO_3^- (PC3 in Table 1). Furthermore, we detected an inverse relationship between AOB and PC4 (higher loadings with NH_4^+), and a positive relationship between AOB and PC3 (higher loadings with NO_3^-). No significant relationship, however, was found between either AOA and PC3 or AOA and PC4, again suggesting AOB was the dominant ammonia oxidizer in our study. Overall, our results suggest that crop rotation shifted the abundance of ammonia-oxidizing microorganisms greater than tillage. The CCC rotation likely possesses a greater nitrification rate and greater potential of N_2O emissions due to elevated copies of AOB compared to AOA. Hink et al. (2018) reported that the AOB oxidation pathway (nitrification) increased N_2O emissions twofold compared to that of AOA. Over four years and from the same study site, Behnke et al. (2018b) found a 6.5 times greater N_2O yield from CCC compared to SSS. Likewise, our results showed a significant increase in the denitrification pathway through *nirK* from the CCC compared to the SSS (Fig. 3B). Elevated levels of *nirK* functional genes (Fig. 3) suggest that pathways responsible for N_2O emissions are enhanced with a monoculture of corn, though more information regarding gene expression and denitrification activity is needed to corroborate this finding. In the meta-analysis by Ouyang et al. (2018), the authors also observed an increase in both nitrifier and denitrifier abundance with increasing N fertilization levels that could help explain N use inefficiencies and intensified rates of N loss in these agricultural systems.

5. Conclusion

The results from this long-term experiment on highly productive soils are one of few to comprehensively research the effects of crop rotation and tillage on soil health as it relates to soil properties and microbial abundances. We analyzed, six microbial target genes involved in the N cycle, and essential soil chemical properties. We found that tillage slightly decreased AOB copy numbers through a reduction in SOM, partially confirming our hypothesis. The use of no till increased AOB copy numbers by reducing the soil pH. We also found that the CCC rotation, which had the lowest pH, reduced copy numbers of AOA and increased copy numbers of AOB; the CS and SC rotations were intermediate compared to the monocultures of CCC and SSS, confirming our hypothesis that CCC will stimulate microbes adapted for conditions of increased inorganic N as reflected by increased abundances of functional genes nirK and amoA (AOB). Fungal ITS increased as more corn entered the rotation due to a reduction in soil pH, favoring fungal growth. The results presented here highlight the risk associated with growing corn following corn; in order to maintain yield levels, more inorganic N is required, thereby intensifying the N cycle causing a dangerous loop, characterized by increased abundance of autotrophic AOB and heterotrophic denitrifiers, and low pH. The result is acidification and potential increases in harmful N_2O emissions. Therefore, a reduction in the number of corn years will help reduce the negative effects of growing corn following corn on soil functional genes. Furthermore, it is critical to soil health in these systems to maintain or increase SOM and pH levels to buffer the negative effects of this intense N cycling and acidification.

CRediT authorship contribution statement

G.D. Behnke: Investigation, Writing - original draft. **M.C. Zabaloy:** Conceptualization, Methodology, Writing - review & editing. **C.W. Riggins:** Methodology, Resources, Writing - review & editing. **S. Rodríguez-Zas:** Conceptualization, Software, Resources. **L. Huang:** Writing - review & editing. **M.B. Villamil:** Conceptualization, Funding acquisition, Project administration, Resources, Data curation, Software, Formal analysis, Writing - review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

This research was partially funded by a HATCH Grant (no. ILLU-802-947) and by Award No. AG 2018-67019-27807 both from the United States Department of Agriculture, USDA-NIFA. We acknowledge Dr. Alvaro Hernandez and Dr. Mark Band from the Roy Carver Biotechnology Center for Functional Genomics lab at the University of Illinois at Urbana-Champaign, for their assistance with primer selection and high throughput qPCR analyses. We are thankful to Mr. Greg Steckel, and Mr. Marty Williams for their contribution in managing the experimental sites, and to Mr. Samuel Kato, for everyday help with field and lab activities.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2020.137514.

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