Soil Health Management Enhances Microbial Nitrogen Cycling Capacity and Activity

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ABSTRACT Soil microbial transformations of nitrogen (N) can be affected by soil health management practices. Here, we report in situ seasonal dynamics of the population size (gene copy abundances) and functional activity (transcript copy abundances) of five bacterial genes involved in soil N cycling (ammonia-oxidizing bacteria [AOB] amoA, nifH, nirK, nirS, and nosZ) in a long-term continuous cotton production system under different management practices (cover crops, tillage, and inorganic N fertilization). Hairy vetch (Vicia villosa Roth), a leguminous cover crop, most effectively promoted the expression of N cycle genes, which persisted after cover crop termination throughout the growing season. Moreover, we observed similarly high or even higher N cycle gene transcript abundances under vetch with no fertilizer as no cover crop with N fertilization throughout the cover crop peak and cotton growing seasons (April, May, and October). Further, both the gene and transcript abundances of amoA and nosZ were positively correlated to soil nitrous oxide (N₂O) emissions. We also found that the abundances of amoA genes and transcripts both positively correlated to field and incubated net nitrification rates. Together, our results revealed relationships between microbial functional capacity and activity and in situ soil N transformations under different agricultural seasons and soil management practices.

IMPORTANCE Conservation agriculture practices that promote soil health have distinct and lasting effects on microbial populations involved with soil nitrogen (N) cycling. In particular, using a leguminous winter cover crop (hairy vetch) promoted the expression of key functional genes involved in soil N cycling, equaling or exceeding the effects of inorganic N fertilizer. Hairy vetch also left a legacy on soil nutrient capacity by promoting the continued activity of N cycling microbes after cover crop termination and into the main growing season. By examining both genes and transcripts involved in soil N cycling, we showed different responses of functional capacity (i.e., gene abundances) and functional activity (i.e., transcript abundances) to agricultural seasons and management practices, adding to our understanding of the effects of soil health management practices on microbial ecology.

KEYWORDS soil health, conservation agriculture, soil nitrogen cycle, soil microbes, quantitative PCR (qPCR), quantitative reverse transcription PCR (qRT-PCR), agroecosystems, denitrification, nitrification, nitrogen fixation, soil microbiology

Nitrogen (N) is a major nutrient for plant growth, and its transformation and availability in soil is a key factor in terrestrial ecosystem productivity (1). Nitrogen fertilizer has been widely used to improve crop yields in agroecosystems, but losses of excess N result in the eutrophication of waterbodies and exacerbate atmospheric concentrations of the greenhouse gas nitrous oxide (N₂O) (2). Conservation agriculture and soil health practices, such as growing cover crops, reducing N fertilization, and...
minimizing tillage, ensure mineral nutrients can be properly used by crops and do not become surplus or deficient (3).

The C-to-N ratios of plant residues can drive the net balance of soil mineral N by affecting microbial mineralization and immobilization of N (4). Cover crops, in addition to the main crop, can take up soil nitrate (NO$_3^-$) to reduce N losses via leaching and denitrification (5). Further, leguminous cover crops can supplement crop N requirements through biological N fixation, thereby reducing exogenous N fertilizer requirements and associated N losses (6). The effects of tillage intensity on N losses through N$_2$O emissions are cropping system specific, but reduced or no tillage generally mitigates N$_2$O emissions compared to conventional tillage, especially in long-term (>10 years) production systems (7, 8).

Conservation agriculture practices can change soil properties, which are important factors influencing microbial functional groups involved in soil N cycling (9). For example, increased soil organic matter (SOM) and soil water content (SWC) under reduced tillage and winter cover cropping can create nutrient-rich anaerobic microsites that favor microbial denitrification (10, 11). N limitation caused by reduced N fertilization may decrease the population size of ammonia-oxidizing bacteria (AOB) (12) but promote the activity of diazotrophs for N fixation (13).

The abundance and expression of genes encoding key enzymes involved in soil N transformations have been widely used to evaluate the abundances and activity of microbial populations involved in specific N cycling processes (14). For example, different microbial assemblages are responsible for N fixation, nitrification, and denitrification (15). Biological N fixation is the reduction of atmospheric N$_2$ to biologically available ammonium. Molecular analysis of N-fixing bacteria often targets nifH genes encoding a subunit of nitrogenase reductase, which catalyzes the reduction of dinitrogen to ammonia (16–18). Nitrification is the process of converting ammonia to nitrate and is one of the major processes contributing to N$_2$O production (19–22) and nitrate leaching (23) in soils. The first and rate-limiting step of nitrification is microbial oxidation of ammonia to hydroxylamine catalyzed by ammonia monooxygenases (AMO). Thus, molecular analysis of ammonia-oxidizing bacteria (AOB) and archaea (AOA) commonly targets amoA genes encoding subunit A of AMO (24, 25). Although AOA may be more numerically abundant than AOB in soil (26), AOB have been shown to be generally more responsive than AOA to soil management practices, such as N fertilization (27–29) and tillage (30), and were shown to be functionally dominant for ammonia oxidation in agricultural topsoils (31). Denitrification is the stepwise reduction of nitrate to nitrite, nitric oxide, nitrous oxide, and dinitrogen gas under anaerobic conditions and is catalyzed by nitrate, nitrite, nitric oxide, and nitrous oxide reductases, respectively (32). Reduction of nitrite to nitric oxide is catalyzed by two structurally different nitrite reductases (NIR) carried by different taxa as follows: (i) Cu-containing NIR (CuNIR) encoded by a single gene nirK, sometimes accompanied by gene nirV, and (ii) cytochrome cd$_1$ NIR (cdNIR) encoded by at least 10 genes (nirSECFDGLHJN) with nirS gene encoding the functional subunits of cdNIR (33). The reduction of N$_2$O to N$_2$ gas under anaerobic conditions and is catalyzed by nitrous oxide reductase (NOS), which is frequently studied by using the marker gene nosZ that encodes the multi-Cu NOS catalytic subunit (34, 35). The variation and the ratio between amoA, nirK, and nirS that are involved in N$_2$O production and nosZ that is involved in N$_2$O reduction have been used to predict system-level N$_2$O emissions (36, 37).

The reported effects of conservation soil management practices on important functional groups involved in N cycling are inconsistent. For example, many studies indicate that reduced or no tillage can increase the abundance of both AOA and AOB amoA (38), nirK (39), nosZ (39, 40), and (nirK + nirS)/nosZ ratios (41). Other studies, however, reported a positive effect of no tillage on AOB amoA (30) but did not find significant effects of tillage on the abundances of AOA amoA (30), nirK (42), or nirS genes (39). Cover cropping may also affect some populations: nirK gene abundances were higher under cereal rye compared to hairy vetch while nirS gene abundances...
were unaffected by cover crop species (43). Nitrogen input can suppress \textit{nifH} abundance when the available N is excessive (44, 45), but it can also improve abundances when available N is limited (46). These inconsistent and sometime contradictory results may be due to a lack of temporal resolution. For example, field trials may not have been conducted long enough to invoke a significant change in the soil system. Moreover, there is seasonal variability in microbial functional dynamics driving N transformations that are not captured if only a single time point sample is taken. Another critical gap in our current understanding of functional group dynamics is the relationship between functional capacity and activity. Many studies have only focused on N cycling functional groups at either the genetic level (i.e., using extracted DNA), which misses changes in activity or expression, or at the transcriptional level (i.e., using extracted RNA), which misses changes in population abundances.

The objectives of our study were to (i) determine the effects of tillage, cover cropping, and N fertilization on abundance and activity of N cycling microbes; (ii) investigate the seasonal dynamics of abundance and activity of N cycling functional groups under long-term conservation soil management; and (iii) identify relationships among the N cycle functional gene and transcript abundances, microbial-controlled N transformation rates, and soil physicochemical parameters. The study was conducted at a long-term (36 years) continuous cotton experiment with different tillage, cover cropping, and N fertilization regimes. To examine the dynamics of soil N cycling functional groups, we used quantitative PCR (qPCR) and real-time quantitative reverse transcription-PCR (qRT-PCR) to target the N cycle genes and transcripts of \textit{nifH}, \textit{AOB amoA}, \textit{nirK}, \textit{nirS}, and \textit{nosZ}. We then explored the seasonal dynamics of soil N cycling populations under these different conservation agricultural management practices and their relationships to soil N pools and fluxes.

RESULTS

Soil properties and ambient temperatures. All of the soil physicochemical parameters and N transformation rates measured in this study showed seasonal dynamics, and most of them were significantly affected by hairy vetch (see Table S2 and S3 in the supplemental material). In particular, increased nitrification and N mineralization rates in vetch plots were mainly observed in cover crop peak season (April) and spring crop transition (May) (Table S3). N fertilization significantly increased the concentration of NO$_3^-$-N, total soil C (TC), total soil N (TN), N$_2$O-N, and the rate of incubated nitrification and N mineralization (Table S3). Compared to conventional tillage (CT), no tillage (NT) significantly increased the concentration of total extractable C (TEC), TC, and TN, which was particularly noticeable in April and October for TEC and in May and October for TC and TN (Table S3). Ambient temperatures during the cotton growing seasons (May and October, 16.1 and 17.2°C, respectively) were higher than during the cover crop growing seasons (November and April, 8.3 and 9.4°C, respectively).

N cycle genes and transcripts. The abundances of N cycle genes and transcripts were influenced differently by agricultural season and soil management practices. In general, for both relative (normalized to 16S rRNA genes and 16S rRNA) and absolute abundances (per gram dry weight soil), genes were mostly affected by only main effects or two-way interaction effects of agricultural season and soil management practices, while transcripts were affected by much more complex interaction effects among season and management practices (Table 1; see also Table S4 and S5 in the supplemental material).

Both relative and absolute abundances of marker genes for N fixation (\textit{nifH}) were significantly affected by agricultural season and tillage (Table 1), with highest abundances during cover crop harvest (April) and under CT (Fig. 1). Moreover, although no significant effect of N fertilization on the relative abundance of \textit{nifH} genes was observed (Fig. 2A), absolute abundance of \textit{nifH} was significantly higher under no N fertilization than N fertilization, especially in no cover (NC) and vetch (V) cropping systems (Fig. 2B). Expression of \textit{nifH} had different and more complex dynamics. N fertilization (67 kg N ha$^{-1}$ [67N]) increased \textit{nifH} transcript relative abundance in NT but decreased...
| Factor               | Abundance | niR | amoA | nirK | niR5 | nosZ | 16S |
|---------------------|-----------|-----|------|------|------|------|-----|
| Season              | N         | 10.97*** | 71.34*** | 56.80*** | 7.91*** | 12.81*** | 49.64*** | 16.04*** | 62.88*** | 41.16*** | 24.29*** |
|                     | A         | 13.44*** | 62.28*** | 57.66*** | 15.97*** | 12.54*** | 30.94*** | 25.48*** | 64.89*** | 31.54*** | 33.34*** | 9.30*** | 15.65*** |
| Tillage             | N         | 9.18**   | 7.12** | 0.04 | 0.02 | 0.57 | 0.03 | 6.71* | 0.78 | 0.51 | 9.87** |
|                     | A         | 49.60*** | 13.20*** | 6.63* | 2.08 | 17.28*** | 2.75 | 59.71*** | 4.36* | 18.30*** | 17.20** |
| Cover               | N         | 2.08     | 4.18* | 88.83*** | 71.70*** | 1.09 | 26.28*** | 2.68 | 14.81*** | 6.29** | 28.50*** |
|                     | A         | 2.21     | 10.97*** | 87.58*** | 52.35*** | 0.86 | 23.39*** | 3.71* | 18.62*** | 7.74*** | 27.22*** |
| Nitrogen            | N         | 1.00     | 0.03 | 0.16 | 0.12 | 2.63 | 0.89 | 18.81*** | 1.22 | 17.69*** | 1.42 |
|                     | A         | 4.10*    | 0.09 | 5.65* | 0.17 | 2.69 | 0.88 | 4.99* | 1.25 | 2.24 | 1.27 | 5.18* |
| Season × till       | N         | 1.33     | 2.91* | 3.72* | 3.74* | 0.99 | 0.42 | 2.47 | 1.11 | 1.77 | 3.39* |
|                     | A         | 1.04     | 3.07* | 2.97* | 2.68* | 0.86 | 1.81 | 1.46 | 0.96 | 1.52 | 3.68* |
| Season × cover      | N         | 1.03     | 2.17* | 1.12 | 4.81*** | 0.70 | 5.12*** | 0.51 | 4.81*** | 0.64 | 7.56*** |
|                     | A         | 0.94     | 1.27 | 1.78 | 4.59*** | 1.82 | 3.37** | 1.31 | 5.53*** | 2.10 | 4.93*** | 0.92 | 2.37* |
| Season × N          | N         | 0.73     | 0.88 | 1.15 | 4.82** | 2.46 | 0.67 | 1.88 | 0.38 | 2.06 | 1.60 |
|                     | A         | 0.10     | 1.60 | 0.83 | 1.74 | 1.64 | 2.35 | 1.07 | 0.92 | 0.74 | 3.87* |
| Till × cover        | N         | 0.43     | 0.61 | 2.34 | 0.15 | 0.04 | 0.79 | 0.34 | 0.77 | 0.04 | 1.89 |
|                     | A         | 0.77     | 1.29 | 2.02 | 1.12 | 0.11 | 0.16 | 0.32 | 1.05 | 0.07 | 0.15 | 0.07 | 1.70 |
| Till × N            | N         | 0.55     | 11.63*** | 0.76 | 9.99** | 0.59 | 20.20*** | 0.03 | 2.48 | 0.05 | 11.71*** |
|                     | A         | 0.02     | 0.56 | 0.06 | 0.43 | 1.09 | 3.34 | 2.31 | 0.07 | 1.39 | 0.29 | 1.55 | 5.97* |
| Cover × N           | N         | 0.20     | 3.86* | 13.66*** | 16.38*** | 1.58 | 1.59 | 4.37** | 0.67 | 7.98*** |
|                     | A         | 0.80     | 2.56 | 5.51*** | 8.78*** | 0.99 | 4.99** | 0.09 | 16.37*** | 3.99* | 3.38* | 3.97* |
| Till × cover × N    | N         | 2.10     | 0.76 | 2.52 | 0.23 | 0.43 | 1.08 | 0.96 | 2.18 | 0.94 | 0.45 |
|                     | A         | 0.20     | 1.05 | 1.04 | 0.93 | 1.79 | 1.79 | 0.13 | 3.56* | 0.06 | 1.74 | 0.38 | 0.84 |
| Season × till × cover| N   | 0.72     | 5.26*** | 1.11 | 6.16*** | 0.94 | 9.88*** | 1.11 | 4.82*** | 0.65 | 4.75*** |
|                     | A         | 1.10     | 3.66*** | 1.78 | 2.33* | 0.99 | 7.46*** | 0.28 | 4.39*** | 0.29 | 1.82 | 1.07 | 2.05 |
| Season × till × N   | N         | 0.23     | 0.29 | 0.29 | 0.38 | 0.17 | 1.43 | 0.89 | 1.59 | 0.98 | 0.44 |
|                     | A         | 0.61     | 0.08 | 0.74 | 0.32 | 2.57 | 0.76 | 2.98 | 1.04 | 2.05 | 0.10 | 1.52 | 0.08 |
| Season × cover × N  | N         | 0.93     | 2.39* | 0.89 | 6.40*** | 0.60 | 3.33** | 0.42 | 1.28 | 0.64 | 4.18*** |
|                     | A         | 1.63     | 2.26* | 1.18 | 2.97** | 0.59 | 2.18* | 1.03 | 1.10 | 0.76 | 2.60* |
| Season × till × cover × N| N | 0.30     | 0.58 | 1.67 | 1.80 | 0.48 | 1.21 | 0.78 | 2.46* | 0.51 | 2.26* |
|                     | A         | 0.29     | 1.90 | 1.27 | 1.84 | 0.64 | 1.82 | 0.95 | 2.94* | 0.59 | 4.49*** | 0.47 | 1.79 |

*ANOVA based on GLIMMIX procedure in SAS. Significance is shown with bold fonts and asterisks. F values are reported. Significance levels are as follows: *, P value ≤ 0.05; **, P value ≤ 0.01; ***, P value ≤ 0.001.

N, normalized; A, absolute.
In general, \( \text{nifH} \) transcript abundances were higher during fall crop transition (November) than other seasons (Fig. 3 and 4). \( \text{nifH} \) transcript abundances were also significantly affected by the interaction of season, cover crop, and tillage (Table 1). The relative abundance of \( \text{nifH} \) transcripts was not significantly different regardless of cover crops or tillage in April but was significantly higher under CT than NT in spring crop transition (May) for NC plots, in cotton peak season (October) for NC and V plots, and in fall crop transition (November) for wheat (W) plots (Fig. 3A). The absolute abundance of \( \text{nifH} \) transcript was significantly higher under CT than NT in April and May for NC plots, in October for V plots, and in November for W plots (Fig. 3B). The interaction of season, cover crop, and N fertilization also significantly affected \( \text{nifH} \) transcripts (Table 1). Fertilizer generally had a positive effect on \( \text{nifH} \) transcripts in NC plots but a negative effect in W plots, especially in October (Fig. 4).

Both relative and absolute abundances of ammonia oxidation gene AOB \( \text{amoA} \) were significantly affected by season combined with tillage (Table 1), with the highest relative abundance in October under both CT and NT and lowest in November under CT (Fig. 1A). The highest absolute \( \text{amoA} \) abundance was in May under CT and in
October under both CT and NT and lowest in November under both CT and NT (Fig. 1B). The amoA gene abundances were also significantly affected by the combination of cover crop and N fertilization (Table 1): N fertilization increased amoA gene relative abundance under V and NC but decreased both relative and absolute abundances under wheat (Fig. 2). Similar to nifH, addition of N fertilization increased relative abundance of amoA transcripts under NT but decreased under CT (Fig. S2A). Their absolute abundance was not affected by the interaction of N fertilization and tillage (Fig. S2B).

In addition, amoA transcript abundances were significantly affected by the combination of season, cover crop, and tillage (Table 1). In April and May, amoA transcript abundances were highest under V in both NT and CT (Fig. 3). After a lag in April and May, NC plots had increased amoA transcripts in October under CT and in November under NT (Fig. 3). The interaction of season, cover crop, and N fertilization also significantly affected amoA transcripts (Table 1). Addition of fertilizer significantly increased amoA transcript relative abundance under W in April and under NC in May, though not to the same level as V (Fig. 4A). In October and November, 67N continued to exhibit increased relative abundance of amoA transcripts under NC; in contrast, 67N caused a decrease under W in October and November and V in November (Fig. 4A).
absolute abundance of *amoA* transcripts showed similar trends to their relative abundance (Fig. 4B).

The abundances of nitrite reduction gene nirK varied by agricultural season (Table 1), with lowest relative and absolute abundances in May and April, respectively (Fig. 1). CT increased absolute abundance rather than relative abundance of nirK genes (Fig. 1). N fertilization significantly increased their absolute abundance under V (Fig. 2B).
The relative abundance of nirK transcripts increased with fertilization in NT plots but decreased under CT (Fig. S2A). In addition, the nirK transcript abundances were significantly affected by the combination of season, cover crop, and tillage (Table 1). In April, May, and October, nirK transcript abundances were generally highest under V, which was more obvious for their absolute abundance (Fig. 3). In November, CT plots had similar levels of nirK transcripts regardless of cover, while NT plots had much more variable abundances between cover crops (Fig. 3). The interaction of season, cover crop, and N fertilization rate also significantly affected both relative and absolute abundances of

**FIG 4** Seasonal dynamics of 16S rRNA normalized transcript abundances of nifH, amoA, nirK, nirS, and nosZ (A) and absolute transcript abundances of nifH, amoA, nirK, nirS, nosZ, and 16S rRNA gene (B) in relation to cover crop treatments and N fertilization rate. Points represent the mean ± standard error (n = 8). NC, no cover; V, vetch; W, wheat; 0N, no N fertilization; 67N, 67 kg N ha⁻¹ fertilization.
nirK transcripts (Table 1). Fertilization generally increased nirK transcripts under W in April and November but decreased in May and October (Fig. 4).

The abundances of nitrite reduction gene nirS were significantly affected by agricultural season and tillage (Table 1), with higher abundances in November and under CT (Fig. 1). nirS gene abundances were also significantly affected by the combination of cover crop and N fertilization rate (Table 1): N fertilization increased nirS gene relative abundance in NC and W plots but had no effect in V plots (Fig. 2A); N fertilization increased nirS gene absolute abundance in W plots but decreased in V plots (Fig. 2B). nirS transcript abundances were significantly affected by the interaction between agricultural season and all three soil management practices (Table 1). The highest transcript relative abundance of nirS was observed in NT-V-0 kg N ha⁻¹ (0N), NT-V-67N, CT-V-0N, and NT-NC-67N in April, May, October, and November, respectively, while the highest absolute abundance was observed in NT-V-0N, CT-V-0N, CT-V-67N, and NT-NC-0N in April, May, October, and November, respectively (Table S4 and S5).

The abundances of nitrous oxide reduction gene nosZ were significantly affected by agricultural season (Table 1), with both relative and absolute abundances highest in October (Fig. 1). Their absolute abundance was higher in CT than NT (P < 0.0001) (Fig. 1B). nosZ gene relative abundance was highest under vetch (P = 0.0024) and 67N treatments (P < 0.0001), while absolute abundance was only increased by 67N under wheat (Fig. 2). As with nirS transcripts, there was a significant interaction between agricultural season and the three soil management practices on nosZ transcript abundances (Table 1). nosZ transcript relative abundance was highest in NT-NC-67N in April and November and CT-V-0N in May and October and lowest in NT-W-0N in April and November, NT-NC-0N in May, and CT-W-67N in October (Table S4). nosZ transcript absolute abundance was highest in CT-V-67N in April and October, CT-V-0N in May, and NT-V-67N in November and lowest in NT-NC-0N in April and May, CT-W-67N in October, and CT-W-0N in November (Table S5).

16S rRNA genes and 16S rRNA. The abundance of 16S rRNA genes showed significant seasonal dynamics (P < 0.0001). Highest abundances were observed in May and lowest in April (Table S5). Compared to NT, CT slightly but significantly promoted 16S rRNA gene abundances from a mean of $2.35 \times 10^9$ copies g⁻¹ dry weight soil in NT to 3.02 $\times 10^9$ copies g⁻¹ dry weight soil in CT (Table S5). 16S rRNA gene copy abundances decreased from $2.92 \times 10^9$ copies g⁻¹ dry weight soil in 0N to 2.43 $\times 10^9$ copies g⁻¹ dry weight soil in 67N (Table S5). 16S rRNA copy numbers were significantly affected by the combination of season and cover crop (P < 0.05), which was lowest in April under NC (9.01 $\times 10^9$ copies g⁻¹ dry weight soil) and highest in October in V (5.74 $\times 10^{11}$ copies g⁻¹ dry weight soil; see Table S5). Moreover, 67N increased 16S rRNA abundances in CT but decreased them in NT (Fig. S2). Unlike N cycle gene transcripts, the abundance of 16S rRNA was not significantly affected by the interaction of season, tillage, and cover crop/N fertilization (Table 1; Fig. 3 and 4).

Cover crop effects on N cycling microbial activity. In general, V plots tended to exhibit the highest N cycle gene transcript abundances across seasons. The positive effect of 67N addition on N cycle gene transcripts was primarily observed in plots with no cover crops (NC), especially during cotton peak season (October) (Fig. 4). It was notable that fertilization did not have a positive effect in V plots, with V-0N exhibiting similarly high or even higher N cycle gene transcript abundances as V-67N, especially during the cotton growing season (May and October) (Fig. 4). We also observed similarly high or even higher N cycle gene transcript abundances under vetch with no fertilizer (V-0N) as no cover crop with N fertilization (NC-67N) throughout the cover crop harvest and cotton growing seasons (April, May, and October) (Fig. 4).

The transcript/gene ratios of N cycle genes also generally remained higher in V plots under the treatment combination of cover crop with tillage and with N fertilization in April, May, and October (Fig. 5; see Table S6 in the supplemental material), reconfirming the enhancing effect of vetch on N cycle gene expressions. Moreover, the seasonal dynamics of transcript/gene ratio in relation to cover crop with tillage/N fertilization was generally similar to the trends in transcript absolute abundances (Fig. 5).
indicating that season and soil management practices had stronger effects at the transcript level compared to the gene abundance level.

**Variability of N cycling microbial functional potential and activity.** Nonmetric multidimensional scaling (NMDS) analysis showed the variability of the nitrogen cycling bacterial populations in terms of functional potential (i.e., based on the five gene relative abundances) and functional activity (i.e., based on the five transcript relative abundances) (Fig. 6). As expected, the statistical dispersion, or variability, of the populations by

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**FIG 5** Seasonal dynamics of transcript/gene ratio of *nifH*, *amoA*, *nirK*, *nirS*, and *nosZ* (B) in relation to cover crops and tillage (A) and in relation to cover crops and N fertilization (B). Points represent the mean ± standard error (n = 8). NC, no cover; V, vetch; W, wheat; NT, no tillage; CT, conventional tillage.
activity was consistently and significantly higher than for functional potential (dispersion index = 0.3926 for transcripts and 0.2168 for genes; \( P < 0.001 \)). Analysis of similarities (ANOSIM) testing showed overall significant differences in functional potential by season (\( R = 0.1748; P = 0.001 \)), tillage (\( R = 0.0222; P = 0.020 \)), and N fertilization (\( R = 0.0401; P = 0.003 \)) but not cover crop. The population distribution by activity was significantly affected by season (\( R = 0.2656; P = 0.001 \)), cover crop (\( R = 0.0495; P = 0.001 \)), and tillage (\( R = 0.0205; P = 0.016 \)) but not fertilization (see Table S7 in the supplemental material).

Relationships of soil N pools and processes and soil properties with functional gene abundance and expression. Correlation heatmaps showed significant correlations among absolute abundances of N cycle genes and their transcripts, transcript/gene ratio, and soil properties (Fig. 7). We observed significant positive correlations among the five gene transcripts and significant positive correlations between N cycle genes and their corresponding transcripts for all gene targets except \( nifH \) (Fig. 7). Moreover, the transcript/gene ratios of N cycle genes were more related to their transcript abundances than gene abundances, reconfirming that transcript variation depended more on changes in expression rather than population sizes.

N cycle genes and their transcripts, as well as their transcript/gene ratios, showed different patterns of correlations with soil properties (Fig. 7). The gene and transcript
abundances of *nifH*, *nirK*, and *nirS* were all positively correlated with SWC (Fig. 7). *amoA* gene and transcript abundances were both significantly and positively correlated with $\text{N}_2\text{O}-\text{N}$, $\text{NO}_3^-\text{N}$, field nitrification rate, and incubated nitrification rate (Fig. 7). Notably, *amoA* gene was positively correlated with $\text{NH}_4^+\text{N}$ ($R = 0.494$) while *amoA* transcript had no relationship with $\text{NH}_4^+\text{N}$, and the transcript/gene ratio of *amoA* was negatively correlated with $\text{NH}_4^+\text{N}$ ($R = -0.260$), indicating that although *amoA* gene abundance was promoted by $\text{NH}_4^+\text{N}$, its expression was lower with higher $\text{NH}_4^+\text{N}$ concentration (Fig. 7). In addition, there were significant positive correlations between $\text{N}_2\text{O}-\text{N}$ and the gene and transcript abundances of *amoA* and *nosZ* (Fig. 7).

**DISCUSSION**

**Seasonal dynamics in abundance and activity of nitrogen cycling populations.**

The primary objective of this study was to investigate the impact of soil health management practices on the seasonal population size and activity of soil bacterial communities involved in N transformations by using *nifH*, *amoA*, *nirK*, *nirS*, and *nosZ* as indicators. We found that the N cycling bacterial community activity, as indicated by gene and transcript abundances, respectively, showed seasonal changes.

Without considering soil management practices, the highest functional activity of diazotrophs, as indicated by *nifH* expression, was in November during the fall crop transition, which might be explained by lower N availability at this time. The inhibitory

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**FIG 7** Heatmap showing correlation among genes, gene transcripts, transcript/gene ratios, and soil properties. SWC, soil water content; TC, total carbon; TN, total nitrogen; TEC, total extractable carbon; TEN, total extractable nitrogen; MBC, microbial biomass carbon; MBN, microbial biomass nitrogen.
effect of increased N availability (NH$_4^+$-N and/or NO$_3^-$-N) on nif$H$ abundance and expression that we observed has been reported in other systems as well (45–48). Diazotrophs will preferentially use easily available N (e.g., nitrate and ammonium), as this takes less energy to assimilate compared to fixing dinitrogen gas (4). Soil freezing-thaw cycles, warming, and N addition can stimulate net N mineralization, increasing N availability (49). Compared to November, the warmer temperatures and N fertilization after cotton planting in May resulted in higher soil N availability during cotton growing seasons, thereby possibly inhibiting the expression of nif$H$. The cotton and winter cover crops take up inorganic and dissolved organic N for growth, leading to increased soil N limitation, especially during the cover crop growing period, which might enhance the competitiveness of diazotrophs and result in their relatively high abundances in April and November. Moreover, N fixation can be affected by soil oxygen content because nitrogenase is an extremely oxygen-sensitive enzyme (4). The high soil water content (SWC) in November may have resulted in lower soil oxygen, contributing to a higher functional activity of diazotrophs. The positive correlation between SWC and nif$H$ abundance in our study was also observed in other studies (50, 51). In addition, the relatively high soil C:N ratio and the decomposition of a large amount of plant residues and roots after cotton harvest season (October) may have led to soil N limitation, stimulating the expression of N fixation genes.

The amo$A$ gene relative and absolute abundances were both highest in October and lowest in November, which corresponded to the highest soil NH$_4^+$-N concentrations in October and lowest in November. Moreover, a significantly positive correlation between amo$A$ gene abundances and NH$_4^+$-N concentrations was observed in our study, indicating that concentrations of NH$_4^+$-N, the substrate for nitrification, influence the abundance of nitrifiers (28, 35). The decrease in amo$A$ gene abundance in November may be due to higher SWC and lower soil oxygen availability because AOB are aerobic bacteria (35). The seasonal dynamics of amo$A$ transcript relative and absolute abundances both depended on the interaction of cover crops with either tillage or N fertilization rate. However, contrary to amo$A$ gene abundance, relative amo$A$ expression (normalized to gene abundance) was higher in November than in October and was negatively correlated to NH$_4^+$-N concentrations, indicating that while population sizes of AOB were down, their functional activity was increased. One hypothesis to explain this unexpected result was that ammonia oxidizers increased expression of their functional genes under oxygen and/or nutrient stress. We observed a significant positive correlation between expression level of amo$A$ and SWC, which was also reported in other studies. For example, Theodorakopoulos et al. found that >70% water-filled pore space favored transcription of the AOB amo$A$ gene (37). A pure culture experiment by Yu and Chandran found that a low level of dissolved oxygen can promote amo$A$ transcription in AOB Nitrosomonas europaee (52). In addition, Bollmann et al. reported that during short-term (2 weeks) starvation, the amo$A$ mRNA of Nitrosospira briensis remained present, and the ammonia-oxidation functionality was maintained at a high level (53).

The abundances of nirS genes and transcripts were greater than those of nir$K$ in all agricultural seasons, which suggested that nir$S$-harboring denitrifiers were more predominant at our field site. Both relative and absolute transcript abundances and expressions of nir$K$- and nir$S$-harboring denitrifiers were highest in November, which is likely due to low soil oxygen level resulting from high SWC at that time. The significantly positive correlations of SWC with the transcript abundances and expression levels of nir$K$ and nir$S$ also support this explanation. Both relative and absolute abundances of nosZ$S$ genes and transcripts were highest in October. However, the N$_2$O-N emissions were highest in both May and October. The not fully consistent seasonal trend between nos$Z$ and N$_2$O-N emission suggests that the typical nos$Z$ (clade I nos$Z$) that we targeted in this study may not independently predict the seasonal dynamics of N$_2$O emission, and atypical nos$Z$ (clade II nos$Z$) may be playing an important role (54, 55). In fact, several studies have reported that atypical nos$Z$ is more abundant than clade I nos$Z$ in agricultural soils (56–58).
Effects of cover crops on abundance and activity of nitrogen cycling populations. Different responses were observed between the two types of cover crops. In general, compared to winter wheat, hairy vetch had significantly higher transcript abundance and transcript/gene ratios for all N cycle genes. The elevated activity of N cycling microbes under vetch was not limited to just the cover crop period (April) but persisted past cover crop termination through the cotton growing season (October). This supports the idea that leguminous cover crops have a lasting stimulation of overall N cycling microbes in agricultural soil. This is consistent with our previous findings from this site, where we documented significantly increased microbial biomass (both total and microbial biomass N) and enzyme activities in the vetch treatments (59). It may be that the biological N fixation rate stimulated by legumes and the low C:N ratio of legume residues supplied more N to the soil, providing a competitive advantage for N cycling populations (6, 60). Winter wheat is a nonleguminous cover crop with high C:N residues that takes up additional N from soil for growth, which may decrease soil N availability and inhibit the functional gene expression of N cycling microbes (except nifH). Some studies observed that leguminous cover crops can promote the release of mineral N and increase denitrification and N2O emissions relative to other cover crops or no cover (43, 61, 62). The relatively high and generally similar level of N cycle gene expression under combinations of hairy vetch with no fertilization and no cover crop with N fertilization supports the idea that, in some cases, N-fixing cover crops can replace N fertilization to maintain the N cycling rate.

Effects of tillage and fertilization on abundance and activity of nitrogen cycling populations. Overall, tillage only affected the relative abundances of genes nifH and nirS, but it affected the absolute abundances of all five functional genes and 16S rRNA genes, with higher abundances under conventional tillage than under no tillage. This may be because no-till treatment can decrease surface soil bulk density, increase soil porosity, and result in higher oxygen content in surface soils (63). The aerobic conditions resulting from conventional tillage may suppress the growth of anaerobic N cycling bacteria, such as N fixers and denitrifiers. Another possible explanation is that conventional tillage makes substrates more available for soil microbes by destroying soil aggregates (64, 65), increasing soil microbial populations, including the N cycling populations.

Compared to no fertilization, N fertilization with 67 kg N ha\(^{-1}\) only significantly increased the relative abundance of genes nirS and nosZ but only when no cover crops and wheat were used. This effect was observed only in wheat plots when absolute abundance was taken into consideration. This is partly in accordance with a meta-analysis by Ouyang et al., which showed that N fertilization had no effect on nifH abundance, but significantly increased the abundance of amoA, nirK, nirS, and nosZ genes, respectively (66). Although some studies using NH\(_4\)NO\(_3\) as N fertilizer also reported that N fertilization can increase AOB diversity, abundance, and activity (30, 67, 68), we did not observe this in our study, likely because similar concentrations of NH\(_4\)\(^+\)-N and field nitrification rates were observed under both fertilizer treatments. Increased abundance of denitrification genes nirS and nosZ with N fertilization corresponded to high NO\(_3\)-N concentration and high N\(_2\)O-N emission in fertilized soil.

Linking the nitrogen cycling groups with nitrogen cycling processes. In this study, we assessed the relationships between abundance and expression of functional genes and soil properties. We were particularly interested in determining whether N\(_2\)O emissions, a potent greenhouse gas, could be correlated to the population size or activity of bacteria involved in N cycling processes responsible for N\(_2\)O production and transformation. Both the absolute abundances of genes and transcripts of amoA and nosZ were positively correlated to N\(_2\)O emissions. This suggests that soil AOB populations might be a main contributor to N\(_2\)O production through nitrification, nitrifier denitrification, or both (69, 70). Likewise, Soares et al. also observed that AOB, rather than denitrifiers, were the main contributors to N\(_2\)O emissions in tropical agricultural soils (71). Moreover, research by Theodorakopoulos et al. suggested that AOB amoA transcript abundances, rather than gene abundances, were a good indicator of N\(_2\)O emissions, and ammonia oxidation was a major process contributing to N\(_2\)O emissions.
However, some studies have found no correlations between N$_2$O emissions and abundance of related N cycle genes (AOB amoA, nirK, nirS, and nosZ) (36, 42). Therefore, the contribution of AOB and denitrifiers to N$_2$O emissions might be site-specific and/or influenced by local soil properties and environmental factors. In addition, although we found significant positive correlation between field and incubated net nitrification rates with absolute abundances of both amoA genes and transcripts, a disconnection between AOB amoA gene abundances and nitrification rates has been observed in some studies (38, 67). This inconsistent result may be site-specific and might be influenced by the proportion of dormant microbes in soils, substrate limitation, microsite heterogeneity, and/or the fact that measured process rates reflect net, rather than gross, rates.

**Conclusions.** In summary, our results showed that the distribution patterns, abundances, and expressions of N cycle functional genes generally changed significantly between seasons and were affected by soil management practices. We demonstrated that leguminous cover crops like hairy vetch can promote functional activity of N cycling populations through the growing season, equaling or exceeding the effects of inorganic N fertilizer. Our findings highlight the fact that both genetic and transcriptional assessments can provide important information about microbial populations and functions. Transcriptional-level study has higher resolution than the genetic level in examining the effects of soil management practices on N cycling communities, with larger variation under different season and management practices observed. In addition, the interaction effects of season and soil management practices on N cycle gene expressions based on relative (normalized by 16S rRNA copies) and absolute (copies per gram soil) transcript abundances showed generally similar trends, indicating that active soil N cycling communities were more responsive than total active soil bacterial populations to agricultural management practices. Together, our work revealed insights into important relationships between microbial functional capacity and activity and associated nitrogen pools and processes in the field, adding to our understanding of the effects of soil health management practices on soil microbial ecology.

**MATERIALS AND METHODS**

**Study site, experimental design, and sampling.** This study was conducted at a long-term continuous cotton conservation agriculture field experiment, established in 1981, at the University of Tennessee West Tennessee Research and Education Center (WTREC) in Jackson, Tennessee. Soils at this site are classified as well-drained Lexington silt loams (fine-silty, mixed, thermic, Ultic Hapludalfs) with a 0 to 2% slope. The main crop was rainfall cotton (*Gossypium hirsutum* L.). The field study implemented a randomized complete block design with split-split plot treatment arrangements in four replicates such that N fertilization rate was the main plot treatment, cover crop was the sub-plot treatment, and tillage was the sub-subplot treatment; a subset of 12 of the 32 treatment combinations was used here (see Fig. S1 in the supplemental material). The 12 cropping system management combinations included 2 types of tillage (conventional tillage [CT], no tillage [NT]); 3 types of winter cover crops (hairy vetch [*Vicia villosa* Roth] [V], winter wheat [*Triticum aestivum* L.] [W], no cover crop [NC]); and 2 levels of NH$_4$NO$_3$ fertilization rates (0, 67 kg N ha$^{-1}$ [0N, 67N, respectively]) (Table 2). For tilled

**TABLE 2** Summary of 12 cropping system treatments used in this study

| Cropping system | Tillage      | Winter cover crop | N fertilization |
|-----------------|--------------|-------------------|-----------------|
| NT-NC-0N        | No tillage   | No cover          | No fertilizer   |
| NT-NC-67N       | No tillage   | No cover          | 67 kg N ha$^{-1}$|
| NT-V-0N         | Hairy vetch  | No fertilizer     |
| NT-V-67N        | Hairy vetch  | 67 kg N ha$^{-1}$ |
| NT-W-0N         | Winter wheat | No fertilizer     |
| NT-W-67N        | Winter wheat | 67 kg N ha$^{-1}$ |
| CT-NC-0N        | Conventional tillage | No cover | No fertilizer |
| CT-NC-67N       | Conventional tillage | No cover | 67 kg N ha$^{-1}$|
| CT-V-0N         | Hairy vetch  | No fertilizer     |
| CT-V-67N        | Hairy vetch  | 67 kg N ha$^{-1}$ |
| CT-W-0N         | Winter wheat | No fertilizer     |
| CT-W-67N        | Winter wheat | 67 kg N ha$^{-1}$ |
plots, tillage was done twice before planting by a standard disc harrow followed by smoothing and breaking up of clods by a triple-K harrow. Field operations for these plots, including cover crop termination, cotton planting, and phosphorus and potassium fertilizer application are described in more detail in previous studies of this site (59, 72).

Subsamples (2.5-cm diameter by 7.5-cm-long cores) of soil were collected from 10 random locations in each experimental sub-subplot (12 cropping systems × 4 replicates = 48 experimental plots), composted, sieved through a 2-mm mesh, and then refrigerated until analysis. Collections were obtained four times corresponding to different stages of crop growth in 2017, including cover crop harvest (April, immediately following cover crop harvest), spring crop transition (May, after cotton planting and before fertilizer application), cotton crop peak (October, before cotton harvest), and fall crop transition (November, after cotton harvest and before winter cover crop planting). Soil properties measured included N2O flux (N2O-N), soil water content (SWC), soil pH, NO3–N, NH4+–N, total extractable N (TEN), total extractable C (TEC), total soil C (TC), total soil N (TN), soil ratio of C to N (CN ratio), microbial biomass N (MBN), microbial biomass C (MBC), field net nitrification rate (field nitrification), field net N mineralization rate (field N mineralization), incubated net nitrification rate (incubated nitrification), and incubated net N mineralization rate (incubated N mineralization). Detailed methods on the measurement of these soil properties are provided in the supplemental material (see Text S1, Methods S1 to S5).

**Soil DNA and RNA extraction.** Soil genomic DNA was extracted from 0.25 g of soil using the DNeasy PowerSoil Kit (Qiagen, Hilden, Germany) according to the manufacturer’s protocol. DNA concentrations were quantified using NanoDrop One spectrophotometry (NanoDrop Technologies, Wilmington, DE). DNA was stored at −20°C.

Soil RNA was extracted using the RNeasy PowerSoil total RNA kit (Qiagen, Hilden, Germany) according to the manufacturer’s protocol. RNA was eluted in RNase-free water and stored at −80°C. RNA quality was checked by 1% agarose gel electrophoresis and NanoDrop One spectrophotometry (NanoDrop Technologies, Wilmington, DE). Samples were checked for DNA contamination by attempting to amplify the N cycle functional genes by PCR and subsequent agarose gel electrophoresis. Only samples that gave a negative electrophoresis result (i.e., no DNA contamination) were used for reverse transcription. cDNA was produced from RNA using SuperScript IV reverse transcriptase (Invitrogen, Paisley, UK) according to the manufacturer’s protocol. Random hexamer primers (Invitrogen) were used at a final concentration of 2.5 μM per reaction. cDNA samples were diluted to a final concentration of 15 ng μl⁻¹ and stored at −20°C.

**Molecular cloning.** Genes *nifH*, *amoA*, *nirK*, *nirS*, and *nosZ* were amplified from extracted soil DNA with previously designed primer pairs and reaction conditions (see Table S1 in the supplemental material). PCR products were purified with QiAquick PCR purification kit (Qiagen), ligated into pCR 4-TOPO vectors (Invitrogen), and transformed into Transform One Shot TOP10 competent cells (Invitrogen) following the manufacturer’s protocol. After shaking at 225 rpm at 37°C for 1 h, cells were spread on LB solid medium containing 50 μg ml⁻¹ ampicillin and cultured overnight. Positive clones were grown in liquid LB medium amended with ampicillin medium overnight, and plasmid DNA was extracted using the PureLink quick plasmid miniprep kit (Invitrogen) according to the manufacturer’s instructions. Plasmid DNA concentrations were determined on a Hoefer DQ 200 fluorometer (Hoefer Inc., San Francisco, CA), which uses a Hoechst dye specific for double-stranded DNA that compares DNA fluorescence with a known standard, and then sequenced with primers M13 Forward (−20)/M13 Reverse to confirm that the correct target gene was cloned. The copy numbers of *nifH*, *amoA*, *nirK*, *nirS*, and *nosZ* genes were calculated using the concentration of the extracted plasmid DNA, size (base pairs) of the plasmids containing cloned products, and the molecular weight of DNA. Ten-fold serial dilutions of the plasmid DNA (10⁻² to 10⁻⁶ copies μl⁻¹) were used during qPCR assays to generate standard curves for quantifying N cycle genes and transcripts in soil samples.

**Quantitative analysis of N cycle genes and gene transcripts.** For both DNA and cDNA samples, qPCR of *nifH*, *amoA*, *nirK*, *nirS*, and *nosZ* were performed on a CFX96 Optical real-time detection system (Bio-Rad, Laboratories Inc., Hercules, CA, USA). The 20-μl qPCR reaction mixture contained 10 μl Maxima SYBR green qPCR master mix (2x) (Thermo Scientific, USA), 1 μl PCR forward and reverse primer (both 10 μM), 2.5 μl DNA or cDNA template, and 5.5 μl nuclease-free water. Primer sets and reaction parameters used are listed in the supplemental material (see Table S1). Following the reactions, a melt curve analysis was performed to confirm the specificity of the PCR product for each real-time PCR amplification. Quantitative PCR of 16S rRNA genes and 16S rRNA was done using Femto bacterial DNA quantification kit (Zymo Research Corp., CA, USA) following the manufacturer’s protocols.

Both absolute abundances (copies per gram dry weight soil) and relative abundances (normalized to 16S rRNA genes or to 16S rRNA) of functional genes and transcripts were analyzed and compared to ensure the reliability of the result. Absolute abundance helped to achieve better understanding of the variation of N cycling bacteria with seasons and management practices, while relative abundance mitigated the impact of DNA and RNA extraction efficiency on data accuracy and revealed the relative changes of N cycling population within the total soil bacteria. The relative abundance of N cycle genes and transcripts used in this study were calculated as follows.

\[
NGA = \frac{\text{N cycle gene copies/μl DNA extract}}{\text{16S rRNA gene copies/μl DNA extract}}
\]
\[ NTA = \frac{N \text{ cycle transcript copies/\mu l RNA extract}}{16S \text{ rRNA copies/\mu l RNA extract}} \]

where NGA is the relative abundance of the gene, and NTA is the relative abundance of the gene transcript.

The transcript-to-gene copy number ratio (transcript/gene ratio) for each gene was also calculated to provide a means to determine whether transcript variation was due to changes in population sizes (i.e., genes) or changes in expression (i.e., transcripts) (73) and was calculated as follows.

\[ \text{Transcript/gene ratio} = \frac{\text{transcript copies/g dry weight soil}}{\text{gene copies/g dry weight soil}} \]

**Statistical analysis.** Statistical analyses to test the effects of agricultural season and management practices on the abundance and expression of N cycle genes were performed in SAS 9.4 (SAS Inst., Cary, NC, USA). The relative and absolute abundances of functional genes and transcripts as well as their transcript/gene ratio were both log transformed to achieve normal distributions of residuals. These log-transformed data were analyzed using a repeated-measures mixed model analysis of variance (ANOVA) within the GLIMMIX procedure. The fixed effects included season, tillage system, cover crop, and N fertilizer rate, as well as all interactions. Random effects included the block, block by nitrogen within cover crop, and the block by tillage by nitrogen within cover crop. Means were compared using a least significant difference (LSD) method. The effects of agriculture practices on N cycling microbes were visualized using scatterplots made by graphing software Origin Pro 2019b.

Pearson correlation analyses were used to determine correlation among genes, gene transcripts, transcript/gene ratios, and soil properties by rcorr function of package Hmisc in R 3.6.1 (the R Foundation for Statistical Computing). Correlation coefficient \( P \) values were then adjusted with a correction for multiple comparisons using the BH method of the Hmisc package. A heatmap based on Pearson correlations was used to visualize the significant positive and negative correlations among measured soil properties, N cycle gene abundance, gene transcript abundance, and transcript/gene ratios, and was performed by the pheatmap package in R 3.6.1.

Nonmetric multidimensional scaling (NMDS) based on Bray-Curtis distances was performed in R 3.6.1 with packages vegan 2.5-5 and phyloseq to reveal the distribution patterns of N cycling functional groups and their activity among seasons and management practices. Analysis of similarities (ANOSIM), a rank-based nonparametric statistical test, was also performed by R with the function anosim in package vegan to compare groups and test the null hypothesis that the similarity between groups is greater than or equal to the similarity within the groups. Dispersion indices were calculated in R with the function betadisper in package vegan to assess the multivariate homogeneity of group dispersions. The function TukeyHSD_betadisper in package vegan was used to calculate Tukey’s honest significant differences between groups. In this study, statistically significant difference was accepted at a \( P \) value of <0.05 unless otherwise noted.

**SUPPLEMENTAL MATERIAL**

Supplemental material is available online only.

**TEXT S1**, DOCX file, 0.02 MB.
**FIG S1**, TIF file, 0.2 MB.
**FIG S2**, TIF file, 0.1 MB.
**TABLE S1**, DOCX file, 0.01 MB.
**TABLE S2**, DOCX file, 0.02 MB.
**TABLE S3**, DOCX file, 0.03 MB.
**TABLE S4**, DOCX file, 0.05 MB.
**TABLE S5**, DOCX file, 0.1 MB.
**TABLE S6**, DOCX file, 0.03 MB.
**TABLE S7**, DOCX file, 0.02 MB.

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We declare no competing interests.
REFERENCES

1. LeBauer DS, Treseder KK. 2008. Nitrogen limitation of net primary productivity in terrestrial ecosystems is globally distributed. Ecology 89:371–379. https://doi.org/10.1890/06-0257.1.

2. Galloway JN, Townsend AR, Erisman JW, Bekunda M, Cai Z, Freney JR, Martinelli LA, Seitzinger SP, Sutton MA. 2008. Transformation of the nitrogen cycle: recent trends, questions, and potential solutions. Science 320:889–892. https://doi.org/10.1126/science.1136674.

3. White PJ, Crawford JW, Díaz Álvarez MC, García Moreno R. 2012. Soil management for sustainable agriculture. Appl Environ Soil Sci 2012:850739. https://doi.org/10.1155/2012/850739.

4. Chapin FS, Matson PA, Mooney HA. 2002. Principles of terrestrial ecosystem ecology. Springer, New York, NY.

5. Parkin T, Kaspar T. 2006. Nitrous oxide emissions from corn-soybean systems in the Midwest. J Environ Qual 35:1496–1506. https://doi.org/10.2134/jeq2005.0183.

6. Yang D, Xiao X, He N, Zhu W, Liu M, Xie G. 2020. Effects of reducing chemical fertilizer combined with organic amendments on ammonia-oxidizing bacteria. sp. NpAV. FEMS Microbiol Lett 139:181–188. https://doi.org/10.1111/j.1365-2486.1996.tb08200.x.

7. van Kessel C, Venterea R, Six J, Adviento-Borbe MA, Linquist B, van der Heijden MG. 2012. Nitrogen limitation of net primary productivity with distinct denitrification potential in prairie soils differing in soil nitrate leaching. Sci Rep 7:9738. https://doi.org/10.1038/s41598-017-08554-1.

8. Feng J, Li F, Zhou X, Xu C, Ji L, Chen Z, Fang F. 2018. Impact of agronomic practices on the effects of reduced tillage systems on CH4 and N2O emissions. Soil Till Res 150:132–139. https://doi.org/10.1016/j.still.2016.12.014.

9. Khalil K, Mary B, Renault P. 2004. Nitrous oxide production by nitrification and denitrification in soil aggregates as affected by O2 concentration. Soil Biol Biochem 36:687–699. https://doi.org/10.1016/j.soilbio.2004.01.004.

10. Cambardella CA, Johnson JM, Varvel GE. 2012. Soil carbon sequestration and climate change. Proc Natl Acad Sci U S A 113:1447–1447. https://doi.org/10.1073/pnas.1617953114.

11. Aitken J, Craig A, Harmsen J, Schoumans J. 2015. Microbial communities with distinct denitrification potential in prairie soils differing in soil nitrate leaching. Sci Rep 7:9738. https://doi.org/10.1038/s41598-017-08554-1.

12. Barta J, Tovar-Moll W, Heinesch B, Aubinet M, Vandenbol M. 2017. Increased expression of the nirK gene downstream of the amoA gene in switchgrass roots. Phytobiomes J. https://doi.org/10.1016/j.phytobiomes.2017.06.010.

13. Feng J, Li F, Zhou X, Xu C, Ji L, Chen Z, Fang F. 2018. Impact of agricultural nitrogen management for sustainable agriculture. Appl Environ Soil Sci 2012:850739. https://doi.org/10.1155/2012/850739.

14. Orr CH, James A, Leifert C, Cooper JM, Cummings SP. 2011. Diversity and activity of free-living nitrogen-fixing bacteria and total bacteria in organic and conventionally managed soils. Appl Environ Microbiol 77:911–919. https://doi.org/10.1128/AEM.01250-10.

15. Akter Z, Pageni BB, Lupwayi NZ, Balasubramanian PM. 2014. Biological nitrogen fixation and nirH gene expression in dry beans (Phaseolus vulgaris L.). Can J Plant Sci 94:203–212. https://doi.org/10.4141/cjps13-200.

16. Babu JC, Buckley DM. 2014. A comprehensive aligned nirH gene database: a multipurpose tool for studies of nitrogen-fixing bacteria. Database (Oxford) 2014:bau001. https://doi.org/10.1093/database/bau001.

17. Liu R, Hu H, Suter H, Hayden HL, He J, Melé P, Chen D. 2016. Nitrification is a primary driver of nitrous oxide production in laboratory microcosms from different land-use soils. Front Microbiol 7:1373. https://doi.org/10.3389/fmicb.2016.01373.

18. Jørgensen MH, Hajima T, Itô A. 2019. Fraction of nitrous oxide production in nitrification and its effect on total soil emission: a meta-analysis and global-scale sensitivity analysis using a process-based model. PLoS One 14:e0219159. https://doi.org/10.1371/journal.pone.0219159.
39. Tatti E, Geyer C, Burton D, Wertz S, Zebarth B, Chantigny M, Filion M. 2015. Tillage management and seasonal effects on denitrifier community abundance, gene expression and structure over winter. Microb Ecol 70:795–808. https://doi.org/10.1007/s00248-015-0591-x.

40. Badagliacca G, Benitez E, Amato G, Badalucco L, Giambello D, Laudicina VA, Ruisi P. 2018. Long-term no-tillage application increases soil organic carbon, nitrous oxide emissions and faba bean (Vicia faba L.) yields under rain-fed Mediterranean conditions. Sci Total Environ 639:330–359. https://doi.org/10.1016/j.scitotenv.2018.05.157.

41. Wang J, Zou J. 2020. No-till increases soil denitrification via its positive effects on the activity and abundance of the denitrifying community. Soil Biol Biochem 142:107706. https://doi.org/10.1016/j.soilbio.2020.107706.

42. Maul JE, Cavigelli MA, Vinary B, Buyer JS. 2019. Cropping system history and crop rotation phase drive the abundance of soil denitrification genes nirK, nirS and nosZ in conventional and organic grain agroecosystems. Agric Ecosystem Environ 279:95–106. https://doi.org/10.1016/j.agee.2018.11.022.

43. Bowen H, Maul JE, Poffenbarger H, Mirsky S, Cavigelli M, Varwood S. 2018. Spatial patterns of microbial denitrification genes change in response to poultry litter placement and cover crop species in an agricultural soil. Biol Fertil Soils 54:769–781. https://doi.org/10.1007/s00374-018-1301-x.

44. Wang C, Zheng M, Song W, Wen S, Wang B, Zhu C, Shen R. 2017. Impact of 25 years of inorganic fertilization on diazotrophic abundance and community structure in an acidic soil in southern China. Soil Bio Biochem 113:240–249. https://doi.org/10.1016/j.soilbio.2017.06.019.

45. Cusack DF, Silver W, McDowell WH. 2009. Biological nitrogen fixation in two tropical forests: ecosystem-level patterns and effects of nitrogen fertilization. Ecosystems 12:1299–1315. https://doi.org/10.1007/s10021-009-9290-0.

46. Lindsay EA, Colloff MJ, Gibb NL, Wakelin SA. 2010. The abundance of mRNAs and nirS genes underscore niche differentiation amongst terrestrial N2O reducing communities. Soil Bio Biochem 42:1971–1981. https://doi.org/10.1016/j.soilbio.2010.04.025.

47. Ouellet A, El Khoury B, Harvey J, Barnoud C, Breitenbach M, Lee JR, Rayburn B. 2012. Unexpected nondenitrification effects on the activity and abundance of the denitrifying community. Soil Biology Biochem 44:406–419. https://doi.org/10.1016/j.soilbio.2011.11.024.

48. Konstantinidis KT, Lohan MI, Prosser IJ, Park CT, DeLong EF. 2006. Unexpected nondenitrification of nitrous oxide by ammonia-oxidizing bacteria and mitigation options from N fertilization in an intensively managed calcareous uvo-aquic soil. Sci Rep 4:3950. https://doi.org/10.1038/srep03950.

49. Soares JR, Casman NA, Kielak AM, Pijl AJ, Carmo JB, Lourenco KS, Laanbroek HJ, Cantarella H, Kuranma EE. 2016. Nitrous oxide emission related to ammonia-oxidizing bacteria and mitigation options from N fertilization in a tropical soil. Sci Rep 6:30349. https://doi.org/10.1038/srep30349.

50. Konstantinidis KT, Lohan MI, Prosser IJ, Park CT, DeLong EF. 2006. Unexpected nondenitrification of nitrous oxide by ammonia-oxidizing bacteria and mitigation options from N fertilization in an intensively managed calcareous uvo-aquic soil. Sci Rep 4:3950. https://doi.org/10.1038/srep03950.

51. Suwing J, van Beusekom R, Zhang X, Gao B, Hu XK, Lu X, Well R, Christie P, Bakken LR, Ju XT. 2014. Ammonia-oxidation as an engine to generate nitrous oxide in an intensively managed calcareous fluvo-aquic soil. Rev Environ Sci Biotechnol 13:349–359. https://doi.org/10.1007/s11157-013-9247-4.

52. Wang H, Li X, Li X, Wang J, Zhang S. 2017. Changes of microbial population and N-cycling function genes with depth in three Chinese paddy soils. PLoS One 12:e019506. https://doi.org/10.1371/journal.pone.019506.

53. Abdalla M, Rueangritsarakul K, Jones M, Osborne B, Helmy M, Roth B, Burke J, Nolan P, Smith F, Williams M. 2012. How effective is reduced tillage-cover-crop management in reducing N2O fluxes from arable crop soils? Water Air Soil Pollut 223:5135–5174. https://doi.org/10.1007/s11270-012-1268-9.

54. Terlaars-van der Nielen L, Lachouani P, Knudsen MT, Ambus P, Boelt B, Gidum R. 2016. Productivity and carbon footprint of perennial grass-forage legume intercropping strategies with high or low nitrogen fertilizer input. Sci Total Environ 541:1339–1347. https://doi.org/10.1016/j.scitotenv.2015.10.013.

55. López-Vázquez A, Cadena-Zapata M, Campos-Magaña S, Zermelo-Gonzalez A, Mendez-Dorado M. 2019. Comparison of energy used and effects on bulk density and yield by tillage systems in a semiarid condition of Mexico. Agronomy 9:189. https://doi.org/10.3390/agronomy9040189.

56. Elliott ET. 1986. Aggregate structure and carbon, nitrogen, and phosphorus in native and cultivated soils. Soil Sci Soc Am J 50:6027–633. https://doi.org/10.2136/sssaj1986.0361599500500000301x.

57. Ashman MR, Hallert PD, Brookes PC. 2003. Are the links between soil aggregate size class, soil organic matter and respiration rate artefacts of the fractionation procedure? Soil Bio Biochem 35:435–444. https://doi.org/10.1016/S0038-0717(02)00295-X.

58. Ouyang Y, Evans SE, Friesen ML, Tiemann LK. 2018. Effect of nitrogen fertilization on the abundance of nitrogen cycling genes in agricultural soils: a meta-analysis of field studies. Soil Bio Biochem 127:71–78. https://doi.org/10.1016/j.soilbio.2018.08.024.

59. Liu S, Coyne MS, Grove JH. 2017. Long-term tillage and nitrogen fertilization: consequences for nitrifier density and activity. Appl Soil Ecol 120:121–127. https://doi.org/10.1016/j.apsoil.2017.07.034.

60. Liu S, Coyne MS, Grove JH. 2018. Tillage, cropping, seasonal and tillage management influence ammonia oxidizing bacterial communities in long-term maize. Appl Soil Ecol 129:98–106. https://doi.org/10.1016/j.apsoil.2018.05.002.

61. Zhu X, Burger M, Doane TA, Horwath WR. 2013. Ammonia oxidation pathways and nitrifier denitrification are significant sources of N2O and NO under low oxygen availability. Proc Natl Acad Sci U S A 110:6328–6333. https://doi.org/10.1073/pnas.1219991110.

62. Huang T, Gao B, Hu XK, Lu X, Well R, Christie P, Bakken LR, Ju XT. 2014. Ammonia-oxidation as an engine to generate nitrous oxide in an intensively managed calcareous fluvo-aquic soil. Rev Environ Sci Biotechnol 13:349–359. https://doi.org/10.1007/s11157-013-9247-4.

63. Soares JC, Casman NA, Kielak AM, Pijl AJ, Carmo JB, Lourenco KS, Laanbroek HJ, Cantarella H, Kuranma EE. 2016. Nitrous oxide emission related to ammonia-oxidizing bacteria and mitigation options from N fertilization in a tropical soil. Sci Rep 6:30349. https://doi.org/10.1038/srep30349.

64. Konstantinidis KT, Lohan MI, Prosser IJ, Park CT, DeLong EF. 2006. Unexpected nondenitrification of nitrous oxide by ammonia-oxidizing bacteria and mitigation options from N fertilization in an intensively managed calcareous uvo-aquic soil. Sci Rep 4:3950. https://doi.org/10.1038/srep03950.

65. Suwing J, van Beusekom R, Zhang X, Gao B, Hu XK, Lu X, Well R, Christie P, Bakken LR, Ju XT. 2014. Ammonia-oxidation as an engine to generate nitrous oxide in an intensively managed calcareous fluvo-aquic soil. Rev Environ Sci Biotechnol 13:349–359. https://doi.org/10.1007/s11157-013-9247-4.

66. Cusack KD, Fitzgerald LA, Cockrell AL, Biffinger JC. 2015. Selection and evaluation of reference genes for reverse transcription-quantitative PCR expression studies in a thermophile bacterium grown under different culture conditions. PLoS One 10:e0131015. https://doi.org/10.1371/journal.pone.0131015.