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Nitrogen fertilizer driven nitrous and nitric oxide production is decoupled from microbial genetic potential in low carbon, semi-arid soil

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Introduction: Nitrous oxide (N₂O) emission from soil is a major concern due to its contribution to global climate change and its function as a loss mechanism of plant-available nitrogen (N) from the soil. This is especially true in intensive agricultural soils with high rates of N fertilizer application such as those on the semi-arid Southern High Plains, USA.

Methods: This study examined emissions of N₂O, pore-space concentrations of N₂O and nitric oxide (NO), soil chemical properties, water content, and the genetic potential for N cycling five years after conservation system and N management implementation.

Results: For these semi-arid soils with low N, carbon, and water contents, large soil N₂O emissions (up to 8 mL N₂O-N m⁻² day⁻¹) are directly related to the application of N fertilizer which overwhelms the N₂O reducing capacity of the soil. When this fertilizer N is depleted, N₂O flux is either low, non-existent, or net-negative and has been observed as early as mid-season for preplant applied N fertilizer (-0.1 mL N₂O-N m⁻² day⁻¹). Soil pore-space gas concentrations (N₂O and NO) remained relatively constant across the growing season (average N₂O: 0.78 μL N₂O L⁻¹ soil air; NO: 3.3 μL NO L⁻¹ soil air, indicating a base-level of N-cycle activity, but was not directly related to surface emissions of N₂O which decreased across the growing season. In addition, genetic potential for N cycle activities increased across the growing season simultaneously with stagnant/reduced N cycle activity. This reflects the difficulty in relating genetic potential to *in-situ* activity in field research.

Conclusion: It is likely that in a nutrient and carbon-poor soil, such as the semi-arid agricultural soil in this study, the microbial processes associated with N cycling are mostly limited by inorganic-N and less directly related to genetic potential at the time of sampling.

KEYWORDS

nitrous oxide, pore-space gases, microbial abundance, semi-arid soils, no-tillage, cover crop

1 Introduction

Nitrous oxide (N_2O) production is one of the major concerns in fertilized and irrigated agroecosystems due to its contribution to the greenhouse effect and its role in the destruction of stratospheric ozone, both of which contribute to its large global warming potential (1, 2). Microbial nitrification and denitrification in natural and managed soils are responsible for 70% of global N_2O emissions (3–5). The controls over these two N cycling processes can be broken down into proximal (primary) and distal (secondary) effects based on their immediacy of impact on the process.

Primary controls over nitrogen gas cycling and N_2O production include the amount of, and interactions between soil carbon (C) (6–8) soil nitrate (NO_3^-) (9–12) and ammonium (NH_4^+) (13), soil oxygen (O_2) (14), and pH (7, 15) and have been well documented in the literature. Briefly, both NH_4^+ -N and NO_3^- -N are the starting substrate for the production of N gases in soil ecosystems, where the oxidation (nitrification) and reduction (denitrification) is directly tied to soil O_2 content with nitrification requiring an aerobic environment and denitrification requiring an anaerobic environment. Furthermore, the use of carbon as an energy source in anaerobic environments enables denitrification to take place (6) while C respiration may create temporary anaerobic microsites which can harbor denitrification activity. Lastly, soil pH directly influences the completion of denitrification through the pH specificity of N_2O reductase activity (16) and other nitrification and denitrification enzymes. Distal controls over N trace gas emissions mostly operate to affect the genetic potential for denitrification and nitrification and can include disturbance (frequency and intensity), soil pH, soil C content, soil moisture and O_2 content, temperature, and predation (7, 15). Understanding genetic potential within a system as a distal control allows for better defining the mechanisms behind soil processes. For example, in the case of semi-arid soils, the genetic potential for specific processes such as denitrification may not be expected due to the low water content of the soil. However, previous studies have shown a strong potential for processes such as N_2O reduction (via *nosZ* clade I and II abundance) in semi-arid lands which is a key step in denitrification (17).

These primary and secondary controls for soil N cycling can be significantly affected by producer decisions regarding soil tillage and nutrient management. Physical disturbances such as tillage can alter soil structure, introduce/bury organic inputs, and increase water infiltration which act to alter the O_2 and C content of the soil while also affecting microbial abundance (18, 19). The application of fertilizer alters the soil chemical composition affecting the rate of nutrient cycling and soil pH in addition to altering the soil microbial community through selection and inhibition based on the added nutrients (20). Furthermore, the timing of these decisions can alter the potential for production and consumption of greenhouse gases (GHGs) and affect their concentration in the soil atmosphere (21–23) especially in semi-arid soils which are less well buffered against environmental change due to low organic matter, water, and nutrient content and thus naturally vary throughout the growing season.

Previous studies have highlighted the importance of N fertilizer application on N_2O emissions from the soil surface in semi-arid agriculture (17) where it was determined that the timing of N fertilizer application altered the temporal pattern of N_2O production. However, surface emissions alone may not fully indicate the level of N-cycle activity related to N fertilizer application. Pore-space, or soil air, concentrations of N gases have been studied in several environments including agricultural production (24) and forests (25) where N gas production was correlated with N fertilizer application and soil N content (respectively). In highly intensive agroecosystems such as those on the semi-arid Southern High Plains, pore-space concentrations will also likely be related to soil N content through N fertilizer application but will likely differ in concentration due to the low N, C, and water content in the semi-arid soils relative to previous studies (24, 25). Strong relationships between pore-space concentrations of N gases and emissions from the soil surface in forest soils have also been reported (26), but soil management decisions may alter this relationship due to alterations in pore-space, microsites, and genetic potential in the soil profile. In the case of semi-arid soils, this has already begun to be evaluated, where strong genetic potential for N_2O consumption was determined in surface soils (23).

The study reported here evaluates the combined effects of conservation soil management (no-tillage and cover cropping) and altered N timing (pre-plant vs. split application) on N gas production in semi-arid soil under cotton (*Gossypium hirsutum* L.) production. In addition, soil chemical parameters that drive N gas production and the genetic potential for N gas production are evaluated to begin to understand the role these proximal and distal controls play in producing N gases in fertilized semi-arid systems. We hypothesized that conservation management practices would alter both proximal and distal controls for N gas production in this system due to the known effects cover-cropping and no-tillage have on soil chemical characteristics and the activity and abundance of microorganisms. We further hypothesized that there will be a combined effect of genetic potential and soil NO_3^- -N on N gas production supporting previous determinations of N_2O emissions being driven by denitrification in these soils. Lastly, we hypothesized that N gas production and the drivers of production will vary seasonally with peak activity during the early growing season for the pre-plant application of N fertilizer which should alleviate N limitation and jumpstart N cycling in this system.

2 Methods

2.1 Site description

Research was conducted in continuous cotton systems at the Texas A&M AgriLife Research Center in Lubbock, TX (33.687°, -101.827°) where conservation systems were implemented in Fall 2014 and sampling was conducted from 2018–2020. Annual precipitation is 481 mm and the 30-year mean annual temperature for Lubbock is 16.1°C (30-year average, 1991–2020) (27). The soil series at the research site is an Acuff loam described as a fine-loamy, mixed, superactive, thermic Aridic Paleustoll (28).

The study was designed as a split plot with the main plot consisting of different conservation practices (tillage systems), and the split plot consisting of N fertilizer treatments differing in their time of application. Conservation practices included no-tillage with a winter wheat cover crop (NTW) and no-tillage winter fallow (NT), compared to a conventional tillage winter fallow (CT) system. Both the NTW and NT systems were introduced in November 2015. The field was in conventional tillage for at least the previous 60 years. Split plot N fertilizer application timings include the following: no-added N (control); 100% of N applied pre-plant (PP); and 40% of N applied PP and 60% applied mid-season (SPLIT). The study consisted of 3 blocks (replicates) for a total of 27 plots, each measuring 15 m in length and four rows wide with 1 m row spacing. Full agronomic information for the three-year study and a timeline for management and sampling in 2020 is included in the supplemental material ([Supplementary Material 1: Methods](#)).

Agronomic information for the final year is briefly presented here. Furrow irrigation of the control plots and all treatment plots was conducted on 03 June, 30 June, 10 August, and 26 August with each event applying about 152 mm of water. Pre-plant N fertilizer was applied 6 May 2020. Mid-season N fertilizer applications were made 21 July 2020 near the initiation of reproductive growth. Application of N fertilizer was made at rate of 168 kg ha⁻¹ as urea-ammonium-nitrate (UAN) and was conducted using a knife and coulter fertilizer applicator mounted to a tractor. Nitrogen fertilizer was applied 10–15 cm from the cotton row at a depth of 15 cm. Nitrate added in the irrigation water amounted to 47 kg N ha⁻¹ in 2020, calculated from the concentration of nitrate-N in the irrigation water, the rate of irrigation applied, and the number of irrigation events (29). Cotton (DP 1820) was planted on 1 June 2020 and harvested on 20 November 2020. Cover crops were planted in November 2019 and terminated on 15 April 2020.

2.2 Soil sampling and analyses

Soil sampling was conducted at three key cotton growth periods: early-season (June, vegetative growth), mid-season (August, peak plant production), and late-season (October, reproductive growth) in 2018, 2019, and 2020. However, only 2020 is presented in the main text due to the inclusion of soil gas and microbial genetic potential measurements being made in conjunction with soil sampling in the final year. In 2020, samples were specifically collected on 24 June, 05 August, and 10 October. Soil samples were collected using 2.5 x 40 cm hand probes to a depth of 20 cm separated into 0–10 and 10–20 cm depths. Six soil cores per plot were collected, homogenized, and stored on ice until a ~25 g subsample could be collected and frozen at -80°C for DNA analysis. The remaining soil was weighed and then dried at 60°C for 7 days to determine gravimetric water content. Soil samples were extracted for soil nitrate-N (NO_3^- -N) and ammonium-N (NH_4^+ -N) using a 1 M KCl (1:10 soil to extractant ratio, 4 g of soil) and analyzed for NO_3^- -N using flow injection spectrometry (FIALab 2600, FIALab Instruments Inc., Bellevue, WA) following cadmium reduction to NO_2^- and for NH_4^+ -N by the Berthelot reaction involving salicylate (30). In addition, soil samples were analyzed for mineralizable C (C_{min}) by a 3-day rewetting incubation with 40 g of air-dried soil sieved at 4 mm (31). Soil pH was determined by a 1:2 dilution of 10 g soil with DI water (32).

2.3 Microbial analyses

Microbial analyses for genetic potential were conducted by quantitative polymerase chain reaction (qPCR) of major bacterial nitrification and denitrification genes and the genetic marker for bacterial abundance (Table 1) for samples collected

TABLE 1 Primer sequences and thermal profiles for total bacterial and bacterial N-cycle functional gene abundances.

Target group	Primer Name	Sequence (5'→3')	Thermal profile	Average Amplification efficiency (R ² > 0.95) (%)	References
16S rRNA	Eub338	ATCATGGTCTGCGCG	95°C for 15 min; 95°C for 1 min, 53°C for 30 s, 72°C for 1 min × 40 cycles	97	(31)
	Eub518	GCCTCGATCAGRTTGTGGTT			
Bacterial <i>amoA</i>	AOB amoA-1F	GGGGWTTCTACTGGTGGT	95°C for 5 min; 94°C for 45 s, 60°C for 45 s, 72°C for 1.5 min × 40 cycles	100	(32)
	AOB amoA-2R	CCCCTCKGSAAAGCCTTCTTC			
<i>nirS</i>	nirS4F	G TSAACG TSAAGGARACSGG	94°C for 2 min; 94°C for 30 s, 58°C for 1 min, 72°C for 1 min × 40 cycles, 72°C for 10 min	104	(33)
	R3cd	GASTTCGGRTGSGTCTTGA			
<i>nirK</i>	nirK876	ATYGGCGGVCA YGGCGA	94°C for 2 min; 94°C for 30 s, 58°C for 1 min, 72°C for 1 min × 40 cycles	95	(34, 35)
	nirK1055	G CYTCGATVAGRTTGTGGTT			
<i>nosZ</i> clade I	nosZ2F	CGCRACGGCAASAAGGTSMSGT	50°C for 2 min, 95°C for 3 min; 95°C for 15 s, 67–62°C for 30 s, 72°C for 30 s; 95°C for 15 s, 62°C for 1 min, 72°C for 1 min × 34 cycles	97	(36)
	nosZ2R	CAKRTGCAKSGCRTGGCAGAA			
<i>nosZ</i> clade II	clade II F	CTIGGICCIYTKAYAC	95°C for 2 min; 95°C for 30 s, 56°C for 30 s, 72°C for 40 s × 40 cycles	90	(37)
	clade II R	GCIGARCARAAITCBGTRC			

in the final year of the study (33–39). DNA was extracted using a Quick-DNA Fecal/Soil Microbe 96 Magbead Kit (Zymo Research, Irvine, CA, USA) with a 30-min, room-temperature drying period prior to final DNA elution. Quantitative polymerase chain reaction was conducted on a Thermo Fisher Quant Studio 5 (Thermo Fisher Scientific Inc., Waltham Mass, US) with gBlock gene fragments specific for each gene assayed (Supplementary Table 1, Integrated DNA Technologies, Inc., Coralville, IA, USA). In addition, quality control qPCR was conducted on the Quant Studio 5 by spiking a known concentration qPCR reaction with extracted DNA from this study and evaluating if any inhibition occurred in a process similar to Hartman et al. (40), where extracted DNA was added to a qPCR assay to quantify the abundance of *Vibrio alginolyticus* with *gyrB* as the gene target (41). A spiked sample with a threshold cycle (C_t) value within 3 standard deviations of the quality control C_t mean was determined to not contain inhibitors. A small number (<25) of samples were determined to inhibit PCR reactions and were diluted 1:10 with sterile water to reduce inhibition and re-analyzed with qPCR as described above. Initial extractions and dilutions were used to conduct qPCR evaluation of the soil for the 16S rRNA gene, *nosZ* clade I and clade II (N₂O reductases). Due to elution volume limitations, additional extractions were conducted, and diluted 1:10 with sterile water, as described above for *nirK* (copper dependent nitrite reductase), *nirS* (cytochrome cd nitrite reductase), and *amoA* (ammonia monooxygenase) qPCR

evaluations from the same soil subsamples as the previous extractions.

2.4 Greenhouse gas emissions and pore-space concentrations

Nitrous oxide emissions and in-ground concentrations of N₂O and NO were collected during the final year of the study using a Gasetm DX4040 portable FTIR gas analyzer (Gasetm Technologies, Helsinki, Finland) integrated with a LiCor 8100A opaque chamber for soil surface emissions. The Gasetm DX4040 was also connected to in-ground pore-space probes (PSPs) to measure in-ground concentrations (42). Soil N₂O emissions and pore-space samplings were conducted within 48 hours of soil sample collection. Soil NO emissions were measured but were not determined to be significant (flux rate below measurable limit) and thus are not included. Pore-space concentration measurements were conducted according to Brummell et al. (43) and McDonald et al. (42). Briefly, PSPs were installed at the beginning of the growing season and then connected to reservoir bottles 24 hours before measurement. On the day(s) of sampling, PSPs with reservoir bottles were integrated into a closed loop system with the Gasetm analyzer for soil N₂O and NO concentration determination. The reservoir bottle increased the system volume to greater than that of the sample cell within the Gasetm, thus eliminating any vacuum being placed on soil pores.

2.5 Statistical analyses

Analyses of variance (ANOVA) for soil chemical parameters was conducted across the three growing seasons within each depth using the lmer function within the lme4 package in R (44) where the initial analysis of variance model included sampling period, conservation system, and N treatment as fixed effects with evaluation of their main effects as well as their combined interactions. Random effects specified in the initial model were rep and the interaction of rep and sampling period to account for repeated measurement of the same research plots over time. The two sampling depths were parsed prior to ANOVA due to the well-known effects of depth on soil parameters, and the desire to evaluate the data in the context of the area from which soil pore-space gases will be measured. In addition, the application of N fertilizer at a depth of 15 cm created a gradient of inorganic-N between the two depths which may have affected the production of greenhouse gases at depth. Consistent over the evaluation of soil parameters was the significant effect of sampling period as a main effect, while only the repeated measure of $\text{NO}_3\text{-N}$ was affected with an interaction of sampling period and N timing. Due to the strong observed effect of sampling period as a main factor, analyses for the final year of the study were split by depth and sampling period to understand within seasonal effects without the variability of the distinct climatic and soil conditions at each sampling period. Analyses of variance of soil chemical parameters for the three-year dataset and final year are presented in the supplementary material ([Supplementary Material 2: Soil Chemical Results](#)). Microbial genetic potential for N cycling was also evaluated by ANOVA within sampling period and depth for the final year of the study ([Supplementary Material 3: Microbial abundance in the final year](#)).

Principal components analysis of the soil parameters was conducted using the prcomp function in the stats package in R (45), where an initial evaluation of the relationship of soil parameters was conducted across all three years of the study, and then separately for the final year to support the use of the final year as a reasonable representative of the variability in soil parameters over the length of the study. Soil parameters were normalized to a range of 0-1 prior to analyses to evaluate the variability between samples without the vast differences in scale using the preProcess function of the caret package in R (46). As mentioned above, soil gas measurements were only collected in the final year of the study due to the use of the first two years as test periods for the soil pore-space probes and subsequent mechanical issues with the FTIR analyzer that prevented full-season evaluation of pore-space gases and surface emissions, further necessitating the final-year analyses. Microbial abundance data was subsequently limited to analysis during the final year of the study as well due to the research team's decision to specifically focus on the final year for the holistic evaluation of the soil biochemical effects of the implemented

treatments on gas production. Principal components analysis of microbial gene abundances was conducted using the same process as the analysis for soil chemical parameters detailed above. However, soil chemical parameter vectors were fit to the ordination of microbial gene data using the envfit function in the vegan package in R (47). Principal components ordinations and contributions to axes were plotted using ggplot2 (48)

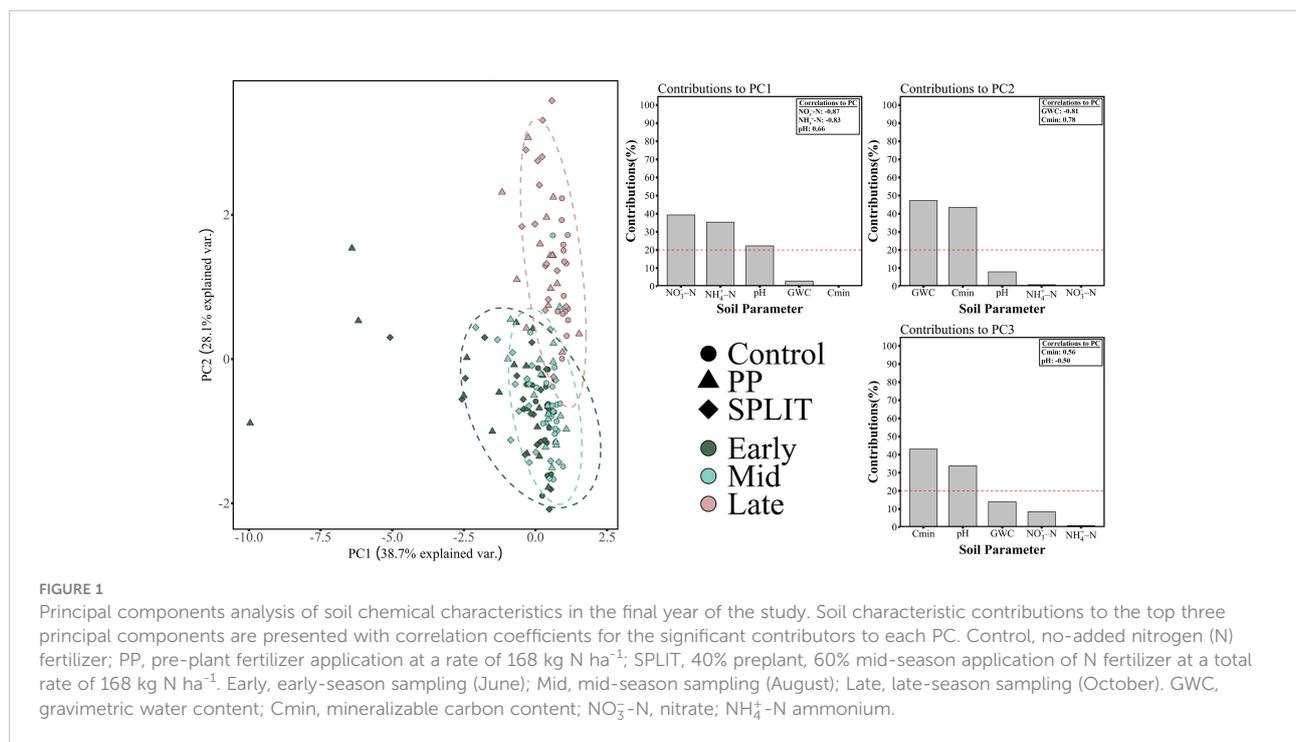
LASSO regression analysis was conducted using the glmnet and cv.glmnet functions within the glmnet package in R (49). The data was split into training and testing datasets at random with a 75%, 25% split resulting in 114 samples for training and 38 samples for testing (46). The model for each gas was initially developed using leave-one-out cross validation with the cv.glmnet function, after which the best model was refined using the glmnet function and the lambda generated by the initial model. The model was then used to predict gas concentration for the testing dataset using the predict function, and the R^2 of the fit was calculated to determine predictive capacity of the model for the new dataset. Lastly, the measured vs. the predicted concentrations for the testing dataset were plotted to visualize the fit of the data to the linear model using ggplot2 (48).

3 Results

3.1 Soil characteristics

Soil chemical analyses were conducted for the combined effect of the implemented treatments and time over the course of the growing season across all three years of the study and are presented in the supplementary material ([Supplementary Material 2: Soil chemical results](#)). Due to significant effects of sampling period as a main effect and minimal interaction with soil chemical parameters, soil chemical characteristics were evaluated within sampling period and depth for the final year of the study where soil chemical analyses were combined with microbial gene abundance and gas sampling. The results of ANOVA for the final year are also presented in the supplementary material (see [Supplementary Material 2.2 Final Year Analyses](#)).

The relationships between soil chemical parameters as they are affected by the implementation of conservation system and altered N timings was addressed using PCA across the entire dataset ([Supplementary Figure 2](#)) and within the final year of the study ([Figure 1](#)) across both depths. It is clear that the data in the final year are representative of the pattern of variance present in the three-year dataset due to the similar pattern in variance for the full dataset and the final year alone and the similar total variance explained by PCA of soil chemical parameters between the final year (66.8%, [Figure 1](#)) and the full three-year dataset (59.2%, [Supplemental Figure 2](#)). For the final year, PC1



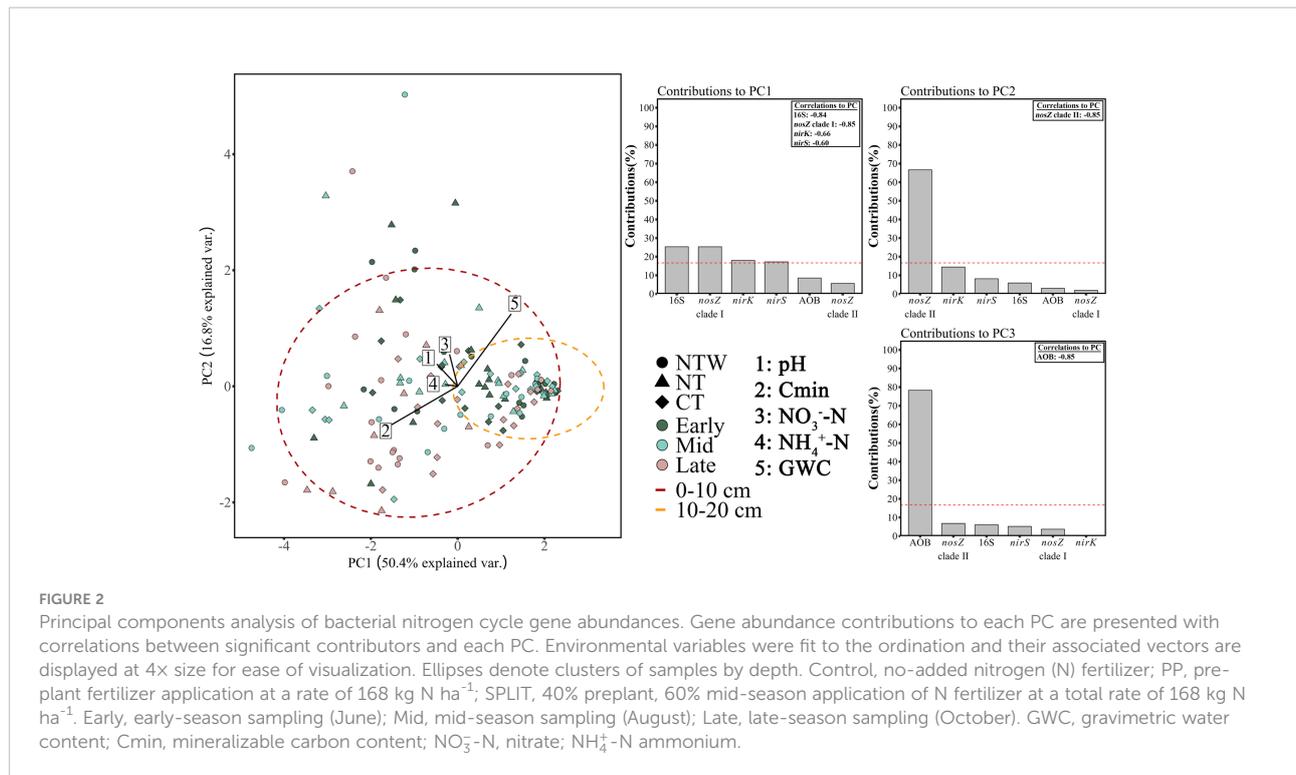
explained 38.7% of the variance in the data where significant contributions to the variability explained included soil NO₃⁻-N, pH, and NH₄⁺-N which each contributed greater than 20% of the variability (Figure 1). PC2 explained 28.1% of the variance in soil chemical parameters where significant contributions included GWC and Cmin content (Figure 1). Lastly, PC3 also explained a moderate proportion of variability, accounting for 14.6% of the variability with both Cmin content and soil pH significantly contributing to the variance explained (Figure 1).

Correlations between the significant contributors to variance explained for each PC are displayed in Figure 1. Both NO₃⁻-N and NH₄⁺-N were negatively correlated with PC1 and soil pH was positively correlated with PC1 (Figure 1); Cmin content was positive correlated with PC2 and GWC was negatively correlated with PC2; and both Cmin content and pH were negatively correlated with PC3. As mentioned above, PCA analyses were conducted across depths and depth was not determined to cluster within the ordination of either soil chemical dataset.

3.2 Microbial abundances

Analyses of variance for genetic abundances are reported in the supplemental material (see [Supplemental Material 5: Microbial abundance in the final year](#)). In brief, bacterial *amoA* (AOB) abundance was affected at all three sampling periods, while *nirS* and *nirK* abundances were unaffected at

any of the three sampling periods. In general, bacterial N cycle gene abundances increased across the growing season, with a distinct increase in AOB abundance from the early- to mid-season sampling. As with soil chemical characteristics, microbial genetic abundances were evaluated by PCA to determine overall relationships of implemented treatments and sampling periods on the microbial genetic potential for N cycle activities (Figure 2). In addition, soil chemical characteristics were fit to the PCA ordination of the microbial gene abundances, and their vectors are presented on Figure 2. It should be noted that vector coordinates were multiplied by 4 for visualization on the figure, but the relationships to the ordination of the microbial gene abundances remain unchanged. In addition, some clustering was observed by depth for microbial gene abundances and ellipses are fit to these clusters. Principal components analysis of microbial gene abundances revealed no distinct clustering by sampling period, conservation system, or N timing. The data was moderately clustered by depth, where the 0-10 cm depth contained much more variability than the 10-20 cm depth (Figure 2). The total variability explained by PCA for gene abundances was 67.2 for PC1 and PC2 combined, where PC1 explained 50.4% of the variance and PC2 explained 16.8% of the variance. In addition, PC3 moderately contributed to variance explained (13.8%). Significant contributors to the variability explained by PC1 included the 16S gene, *nosZ* clade I, *nirK*, and *nirS* where all genes were negatively correlated with PC1 (Figure 2). Only *nosZ* clade II significantly contributed to the



variance explained by PC2, where clade II was positive correlated with PC2 (Figure 2). Lastly, only AOB significantly contributed to the variance explained by PC3, where AOB was positively correlated with the axis (Figure 2).

For the analysis fitting soil chemical parameters to the ordination of genes in Figure 2, only Cmin content and GWC were determined to be significant, with nearly antithetical relationships to the ordination data (Figure 2).

3.3 Nitrous oxide emissions

Nitrous oxide emissions were evaluated at each soil sampling event to assess the impacts of conservation system and N timing on production of N₂O from the soil-atmosphere interface. At the early-season sampling, N timing significantly affected N₂O emissions ($p=0.002$, Figure 3) where emissions from the PP treatment (8071 $\mu\text{g N}_2\text{O-N m}^{-2} \text{d}^{-1}$) were greater than from the SPLIT treatment (1697 $\mu\text{g N}_2\text{O-N m}^{-2} \text{d}^{-1}$) and the control (-218 $\mu\text{g N}_2\text{O-N m}^{-2} \text{d}^{-1}$).

At the mid-season sampling, N timing affected N₂O emissions ($p=0.002$, Figure 3) where emissions from the SPLIT treatment (2307 $\mu\text{g N}_2\text{O-N m}^{-2} \text{d}^{-1}$) were greater than from the PP treatment (-106 $\mu\text{g N}_2\text{O-N m}^{-2} \text{d}^{-1}$) and the control (-362 $\mu\text{g N}_2\text{O-N m}^{-2} \text{d}^{-1}$).

No net production or consumption of N₂O was determined at the late-season sampling (Figure 3 and Supplementary Table 12,13).

3.4 Soil pore-space concentrations of nitrogen

Pore-space concentrations of N₂O and NO were analyzed for each sample date at a depth of 7.5 cm and 15 cm. At the early-season sampling at the 7.5 cm depth, pore-space concentration of N₂O was affected by N timing ($p=0.041$, Figure 4 and Supplementary Table 13) where the PP treatment had greater N₂O concentration (1.2 $\mu\text{L N}_2\text{O L}^{-1}$) than the control (0.88 $\mu\text{L N}_2\text{O L}^{-1}$), but not the SPLIT treatment (1.0 $\mu\text{L N}_2\text{O L}^{-1}$). No treatment effects were determined for NO at 7.5 cm or for either N₂O or NO at 15 cm at the early season sampling (Figure 4 and Supplementary Table 12). Pore-space concentrations of NO and N₂O were unaffected at both 7.5 cm or 15 cm depth for the mid- and late-season samplings (Figure 4 and Supplementary Tables 12, 13). Across the growing season, pore-space N₂O concentrations consistently decreased at both the measured depths, while NO concentrations increased from the early- to mid-season samplings and then decreased at the late season sampling at the 7.5 cm depth and were unchanged from

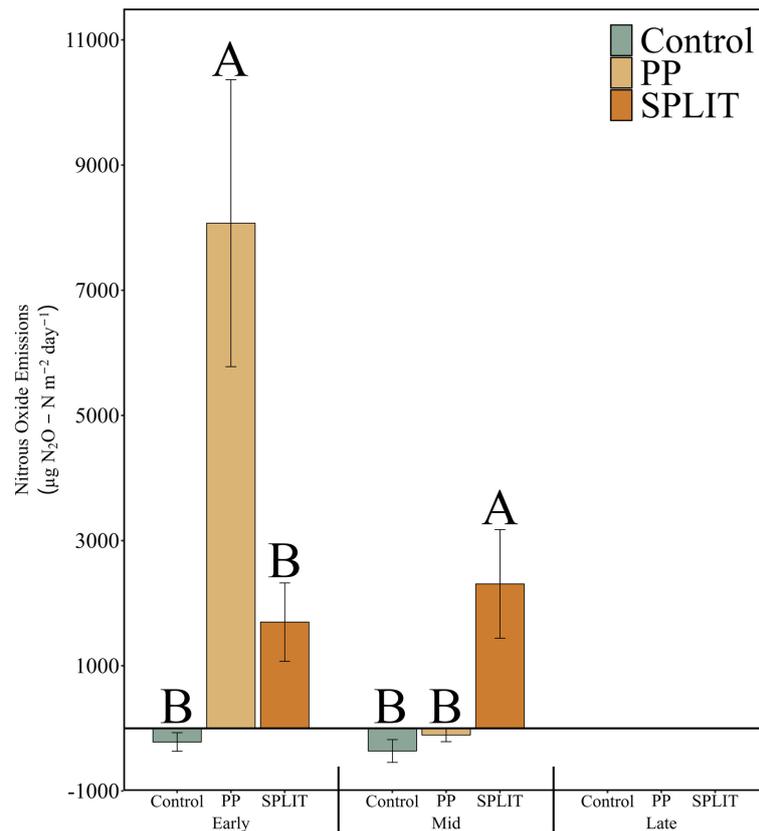


FIGURE 3

Nitrous oxide emissions ($\mu\text{g N}_2\text{O-N m}^{-2} \text{ day}^{-1}$) measured at the soil-atmosphere interface in 2020 at the early growth period (June), mid-season growth period (August), and late growth period (October) of a cotton cropping system. Data was collected at a single timepoint within each growth stage in conjunction with soil sampling and pore-space gas concentrations. Control, no added N fertilizer; PP, 100% pre-plant application of N fertilizer (168 kg ha^{-1}); SPLIT, 40% PP 60% mid-season application of N fertilizer. Error bars are standard error. LSD letters are compared within sampling period and are different at $\alpha=0.05$.

early- to mid-season, but decreased at the late-season sampling at the 15 cm depth.

3.5 Regression modeling of soil gas production

Least absolute shrinkage and selection operator (LASSO) regression modeling was conducted for pore-space concentrations of N_2O and NO to determine the best representation of the drivers of each gas' production in these semi-arid soils. The models developed for each gas represent the most parsimonious combination of soil chemical parameters and microbial gene abundances that can predict the concentration of N_2O and NO in the soil pore-space. Model coefficients for each gas within each sampling period are reported in Table 2, and predictive capacity of the model is presented in Figure 5.

The model generated for the prediction of pore-space N_2O concentration performed moderately, with an R^2 of 0.46 for the measured vs. predicted concentration (Figure 5). The model coefficients for pore-space N_2O included GWC, pH, and $\text{NO}_3^- \text{-N}$ (Table 2). The model generated for prediction of pore-space NO performed poorly compared to N_2O with an R^2 of 0.20 (Figure 5). The model coefficients for pore-space NO included GWC and AOB abundance (Table 2).

4 Discussion

4.1 Variability in soil chemical composition and genetic potential

Soil chemical characteristics were analyzed across the 3-year study and separately within the final year, where variability in the final year was determined to be similar to that of the entire

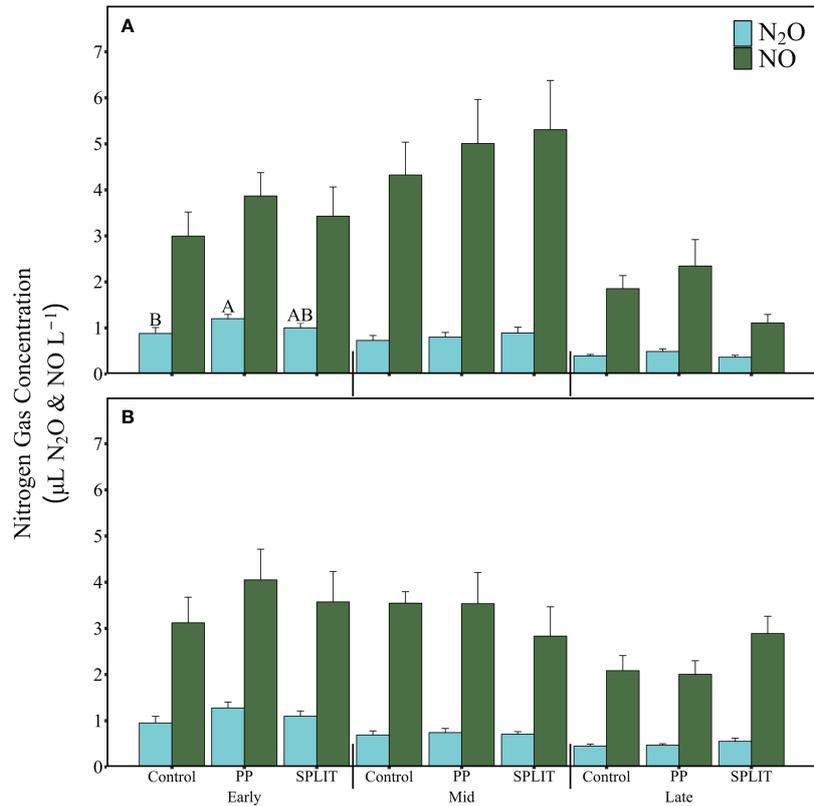


FIGURE 4 Average nitrogen (N) gas concentrations of soil pore-space N gases (N₂O and NO, μL L⁻¹) for N timing treatments at each sampling period. **(A)** 7.5 cm depth; **(B)** 15 cm depth. Control, no-added N; PP; 100% pre-plant N fertilizer application; SPLIT 40% pre-plant 60% mid-season application of N fertilizer. LSD letters are different at p < 0.05 within each sampling period and N gas, error bars represent standard error.

TABLE 2 Model coefficients for LASSO regression analysis of pore-space N₂O and NO concentration.

Variable	Coefficients	
	Pore-Space N ₂ O	Pore-Space NO
Intercept	0.48	1.51
16S	.	.
AOB	.	1.78
<i>nirK</i>	.	.
<i>nirS</i>	.	.
<i>nosZ</i> clade I	.	.
<i>nosZ</i> clade II	.	.
Cmin	.	.
GWC	0.53	2.43
pH	-0.09	.
NO ₃ ⁻ -N	0.34	.
NH ₄ ⁺ -N	.	.

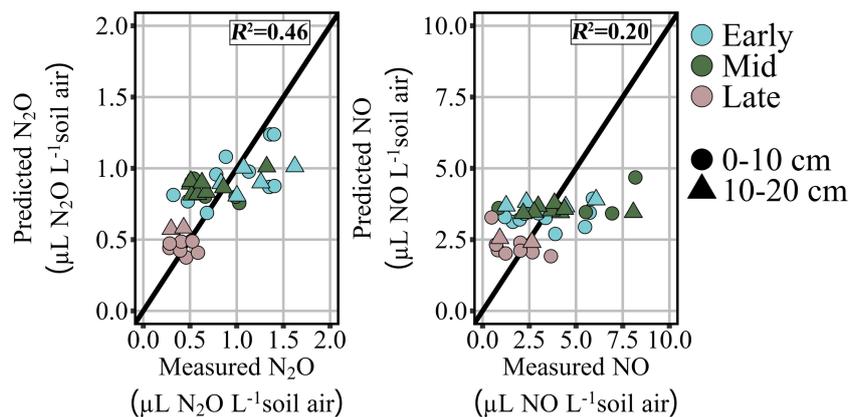


FIGURE 5

Least absolute shrinkage and selection operator (LASSO) regression model prediction of testing data for pore-space concentration of nitrous oxide ($\mu\text{L N}_2\text{O L}^{-1}$ soil air) and pore-space concentration of nitric oxide ($\mu\text{L NO L}^{-1}$ soil air).

three-year dataset. Principal components analysis confirmed the strong effect of sampling period on soil chemical conditions, where the early- and mid-season samplings clustered together, with the late-season sampling period moderately separated. Sampling period separation was most apparent along PC2, where the extremely dry soil conditions separated the late-season sampling from the two samplings earlier in the growing season. There was no clear clustering by conservation system or N treatment, likely due to the strong effect of climate and treatment implementation which resulted in temporal clustering by sampling period. We hypothesized that soil management practices would impact soil chemical parameters and water content of the soil, which was confirmed through ANOVA, but did not result in clear patterns of separation with PCA, suggesting a larger overall effect of either climate, or another unmeasured factor.

Principal components analysis of genetic potential generally did not reveal any distinct clustering of implemented treatments or patterns in gene abundance through the growing season for the N cycling community as a whole. Interestingly, the contributions to each PC of the microbial gene abundance PCA were distinct. PC1 was associated with the overall bacterial population and the population of typical denitrifiers (*nirS/K* and *nosZ* clade II), PC2 was highly driven by the abundance of *nosZ* clade II which is known to be highly abundant in the soils in this study (23), and PC3 was affected by AOB abundance. The application of environmental variables to the ordination revealed a significant relationship between the variability in genetic potential and Cmin and GWC. Although no treatments clustered with these parameters, the significant relationships suggest some distal control of C and water on the genetic potential for N cycling in this semi-arid soil. However,

there was clearly an additional unmeasured factor driving the variability in genetic potential observed in these semi-arid soils, although it must be noted that the relationship between soil chemical changes and microbial gene abundance may not be linear due to genetic potential including active, dormant, or even relic DNA.

4.2 Trends in nitrogen gas production in semi-arid agriculture

A strong relationship between pore-space N_2O and NO and emissions from the soil surface was expected, as was previously reported in forest soils (26); however, no significant relationship was found in our study due to the sporadic nature of significant positive emissions of N_2O . A reduction in N_2O concentrations in the soil pore-space observed with the progression of the growing season at both depths was likely related to diminishing N resources. The same reduction was also observed for N_2O emissions but emissions and pore-space concentrations were not correlated (analysis not reported). Compared to previous measurements in agricultural fields (24) and forests (25), the concentration of NO in the soil pore-space was much greater for this semi-arid agricultural soil. We suspect that these high NO concentrations are a consequence of greater N fertilizer input than previous studies. Increases in gas production aligned with reported trends in N gas production in fertilized systems (10–12), and with seasonal variation in gas production (25). Increased NO concentrations at the mid-season sampling likely also reflect general increases in microbial activity due to increased temperatures where NO concentration wasn't affected by the application of N fertilizer.

4.3 Relating soil chemistry and genetic potential to nitrogen gas production

It was hypothesized that denitrification was driving the production of N gas production in these semi-arid soils due to the large abundance of *nosZ* clade I and II genes previously observed (23). LASSO regression analysis produced a model supporting this hypothesis for N₂O pore-space concentration where the model featured positive coefficients for GWC and NO₃⁻-N, and a negative coefficient for pH. Both GWC and NO₃⁻ are known drivers of N₂O production (9–12), and their inclusion in the model as positive coefficients supports the idea that the pore-space N₂O measured is resulting from denitrification activities. When considering the timing of N₂O emissions and the method of application (liquid UAN knifed into the soil), it is not surprising that denitrification is the main driver of excess gas production that translates to enrichment of N₂O in the pore-space and non-directly to emission of N₂O from the soil surface. Furthermore, the inclusion of pH in the model is key, as the effects of pH on N₂O reduction capacity are well known (10). The regression model produced for N₂O pore-space concentrations was moderately predictive, resulting in an R² of 0.46 for the testing dataset. Basing the prediction of pore-space gas on such a small set of field measurements (and only five total soil chemical parameters) likely resulted in reduced predictive power. However, the moderate predictivity of the model does not diminish the lack of genetic potential contribution to the model for N₂O in the pore-space.

In contrast to the denitrification driven concentration of N₂O in the soil pore-space, the LASSO regression model for NO concentration featured indicators of nitrification. Increased ammonia oxidation by AOB could have resulted in pooling of NO in the soil pore-space if it overwhelmed the oxidizing and reducing capacity of the N cycling population (13). The addition of GWC to this model indicates the controlling nature of water in this semi-arid soil. However, it must be noted that the model was poorly predictive, producing an R² of 0.2 for the testing dataset comparison of measured and predicted pore-space NO concentrations. Due to the poor performance of the model, we cannot clearly define the production of NO as a nitrification activity, especially without the inclusion of NH₄⁺ in the model. Despite the lack of connection to genetic potential these models represent early indications of distinct N-cycle pathways producing each of these important GHGs and further represent the clear differences between N gas production in semi-arid lands and mesic systems where carbon plays a stronger role in N gas production (6, 7, 15).

4.4 Genetic potential is generally decoupled from gas production mechanisms

To further consider the interactions of soil chemistry, genetic potential, and N gas production we will examine the PP timing of

N fertilizer application. Emissions and pore-space concentrations of N₂O peak during the early-season sampling for the PP timing due to the recent application of N fertilizer (10–12) supporting our hypothesis that N fertilizer application would relieve the poor N content of the soil, jumpstart N cycling, and overwhelm the N₂O reducing capacity of the soil. By the mid-season sampling, net consumption of N₂O was recorded for the PP timing and was likely related to the loss of soil NO₃⁻-N by plant and microbial consumption. Low inorganic-N concentrations are known to be associated with N₂O consumption (50–52), and by mid-season, NO₃⁻-N was greatly reduced compared to the early-season.

Despite the reduction in emissions, and pore-space concentrations, genetic potential for these pathways increased from the early- to mid-season samplings in the PP timing. We suspect there are two potential reasons for this seemingly antithetical outcome. First, we suspect the genetic abundance increase measured at the mid-season point may reflect the past increase in N cycle gene abundance and subsequent N cycle activity which resulted in the loss of NO₃⁻ from the soil, and reduced emissions at the mid-season sampling period. This is supported by the abundance of *nosZ* clade II, which increased from early- to mid-season reflecting a need for N₂O-reductase in the preceding months where high N cycle activity produced more N₂O and thus increased the need for this gene. The high level of N cycle activity would also thus be reflected in greater abundances of other N cycle organisms from the early- to mid-season sampling periods which we also observed.

A second potential reason for this outcome follows similar logic to the first where a significant loss of soil NO₃⁻-N occurred between the early- and mid-season sampling, related to plant and microbial consumption. However, the loss of soil N would have selected for non-traditional N₂O reducers as has been seen in several environments (26, 51, 52). In this scenario, the reduced NO₃⁻ is indicative of an active population of N cycling organisms and an active crop that is consuming soil inorganic-N. With continually reducing N resources, the plant may be selecting for organisms that can mineralize organic matter and supplement the N supply. For AOB, this would likely be a highly plausible reason for the increase in abundance as there would be fresh NH₄⁺ continually being released from the soil matrix. However, for the other genetic abundance increases, this is less plausible than the reflection of past activity and environmental selection resulting in the measured community abundance as explained above. Furthermore, due to environmental selection and plant selection, one would expect to see a decrease in genetic potential for N cycle activities between the mid- and late-season samplings, which was not reflected in this study.

The lack of reduction in genetic potential over the growing season further reflects the difficulty in using gene abundance to predict microbial activity. When evaluating microbial processes that aren't consistently active in the study system, the stable nature of DNA in the environment and the potential for

dormant or inactive microbial species, clouds the connection between genetic potential and actual environmental outcomes.

5 Conclusions

For semi-arid agricultural soils with low organic C and limited water, N cycle activity is likely exclusively driven by the application of N fertilizer, with N₂O emissions peaking following application, and returning to no emissions or consumption of N₂O once the fertilizer NO₃⁻-N is depleted. This is in contrast to high concentrations of N₂O and NO within the soil pore-space which indicated N cycle activity through the mid-season sampling. For producers considering the environmental impact of N fertilization and soil management, any steps taken to reduce bulk applications of N fertilizer may thus reduce gaseous N losses from the soil without negatively affecting N cycling activity within the soil matrix. Regression modeling of pore-space concentrations revealed distinct drivers of N₂O and NO where N₂O is associated with drivers for denitrification and NO is associated with the genetic potential for nitrification. The model for N₂O was moderately predictive and would likely be greatly improved by the inclusion of measures of active microbial processes which better reflect the connection to active denitrifiers.

Consistent NH₄⁺-N concentrations throughout the growing season in conjunction with the abundant ammonia oxidizing population indicate N mineralization as a key process to supplement low N conditions in these soils but its effect on N gas production is unclear. It is possible that under reduced inorganic-N conditions the crop selects for N mineralization activities which increase the concentration of NO in the pore-space through N mineralization and ammonia-oxidation. Furthermore, it was clear that genetic potential was not reflective of current microbial activity at this semi-arid study site but may indicate past N cycle activity. It would be interesting to apply this research approach in more consistent N₂O producing conditions to evaluate the effectiveness of regression analyses and machine learning to predict N₂O flux out of the soil. However, for semi-arid soils that often rely on microsites for anaerobic activity, there likely a host of conditions that would need further consideration to improve predictability of N₂O emission.

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Data availability statement

The original contributions presented in the study are included in the article/[Supplementary Material](#). Further inquiries can be directed to the corresponding author.

Author contributions

MM: Data acquisition, data interpretation, and writing of the manuscript. KL: Conception and design, data interpretation, and writing of the manuscript. PD, TB: Writing of the manuscript. BH: Data acquisition and writing of the manuscript. TG: Data interpretation and writing of the manuscript. All authors contributed to the manuscript and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fsoil.2022.1050779/full#supplementary-material>

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Supplementary Material

Supplementary Material: Nitrogen Fertilizer Driven Nitrous and Nitric Oxide Production is Decoupled from Microbial Genetic Potential in Low Carbon, Semi-arid Soil

1 Methods

1.1 2018

Four irrigation events were conducted in 2018, with 152 mm of irrigation applied at each event on 26 May, 23 June, 21 July, and 6 August. Fertilizer application was made on 19 May, and 16 July at a total rate of 168 kg N ha⁻¹ as UAN (32-0-0). Cotton (DP 1518) was planted at a rate of 124000 seed ha⁻¹ on 24 May 2018 and harvested on 16 November 2018. Herbicide was applied for chemical control of weeds several times during the growing season including pre-emergent application on 24 April; glyphosate application on 22 May, 10 July, and 13 August; and dicamba application on 21 June, 10 July, and 13 August. Conventional tillage plots were cultivated on 19 July. Due to multiple failings of the cover in winter 2017, the cover crop for 2018 was switched to triticale (*Triticale hexaploide* Lart), planted in February 2018, and terminated on 24 April. The cover crop was well established, but low biomass due to the short growing season.

1.2 2019

In 2019, furrow irrigation was conducted twice at a rate of 152 mm on 26 June and 30 July. Fertilizer was applied on 17 May and 15 July at a total rate of 168 kg ha⁻¹ as UAN. In addition, as part of the total rate of fertilizer N application, 10-34-0 phosphorus fertilizer was applied on 15 May to alleviate potential phosphorus deficiency. Cotton (DP 1820 B3XF) was planted on 29 May 2019 at a rate of 124000 seed ha⁻¹ and harvested on 5 November 2019. Herbicide was applied several times during the growing season including 2,4-D on 28 March; glyphosate on 18 April, 25 April, pre-emergent application of S-metolachlor on 10 June, and dicamba application on 16 July and 20 August. Conventional tillage plots were cultivated on 22 May. Cover crop for 2019 was wheat (*Triticum aestivum*), planted in November 2018 and terminated on 18 April. Cover crop biomass was better in 2019, with a full coverage of no-tillage + cover cropped plots.

1.3 2020

Herbicide was applied for the control of weeds in 2020 including 2,4-D amine on 26 February and 31 March; glyphosate and Flumioxazin on 04 May; and dicamba on 17 June, 19 July, and 15 August. Conventional plots were not tilled for weed control but were intermittently controlled for weeds by hand-hoeing. The cover crop was planted in November 2019 and terminated on 16 April. Cover crop biomass was good in 2020 with full coverage. A timeline of general management and sampling events is presented in Supplementary Fig. 1.

1.4 Soil Sampling

Soil sampling was conducted in 2018 and 2019 in the same manner as described for 2020. In 2018, soil samples were collected on 6 June, 24 August, and 1 November for the early-, mid, and late-season samples respectively. For the 2019 growing season, soil samples were collected on 14 June, 14 August, and 24 October. Finally, in 2020 soil sampling was conducted on 24 June, 05 August, and 10 October. Gas sampling was conducted intermittently across 2018 and 2019 due to mechanical errors with equipment and testing of pore-space probe method.

2 Soil chemical results

2.1 Three-year dataset

2.1.1 0-10 cm Soil Depth

Soil chemical parameters were analyzed using analyses of variance with consideration for the repeated measurement across the growing season for all three years of soil chemical data collected. At the 0-10 cm depth, a significant effect of sample period on the measured values for soil pH ($p=0.027$), gravimetric water content (GWC, $p=0.005$), soil nitrate (NO_3^- -N) concentration ($p=0.002$), and soil ammonium (NH_4^+ -N) concentration ($p=0.022$) was determined. Seasonal means were separated by Fisher's protected LSD and it was determined that soil pH was greater at the late-season sampling (7.81) compared to the early- (7.51) and mid-season (7.54) samplings. Gravimetric water content was determined to be greater at the early-season sampling (11.93 g water 100 g⁻¹ soil) compared to the mid- (9.53 g water 100 g⁻¹ soil) and late-season (8.63 g water 100 g⁻¹ soil) samplings. Lastly, both NO_3^- -N and NH_4^+ -N concentration were reduced at the late-season sampling (NO_3^- -N: 4.56 mg N kg⁻¹ soil; NH_4^+ -N: 4.5 mg N kg⁻¹ soil) compared to the early- (NO_3^- -N: 24.67 mg N kg⁻¹ soil; NH_4^+ -N: 13.7 mg N kg⁻¹ soil) and mid-season (NO_3^- -N: 21.74 mg N kg⁻¹ soil; NH_4^+ -N: 13.4 mg N kg⁻¹ soil) samplings. These patterns follow typical expectations for the behavior of the measured soil chemical parameters where pH is generally decreased when greater inorganic N is present, and the soil water content is generally increased at the beginning of the year compared to the mid-and late season samplings.

Treatment effects over the course of the study at the 0-10 cm soil depth were present for soil pH (N timing: $p<0.001$), Cmin (Conservation system: $p=0.026$), and NO_3^- -N concentration (Conservation system: $p<0.001$; N timing: $p=0.001$). Across the three sampling events, soil pH was increased for the control (7.93) compared to the PP (7.41) and SPLIT (7.52) treatments. Mineralizable carbon content was increased for the NTW system (84.2 mg CO₂-C kg⁻¹ soil) compared to the CT system (67.4 mg CO₂-C kg⁻¹ soil), where the NT system (74.1 mg CO₂-C kg⁻¹ soil) was not different than either. Lastly, NO_3^- -N concentration was increased for the CT system (25.5 mg N kg⁻¹ soil) compared to the NTW (13.3 mg N kg⁻¹ soil) and NT (12.2 mg N kg⁻¹ soil) systems and was also greater for the PP (18.86 mg N kg⁻¹ soil) and SPLIT (22.68 mg N kg⁻¹ soil) N timings compared to the control (9.43 mg N kg⁻¹ soil). Similar to sampling period effects, the main effects of conservation system and N treatment on soil chemical parameters follow expected patterns, especially in the case of the relationship between soil pH and inorganic-N where there is a clear negative relationship between the application of N fertilizer and soil pH.

Finally, for the overall analysis at the 0-10 cm soil depth, only NO_3^- -N concentration was influenced by the repeated measure of N treatment over time ($p=0.008$), where the PP timing at the early-season sampling and the SPLIT timing at the mid-season sampling had greater NO_3^- -N than all N timings at any month with the exception of the SPLIT timing at the early-season sampling (Supplementary Table 2). It follows convention that inorganic-N concentrations would be differentially affected by the timing of N fertilizer application as it relates to the measurement of NO_3^- -N during the growing season, however this pattern was not observed for the concentration NH_4^+ -N which was unaffected by N timing.

2.1.2 10-20 cm Soil Depth

Soil chemical parameters at the 10-20 cm depth were affected similarly to the 0-10 cm depth where sampling period as a main factor affected soil pH ($p=0.019$) and GWC ($p=0.003$). Soil pH was greatest at the late-season sampling (7.97) compared to the early- (7.71) and mid-season samplings (7.71). Gravimetric water content was greatest at the early-season sampling (13.6 g water 100 g⁻¹ soil) compared to the mid- (10.7 g water 100 g⁻¹ soil) and late-season samplings (11.3 g water 100 g⁻¹ soil).

Treatments as main factors affected soil pH (conservation system: $p=0.001$; N timing: $p<0.001$), GWC (conservation system: $p=0.033$), and NO_3^- -N concentration (conservation system: $p<0.001$; N timing: $p=0.001$) at the 10-20 cm depth. Soil pH was increased for the NTW (7.85) and NT (7.86) systems compared to the CT system (7.68) and was also increased for the control (8.05) compared to the PP (7.69) and SPLIT (7.65) timings. Gravimetric water content was increased for the NT system (12.5 g water 100 g⁻¹ soil) compared to the CT system (11.2 g water 100 g⁻¹ soil), while the NTW system (11.9 g water 100 g⁻¹ soil) was not different than either the CT or NT systems. The concentration of NO_3^- -N was increased for the CT system (17.86 mg kg⁻¹ soil) compared to the NTW (7.44 mg kg⁻¹ soil) and NT (8.63 mg kg⁻¹ soil) systems. In addition, NO_3^- -N concentration was increased for the PP (13.72 mg kg⁻¹ soil) and SPLIT (14.76 mg kg⁻¹ soil) timings compared to the control (5.45 mg kg⁻¹ soil). The interaction of conservation system and N timing also affected soil pH across all three study years and sampling periods ($p=0.016$; Supplementary Table 3) where pH was increased for the control within all conservation systems (NTW-control: 8.06; NT-control: 8.03; CT-control: 8.05) compared to the treatments where N fertilizer was applied in all conservation systems. In addition, the SPLIT timing in the NTW system (7.79) had greater pH than the PP and SPLIT timings within the CT system (CT-PP: 7.59; CT-SPLIT: 7.39). Furthermore, the SPLIT timing in the NT system (7.78) and the PP timing in the NTW and NT systems (NTW-PP: 7.70; NT-PP: 7.76) had greater pH than the SPLIT timing in the CT treatment.

For the overall analysis at the 10-20 cm depth, the repeated measure of GWC, NO_3^- -N concentration and NH_4^+ -N concentration were affected by the interaction of sampling period and conservation system (GWC, Supplementary Table 4) and N timing (NO_3^- -N and NH_4^+ -N concentrations, Supplementary Table 5). Gravimetric water content was greater for all conservation systems at the early-season sampling and for the NT system at the late-season sampling compared to the water content measured for all conservation systems at the mid-season and the NTW and CT at the late-season sampling (Supplementary Table 4). The concentration of NO_3^- -N was greater for the SPLIT timing at the mid-season sampling and the PP timing at the early-season sampling compared to the control at all sampling periods, the PP timing at the mid-

and late-season samplings, and the SPLIT timing at the late-season sampling (Supplementary Table 5). Lastly, the concentration of NO_3^- -N in SPLIT timing at the early-season sampling was greater than the control at the late-season sampling, but not different than the PP timing at the early-season sampling or the SPLIT timing at the mid-season sampling (Supplementary Table 5). The concentration of NH_4^+ -N was greater for the PP timing at the early-season sampling compared to the control at the early and late-season sampling periods, the PP timing at the mid- and late-season samplings, and the SPLIT timing at the early- and late-season samplings (Supplementary Table 5). The SPLIT timing and the control at the mid-season sampling (Supplementary Table 5) were not different than any of the N timings at any of the sampling periods.

2.1.3 Principal Components Analysis

Across the three-year dataset, the total variance explained was 59.2% where PC1 explained the 38.7% of the variance and PC2 explained 20.5% of the variance. Contributions to the variability explained by PC1 included soil NO_3^- -N, pH, and NH_4^+ -N which contributed greater than 20% of the variability each (Supplementary Fig. 2). Only GWC significantly contributed to the variance explained by PC2 across the three-year dataset (Supplementary Fig. 2). Lastly, PC3 explained 18.7% of the variability in soil chemical parameters across the three study years, where Cmin content was the only significant contributor (Supplementary Fig. 2). Correlations between the significant contributors to variance explained for each PC are displayed in Supplementary Fig. 2 where NO_3^- -N and NH_4^+ -N were positively correlated with PC1 in contrast to soil pH which was negatively correlated with PC1; GWC was negatively correlated with PC2; and Cmin content was positively correlated with PC3 (Supplementary Fig. 2).

2.2 Final year analyses

2.2.1 Early-season sampling

Gravimetric water content was affected by conservation system at the 0-10 cm depth at the early season sampling ($p=0.001$) where the NTW and NT systems had greater water content than the CT system (Supplementary Table 6). Mineralizable C was affected by N timing at the 0-10 cm depth early in the growing season ($p=0.024$) where the PP treatment had greater Cmin than the NT system (Supplementary Fig. 3). At both the 0-10 cm and 10-20 cm depth soil pH and NO_3^- -N were affected by N timing ($p=0.002$, $p=0.032$, respectively). Soil pH was reduced for the PP treatment compared to the control at both depths while NO_3^- -N was increased for the PP treatment compared to the control at both depths (Supplementary Fig. 3).

2.2.2 Mid-season sampling

Soil pH, GWC, and NO_3^- -N were affected by conservation system at the mid-season sampling at the 0-10 cm depth ($p=0.006$, $p=0.038$, $p=0.047$, respectively). Soil pH was increased in the NTW system compared to the NT system, GWC was increased for the NTW and NT systems compared to the CT system, and NO_3^- -N was increased for the CT system compared to the NT system at the 0-10 cm depth (Supplementary Table 6).

2.2.3 Late-season sampling

At the late-season sampling at the 0-10 cm depth, GWC, Cmin, NO_3^- -N, and NH_4^+ -N were affected by conservation system ($p=0.015$, $p=0.001$, $p=0.034$, $p=0.001$ respectively).

Mineralizable C was greater for the NTW system compared to the NT and CT systems, GWC was greater for the NTW system compared to the NT system, NO_3^- -N and NH_4^+ -N were greater for the NTW system compared to the CT system, and NH_4^+ -N was also greater for the NT system compared to the CT system (Supplementary Table 7). Soil pH and NH_4^+ -N were affected by N timing at the 0-10 cm depth at the late-season sampling ($p < 0.001$, $p < 0.001$, respectively) where soil pH was decreased for the applied N treatments (PP and SPLIT) compared to the control, and NH_4^+ -N was increased for the applied N treatments compared to the control (Supplementary Fig. 3). In addition, C_{min} was affected by the interaction of conservation system and N timing ($p = 0.006$, Supplementary Table 6). Due to excessively dry soil conditions at the soil surface, the NT system could not be sampled for the 10-20 cm depth at the late-season sampling. Statistical analysis was thus only conducted between the NTW and CT systems (including N timing and interaction effects). Despite this limitation, conservation system was determined to affect GWC at the 10-20 cm depth at the late-season sampling ($p = 0.005$) where GWC was greater for the CT system compared to the NTW system (Supplementary Table 6). Nitrogen timing affected GWC and soil pH at the 10-20 cm depth ($p = 0.005$, $p = 0.004$, respectively) where soil pH was increased for the control compared to the applied N treatments and GWC was greater for the control compared to the SPLIT treatment (Supplementary Fig. 3).

3 Microbial abundance in the final year

No effects of implemented treatments on 16S rRNA, *nirK*, *nirS*, *nosZ* clade I, or *nosZ* clade II abundances were determined at the early- or mid-season sampling periods at either the 0-10 cm or 10-20 cm depth (Supplementary Table 8). The abundance of bacterial *amoA* (AOB) was affected by N timing at the early-season sampling at the 10-20 cm depth and at the 0-10 cm depth at the mid-season sampling, where the SPLIT timing had greater AOB abundance than the control at the early-season sampling, and the PP timing had greater AOB abundance than the control at the mid-season sampling (Supplementary Tables 8,9). At the late-season sampling, at the 0-10 cm and 10-20 cm depths, conservation systems affected *nosZ* clade I abundances where in both cases, the NTW system had greater gene abundance than the NT and CT systems (Supplementary Tables 8,9). In addition, the abundance of *nosZ* clade I was greater in the NT system than the CT system at the 0-10 cm depth. The abundance of AOB was affected by N timing at the 0-10 cm and 10-20 cm depth at the late-season sampling where the abundance of AOB was greater for the SPLIT timing than the control, (Supplementary Tables 8,9). The interaction of conservation system and N timing affected *nosZ* clade I abundance at the 0-10 cm depth at the late-season sampling where the PP timing within the NTW system had a larger clade I population than all other N timing and conservation system combinations except the control within the NTW and NT systems (Supplementary Tables 8,10). At the 10-20 cm depth at the late-season sampling, conservation system (only comparing CT vs. NTW) affected the abundances of 16S rRNA, *nosZ* clade I, and *nosZ* clade II, where the NTW system had greater abundance than the CT system for each gene (Supplementary Tables 8,9). The interaction of conservation system and N timing also affected AOB abundance at the 10-20 cm depth at the late-season sampling, where the SPLIT timing within the CT system had a larger AOB population than the PP timing and the control within the CT system (Supplementary Tables 8,11). No effects of implemented treatments were determined for *nirK* or *nirS* abundance at either depth at any of the sampling events (Supplementary Table 8).

4 Supplementary Tables and Figures



Supplementary Figure 1: Timeline of management and sampling events in 2020.

Supplementary Table 1. gBlock sequences for qPCR standard curves

Gene Name	Organism (Accession #)	Sequence (5' → 3')
16S rRNA	<i>Flavobacterium aquatile</i> (MG897136.1)	ACTGAGACACGGACCAGACTCTACGGGAGGCAGCAGTGAGGAATATTGGACAATGGGCGCAAGCCTGATCCAGCCATGCCGCGTGCAGGAAGACGGTCCTATGGATTGTAAACTGCTTTTATACAGGAAGAAACACTCCGACGTGTCGGAGCTTGACGGTACTGTAGGAATAAGGATCGGCTAACTCCGTGCCAGCAGCCGGTAATACGGAGGATCCAAGCGTTAT
Bacterial <i>amoA</i>	<i>Nitrosomonas europaea</i> (KU747122.1)	GAGTGGGTGACACGTTACTGGGATTCTACTGGTGGTCACTACCCCATCAACTTCGTAACACCGGGCATTATGCTTCCGGGTGCGGTGATGCTGGACTTCACGCTGATCTGACACGCAACTGGCTGGTGACGGCTCTGGTTGGAGGTGGATTCTTCGGTCTGCTGTTCTATCCGGGTAACGGCCGATTTTTGGACCAACCCATTTGCCAATCGTTGTAGAAGGCACATTGCTGTGATGGCTGATTACATGGGACATCTGTATGTTCTGTACAGGTACACCCGAGTATGTTCGTCATATTGAGCAAGTTACTGCGTACCTTTGGTGGTCAACACAGTATTGCAGCATTCTTCTGCGTTCGTATCAATGTTGATGTTACC GTATGGTGGTATCTTGGAAAAGTTTACTGTACAGCCTTTTCTACGTTAAAGGTAAGAGGTCGTATCGTACATCGCAATGATGTTACCCGATTCTGTGAAGAAGGCTTCCAGAGGGATCAAATAAAATGGGTATC
nirS	<i>Pseudomonas aeruginosa</i> (CP034908.2)	CGCTACACCATCGCGCGCCTACTGGCCGCCGAGTTGCGGATCATGGACGGGAGACCTGGAACCGAAGCAGATCGTCTCCACCCGCGCATGACCGTAGACACCCAGACTACCACCCGGAACCGCGCTGGCGGCGATCATCGCTCCACGAGCACCCGAGTTCATCGTCAACGTGAAGGAGACCGGAAGGTCTGCTGGTCAACTACAAGGATATCGACAACCTCACCGTCAACAGCATCGGTGCGGCGCCGTTCTCCACGACGGCGGTGGGACAGCAGCCACCGTACTTCATGACCGCCGCAACAACCTCCAACAAGTTGCGGTGATCGACTCCAAGGACCGTCCGCTGTCGGCCCTGGTGCAGTCCGCAAGACCCCGCGCCGCGTGGCCCAACTTCGTGCATCCCAAGTACGGCCCGGTGTGGAGCACCAGCCACCTGGGCGACGGCAGCATCTCGTGATCGGCACCCGATCCGAAGAACCATCCGCAGTACGCCTGGAAGAAAGTCGCCGAACTACAGGGCCAGGGCGGGCTCGCTGTTTCAAGACCCATCCGAAGTCTCGCACCTTACGTC
nirK	<i>Rhizobium etli</i> (CP000138.1)	GCGACCAAGGCCGGCGTCTTCGTCTATCACTGCGCCCTCCCGCATGGTGGCGTGGCAGTCAATCGGGCATGAACGGCGCCATCATGGTCTGCGCGTGAAGGTCTGAAGGACGGCAAGGGCAAGCCGCTCGTCTACGACAAGTCTATTATGTGCGGCAACAGGACTTTTATGTTCCGCGCGACGAAACGGGCAAAATCAAGAAATACGACAGCCTGGCGAGGCCTACGATGAGACGGTTCGGCCGATGAAGACGCTAACGCCGACCCATATCGTCTTCAA CGGGCGGGTTCGAGCCCTAACCGCGAGAAATGCGATGACGGCAAGGTTCGGCGAAAACGGTGTGATCGTTCATCTCAGGCCAACCGCGACACCCGGCCCATCTGATCGGGGGCATGGCGAATATGCTGGGGCAGCCGCAAAATTCCTCAACCCACCGGACACGGATCAGGAAACCTGGTTCATTCCCGCGGCACGGCGGGTGGCGCCCTTTATCTTCGCTCAGCCTGGCATCTATGCCATCGTGAACCACAACCTTATCGAGGCCCTTCGAGTGGGGCGGCGGCCA
nosZ clade I	<i>Pseudomonas fluorescens</i> (AF197468.1)	ACGATCCGTTCTTCGCCGACACCGTGGCCCTCGCGAAGAAGGACGGCATCAACCTCGAAACCGACAACAAGGTATCCGCGACGGCAATAAGGTGCGGGTGATGACGTGCGATGGCGCCGGCCTATGGCCTGACGGAGTTCACCGTCAAGCAAGGCAACGAGGTACCGTGACCATCACCAACATCGACAGATCGAAGACGTGCCACGGTTTTGTATGACCAACCACGGCGCCAGCATGGAGATCAGCCCGCAACAGACCTCATCCATCACTTTACCGCCGACAAGCCGGCTTGCATTGGTACTACTGCAGTTGGTCTGCCACGCCCTGCACATGGAAATGGTCGGCCGCA
nosZ clade II	<i>Gemmatimonas aurantiaca</i> (AP009153.1)	CATCTGGCCGGCGAAGTGAAGGATCTGGCCTCGGCCCTGCACACCGAGTTCGATGGCAAGGGCTACGCCTACACGTCGCTGTTTCTCCAGCGAGATCGTGAAGTGAAGCTGGGTACGTGGGAGATCGTGCATCGGGTGCCGACGTACTACTCGATCGGTACCTGATGATTCCGGCGGCGCCACACCAAGCCGGCGGCAAGTATGTCGTCGCGCTCAACAAGATCAGCAAGGATCGGTACTGCCACCGGTCGGAGCTCACGCAATCGGCGCAGCTCTACGACATCTCGGGCGACAAGATGAAGCTGCTGCTCGACTTCCCAGCACGGGCGAACCGCACTACGGCAACCGGATCGAAGCCGCAAGATCGAAGCTGCAGAAAGTTCTTACGCTGGCCGAGAACCAGGATCCGCAAGGTGACGAAAGGCCAACGTTGAAGCGGGTGTGAGCCGGAACGGCAAGGTGGTGCACGTGCCGATGGCAGCCATCCGTTGCACTTCCGCCCCGACAACATCGAAGGAGTGCAGGTGGGTGACACGGTGTCTTCCACGTCA CCAACCTCGAGCAGGACTGGGACATCCCGCATGGCTTGGCATCGTACAGGTCAGCCACTTCGGAGTTGCTGGTGTATGCCGGGTGAAACCCGACGTTGCGCTGGGTGCCGAAGCAGATCGGCGTGTATCCGATGTAATGCACCGACTTCTGCTCGGCGTGCACCAGGAAATGTCCGGCTACGTGCGCGTCTCCCCG

Supplementary Table 2. Repeated Measures effects on soil NO₃⁻-N concentration at the 0-10 cm depth for the timing of N fertilizer application. (Letters denote means separation by Fisher's LSD at $\alpha=0.05$)

Sampling Period	N timing	NO ₃ ⁻ -N mg kg ⁻¹	
Early	Control	13.16	CD
	PP	33.14	A
	SPLIT	27.7	AB
Mid	Control	12.98	CD
	PP	16.37	BC
	SPLIT	35.88	A
Late	Control	2.17	D
	PP	7.07	CD
	SPLIT	4.45	CD

¹ Early: June; Mid, August; Late, October

² Control, no added N fertilizer; PP, pre-plant application of N fertilizer at 168 kg ha⁻¹; SPLIT, 40% preplant and 60% mid-season application of N fertilizer at a total rate of 168 kg ha⁻¹. All N fertilizer applied as UAN.

Supplementary Table 3. Conservation System and N timing interaction effects on soil pH at the 10-20 cm depth across the three-year dataset. (Letters denote means separation by Fisher's LSD at $\alpha=0.05$)

Conservation System ¹	N Timing ²	pH	
NTW	Control	8.06	A
	PP	7.7	BC
	SPLIT	7.79	B
NT	Control	8.03	A
	PP	7.76	BC
	SPLIT	7.78	BC
CT	Control	8.05	A
	PP	7.59	C
	SPLIT	7.39	D

¹ NTW, no-tillage with a winter-wheat cover crop; NT, no-tillage winter fallow; CT, conventional tillage winter fallow

² Control, no added N fertilizer; PP, pre-plant application of N fertilizer at 168 kg ha⁻¹; SPLIT, 40% preplant and 60% mid-season application of N fertilizer at a total rate of 168 kg ha⁻¹. All N fertilizer applied as UAN.

Supplementary Table 4. Repeated measures analysis of gravimetric water content (GWC) as affected by conservation system across the three-year dataset. (Letters denote means separation by Fisher's LSD at $\alpha=0.05$)

Sampling Period ¹	Conservation System ²	GWC	
		g water 100 g ⁻¹ soil	
Early	NTW	14.2	A
	NT	13.44	A
	CT	13.21	A
Mid	NTW	11.03	B
	NT	10.71	B
	CT	10.42	B
Late	NTW	10.45	B
	NT	13.5	A
	CT	9.88	B

¹ Early: June; Mid, August; Late, October

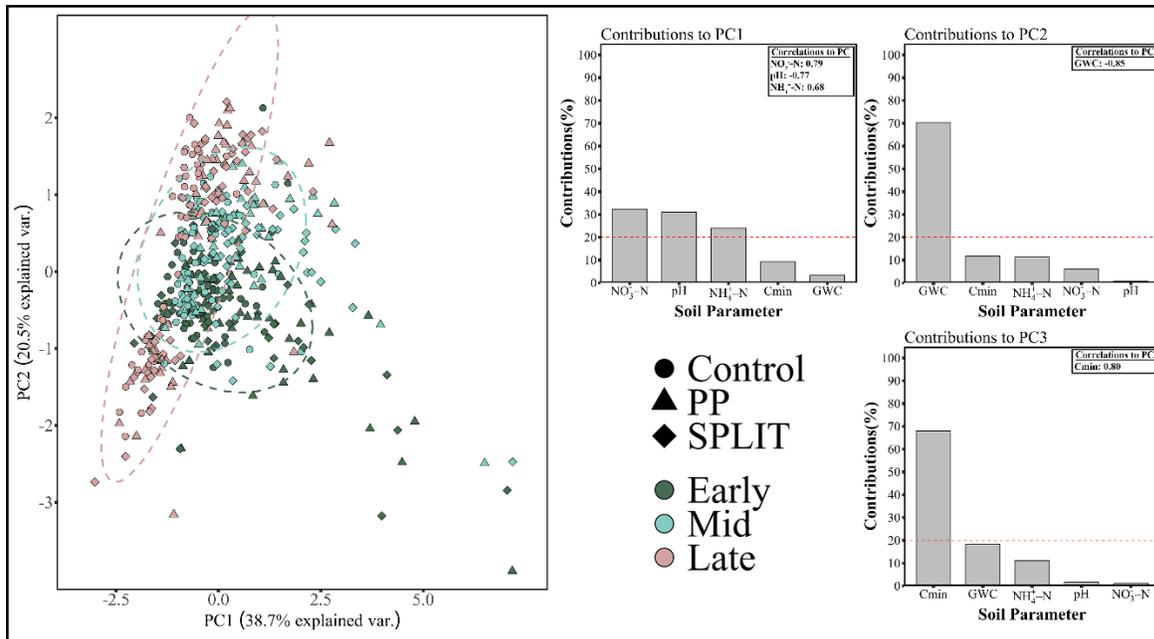
² NTW, no-tillage with a winter-wheat cover crop; NT, no-tillage winter fallow; CT, conventional tillage winter fallow

Supplementary Table 5. Repeated measures analysis of soil nitrate (NO₃⁻-N) and ammonium (NH₄⁺-N) affected by timing of N fertilizer application across the three-year dataset. (Letters denote means separation by Fisher's LSD at $\alpha=0.05$)

Sampling Period ¹	N timing ²	NO ₃ ⁻ -N		NH ₄ ⁺ -N	
		mg kg ⁻¹		mg kg ⁻¹	
Early	Control	6.01	BC	3.58	B
	PP	22.2	A	10.63	A
	SPLIT	13.54	AB	4.39	B
Mid	Control	7.19	BC	6.16	AB
	PP	10.71	BC	4.96	B
	SPLIT	22.31	A	5.71	AB
Late	Control	3.16	C	3.49	B
	PP	8.24	BC	3.69	B
	SPLIT	8.42	BC	4.04	B

¹ Early: June; Mid, August; Late, October

² Control, no added N fertilizer; PP, pre-plant application of N fertilizer at 168 kg ha⁻¹; SPLIT, 40% preplant and 60% mid-season application of N fertilizer at a total rate of 168 kg ha⁻¹. All N fertilizer applied as UAN.



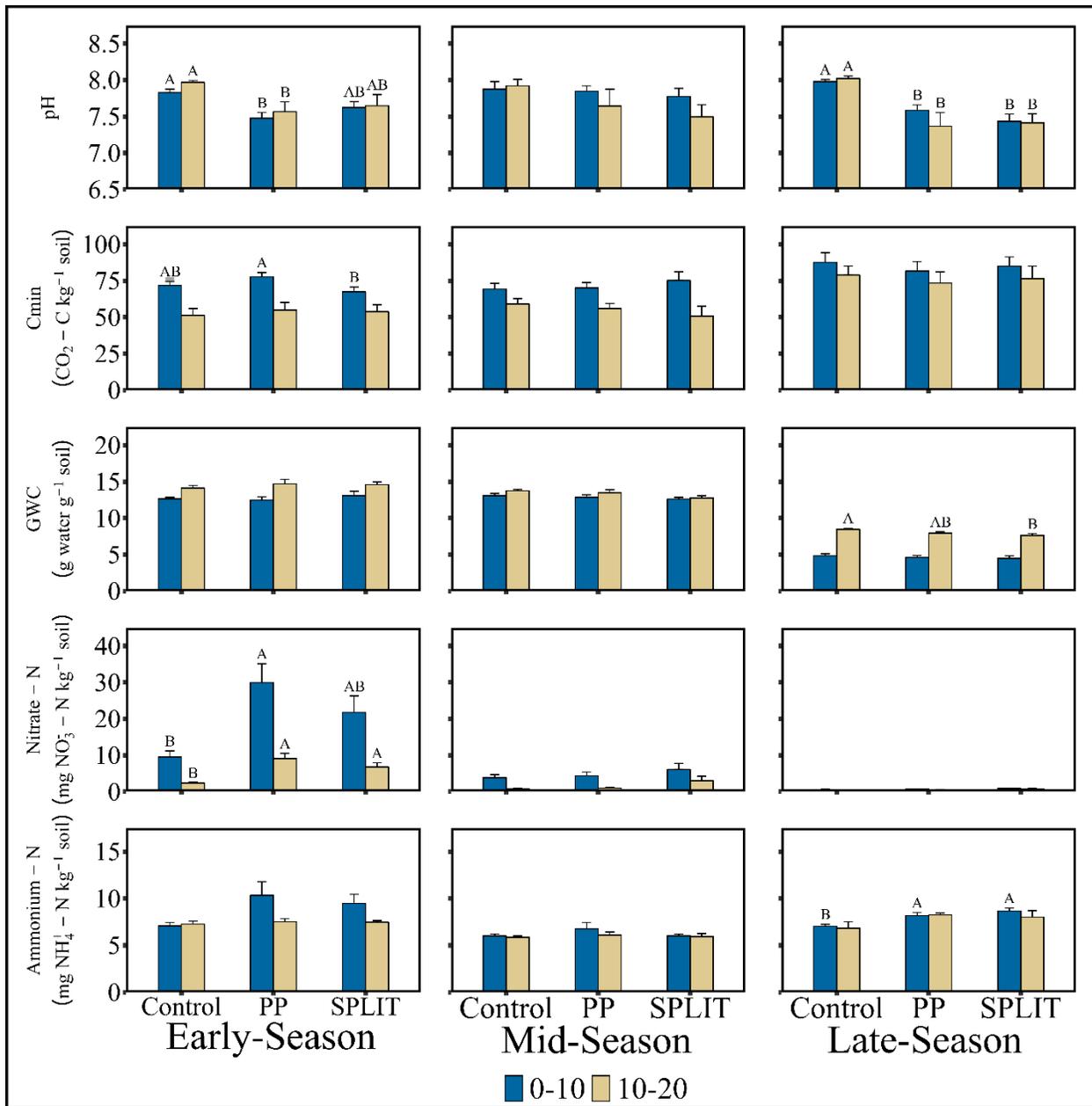
Supplementary Figure 2. Principal components analysis of soil chemical characteristics across the three-year study. Soil characteristic contributions to the top three principal components are presented with correlation coefficients for the significant contributors to each PC. Control, no-added nitrogen (N) fertilizer; PP, pre-plant fertilizer application at a rate of 168 kg N ha⁻¹; SPLIT, 40% preplant, 60% mid-season application of N fertilizer at a total rate of 168 kg N ha⁻¹. Early, early-season sampling (June); Mid, mid-season sampling (August); Late, late-season sampling (October). GWC, gravimetric water content; Cmin, mineralizable carbon content; NO₃⁻-N, nitrate; NH₄⁺-N ammonium.

Supplementary Table 6. Mean conservation system soil chemical characteristics at each sampling period at the 0-10 cm and 10-20 cm depths in 2020.

Sampling Period	Depth	Conservation System ¹	pH	Cmin ² mg CO ₂ -C kg soil ⁻¹	GWC g water 100 g soil ⁻¹	NO ₃ ⁻ mg kg soil ⁻¹	NH ₄ ⁺ mg kg soil ⁻¹					
Early-Season Sampling	0-10 cm	NTW	7.5	75.5	13.5	A ³	21.5	9.5				
		NT	7.6	69.8	13.2	A	18.6	8.9				
		CT	7.4	71.7	11.5	B	21.0	8.5				
	10-20 cm	NTW	7.7	51.3	15.1		6.2	7.2				
		NT	7.8	52.8	13.8		7.2	7.5				
		CT	7.7	56.0	14.5		4.8	7.6				
Mid-Season Sampling	0-10 cm	NTW	8.0	A	74.1	13.3	A	5.0	AB	6.7		
		NT	7.6	B	71.1	13.2	A	2.6	B	6.0		
		CT	7.9	AB	69.4	12.1	B	6.7	A	6.1		
	10-20 cm	NTW	7.8		57.9	13.5		1.2		6.0		
		NT	7.6		59.8	13.5		1.5		6.1		
		CT	7.7		48.1	12.9		2.2		5.8		
Late-Season Sampling	0-10 cm	NTW	7.6		101.7	A	5.1	A	0.9	A	8.5	A
		NT	7.7		72.0	B	4.0	B	0.7	AB	8.1	A
		CT	7.8		80.6	B	4.9	A	0.6	B	7.3	B
	10-20 cm	NTW	7.8		82.0		7.7	B	0.7		7.3	
		-	-		-		-		-		-	
		CT	7.5		70.6		8.3	A	0.6		8.2	

¹ NTW, no-tillage with winter wheat cover crop; NT, no-tillage winter fallow; CT, conventional tillage winter fallow.

² Cmin, mineralizable carbon content; GWC, gravimetric water content; NO₃⁻-N, soil nitrate concentration; NH₄⁺-N, soil ammonium concentration



Supplementary Figure 3. Nitrogen timing averages for soil chemical characteristics at each sampling period for the 0-10 cm and 10-20 cm depth in 2020. Control, no added N fertilizer; PP, 100% pre-plant application of N fertilizer (168 kg ha⁻¹); SPLIT, 40% PP 60% mid-season application of N fertilizer. LSD letters are compared within sampling period and depth and are different at $\alpha=0.05$.

Supplementary Table 7. Interaction effects on Mineralizable Carbon (Cmin) content within the 0-10 cm depth at the late-season sampling in 2020

Con. System ¹	Treatment ²	Cmin ³	LS-MEANS
		mg CO ₂ -C kg ⁻¹ soil	<i>p</i> <0.05
NTW	Control	96.4	abc
	PP	104.4	c
	SPLIT	104.3	c
NT	Control	65.7	a
	PP	68.7	ab
	SPLIT	81.7	abc
CT	Control	100.8	bc
	PP	72.2	abc
	SPLIT	69	ab

¹ Conservation system: NTW, no-tillage with winter wheat cover crop; NT, no-tillage winter fallow; CT, conventional tillage winter fallow.

² Control, no-added N fertilizer; PP, 100% pre-plant N fertilizer application (168 kg ha⁻¹); SPLIT, 40% PP, 60% mid-season (21 July) application of N fertilizer (168 kg ha⁻¹ total)

³ Cmin, mineralizable carbon

Supplementary Table 8. Analysis of variance (ANOVA) values for gene abundances in 2020

Sampling Period	Depth	16S rRNA			AOB			<i>nirK</i>			<i>nirS</i>			<i>nosZ</i> Clade I			<i>nosZ</i> Clade II		
		Con. System ¹	N treatment	CS × NT ²	Con. System	N treatment	CS × NT	Con. System	N treatment	CS × NT	Con. System	N treatment	CS × NT	Con. System	N treatment	CS × NT	Con. System	N treatment	CS × NT
Early-Season Sampling	0-10 cm	0.507	0.374	0.621	0.618	0.063	0.657	0.206	0.451	0.827	0.744	0.127	0.469	0.944	0.257	0.429	0.452	0.621	0.182
	10-20 cm	0.896	0.616	0.205	0.589	0.022	0.132	0.201	0.624	0.126	0.441	0.508	0.242	0.649	0.577	0.182	0.068	0.078	0.082
Mid-Season Sampling	0-10 cm	0.880	0.961	0.315	0.144	0.044	0.096	0.210	0.958	0.169	0.106	0.646	0.567	0.338	0.822	0.307	0.950	0.147	0.584
	10-20 cm	0.457	0.657	0.905	0.283	0.256	0.362	0.395	0.541	0.484	0.815	0.795	0.995	0.890	0.783	0.767	0.595	0.224	0.835
Late-Season Sampling	0-10 cm	0.143	0.126	0.256	0.057	0.017	0.247	0.276	0.976	0.225	0.341	0.110	0.516	<0.001	0.564	0.006	0.233	0.670	0.926
	10-20 cm	0.007	0.710	0.422	0.533	0.011	0.006	0.107	0.552	0.442	0.055	0.740	0.487	0.002	0.156	0.083	0.046	0.129	0.302

¹Conservation System

²Conservation system and N treatment interaction

Supplementary Table 9. Total bacterial (16SrRNA) and Nitrogen cycle gene abundances within each growth period in 2020 at 0-10 cm and 10-20 cm depth

Sampling Period	Depth	Conservation System ¹	N Treatment ²	16S rRNA	AOB	<i>nirK</i>	<i>nirS</i>	<i>nosZ</i> Clade I	<i>nosZ</i> Clade II	
				count g ⁻¹ dry soil						
Early-Season Sampling	0-10 cm	NTW	-	4.77E+07	1.03E+06	6.07E+05	3.17E+05	1.05E+06	3.59E+08	
		NT	-	4.44E+07	7.37E+05	9.85E+05	2.60E+05	1.06E+06	2.12E+08	
		CT	-	5.92E+07	6.82E+05	5.73E+05	2.99E+05	9.74E+05	1.59E+08	
		-	Control	5.87E+07	2.95E+05	8.91E+05	3.85E+05	1.17E+06	3.09E+08	
		-	PP	5.24E+07	9.14E+05	5.77E+05	2.43E+05	1.16E+06	2.66E+08	
		-	SPLIT	4.01E+07	1.24E+06	6.97E+05	2.48E+05	7.56E+05	1.55E+08	
	10-20 cm	NTW	-	2.22E+07	6.09E+04	7.23E+04	7.46E+04	3.96E+05	5.35E+07	
		NT	-	2.52E+07	5.94E+04	1.66E+05	1.41E+05	5.23E+05	2.56E+08	
		CT	-	2.00E+07	8.77E+04	1.86E+05	1.13E+05	2.54E+05	5.41E+07	
		-	Control	2.66E+07	2.03E+04	B 1.13E+05	1.36E+05	5.62E+05	2.47E+08	
		-	PP	2.46E+07	7.20E+04	AB 1.36E+05	7.67E+04	3.42E+05	8.87E+07	
		-	SPLIT	1.61E+07	1.16E+05	A 1.75E+05	1.16E+05	2.69E+05	2.78E+07	
Mid-Season Sampling	0-10 cm	NTW	-	6.56E+07	7.15E+06	1.04E+06	4.43E+05	1.19E+06	4.65E+08	
		NT	-	6.18E+07	6.16E+06	5.80E+05	2.91E+05	8.62E+05	4.01E+08	
		CT	-	6.93E+07	2.81E+06	8.31E+05	6.01E+05	1.00E+06	3.87E+08	
		-	Control	6.67E+07	2.21E+06	B 7.80E+05	4.56E+05	1.01E+06	2.60E+08	
		-	PP	6.31E+07	8.19E+06	A 8.14E+05	3.76E+05	9.54E+05	7.32E+08	
		-	SPLIT	6.69E+07	5.73E+06	AB 8.52E+05	5.03E+05	1.09E+06	2.61E+08	
	10-20 cm	NTW	-	1.29E+07	1.60E+06	1.66E+05	1.42E+05	1.43E+05	5.58E+07	
		NT	-	1.29E+07	4.50E+05	1.79E+05	1.17E+05	1.81E+05	1.21E+08	
		CT	-	1.84E+07	7.45E+05	4.62E+05	1.59E+05	1.75E+05	1.14E+08	
		-	Control	1.73E+07	3.14E+05	1.62E+05	1.52E+05	1.32E+05	8.52E+07	
		-	PP	1.41E+07	9.22E+05	4.20E+05	1.53E+05	1.87E+05	4.10E+07	
		-	SPLIT	1.28E+07	1.55E+06	2.24E+05	1.13E+05	1.80E+05	1.64E+08	
Late-Season Sampling	0-10 cm	NTW	-	5.48E+07	8.26E+06	1.19E+06	4.68E+05	1.49E+06	A 4.71E+08	
		NT	-	5.38E+07	6.97E+06	8.18E+05	5.94E+05	1.08E+06	B 2.19E+08	
		CT	-	3.96E+07	3.32E+06	8.29E+05	4.06E+05	6.67E+05	C 1.71E+08	
		-	Control	5.93E+07	2.95E+06	B 9.78E+05	6.14E+05	1.12E+06	3.78E+08	
		-	PP	4.23E+07	6.28E+06	AB 9.29E+05	5.19E+05	1.13E+06	2.65E+08	
		-	SPLIT	4.66E+07	9.32E+06	A 9.31E+05	3.35E+05	9.87E+05	2.19E+08	
	10-20 cm	NTW	-	3.35E+07	A 3.30E+06	9.41E+05	3.80E+05	8.32E+05	A 2.94E+08	A
		-	-	-	-	-	-	-	-	-
		CT	-	1.15E+07	B 2.83E+06	4.91E+05	1.49E+05	1.72E+05	B 5.84E+07	B
		-	Control	1.90E+07	1.31E+06	B 5.69E+05	3.19E+05	3.19E+05	3.67E+07	
		-	PP	2.57E+07	3.14E+06	AB 6.70E+05	2.57E+05	7.21E+05	3.23E+08	
		-	SPLIT	2.27E+07	4.74E+06	A 9.09E+05	2.17E+05	4.66E+05	1.69E+08	

¹ Conservation system: NTW, no-tillage with winter wheat cover crop; NT, no-tillage winter fallow; CT, conventional tillage winter fallow.

² Control, no-added N fertilizer; PP, 100% pre-plant N fertilizer application (168 kg ha⁻¹); SPLIT, 40% PP, 60% mid-season (21 July) application of N fertilizer (168 kg ha⁻¹ total)

Supplementary Table 10. Interaction effects on *nosZ* clade I abundance within the 0-10 cm depth at the late-season sampling.

Con. System ¹	N Treatment	<i>nosZ</i> clade I count g ⁻¹ dry soil	LS- MEANS <i>p</i> <0.05
NTW	Control	1.32E+06	AB
	PP	2.09E+06	A
	SPLIT	1.07E+06	B
NT	Control	1.29E+06	AB
	PP	9.05E+05	B
	SPLIT	1.05E+06	B
CT	Control	7.64E+05	B
	PP	3.98E+05	B
	SPLIT	8.38E+05	B

¹Conservation System

Supplementary Table 11. Interaction effects on AOB abundance within the 10-20 cm depth at the late-season sampling.

Con. System ¹	Treatment	AOB count g ⁻¹ dry soil	LS- MEANS <i>p</i> <0.05
NTW	Control	2.16E+06	AB
	PP	4.88E+06	AB
	SPLIT	2.87E+06	AB
CT	Control	4.57E+05	B
	PP	1.41E+06	B
	SPLIT	6.62E+06	A

¹Conservation System

Supplementary Table 12. Analysis of variance (ANOVA) for nitrous oxide (N₂O) emissions, pore-space N₂O concentration and pore-space nitric oxide (NO) concentration within each sampling period in 2020

Depth	Gas Type	Sampling Period	Con.	N	CS × NT ²
			System ¹	treatment	
ANOVA (<i>p</i> -values<0.05)					
Surface	N ₂ O Emissions	Early-Season	0.508	0.002	0.768
		Mid-Season	0.351	0.002	0.152
		Late-Season	-	-	-
7.5 cm	N ₂ O Pore-Space Concentrations	Early-Season	0.545	0.041	0.122
		Mid-Season	0.569	0.426	0.312
		Late-Season	0.370	0.100	0.868
15 cm	N ₂ O Pore-Space Concentrations	Early-Season	0.775	0.099	0.143
		Mid-Season	0.727	0.844	0.463
		Late-Season	0.560	0.190	0.308
7.5 cm	NO Pore-Space Concentrations	Early-Season	0.928	0.328	0.301
		Mid-Season	0.710	0.751	0.384
		Late-Season	0.303	0.098	0.841
15 cm	NO Pore-Space Concentrations	Early-Season	0.989	0.350	0.225
		Mid-Season	0.625	0.629	0.741
		Late-Season	0.471	0.111	0.489

¹Conservation System

²Conservation system and N treatment interaction

Supplementary Table 13. Nitrous oxide (N₂O) emissions, N₂O pore-space concentrations, and nitric oxide (NO) pore-space concentrations collected at the Early-, Mid-, and Late-Season samplings in 2020

Sampling Period	Conservation System ¹	N Treatment ²	N ₂ O-N Emissions	N ₂ O-N Emissions Standard Error	N ₂ O Pore-space Concentration 7.5 cm	N ₂ O Pore-space Concentration 7.5 cm Standard Error	N ₂ O Pore-space Concentration 15 cm	N ₂ O Pore-space Concentration 15 cm Standard Error	NO Pore-space Concentration 7.5 cm	NO Pore-space Concentration 7.5 cm Standard Error	NO Pore-space Concentration 15 cm	NO Pore-space Concentration 15 cm Standard Error
			(μg N ₂ O-N m ⁻² h ⁻¹)	(μL N ₂ O L ⁻¹)	(μL N ₂ O L ⁻¹)	(μL NO L ⁻¹)	(μL NO L ⁻¹)	(μL NO L ⁻¹)				
Early-Season Sampling	NTW	Control	0.00	0.00	0.88	0.31	0.94	0.32	2.09	1.49	2.26	1.50
		PP	273.11	100.95	1.16	0.11	1.29	0.15	4.01	1.05	4.21	1.11
		SPLIT	34.95	22.16	0.59	0.10	0.63	0.10	1.60	0.23	1.62	0.19
	NT	Control	-27.26	14.59	1.31	0.17	1.28	0.28	4.40	0.90	3.83	0.94
		PP	472.44	288.33	1.10	0.22	1.17	0.23	2.58	0.43	2.74	0.58
		SPLIT	120.78	62.81	1.19	0.12	1.38	0.24	4.61	0.91	5.59	1.42
	CT	Control	0.00	0.00	0.99	0.25	1.06	0.26	3.76	1.76	3.99	1.87
		PP	263.37	47.10	0.93	0.11	1.04	0.11	3.44	1.18	3.51	1.15
		SPLIT	56.43	41.82	1.08	0.21	1.20	0.26	3.08	0.48	3.22	0.49
Mid-Season Sampling	NTW	Control	-16.84	16.84	0.76	0.25	0.60	0.16	4.60	1.67	3.14	0.56
		PP	0.00	0.00	0.76	0.19	0.83	0.22	3.68	1.10	3.70	0.52
		SPLIT	153.92	57.60	0.66	0.19	0.64	0.06	4.69	1.33	3.80	0.19
	NT	Control	-28.42	14.33	0.72	0.14	0.78	0.10	3.26	0.57	3.52	0.76
		PP	-13.29	13.29	0.82	0.12	0.80	0.26	5.37	1.40	4.67	1.70
		SPLIT	123.17	81.34	0.85	0.30	0.65	0.09	6.39	2.48	2.42	0.85
	CT	Control	0.00	0.00	1.11	0.24	0.76	0.05	7.50	2.10	3.25	1.76
		PP	0.00	0.00	0.62	0.13	0.62	0.02	3.69	2.13	2.78	0.98
		SPLIT	11.40	11.40	0.94	0.21	0.74	0.17	4.73	0.95	2.46	0.78
Late-Season Sampling	NTW	Control	-	-	0.33	0.02	0.40	0.07	1.72	0.22	2.00	0.82
		PP	-	-	0.41	0.01	0.55	0.05	1.47	0.05	2.64	0.41
		SPLIT	-	-	0.44	0.09	0.40	0.08	2.38	0.83	1.61	0.43
	NT	Control	-	-	0.43	0.05	0.49	0.09	1.50	0.29	2.03	0.78
		PP	-	-	0.49	0.09	0.43	0.01	2.61	0.84	1.87	0.15
		SPLIT	-	-	0.55	0.15	0.49	0.06	2.93	1.61	2.12	0.62
	CT	Control	-	-	0.38	0.05	0.47	0.08	0.82	0.40	2.12	0.46
		PP	-	-	0.33	0.06	0.55	0.14	1.22	0.31	3.27	0.76
		SPLIT	-	-	0.39	0.11	0.64	0.15	1.30	0.27	3.27	0.67

¹ NTW, no-tillage with winter wheat cover crop; NT, no-tillage winter fallow; CT, conventional tillage winter fallow.

² Control, no-added N fertilizer; PP, 100% pre-plant N fertilizer application (168 kg ha⁻¹); SPLIT, 40% PP, 60% mid-season (21 July) application of N fertilizer (168 kg ha⁻¹ total)