CONSERVATION MANAGEMENT AND NITROGEN FERTILIZER TIMING EFFECTS ON MICROBIAL PRODUCTION OF NITROUS OXIDE AND NITRIC OXIDE IN A SEMI-ARID ENVIRONMENT

A Dissertation

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ABSTRACT

Due to the potency of nitrous oxide (N_2O) as a contributor to the greenhouse effect, and the relationship between N_2O emissions agricultural soil management, understanding N_2O dynamics within agricultural production is an important research topic. This is especially true in expanding semi-arid areas where best management practices for reducing erosion and increasing soil health may also increase conditions favorable for denitrification, one of the major pathways for N_2O production.

The purpose of this series of studies was to elucidate the effects best management practices have on soil chemical and biological characteristics associated with nitrogen (N) gas cycling in low carbon content semi-arid soils. In addition, active microbial population dynamics were evaluated to better characterize the biological changes conservation tillage may induce in semi-arid systems.

Initial evaluations determined significant effects of the timing of N fertilizer on net N_2O emissions from the soil surface, where N_2O emissions closely followed N fertilizer application. Net consumption of N_2O was also determined during the initial analysis which was attributed to the abundant N_2O-reducing population(s) present in semi-arid soils. As the implemented systems matured, N fertilizer timing effects on soil chemical and biological parameters and N_2O emissions were determined to vary with the time of year sampled, indicating dynamic seasonal changes related to when N fertilizer is applied. However, the effects determined within sampling periods did not always result in changes in pore-space N_2O and NO concentrations. Metatranscriptomic analysis

revealed some differences in gene expression across conservation systems. Abundant nitrifier transcripts associated with nitrogen metabolism were identified, supporting the role of nitrification in production of N gases in semi-arid agricultural soils.

From the results of this set of studies, it is clear that the mechanisms behind N_2O emissions and N gas cycling are highly complex in semi-arid systems, although the application of N fertilizer seems to have the greatest control over the timing and rate of N gas cycling and N_2O production. It is yet to be determined the exact circumstances leading to the prevalence of N_2O production and consumption in low C content semi-arid soils, but it is likely a combination of factors including many of those evaluated within these experiments.

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NOMENCLATURE

GHG	Greenhouse gas
NTW	No-tillage with a winter wheat cover crop
NT	No-tillage winter fallow
CT	Conventional tillage winter fallow
PP	100% Pre-plant nitrogen fertilizer application
SPLIT	40% pre-plant 60% mid-season nitrogen fertilizer application
MS	Mid-season nitrogen fertilizer application
STB	100% PP nitrogen fertilizer application with stabilizer product
PSP	Pore-space probe
MLRA	Major land resource area
Cmin	Mineralizable carbon
GWC	Gravimetric water content
CO2E	Carbon dioxide equivalent gas emissions
Veg	Vegetative growth period, June
Peak	Peak plant production, August
Repro	Reproductive growth period, October
NAR/NAP	Nitrate reductase
nirS/nirK	Nitrate reductase genes
NOR	Nitric oxide reductase
qnor/*cnor*	Nitric-oxide reductase genes

nosZ clade I/II	Nitrous oxide reductase genes
N_2OR	Nitrous oxide reductase enzyme
AOA	Ammonia oxidizing archaea
AOB	Ammonia oxidizing bacteria
HAO	Hydroxylamine oxidoreductase enzyme
AMO	Ammonia monooxygenase enzyme
GWP	Global warming potential
OM	Organic matter
C	Carbon
N	Nitrogen
SHP	Southern High Plains
UAN	Urea-ammonia-nitrate
a.i.	Active ingredient
qPCR	Quantitative polymerase chain reaction
C_t	Threshold cycle
ASV	Amplicon sequence variant
SRA	Sequence Read Archive
N_{inorg}	Inorganic nitrogen
PC	Principal components
C_T	Total concentration of gases
NMDS	Non-metric multidimensional scaling analysis
BD	Bulk density

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1. INTRODUCTION

Nitrous oxide (N_2O) is the product of several microbial processes and can be derived from specific chemical reactions and fossil fuel combustion. Nitrous oxide is a trace gas in the atmosphere with a concentration of about 334 ppb in 2018 (EPA, 2020) but has a global warming potential (GWP) nearly 300 times that of carbon dioxide (CO_2) making it of particular interest for the industries responsible for its emission. Nitrous oxide's large GWP is partially due to its dual function in the atmosphere where it can act as a radiative force and contribute to the greenhouse effect and also be broken down to nitric oxide(s) which can degrade ozone in the stratosphere (Ravishankara et al., 2009; IPCC, 2013). In addition to its dual functionality as a GHG, N_2O is long-lived in the atmosphere, with an atmospheric lifetime of about 116 years (Prather et al., 2015). The major sources of N_2O include nitrification and denitrification by bacteria and fungi in soil and the oceans, with agricultural soils accounting for about 78% of N_2O emissions in the US in 2018 (EPA, 2020). Within agricultural soils, the natural microbial population participates in the global nitrogen (N) cycle at a more accelerated rate due to the application of N fertilizers, thus stimulating the process related to the production and release of N_2O. Mitigation N_2O and other greenhouse gas emissions has been a major concern for agricultural researchers for the last few decades with various soil management recommendations being made to reduce the rate of N_2O and CO_2 emissions (Malhi et al., 2006; Halvorson et al., 2008) along with proper N management practices (Shelton et al., 2017). The focus of this review will be the major microbial processes responsible for the production and consumption of N_2O and how the controls over these

microbial processes can be affected by soil and nutrient management in agricultural soils.

1.1. Nitrous oxide production and consumption

1.1.1. Nitrification

Nitrification is the multi-step oxidation of ammonia (NH_3) to nitrate (NO_3^-) by autotrophic bacteria and archaea throughout nature (Stein and Klotz, 2016;Kuypers et al., 2018) and is among the principal pathways leading to N loss from soil environments. Classically, nitrification is a multi-step process where NH_3 (sometimes notated as NH_4^+) is converted to nitrite (NO_2^-) by ammonia oxidizing bacteria (AOB) and archaea (AOA), followed by NO_2^- oxidation to NO_3^-, with several intermediates during the first stage including the obligate oxidation of NH_3 to hydroxylamine (NH_2OH). Due to uncertainties regarding AOA nitrification activity (Lancaster et al., 2018), we have chosen to review the literature surrounding AOB-mediated nitrification as it relates to N_2O and nitric oxide (NO) production.

Within the classical nitrification framework, it has long been understood that both N_2O and NO may be produced as metabolic products of NH_3 oxidation (Tortoso and Hutchinson, 1990). The regulation of the processes by which N_2O and NO can be produced from nitrification was reviewed by Firestone and Davidson (1989) where they conceptualized the loss of NO and N_2O from nitrification as a “leaky pipe”. However, in their review, Firestone and Davidson (1989) suggested that NO could be produced via the oxidation of NH_2OH and through the reduction of NO_2^- to NO. The production of NO as an obligate intermediate of the oxidation of NH_2OH to NO_2^- was recently

confirmed to occur in AOB and methanotrophs (Maalcke et al., 2014; Caranto and Lancaster, 2017; Versantvoort et al., 2020).

The confirmation of NO as the enzymatic product of hydroxylamine oxidoreductase (HAO) in AOB was conducted under both aerobic and anaerobic conditions by Caranto and Lancaster (2017), who proposed this reaction as the basis for some of the N_2O production previously associated with nitrification. Their proposal suggested that if the rapid oxidation of NH_2OH to NO via HAO out-paced the oxidation of NO to NO_2^-, then NO release or reduction to N_2O may occur, likely due to the potential toxicity of NO to the cell (Zumft, 1997). Versantvoort et al. (2020) also characterized the oxidation of NH_2OH to NO in methanotrophic bacteria which are known to oxidize ammonia (NH_3) as well as methane (CH_4) in environments where they both occur. In methanotrophs, NH_2OH inhibits methanol dehydrogenase, and thus the reduction of NH_2OH is necessary, but not necessarily metabolically relevant (Versantvoort et al., 2020). Reduction of the NO produced during the oxidation of NH_2OH in AOB and methanotrophs is conducted by the NO reductase enzyme which is also associated with the denitrification pathway and has been found in nearly all AOB (Zorz et al., 2018) and is commonly found in methanotrophs (Versantvoort et al., 2020). In addition to the potential production of N_2O through the newly characterized obligate oxidation of NH_2OH to NO, it has been recently determined that NH_2OH may be directly oxidized to N_2O by cyt P460 (Caranto et al., 2016), thus adding another potential pathway for N_2O production during nitrification. Finally, Kool et al. (2011) summarized the various pathways of N_2O production in soils, including a rough

framework for the NH_2OH oxidation to N_2O and NO confirmed by Caranto et al. (2016) and is summarized in Fig. 1. Kool et al. (2011) demonstrated that nitrifier denitrification, starting with the reduction of NO_2^- to NO, may be a stronger contributor to N_2O production in dry soils than previously expected. With these recent discoveries, we now have a better understanding of NH_2OH oxidation, with the resultant product being NO as originally hypothesized by Firestone and Davidson (1989) as well as better understanding regarding the contribution of nitrifier-led soil process contributing to N_2O production in soil (Wrage-Mönnig et al., 2018).

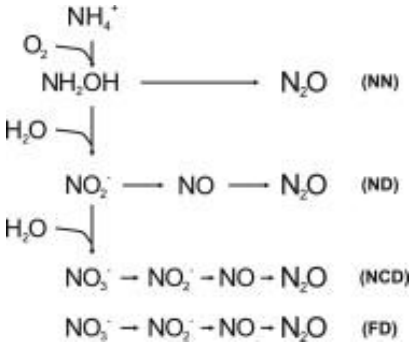

Figure 1.1 Pathways for N_2O production as reviewed by Kool et al. (2011). NN, nitrifier nitrification (further demonstrated by Caranto et al. (2016) and Caranto and Lancaster (2017)); ND, nitrifier denitrification; NCD, nitrification-coupled denitrification; FD, fertilizer denitrification.

It is important to note that even with the recent revelations regarding NH_2OH oxidation, N_2O production can only occur during nitrification with the aid of genes (and homologs) typically associated with denitrification. In agricultural soils with NH_4^+ and NH_3 application, it is possible that rapid oxidation to NH_2OH will occur, which can result in N_2O production as discussed here. Mitigation of N_2O production may be best served by considering not only the controls over anaerobic denitrification, but also

considering those that would impact aerobic nitrifier denitrification (Kool et al., 2011). It is interesting to note that under the new concept regarding the obligate intermediates of NH_3 oxidation to NO_2^-, there is still considerable debate as to the enzymatic catalyst of NO oxidation to NO_2^-, with suggestions including the copper containing protein NcyA encoded by the *ncyA* gene found in some AOB species and the denitrification enzyme NirK (Zorz et al., 2018).

In addition to being potentially directly involved with N_2O production under certain soil conditions, nitrification is also responsible for the oxidation of soil-stable NH_4^+ to water soluble NO_3^- which can increase N_2O emissions through several regulatory methods that will be discussed later in this review. Not only would increasing NO_3^- concentrations affect local microbial populations and N_2O emissions, but due to the solubility of NO_3^- it may affect the N cycle off-site through runoff and leaching (Eghball et al., 2000). Because of these potential N loss pathways, it could be said that the most important step towards N loss from soil is the conversion of organic N to NH_4^+ and NH_3 and subsequent oxidation to NH_2OH, NO, N_2O, NO_2^-, and NO_3^- through nitrification.

1.1.2. Denitrification

Denitrification is the microbial process responsible for the reduction of NO_3^- to inert N_2 gas via several intermediate reactions (Zumft, 1997). Denitrification is an anaerobic process found in most terrestrial environments across the globe where it is activated under low O_2 conditions introduced by precipitation, irrigation, or increased soil respiration (Knowles, 1982; Linn and Doran, 1984; Wu et al., 2017). The activation

of denitrification under anaerobic conditions is both a function of when it is needed for energy production, and when the O_2 sensitive enzymes are viable in the cell cytoplasm and periplasm (Betlach and Tiedje, 1981; Lloyd, 1993). Both bacteria and fungi participate in denitrification reactions in the soil (Knowles, 1982; Shoun and Tanimoto, 1991), although with different endpoints and efficiencies. Bacterial denitrification can be considered "complete" denitrification due to the ability of bacteria to convert the intermediate N_2O into the final inert N_2 gas (Thomson et al., 2012), a reaction not present in fungal denitrification (Shoun et al., 2012). Fungal denitrification may not be complete denitrification as originally described, but it has been reported to be prevalent in agricultural soils, with up to 72% of soil N_2O coming from fungal origin in some studies (Chen et al., 2015). It has been determined that bacterial denitrification may be more of a community-based process than previously thought (Zumft, 1997), which is in line with other research indicating some microbial organisms may be harboring genes from the denitrification cycle as a bet-hedging strategy to survive brief anoxic periods (Lycus et al., 2018). The potential community-based approach to denitrification by a microbial population may complicate our understanding of the size of denitrifying populations if care is not taken to evaluate the process at several points.

To understand the importance of denitrification as a whole, we must examine each step of the process, its controls, and its outputs. Denitrification, like nitrification, is a major pathway for N loss from soil and thus controlling, mitigating, and evaluating it is imperative for both environmental and agricultural sustainability. There are several fates of NO_3^- in soil that are both biologically and environmentally relevant as mentioned

above, with the most preferred method of NO_3^- consumption, other than plant uptake, being microbial reduction through denitrification. The first step of denitrification is the reduction of NO_3^- to NO_2^- and is conducted as anaerobic respiration in all three domains of life and anoxic environments (Kuypers et al., 2018). This dissimilatory step to beginning denitrification is catalyzed by the NAR or NAP enzymes, encoded by the *nar* gene cluster, and takes place in the cytoplasm or periplasm, respectively (Potter et al., 1999; Kuypers et al., 2018). Often, NO_3^- reduction is necessarily coupled with electron donors such as organic matter, methane, or sulfur compounds to aid in energy conservation, and thus the presence of a suitable electron donor is a strong regulator of NO_3^- reduction to NO_2^-.

Following the reduction of NO_3^- to NO_2^-, the next step in the denitrification pathway is the reduction of NO_2^- to NO in the periplasm via one of two unrelated nitrite reductase (NIR) enzymes (Braker et al., 1998; Graf et al., 2014; Kuypers et al., 2018). The most abundant form of the NIR enzyme is the haem-containing cd_1-NIR encoded by *nirS* which is present in about 70% of denitrifying bacteria (Braker et al., 1998; Graf et al., 2014). The Cu-NIR form of the NO_2^- reducing enzymes is less abundant, being only present in about 30% of the denitrifying population, is encoded by *nirK*, and uses copper as its metal cofactor (Braker et al., 1998;Graf et al., 2014). The Cu-NIR form may also be responsible for the oxidation of NO to NO_2^- during nitrification (Caranto and Lancaster, 2017). Although they may be found together in the same organism, the NIR enzymes most often replace the other in function when introduced into a genome (Enwall et al., 2010;Kuypers et al., 2018). Often *nirS* and *nirK* are used as genetic

markers for determining the size of a denitrifying population (Braker et al., 1998), although this may be complicated due to the presence of *nirK* homologs in AOA and their potential association with bet-hedging organisms (Bartossek et al., 2010; Lycus et al., 2018).

As NO concentrations build up in the cell it will become toxic to the microbe and either need to be expelled or further reduced (Zumft, 1997). The reduction of NO to N_2O in bacteria is mediated by a set of haem-copper oxidase (NOR) enzymes produced from two different genes, *cnor* and *qnor* (Braker and Tiedje, 2003). These two forms of the bacterial NOR enzyme differ in their sourcing of electrons with quinol providing electrons for *qnor* and cytochrome-c for *cnor* (Dandie et al., 2007). They also differ in their presence within bacterial denitrifying populations, where *cnor* is typically found with true denitrifiers and *qnor* is found with some denitrifiers but also among organisms that require the ability to reduce NO due to its toxicity to microbial life (Braker and Tiedje, 2003). In addition to bacterial reduction of NO to N_2O, the NOR enzymes are also notably present in fungi and are encoded by the gene *p450nor* which sources its electrons from NADH (Shoun and Tanimoto, 1991;Thomson et al., 2012;Higgins et al., 2016). Fungal denitrification, unlike bacterial, terminates with the reduction of NO to N_2O and thus may be a large contributor to N_2O emissions (McLain and Martens, 2006;Novinscak et al., 2016). Due to N_2O's impact on the environment as a greenhouse gas, the reduction of NO to N_2O is a key point for potential regulation and mitigation of that process. Reduction of NO to N_2O is conducted on the cell membrane, contributing

to the proton motive force and energy conservation as well as producing energy for the cell (Kuypers et al., 2018).

The final step of denitrification is the reduction of N_2O to N_2 in the periplasm by the N_2O reductase enzyme (N_2OR) (Jones et al., 2013). This enzyme is encoded by two different clades of the *nosZ* gene (clade I and clade II) and is only found in bacteria (Thomson et al., 2012; Jones et al., 2014). The two clades of N_2OR differ in several respects, namely the method of transport of the protein across the cell membrane to the periplasm and in what type of organisms they are found. Clade I N_2OR enzymes are most often found in "typical" denitrifiers where they use twin-arginine translocation (TAT) to move the protein across the cell membrane (Sanford et al., 2012). The clade I version of *nosZ* has been reported to co-occur with *nirK/S* containing organisms about 83% of the time (Hallin et al., 2018). In contrast, clade II organisms comprise a wide diversity of microbial species, are ubiquitous is soil, are less likely to be associated with full denitrification gene suites and use a more efficient general secretion route (Sec) pathway for protein transportation across the cell membrane (Sanford et al., 2012; Jones et al., 2013; Graf et al., 2014; Jones et al., 2014; Higgins et al., 2016; Lourenço et al., 2018). There are a few theories regarding the large abundance of clade II organisms in the soil with current ideas leaning towards the use of the enzyme as a bet-hedging strategy for microbes in environments with fluctuating levels of oxygenation (Lycus et al., 2018). In typical denitrification, the genes are activated in sequential order following the removal of O_2 from the environment with NAR enzymes being activated 2-3 hours after O_2 depletion, followed by NIR at 2-12 hours, and finally NOR at 24-48 hours

(Saggar et al., 2013). The issue for many microbes in soil environments lies with the expense of energy for the production of these enzymes and the often-short period of anaerobicity in their environment. Thus, some microbes have potentially harnessed the high energy production and efficiency of the clade II version of N_2OR to reduce N_2O in their environment during these shorter anaerobic periods, thus saving energy while also allowing themselves to still conduct cellular processes.

As mentioned above, clade II organisms are often not associated with full denitrification gene suites. In fact, about 51% of clade II organisms contain the clade II version of *nosZ* and no other denitrification genes (Graf et al., 2014), including common soil bacteria such as *Gemmatimonas aurentiaca* (Kuypers et al., 2018). The bet-hedging theory by Lycus et al. (2018) helps explain the ecological relevance of organisms solely containing *nosZ* clade II, especially when considered with the ability of these microbes to consume N_2O produced by other organisms (Domeignoz-Horta et al., 2016). Evidence has shown that N_2O consumption is enhanced with other environmental situations as well with the most studied of these being the occurrence of N_2O consumption during periods of low soil inorganic-N concentrations (Ryden, 1981; Minami, 1997; Butterbach-Bahl et al., 1998; Rosenkranz et al., 2006; Kroeze et al., 2007). During this period, several factors may be affecting the microbes' ability to produce energy, including reduced N-cycle processes and reduce soil respiration with inadequate N resources. The ability of a microbe to use a relatively abundant soil and atmospheric gas to produce energy would thus be advantageous and ecologically relevant (Hallin et al., 2018) and give support the ubiquitous nature of this ability.

1.2. Mitigation strategies: controls over N_2O production and consumption

Nitrous oxide flux from soil is an undesirable exit point for N from the soil ecosystem. As a product of nitrification and denitrification, it is a naturally occurring process that is affected by various chemical, physical, and biological characteristics of the environment. Chemical, physical, and biological controls over the potential, rate, and efficiency of denitrification are important to consider when evaluating best practices for mitigating N_2O emissions and increasing N_2O consumption. The controls can be divided into two categories, proximal controls related to the function and efficiency of denitrification in soil, and distal controls over the structure of the microbial community which mediate denitrification (Fig. 1.1). The scope of this review is focused on the controls specific to N_2O production via denitrification as evaluated in the literature, which would also necessarily control N_2O production via nitrification due to the use of homologs to denitrification genes and process to reduce nitrification intermediates to N_2O.

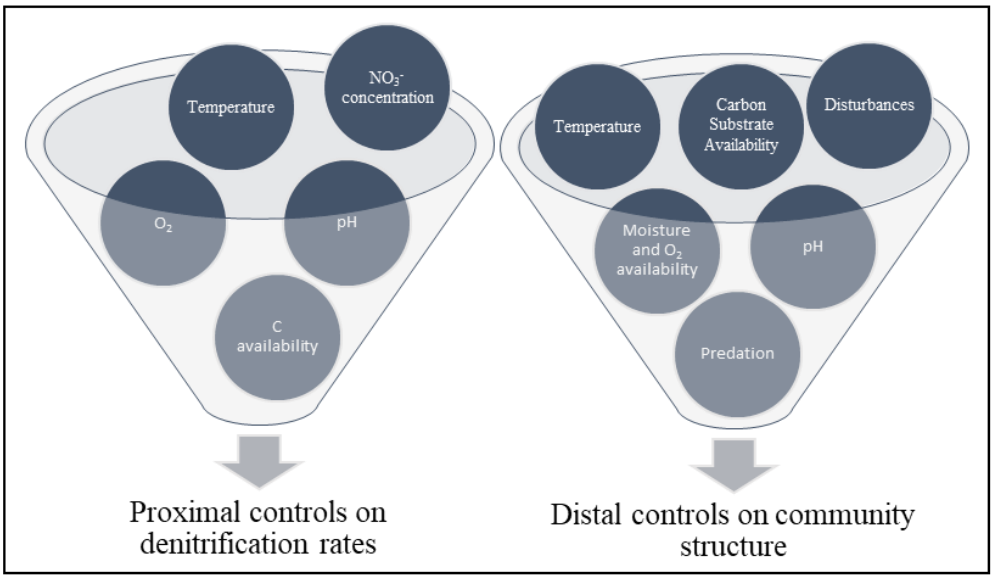

Figure 1.2 Proximal and distal controls over denitrification rates and community structure. Modified from Wallenstein et al. (2006).

1.2.1. Proximal controls

Proximal controls are factors that directly influence the rate, efficiency, and product of denitrification in real time. Of these, the presence or absence of O_2 has been often considered the deciding factor for whether denitrification will proceed (Zumft, 1997). This notion has been challenged in recent years, but still holds as one of the main ways in which we may accurately predict whether denitrification will occur due to the O_2-sensitive nature of many of the enzymes involved (Zumft, 1997). Oxygen concentrations in the soil may be reduced by several events that have been shown to trigger denitrification including precipitation, irrigation, and high rates of soil respiration. The first two of these, precipitation and irrigation, are physical events that

suppress the flow of O_2 into the soil and create an anoxic environment as the O_2 present is used without replacement. As organic carbon is mineralized and CO_2 is released, microbes use O_2 as a terminal electron acceptor. If there is a large amount of organic carbon present, or rates of breakdown are increased (such as with the addition of organic residue, a wetting event, or N fertilizer application), then the microbes may consume the majority of O_2 present creating a brief anoxic period which can trigger the activation of denitrification in a similar manner to soil saturation (Wu et al., 2017). To manage for such O_2 limited situations and reduce the potential for denitrification to occur in a soil ecosystem is complicated as rainfall and the rate of soil respiration are not able to be controlled on an ecosystem scale. However, improved irrigation methods that reduce the level or time of soil saturation may aid in the reduction of N_2O emissions from some agricultural production systems.

A second major proximal control over the occurrence of denitrification in the soil is the presence and concentration of NO_3^- (Robertson, 1989). Increased concentrations of NO_3^- have been determined to increase N_2O emissions *in-situ*, a process that is related to both greater production of N_2O and the preferential scavenging of electrons by the NOR enzymes relative to the N_2OR enzymes (Highton et al., 2020). Without NO_3^- present, N_2O production is reduced, and N_2O consumption may increase (Ryden, 1981; Minami, 1997; Butterbach-Bahl et al., 1998; Rosenkranz et al., 2006; Kroeze et al., 2007) as a potential input of N into the microbial system, and as a means of energy production for the microbe. Managing for NO_3^- concentration in the soil in agricultural settings mostly involves choosing the best management practices including the right time, right place,

right amount, and right type of fertilizer (Smith et al., 2008). If N fertilizer is applied at the right time for crop production, at a rate that will provide enough N to the plant with negligible excess, is of the right type for efficient plant uptake, and is applied where the plant has greatest access to the fertilizer, then the potential for denitrification and N_2O emissions from the soil will likely be reduced. However, this is not possible in all scenarios as agricultural production systems are complicated and thus some management strategies may be preferentially focused on over others.

Carbon availability can also exhibit strong controls over denitrification partially due to the creation of anoxic sites within the soil as mentioned above, but more proximally due to the need for carbonaceous molecules to provide energy for microbes conducting denitrification. Over the entire process, denitrification requires up to three parts carbon for every part N reduced from NO_3^- to N_2 (Shah and Coulman, 1978). A distinct lack of carbon compounds in the soil can thus decrease the potential for denitrification to occur and reduce N_2O production (and consumption). However, in most agricultural systems, this is not an ideal situation as the benefits associated with greater soil fertility and increased carbon inputs (Paustian et al., 2019) outweigh the potential contribution to increased N_2O emissions.

Temperature and pH are the final two well described proximal controls over denitrification rates. Both temperature and pH are known to affect microbial processes generally, but also have specific effects on the rate of chemical reactions and microbial activity as it relates to denitrification and N_2O production and consumption. In the case of temperature, like most microbial processes, denitrification rates have been determined

to peak somewhere between 20°C and 30°C (Saleh-Lakha et al., 2009). Denitrification has been associated with freeze-thaw cycles in northern climates, however it has been determined to have a lower temperature limit at or near 0°C (Holtan-Hartwig et al., 2002). It is known that every enzyme has an optimal pH range of function, for N_2O production (*nirS* and *cnorB* gene expression) this range appears to be between 6 and 8 (Saleh-Lakha et al., 2009). However, N_2O consumption (N_2OR activity) has been determined to be active in acidic conditions but is unlikely to be properly transcribed or translated except at a neutral to slightly basic pH (Liu et al., 2014; Samad et al., 2016). This is an important distinction for agricultural soils as acidic conditions can be present in microsites around N fertilizer inputs, where denitrification is also likely to be most active.

Overall, the relationship between proximal controls and managing N_2O production and consumption in agricultural soils is fairly straightforward. Best management practices for many producers already rely on many of the principles that would aid in reducing N_2O emissions. The key for future mitigation will be determining what soil management practices can increase N_2O consumption in agricultural soils while maintaining agronomic and economic sustainability.

1.2.2. Distal controls

Distal controls over denitrification can be more complicated in their relationship to actual N_2O production and consumption in the soil. Many of these distal controls featured in Fig. 1 relate to the selection of microbial species for the environment. The genetic potential for denitrification to occur is the most important distal control over

denitrification activity in the soil. Without the specific genes present in the microbial population, there is no potential for these processes to occur. This leads into the selection of microbial species through the distal factors mentioned in Fig. 1. It is well known that microbial species can be specialized for their specific environment as exemplified by the discovery of extremophiles in particularly hot, cold, acidic, and alkaline environments (Pikuta et al., 2007). As such, it stands that temperature and pH can affect soil microbial diversity, and thus the potential for denitrification to occur in a particular soil due to potential gene loss in environments that would not require denitrification for survival (Zhou et al., 2016; Hallin et al., 2018).

Similar to the proximal control O_2 exerts on denitrification, O_2 and moisture content can select for a microbial community capable of surviving in those particular circumstances. The distal control of O_2 and moisture may be especially apparent in the cases where the environment transitions between aerobic and anaerobic conditions frequently, possibly selecting for bet-hedging microbes (Lycus et al., 2018). Organic carbon resources also perform a distal form of control over the ability to denitrify in soil, where soils with large amount of carbon are more likely to contain denitrifiers due to increased diversity and the selection of microbes capable of surviving under respiration induced anoxic conditions (Robertson, 1989; Zhou et al., 2002; Wu et al., 2017).

Finally, disturbances can have a large distal control over microbial community structure and thus denitrification rates. In agricultural systems the most obvious example of a disturbance induced control is the reduction of fungal biomass often associated with tillage (van Groenigen et al., 2010). For denitrification specifically, this may be a

beneficial result as fungal organisms cannot reduce N_2O to N_2 and thus release N_2O as their final product (Thomson et al., 2012). Tillage may also reduce denitrification through soil drying and greater use of carbon resources associated with mixing surface residue into the soil, reducing C available for denitrification, although potentially increasing gross CO_2 emissions (Franzluebbers et al., 1995). However, like the proximal control of C availability over denitrification rates, when examining the system as a whole, the benefits associated with the practice (tillage) do not outweigh the potential detriments in many cases, which may include greater soil erosion, reduced microbial populations, greater leaching, and reduced organic carbon resources.

1.3. Conclusion

It is clear that changes in environmental conditions driven by agronomic practices have a significant effect on the potential and activity of the denitrification community. Increasing soil carbon resources, water holding capacity, and reducing disturbances will likely increase N_2O emissions from agricultural soils. However, it is possible that N_2O consumption is also increased within the same agronomic practices as a bet-hedging strategy for soil microbes. The balance between N_2O production and consumption will thus be a key determinant for the environmental sustainability of these systems. In addition, the economic, environmental, and agronomic sustainability must be considered to understand the holistic impact and better guide producer decisions related to their soil and nutrient management.

1.4. References

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2. NITROUS OXIDE CONSUMPTION POTENTIAL IN A SEMI-ARID AGRICULTURAL SYSTEM: EFFECTS OF CONSERVATION SOIL MANAGEMENT AND NITROGEN TIMING ON NOSZ MEDIATED N2O CONSUMPTION

2.1. Summary

Agricultural soils account for less than 10% of the total greenhouse gas (GHG) emissions in the USA but about 75% of nitrous oxide (N_2O) emissions. Soil conservation practices, such as no-tillage, have the potential to mitigate GHG emissions. We examined the short-term consequences of no-tillage with a winter wheat cover crop (NTW) and no-tillage winter fallow (NT) on N_2O emissions, N_2O reducing bacterial populations, and overall soil bacterial abundance during the summer growing season in the southern Great Plains, USA. Conservation practices were coupled with nitrogen (N) fertilizer application timing (100% pre-plant, 100% mid-season, 40% pre-plant 60% mid-season, 100% pre-plant with N stabilizer). In addition, N_2O emissions were measured to determine any functional effects of altering N fertilizer timing and changing bacterial populations. The combination of N treatment and conservation practice affected *nosZ* clade II abundance in the second year of the study. Diversity of *nosZ* clade II was evaluated to determine effects on non-typical N_2O reducers which were highly abundant in this study. No *nosZ* clade II diversity effects were determined, although some clustering of conservation system and N treatments was observed in the second year. Nitrogen treatment affected N_2O-N emissions during the summer of both years, likely related to overall increased microbial activity and N fertilizer application. Negative

fluxes (consumption) of N_2O-N were observed in every treatment and tillage combination and were most pronounced in the control (0 kg N ha^{-1}). Negative fluxes are likely due to a combination of low inorganic-N concentrations at various points during the year and a robust clade II population driving N_2O consumption. Altering conservation system and the timing of N fertilizer application affects the microbial community and will likely continue to select for unique communities as the system matures. This will also likely further impact N_2O emissions from the system and may increase the rate and frequency of N_2O consumption.

2.2. Introduction

Nitrous oxide (N_2O) is a long-lived atmospheric trace gas that has increased its concentration by 20% since preindustrial time (Butterbach-Bahl et al., 2013), has a global warming potential (GWP) approximately 300 times that of CO_2 (IPCC, 2007), and plays an important role in stratospheric ozone depletion (Griffis et al., 2017). Soil is the major global source of nitrous oxide (N_2O), accounting for about 78% of N_2O emissions in the USA in 2018 (EPA, 2020) where the predominant pathways for production are denitrification and nitrification (Bremner, 1997; Barnard et al., 2005; Mørkved et al., 2007; Reay et al., 2012). Denitrification most often occurs in anaerobic environments (Knowles, 1982; Linn and Doran, 1984) while nitrification is an aerobic process. How N_2O is produced is thus often dictated by soil moisture content (Linn and Doran, 1984; Fentabil et al., 2016), with soil texture and organic matter (OM) content impacting soil water holding capacity, and consequently N_2O production (Firestone and

Davidson, 1989; Bremner, 1997). Increased soil moisture and O_2 content affect nitrification and denitrification processes differently. With denitrification, the genes and enzymes responsible are typically O_2sensitive and thus increased soil moisture and reduced O_2would enhance this process and N_2O production (Betlach & Tiedje, 1981; Lloyd, 1993). For nitrification where reduced O_2conditions are present, the oxidation of NH_3 to NO can be the terminus of the nitrification process, resulting in a potential buildup of NO for soil microbes (Caranto & Lancaster, 2017). This NO may then be reduced to N_2O by those microbes, or through release to the environment and subsequent use by denitrifiers. With the oxygen sensitive nature of the enzymes responsible for N_2O reduction, production of N_2O from NO produced through the first step of nitrification in a mostly aerobic soil usually results in N_2O as the final product (Lloyd, 1993).

However, soil carbon (C) content is a strong control over N_2O production, compared to soil water, in semi-arid lands which cover approximately 35% of the terrestrial surface (McLain and Martens, 2006; Barton et al., 2008). In semi-arid lands, additional C inputs are derived from conservation practices such as cover cropping and no-tillage, which are most often implemented to mitigate eolian erosion and increase soil health factors. These practices have been shown to substantially decrease eolian losses (Zobeck & Van Pelt, 2011), but still only about 38% of cropped area used conservation tillage and 6% used cover crops in Texas in 2012 (NASS, 2012). Studies aimed at understanding the impact these changes have on crop productivity and carbon dioxide emissions in semi-arid areas have been conducted (Keeling, Segarra, & Abernathy, 1989; Lewis et al., 2018; McDonald, Lewis, & Ritchie, 2020; McDonald et al., 2019). In

more temperate climates, conservation tillage practices have been reported to not affect N_2O emissions, while in dry climates conservation practices have been reported to generally decrease N_2O emissions (Kessel et al., 2013). However, the effect of these conservation systems on N_2O reduction and reduction potential is less understood. Conservation practices can increase soil respiration, potentially leading to anaerobic microsites and influencing the production and consumption of N_2O and other greenhouse gases (Malhi et al., 2006; McLain and Martens, 2006; Barton et al., 2008; Halvorson et al., 2008; Smith et al., 2008). While anaerobic conditions are likely to increase with cover cropping and no-tillage, increased soil respiration, as brought about by the introduction of inorganic fertilizer and increased carbon inputs will also increase the growth and activity of denitrifiers in an otherwise "aerobic" agricultural soil (Wu et al., 2017). The increased potential for denitrification to occur under no-tillage is further supported by a meta-analysis regarding no-tillage effects on the abundance and activity of denitrifying communities (Wang and Zou, 2020). However, it is also under these conditions that the potential for N_2O mitigation can be derived through increasing the abundance and activity of clade II N_2O-reducing bacteria in anaerobic microsites.

The bacteria capable of N_2O reduction employ the nitrous oxide reductase (N_2OR) enzyme produced from two clades of the *nosZ* gene, clade I and clade II, both of which have been shown to mediate N_2O consumption, especially clade II (Jones et al., 2014). Clade II bacteria tend to be more abundant, diverse, and less likely to be associated with other denitrification genes (Jones et al., 2013; Jones et al., 2014; Higgins et al., 2016). These bacteria are hypothesized to use the reduction of N_2O to nitrogen gas

(N_2) as a bet-hedging strategy to survive brief periods of anoxic conditions such as those present in agricultural soil under irrigation, or where anaerobic microsites are expected (Lycus et al., 2018). In addition to environmental selection via fluctuations in soil O_2, low soil inorganic-N concentrations have been shown to increase N_2O consumption in soil (Ryden, 1981; Minami, 1997; Butterbach-Bahl et al., 1998; Rosenkranz et al., 2006; Kroeze et al., 2007). With N fertilizer application, an increase in soil N_2O emissions is to be expected due to overall increased N cycling as well as through competition for electrons between N_2OR and NO3- reducing enzymes (Barnard et al., 2005; Mania et al., 2014; Shelton et al., 2017). Thus, best management practices for mitigating N_2O emissions should include evaluation of N fertilizer application practices in addition to soil conservation strategies. Selecting for a system that can increase the potential for anaerobic microsites where N_2O reduction can occur while also reducing the amount of labile N for microbial use is challenging and imperative for economic and environmental sustainability in existing and developing semi-arid agricultural soil. Previous reviews have discussed the importance of N_2O consumption in soils, related to global greenhouse gas accounting, where a large potential for N2O consumption and understanding of the practices that enhance this capability could have significant impacts on reducing the effect of agricultural N_2O emissions (Chapuis-Lardy et al., 2007; Hallin et al., 2018). This research aimed to quantify the effects of implementing conservation practices coupled with N fertilizer management (timing of fertilizer application) on the abundance of N_2O-reducing genes, the diversity of the clade II population, and N_2O emissions over a two-year period in continuous cotton production on the Southern High Plains of Texas

(SHP, MLRA 77C). By examining these systems shortly after implementation, we can evaluate the immediate effects of these conservation practices on N_2O flux, while also providing valuable information for other factors that are considered with suggested changes in agronomic production. This study involved 15 unique treatment combinations of conservation practices and timing of fertilizer application for evaluation of N_2O-reducing potential, soil N resources, and in-situ N_2O production and consumption. In addition, the diversity of clade II N_2O reducers was evaluated in a subset of treatment combinations that are most commonly implemented in the study area to determine if implemented conservation practices and N fertilizer management selected for unique N_2O-reducing communities. It was hypothesized that N_2O consumption would be greatest where N fertilizer application was split or removed in a no-tillage system with a winter wheat cover crop and would be driven by an increased N_2O-reducing bacteria population. It was also hypothesized that commonly used treatment combinations would result in unique clade II communities due to their differential impacts on soil C and N inputs.

2.3. Material and Methods

2.3.1. Study Area and Cropping System

This study was conducted at the Texas A&M AgriLife Research and Extension Center in Lubbock, Texas (33.687°, 101.827°). The 30-year (1991-2020) temperature and rainfall averages for this area were 16.1°C and 481 mm, respectively (NOAA, 2021). The soil was an Acuff loam described as fine-loamy, mixed, superactive, thermic Aridic Paleustolls (U.S. Department of Agriculture, 2016). The study design was a split

plot with conservation practice as the main plot and N fertilizer application as the split plot arranged in randomized complete blocks. Main plot conservation treatments included the following: no-till with a winter wheat cover crop (NTW), no-till winter fallow (NT) and conventional tillage winter fallow (CT). Split plot N fertilizer application timings were 1) no-added N (control); 2) 100% of N applied in a pre-plant application (PP); 3) 100% of N applied mid-season (MS) at the first reproductive growth of the cotton crop; 4) 40% of N applied PP and 60% MS applied (SPLIT); and 5) 100% of N applied PP with a N stabilizer product (STB). The stabilizer product used was Limus® Nitrogen Management (N-butyl-thiophosphoric triamide and N-Propyl-thiophosphoric triamide, BASF Corporation, USA) a dual action urease inhibitor. Tillage main plots were randomly assigned to four rows (1-m row spacing) within each of the three blocks (replicates), and the N treatments were randomly arranged within each main plot, with each of the 5 N treatments were replicated within each tillage system. There were 45 plots measuring 15 m in length. Prior to seeding the cover crop in fall 2015, this area was under conventional tillage, winter fallow management for at least the last 60 years.

Nitrogen fertilizer was applied via knife injection using a coulter fertilizer applicator at a total rate of 168 kg N ha^{-1} placed 10-15 cm from the cotton row, as urea ammonium nitrate (UAN-32, 32-0-0). Preplant N treatments were applied on 10 May 2016 and 11 May 2017, and MS applications on 13 July 2016 and 20 July 2017. Wheat (TAM 304) was planted on 25 January 2016, and 22 November 2016 at a seeding rate of 67 kg ha-1 (19 cm row spacing). The planting on 25 January 2016 was a re-plant after a

failed stand due to low soil moisture and precipitation at planting in November 2015. Glyphosate [N-phosphonomethyl glycine] at 2.2 kg active ingredient (a.i). ha^{-1} in 2016 and at 3.5 kg a.i. ha^{-1} in 2017 was used to chemically terminate the wheat cover crop on 13 April 2016 and 20 April 2017. Cotton (Delta-Pine 1321) was planted on 26 May 2016 and 6 June 2017 at a rate of 123,553 seeds ha^{-1} and harvested on 14 November 2016 and 15 November 2017. Furrow irrigation (152 mm) occurred on 1 July 2016, 27 July 2016, 13 August 2016, 6 June 2017, and 30 July 2017. The full field management procedure was reported previously (McDonald et al., 2020). Climate data for the area was collected from the National Oceanic and Atmospheric Administration weather station 2 km south of the research site at the Lubbock International Airport, TX, US.

2.3.2. Soil Analysis

Soil samples were collected prior to N fertilizer application on 8 April 2016, and prior to the in-season mid-season application of N fertilizer in each year (1 July 2016 and 19 July 2017) using a 5.1 cm diameter Giddings probe (Giddings Machine Company, Windsor, CO). Pre-season soil analysis was reported previously (Table S2.1, McDonald et al. 2019). In-season soil samples were collected to a depth of 60 cm and subdivided into three increments: 0-15, 15-30, and 30-60 cm. A representative soil sample for each plot was collected by compositing two cores from each plot. Data from the 15-30 cm and 30-60 cm depth are not presented here. Sub-samples (50 ml) of the in-season soil samples (0-15 cm depth) were stored at -80ºC until DNA extraction for microbial analysis. The remainder of the in-season samples were mechanically ground to

pass a 2-mm mesh screen after drying at 60ºC for seven days. Soil samples were then stored at room temperature until nutrient analysis.

The samples collected in-season were analyzed for ammonium (NH_4^+-N) and NO_3^-- N by extracting with 2M KCl using a 1:5 soil to extractant ratio (5 g soil and 25 mL 2 M KCl). Extracted samples were then analyzed for NH_4^+-N by the Berthelot reaction involving salicylate and NO_3^- by cadmium reduction prior to analysis using flow injection spectrometry (FIAlab 2600, FIAlab Instruments Inc., Bellevue, WA; Keeney and Nelson (1982).

2.3.3. N_2O Flux Measurements

Full details of the gas sampling procedure are outlined in McDonald et al. (2019). Gas samples were collected monthly as well as 1, 3, and 7 days post-fertilizer application (weather permitting) in both years (13 samplings from April 2016-March 2017; 12 samplings from April 2017-December 2017). A Gasmet DX-4040 portable FTIR (Fourier Transform Infrared) multi-gas analyzer (Gasmet Technologies, Helsinki, Finland) integrated with a 20 cm diameter Li-Cor survey gas chamber (Li-8100-103, Li-Cor Biosciences, Lincoln, NE USA) was used for in-situ gas flux analysis. A PVC collar (19.5 cm diameter, 11 cm height) was placed between the 2nd and 3rd row of each plot at least 10 hours prior to sampling at a depth of at least 3 cm below the soil surface. During sampling, the Li-Cor chamber was deployed on each collar for 8 minutes, with a 20 second sampling time to yield 24 samples per plot. Linear regression of N_2O concentration over the deployed time was conducted and fluxes with an r^2 of >0.7 were considered significant. The slope of the trendline was then calculated and used to

determine the soil gas flux with the ideal gas law (McDonald et al. 2019). The minimal detectable concentration of N_2O for the Gasmet DX4040 is 7 µL L^{-1} (J. Cornish, Gasmet Technologies, personal communication, 8 November 2016). The data was sorted into seasons according to the major cotton growing periods of the year where Spring was April through May, Summer was June through September, and Fall/Winter was October through March. These seasonal determinations coincide with pre-plant field operations for the spring, the major growing season in the summer, and the harvest and post-harvest season for the Fall/Winter on the SHP, respectively. Cumulative fluxes were determined from cover crop termination (13 April 2016, 20 April 2017) through cotton harvest (14 November 2016 and 15 November 2017). Calculation of cumulative emissions was determined by averaging the two most recent daily flux rates and extrapolating over the time between the two flux rate measurements. Fluxes of N_2O for this study are considered baseline rates, due to complications in gas measurement following major rainfall and furrow irrigation events related to field access and equipment capabilities. However, these moisture events are infrequent on the SHP (29 days with >10 mm across 2016 and 2017, Menne et al., 2012a; Menne et al., 2012b) and thus "dry" measurements would represent the most common soil flux rate. Cumulative emission calculations estimated the total baseline N_2O flux during the crop growing season to determine potential treatment differences in this semi-arid agricultural system and likely underestimate total N_2O flux across the year due to the inability to measure post-wetting emission events. Winter fluxes of N_2O were measured between the first and second year

of the study and were determined to be low, or negative and thus likely do not significantly contribute to total N_2O emissions from the treatments evaluated here.

2.3.4. Microbial Analysis

In addition to in-season soil chemical measurements, microbial analyses were performed for the 0-15 cm depth and included: qPCR of the 16S, *nosZ* clade I, and *nosZ* clade II genes; sequencing of the *nosZ* clade II genes. DNA from soil samples was extracted using DNeasy PowerSoil DNA isolation kits (Qiagen, Germantown, MD, USA) according to manufacturer protocol with a 5 min incubation at 2-8ºC following solution C2 addition. A NanoDrop spectrometer (ND-1000, NanoDrop Technologies, LLC, DE, USA) was used to assess DNA purity after extraction. Quantitative polymerase chain reaction (qPCR) was conducted on extracted DNA to determine overall bacterial abundance as well as the abundance of both clades of the *nosZ* gene. Extracted DNA was quality checked for qPCR inhibitors in a process similar to Hartman, Coyne, and Norwood (2005) where a spiked sample with a threshold cycle (C_t) value within three standard deviations of the quality control C_t mean was determined to not contain inhibitors. Quality control was conducted by spiking a qPCR assay with extracted DNA from the collected soil samples. The assay chosen quantified the abundance of *Vibrio alginolyticus* with *gyrB* as the gene target (Zhou et al., 2007), and no added DNA from collected soil samples inhibited the reaction. Quality control qPCR was conducted on a Bio-Rad CFX96 Touch Real-Time PCR Detection System (Bio-Rad Laboratories, Inc, Hercules, CA, US). Non-quality-control qPCR was conducted using an Eppendorf Mastercycler epgradient realplex^2 (Eppendorf North America, Hauppauge,

NY, US) with the programs outlined in Table 2.1. Each program began with a denaturing step at 95^{o}C with the 16S and *nosZ* clade I running for 15 min and the *nosZ* clade II running for 5 min. Both the 16S and clade II programs run for 40 cycles while the clade I program was a touchdown program from 67 to 62^{o}C where it then ran for 40 cycles at 62^{o}C.

Table 2.1 Quantitative polymerase chain reaction (qPCR) primers and cycling profiles for total bacterial abundance and nitrous oxide reductase clade I and II.

Target Group	Primers	Primer Sequence	PCR Cycling Profile	Reference
16S rRNA	Eub338	5'- ACTCCTACGGGAGGCAGCAG -3'	95°C/15 min; 95°C/60 s, 53°C/30s. 72°C/60s × 40 cycles	(Fierer et al., 2005)
	Eub518	5'- ATTACCGCGGCTGCTGG -3'		
Nitrous oxide reductase clade I	nosZ2F	5'- CGCRACGGCAASAAGGTSMSSGT -3'	95°C/5 min; 95°C/15s, 67-62°C/30S, 72°C/30s; 95°C/15s, 62°C/30S, 72°C/30s × 34 cycles	(Henry et al., 2006)
	nosZ2R	5'- CAKRTGCAKSGCRTGGCAGAA -3'		
Nitrous oxide reductase clade II	nosZIIF	5'- CTIGGICCIYTKCAYAC -3'	95°C/30 s; 95°C/15s, 54°C/30s, 72°C/45s, 78°C/10s × 40 cycles	(Jones et al., 2013)
	nosZIIR	5'- GCYTCGATVAGRTTRTGGTT -3'		

Sequences of *nosZ* Clade II were amplified with the primers listed in Table 2.1 and determined with a Pac-Bio Sequel (MR DNA, Shallowater, TX) due to the length of the *nosZ* clade II gene (~700 bp). Sequence depth was about 5000 sequences per sample.

Sequences were trimmed with cutadapt (Martin, 2011), and denoised and dereplicated using DADA2 in R (Benjamin J. Callahan et al., 2016; Benjamin J Callahan et al., 2019). Sequences were then uploaded to qiime2 (Bolyen et al., 2019) and low abundance sequences were removed prior to analysis (minimum 5 sequence occurrence and present in at least 2 samples). Downstream analysis was conducted in qiime2 including taxonomic classification using a Naïve Bayes classifier trained on about 5000 *nosZ* sequences from both the National Center for Biotechnology Information (NCBI) database and the FunGene repository (Fish et al., 2013). Reference sequence taxonomy was downloaded from NCBI via the Entrez Direct module (Kans, 2020) and annotated to emulate the 16S Greengenes taxonomy file. Bray-Curtis dissimilarity PCoA plots were constructed in R with ggplot2 (Wickham, 2016). The eight most abundant amplicon sequence variants (ASVs) were chosen for further analysis where each ASV individually represented at least 2% of the total number of sequences (27408 total sequences) and together amounted to approximately 40% of the total sequence count. These eight abundant ASVs were chosen for Basic Local Alignment Search Tool (BLAST).

2.3.5. Statistical Analyses

Data was analyzed using Proc GLIMMIX at a significance level of α=0.1 for soil N concentrations, N_2O emissions, and microbial analysis using SAS version 9.4 (SAS Institute Inc., Cary, NC). Statistical analysis of N_2O flux rate determined a year interaction effect with N treatment (p=0.015), so data was analyzed within year. In addition, an interaction of season (fall/winter, spring, and summer) and N treatment was determined for N_2O flux rate in 2016 (p=0.003), so data was analyzed within season for

both years of the study. Analysis of cumulative emissions was conducted for emissions occurring between cover crop termination (13 April 2016, 20 April 2017) through cotton harvest (14 November 2016 and 15 November 2017) for each year of the study due to a significant year interaction with N treatment (p=0.025). Soil inorganic N analysis was conducted for the 0-15cm depth, and a year interaction with N treatment was determined (p<0.001) so NO_3^--N and NH_4^+-N were analyzed within year. For all analysis of variances, main-plot treatments (NTW, NT, CT) as well as split-plot treatments (control, PP, MS, SPLIT, STB) were treated as fixed effects and replication and replication by conservation system was treated as a random effect. Fisher's protected LMS was used to separate means of significant effects at α=0.1, unless otherwise stated. Correlation analysis was conducted using Pearson's correlation, proc CORR, and regression analysis, proc RSREG and proc REG (SAS, 2013) for determination of any correlation or relationship between soil chemical (N concentrations), biological (microbial abundance and diversity), cover crop biomass, and N_2O-N flux rates and cumulative emissions. Bray-Curtis dissimilarity was determined in qiime2 (Bolyen et al., 2019); principal coordinates analysis plots were conducted in R with ggplot2 (Wickham, 2016); PERMANOVA and PERMDISP were conducted in R with the vegan package (Oksanen et al., 2019). Average sequence abundance within treatment for the eight most abundant features was calculated across both years due to no significant interactions of sequence percentage within year (p<0.05).

2.3.6. Accession Numbers

Demultiplexed, dereplicated, and denoised sequences were uploaded to the Sequence Read Archive (SRA) database under the accession number: PRJNA612879.

2.4. Results

2.4.1. Climatic Conditions

The climatic conditions collected in 2016 were more typical for the SHP, with average temperatures of 29ºC and 25ºC in July and August, respectively, and cooling off to an average temperature of 12ºC in November. Monthly precipitations of about 93 mm and 77 mm occurred in May and August, respectively. Monthly precipitation in 2017 did not reach greater than 35 mm until June, although monthly precipitation was about 148 mm in July and 123 mm in August. Average temperature in May 2017 was about 21ºC, but with a maximum temperature of about 39ºC, which was greater than the maximum temperature in August 2016. The hot start to the 2017 growing season continued into June where the average temperature was about 27ºC, with a maximum temperature of about 44ºC. July 2017 had a similar average temperature as June 2017, although the maximum temperature reached was only about 38ºC (Fig. 2.1).

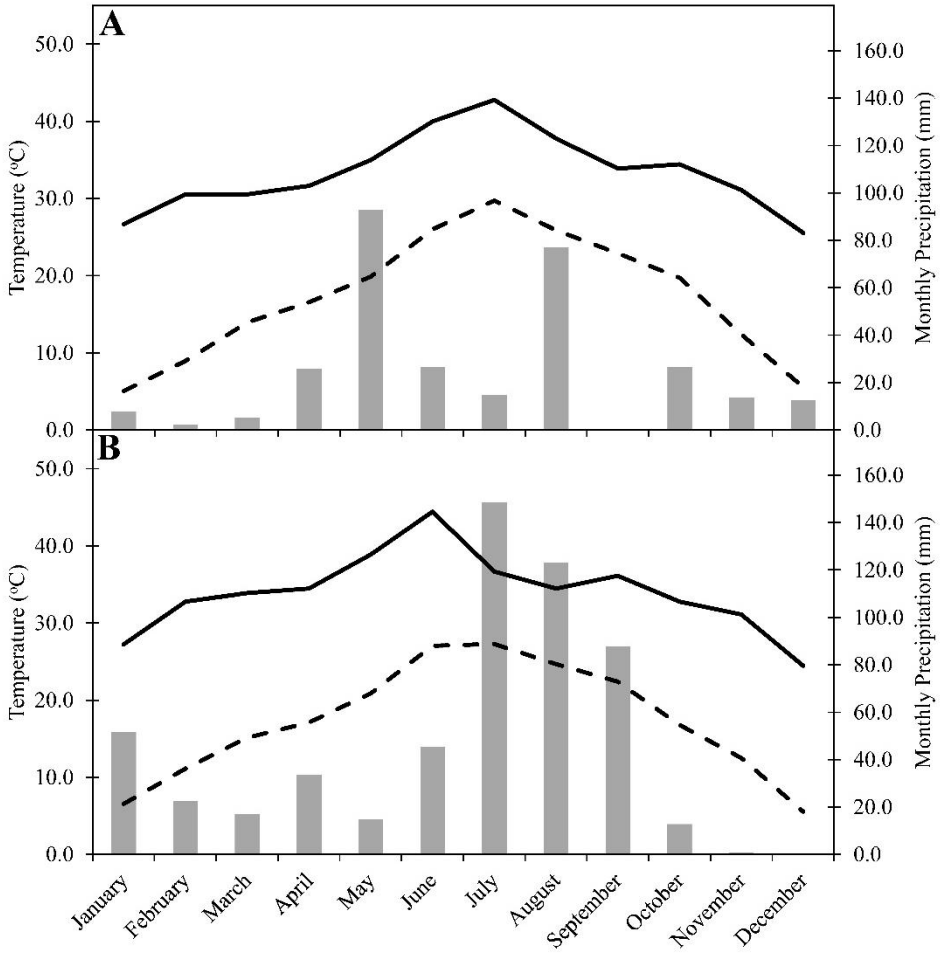

Figure 2.1 Monthly maximum temperature (ºC), monthly mean temperature (ºC), and monthly cumulative precipitation in (A) 2016 and (B) 2017. Data was collected from the National Oceanic and Atmospheric Administration weather station 2 km south of the research site at the Lubbock International Airport, TX, US (GHCND:USW00023042).

2.4.2. Soil Mineral Nitrogen Content

Conservation system did not affect NO_3^--N or NH_4^+-N concentrations in 2016 at the 0-15 cm depth (Table S2.2). Conservation system affected NO_3^--N concentrations in 2017 (p=0.089), with the NTW system having lower concentrations than the CT systems at 0-15 cm. Nitrogen treatment affected NO_3^--N concentrations in 2016 (p=0.002) and

2017 (p=0.002) with concentrations for the PP, SPLIT, and STB treatments being greater than the MS treatment and the control in 2016. In 2017, the PP treatment had greater concentrations of NO_3^--N than the MS and SPLIT treatments and the control, with the STB treatment also having greater NO_3^--N than the MS treatment and the control. The interaction of conservation system and N treatment also affected NO_3^--N concentrations in 2017 (p=0.021, Table 2.2). Main plot and split-plot differences were not determined for in-season NH_4^+-N levels in either year (Table S2.2). Many of the samples contained NH_4^+-N concentration lower than the detectable limit, which may have led to the lack of differences present within either year (analysis included a zero standard and thus zero values for NH_4^+ were treated as zero in statistical analysis). However, there was an interaction effect on NH_4^+-N concentrations in 2017 (p=0.078, Table 2.2). Total inorganic N (N_{inorg}) was calculated as the sum of NO_3^--N and NH_4^+-N. No differences in N_{inorg} were determined for main plot, or the interaction in 2016 (Table S2.2). In both 2016 and 2017, both N treatment significantly affected N_{inorg} (p=0.005, p=0.003, respectively), with the PP and STB treatments having greater N_{inorg} concentrations than the MS treatment and the control in both years. The interaction of conservation system and N treatment affected N_{inorg} concentrations in 2017 (p=0.013, Table 2.2).

Table 2.2 Nitrate-N (NO_3^--N) and ammonium-N (NH_4^+-N) concentrations (0-15 cm depth) prior to mid-season (MS) N fertilizer application

Year	Tillage System[a]	N Timing[b]	NO_3^--N		NH_4^+-N		N_{inorg}[d]	
			mg kg^{-1}	SD[c]	mg kg^{-1}	SD	mg kg^{-1}	SD
2016	NTW	Control	5.7	3.6	8.9	6.7	14.7	8.3
		PP	38.0	26.4	25.5	24.0	63.5	48.9
		SD	0.0	0.1	14.4	0.1	14.5	0.1
		SPLIT	23.7	24.6	7.9	4.1	31.6	27.4
		STB	41.4	34.6	20.2	23.8	61.5	47.4
	NT	Control	4.8	4.7	7.2	6.2	12.0	5.5
		PP	42.2	24.2	14.0	8.3	56.2	32.2
		SD	5.3	2.9	4.9	3.0	10.2	4.6
		SPLIT	23.3	15.3	5.4	2.8	28.6	12.5
		STB	41.1	14.7	11.0	7.7	52.2	18.8
	CT	Control	16.1	14.4	5.3	5.8	21.4	13.9
		PP	34.3	20.6	17.6	13.6	51.9	31.7
		SD	23.2	25.0	11.7	3.5	34.9	21.7
		SPLIT	43.6	15.3	10.5	9.2	54.1	19.8
		STB	29.6	16.5	9.2	5.7	38.7	22.1
2017	NTW	Control	3.5 ef[e]	5.2	3.2 bc	2.8	6.7 de	7.3
		PP	18.1 c-f	12.8	2.3 bc	0.6	20.4 a	12.8
		SD	0.5 f	0.9	0.2 c	0.3	0.7 cde	1.2
		SPLIT	12.1 def	12.2	5.3 c	9.2	17.4 ab	21.2
		STB	28.5 b-e	45.9	3.7 bc	6.4	32.2 de	52.2
	NT	Control	4.1 ef	6.2	1.7 c	3.0	5.8 de	5.2
		PP	37.9 a-d	33.5	2.5 bc	3.6	40.4 abc	34.0
		SD	1.4 f	2.1	0.0 c	0.0	1.4 e	2.1
		SPLIT	2.2 f	2.1	0.0 c	0.0	2.2 e	2.1
		STB	41.3 abc	34.3	13.1 a	16.5	54.4 ab	46.3
	CT	Control	4.4 ef	1.9	0.3 c	0.5	4.7 de	1.7
		PP	63.0 a	35.3	5.2 bc	2.3	68.2 cde	33.3
		SD	17.4 c-f	6.7	0.0 c	0.0	17.4 e	6.7
		SPLIT	49.8 ab	5.7	8.1 ab	8.4	57.9 cde	14.1
		STB	4.3 ef	4.6	0.0 c	0.0	4.3 bcd	4.6

[a] NTW, no-till with winter wheat cover; NT, No-till winter fallow; CT, conventional tillage winter fallow

[b] Control, 0 added nitrogen (N) fertilizer; PP, 100% preplant; MS, 100% mid-season; SPLIT, 40% preplant, 60% mid-season; STB, 100% preplant with N stabilizer

[c] SD, Standard Deviation

2.4.3. Microbial Abundance

Abundance of 16S genes in the 0-15 cm depth of soil was not affected by year interactions with conservation system, N treatment, or the interaction of conservation system and N treatment, and thus years were combined for further analysis. Conservation system (p=0.932), N treatment (p=0.608), and interaction effects (p=0.917) were not significant when years were averaged. The abundance of 16S gene copies was also analyzed by year due to significant year effects determined for other measured parameters. There was no difference in 16S gene abundance due to conservation system, N treatment, or their interaction in 2016 (Table S2.3). In 2017, there were also no conservation system or interaction effects (Table S2.3), although N treatment did affect 16S abundance (p=0.004). The control treatment ($5.43*10^8$) was determined to have a greater 16S abundance than the SPLIT ($4.21*10^8$) and PP treatments ($3.98*10^8$) in 2017, as well as the MS treatment ($5.12*10^8$) having a greater abundance than the PP treatment. Clade I abundance was not affected by N treatment, conservation system, or the interaction of N treatment and conservation system (Table S2.3). Clade II was affected by the interaction of conservation system and N treatment in 2017 (p=0.081, Table 2.3), but not conservation system or N treatment (Table S2.3).

Table 2.3 Total bacteria, nosZ clade I, and nosZ clade II abundance in 2016 and 2017

Year	Conservation system[a]	N Treatment[b]	16S Abundance (copies gram soil^{-1})	Clade I Abundance (copies gram soil^{-1})	Clade II Abundance (copies gram soil^{-1})	
2016	NTW	Control	3.19E+08	5.18E+05	1.42E+08	
		PP	2.65E+08	4.06E+05	1.43E+08	
		MS	2.29E+08	4.29E+05	1.52E+08	
		SPLIT	2.97E+08	4.48E+05	1.78E+08	
		STB	3.58E+08	6.47E+05	2.34E+08	
	NT	Control	2.48E+08	4.42E+05	2.04E+08	
		PP	2.45E+08	3.97E+05	1.68E+08	
		MS	2.51E+08	5.38E+05	2.12E+08	
		SPLIT	1.78E+08	2.60E+05	1.10E+08	
		STB	2.34E+08	3.50E+05	1.46E+08	
	CT	Control	1.94E+08	4.14E+05	8.85E+07	
		PP	2.21E+08	3.61E+05	1.78E+08	
		MS	2.06E+08	2.87E+05	1.82E+08	
		SPLIT	2.49E+08	3.43E+05	1.34E+08	
		STB	2.51E+08	6.09E+05	2.50E+08	
2017	NTW	Control	5.42E+08	1.13E+05	7.80E+07	abcd[c]
		PP	3.12E+08	8.78E+04	8.55E+07	abc
		MS	4.88E+08	1.17E+05	6.84E+07	abcd
		SPLIT	4.78E+08	8.64E+04	5.71E+07	abcd
		STB	4.39E+08	9.73E+04	5.32E+07	bcd
	NT	Control	5.71E+08	1.33E+05	9.44E+07	a
		PP	4.54E+08	1.16E+05	4.76E+07	cd
		MS	5.47E+08	1.48E+05	7.25E+07	abcd
		SPLIT	3.44E+08	8.68E+04	4.83E+07	cd
		STB	4.55E+08	1.13E+05	4.50E+07	d
	CT	Control	5.16E+08	1.40E+05	8.05E+07	abcd
		PP	4.29E+08	7.50E+04	5.75E+07	abcd
		MS	5.00E+08	1.15E+05	6.03E+07	abcd
		SPLIT	4.42E+08	1.08E+05	8.94E+07	ab
		STB	5.80E+08	1.51E+05	9.38E+07	a

[a] NTW, no-till with winter wheat cover; NT, No-till winter fallow; CT, conventional tillage winter fallow

[b] Control, 0 added nitrogen (N) fertilizer; PP, 100% preplant; MS, 100% mid-season; SPLIT, 40% preplant, 60% mid-season; STB, 100% preplant with N stabilizer

[c] LMS letters should be compared across all tillage and N treatments

With no main plot or split plot effects determined within each year, except for the interaction effect on clade II abundance in 2017, N_2O-reducing population abundance was summarized across treatments. Clade I abundance in 2016 and 2017 was about 3.950×10^5 and 1.12×10^5 gene copies g $soil^{-1}$, respectively. Clade II abundance in 2016 was about 1.68×10^8 and 6.88×10^7 gene copies g $soil^{-1}$ in 2017 (Fig. 2.2). The relative abundance of clade I was about 0.16% 16S abundance in 2016 and about 0.02% 16S abundance in 2017. The relative abundance of clade II was about 67% of the 16S abundance in 2016 and 14% of 16S abundance in 2017. No correlations were detected between microbial abundance and soil N concentrations (α=0.05, Table S2.4).

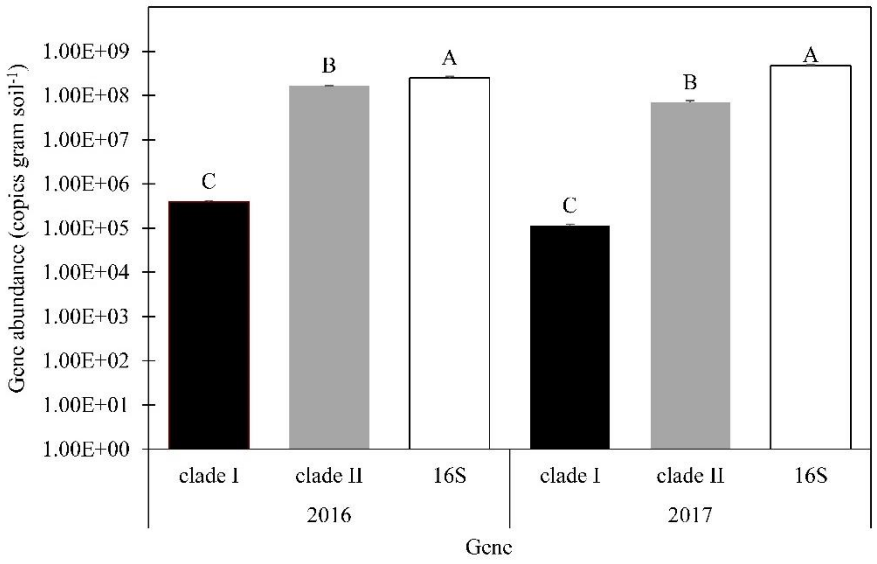

Figure 2.2 Average abundance of nosZ clade I clade II and 16S in 2016 and 2017. Error bars represent standard error. LMS letters are different within year at p<0.05.

2.4.4. Microbial Diversity

Due to the much larger abundance of clade II compared to clade I organisms present, sequence analysis of *nosZ* clade II was conducted on a subset of the in-season samples for 2016 and 2017. The subset included the PP treatment and the control from the CT and NTW systems, which were selected for the greatest potential differences and most common agronomic practices. All three replicates of each combination were sequenced for a total of 24 samples. It was determined that alpha diversity was not affected by conservation system or N treatment (data not shown).

No differences in distance within groups were determined to be significant, so PERMANOVA was conducted to evaluate Bray-Curtis dissimilarity. It was determined that no factors (Year, Tillage, Treatment) or their interactions affected dissimilarity of the microbial communities (α=0.05, Table 2.4). Furthermore, dissimilarity was analyzed with principal coordinates analysis and no distinct clustering occurred with treatment and tillage combinations over the two years (Fig. 2.3), clustering occurs with similar values of dissimilarity, meaning a treatment with similar dissimilarity (across replication) compared to the rest of the treatments would be more tightly clustered. The principal coordinates axes combined to explain 35% of the variability within the data (PC1: 22%, PC2: 13%; Fig. 2.3).

Table 2.4 PERMANOVA results for Bray-Curtis dissimilarity and Unweighted Unifrac distance

Factor	Bray-Curtis dissimilarity	Unweighted Unifrac distance
	----------*p*-value----------	
Year	0.690*	0.156
Tillage	0.257	0.107
Treatment	0.855	0.310
Year*Tillage	0.772	0.847
Year*Treatment	0.122	0.069
Tillage*Treatment	0.746	0.650
Year*Tillage*Treatment	0.430	0.909

*$p<0.05$ significant

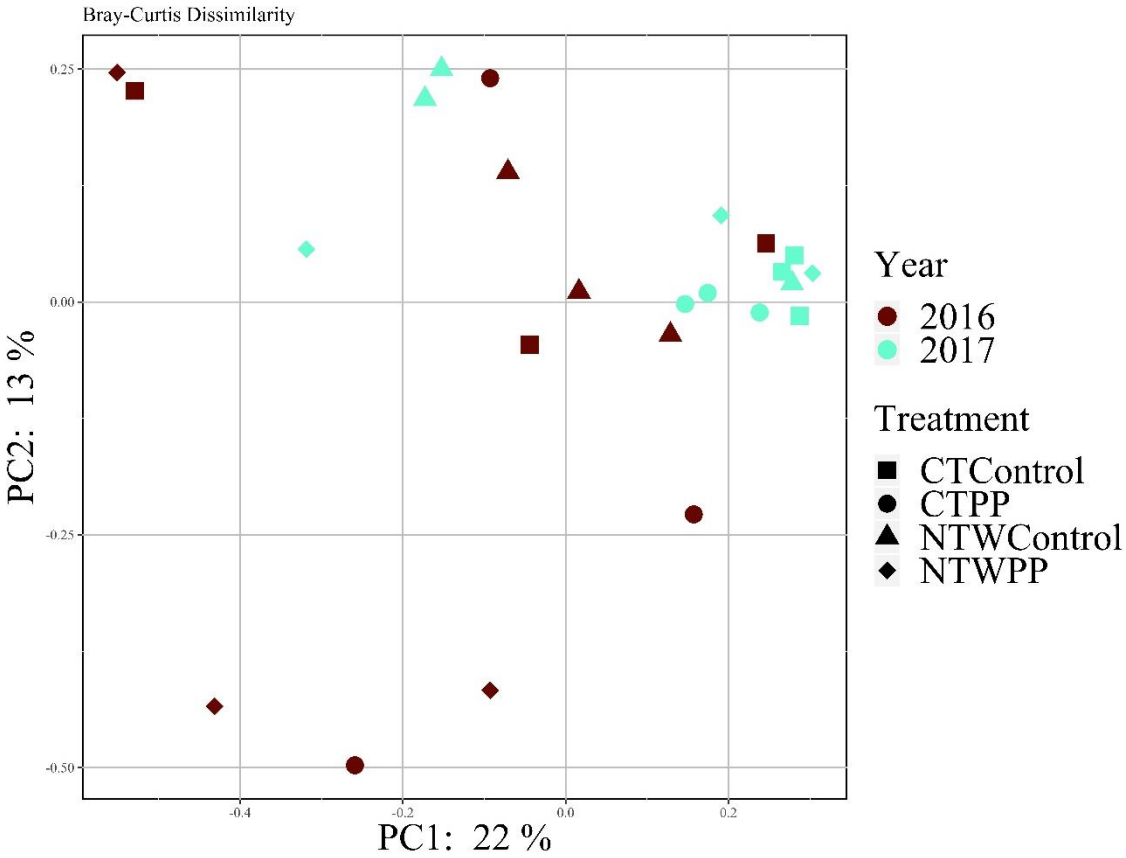

Figure 2.3 Bray-Curtis dissimilarity of microbial communities in 2016 and 2017. CT, conventional tillage; NTW, no-tillage with winter wheat cover crop; PP, pre-plant N application; Control, no-added N fertilizer.

Classification of ASVs with the NCBI and FunGene databases resulted in few sequences having deeper classification than domain. In general, there is less taxonomic information for functional genes such as *nosZ* clade II, leading to many of the samples being classified as "environmental samples", pointing to similar sequences within the databases that have yet to be classified. The most closely related taxonomically defined matches (≥75% match over >90% of the feature length, or closest match <75%) are reported for each of the eight most abundant ASVs (Table S2.5) to provide some insight into the microbial identity (Table 2.5). No year, treatment, tillage, or interaction effects were determined for the treatment abundance of any ASV (α=0.05).

Table 2.5 Abundance of dominant Amplicon Sequence Variants (ASV) and closest taxonomic match

Sequence Number	# of samples	ASV Count	% Total ASVs	Range of % of each Treatment	Taxonomy of Closest Match (Accession Number)	Query Cover	% Identity
1	20	2539	9.3%	0 - 26.3%	*Gemmatirosa kalamazoonesis* (CP007129.1)	99%	74%
2	10	2135	7.8%	0 - 72.1%	*Rhodobacter* (CP017781.1)	82%	69%
3	19	1882	6.9%	0 - 19.5%	*Cyclobacteriaceae* (CP058703.1)	99%	75%
4	16	1527	5.6%	0 - 16.1%	*Flavisolibacter tropicus* (CP011390.1)	99%	75%
5	14	1127	4.1%	0 - 10.7%	*Flavisolibacter tropicus* (CP011390.1)	99%	75%
6	2	591	2.2%	0 - 51.8%	*Gemmatirosa kalamzoonesis* (CP007129.1)	99%	76%
7	10	575	2.1%	0 - 9.9%	*Flavisolibacter* sp. (CP037755.1)	93%	76%
8	11	573	2.1%	0 - 8.1%	*Gemmatirosa kalamazoonesis* (CP007129.1)	99%	74%

2.4.5. Nitrous Oxide Flux Rates

Nitrous oxide emissions were analyzed within season for each year of the study. No differences were found between conservation systems, N treatments, or their interactions for the Spring and Fall seasons in both years of the study (Table S2.6). However, N_2O-N flux was affected by N treatment in the Summer of 2016 (p=0.013) and 2017 (p=0.076) with all the N treatments having a greater flux than the control in Summer 2016 (Fig 2.4A) and all N treatments except the PP treatment having a greater flux than the control in Summer 2017 (Fig. 2.4B). Negative fluxes and no N_2O flux were determined in the MS treatment and the control, respectively, in the spring of 2016 (Fig. 2.4A). Nitrous oxide consumption was also recorded in the Fall/Winter of both years for all treatments with the exception of the PP treatment in 2017 (Fig. 2.4A,B). No correlation between 16S, clade I, or clade II abundance and summer N_2O flux rate was determined (α=0.05)

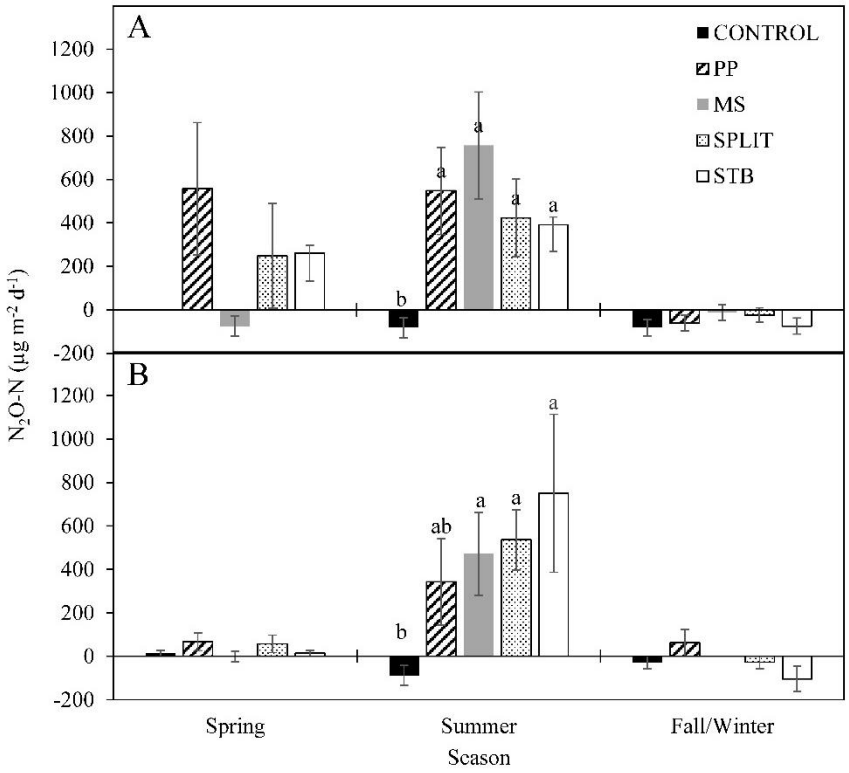

Figure 2.4 Nitrous oxide emissions in (A) 2016 and (B) 2017 averaged by season. PP, pre-plant nitrogen (N) fertilizer application; MS, mid-season N fertilizer application (applied at pinhead square); SPLIT, 40% PP 60% MS N fertilizer application; STB, 100% PP application with N stabilizer product. Spring, April – May; Summer, June – September; Fall/Winter, October – March. Seasonal treatment means with the same letter are not different at α=0.1. Error bars are standard error.

2.4.6. Cumulative Emissions

Nitrogen treatment impacted average growing season cumulative emissions in 2016 with the N treatments producing a greater average cumulative flux than the control (p=0.027, Fig. 2.5). The control had net negative cumulative emissions of N_2O-N in both years of the study (Fig. 2.5). Although not statistically different, cumulative emissions were lower for the STB treatment in 2016. No conservation system or conservation

system and N treatment interaction effects were determined in either year of the study (Table S2.7). In 2017, N treatment did not affect cumulative N_2O-N emissions. Emissions during the second year of the study were greatly increased in the STB treatment compared to the first year while the rest of the N treatments were slightly reduced

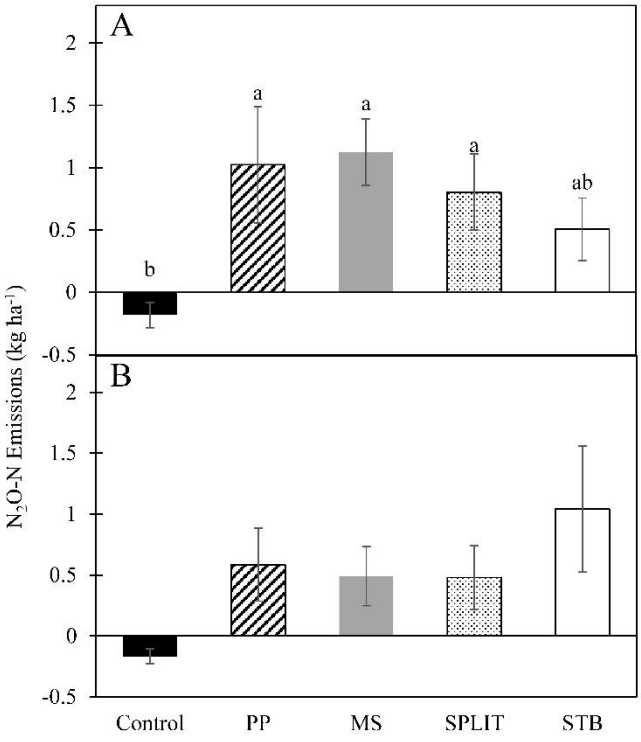

Figure 2.5 Average cumulative N2O-N emissions from cover crop termination to cotton harvest in (A) 2016 and (B) 2017. LSM letters are different at p < 0.05, error bars represent standard error. PP, pre-plant nitrogen (N) fertilizer application; MS, mid-season N fertilizer application (applied at pinhead square); SPLIT, 40% PP 60% MS N fertilizer application; STB, 100% PP application with N stabilizer product.

2.5. Discussion

Results of this study indicate that altering the timing of N fertilizer application will affect the microbial community, which may alter N_2O emissions. In the second year of N treatment implementation, bacterial abundance was reduced for the PP treatment compared to treatments with no N applied prior to sampling, the control and MS treatment. In addition, the SPLIT treatment had reduced bacterial abundance compared to the control. Both of these observations indicate potentially deleterious effects of high rates of N fertilizer application on overall bacterial abundance on the SHP, although other research suggests the deleterious effect of N fertilizer application may be less pronounced in agricultural systems where application is more common (Geisseler & Scow, 2014).

The reduction in the overall bacterial population due to N treatment did not translate to the N_2O-reducing community, but the interaction of N treatment and conservation system did affect clade II abundance in the second year of the study (Table 2.3). In addition, it was clear that clade II greatly outnumbered clade I, suggesting that N_2O reduction potential lies mostly within the more diverse, abundant, and efficient form of N_2O reductase (Sanford et al., 2012). Previous research supports greater clade II abundance in soil (Domeignoz-Horta et al., 2015; Jones, Graf, Bru, Philippot, & Hallin, 2013; Sanford et al., 2012), with clade II being associated with a lifestyle strategy involving the survival of anoxic conditions in a more energetically favorable way (Lycus et al., 2018). In agricultural soil, oxygen concentrations may change rapidly due to precipitation or irrigation events, as well as pore space O_2 concentrations changing due

to soil respiration so a more efficient survival mechanism such as clade II activation is helpful. Although there are no clear patterns across the entire study related to conservation system and N treatment, the most apparent differences in clade II abundance were within the NT system in 2017 where nutrient stratification and low soil C would both be expected. Where N fertilizer was applied before the season (PP, SPLIT, STB) clade II abundance was reduced and where N was not applied for at least 1 full year, clade II abundance was increased (MS, control). Previous research indicates that N_2O consumption can be enhanced during periods of low soil inorganic N (Butterbach-Bahl, Gasche, Huber, Kreutzer, & Papen, 1998; Kroeze, Bouwman, & Slomp, 2007; Rosenkranz et al., 2006) and thus would be expected for the control and MS treatment. Nitrous oxide consumption would thus likely be driven by clade II microbes in N limited environments. Nitrate can act as a proximal control over N_2O consumption where high concentrations can negatively affect the production of the N_2OR enzyme through competition for electrons (Highton et al., 2020). This was observed in the PP and STB treatments in 2017, where increased soil NO_3^--N due to pre-season application acted as a distal control, reducing the N_2O-consuming population at the time of sampling (Table 2.2). A similar but less distinct pattern was present for the CT system, while in the NTW system there was no apparent pattern (Table 2.2). The lack of a pattern in the NTW system could be due to several biological and physical phenomena including overall reduced soil inorganic N prior to the start of the season due to wheat cover crop use of residual soil N (Lyons et al., 2017). This would likely enhance the N_2O-reducing population over the winter and may conceal any effects on clade II abundance in-season

and will likely increase as the system matures and selects for a specific microbial community. The NTW system also encourages water infiltration, soil aeration, and soil C resources, all of which would have differential effects on anaerobic microbial processes further complicating any patterns within that system. Wang et al. (2021) determined greater clade II abundance with long term conservation tillage practices likely leading to greater moderation of N_2O emission within those systems, supporting previous determinations of conservation practice effects on N_2O emissions in systems in place for ≥10 years (Kessel et al., 2013). An increased population of clade II organisms with conservation tillage was not seen in this study, nor were N_2O reductions for conservation practices. However, this study comprised the first two years of conservation practice implementation for the research site, and it is possible that as these systems mature similar patterns will emerge. Abundance of *nosZ* clade I genes was correlated with the amount of aboveground wheat cover crop biomass (data not shown) produced in the NTW system during this study (p=0.005, McDonald et al., 2019), where clade I abundance linearly increased with increasing wheat biomass (p=0.0025, r^2 = 0.56). However, there was no relationship between *nosZ* clade II abundance and wheat biomass (p=0.100). The correlation between clade I abundance and wheat biomass was expected, as greater wheat residue would increase conditions favorable for denitrification, selecting for more typical, complete, denitrifiers such as those associated with *nosZ* clade I.

To further elucidate any patterns in potential N_2O consumption, sequence analysis was conducted for a subset of the conservation system and N treatments within

both years of the study. The subset included the PP treatment and the control from the CT and NTW systems, which were selected for the greatest potential differences and most common agronomic practices. Although no year, N treatment, conservation system, or interaction effects were determined for Bray-Curtis dissimilarity (Table S2.5), there is clearly some clustering of treatment and tillage combinations in the second year of the study (Fig. 2.3), indicating potential development of unique N_2O-reducing communities as also indicated with the significant conservation system and N treatment interaction for clade II abundance in the second year. In a previous study, homologs of *nosZ* were found in 12% of sequenced bacterial genomes (Graf, Jones, & Hallin, 2014), and while no significant classifications could be made from *nosZ* sequences alone in our study, the individual query of the most abundant features allowed for some taxonomic evaluations to be made from fully sequenced soil microbial genomes. These classifications included *Gemmatimonadetes* which are among the most abundant N_2O reducers in soil (Jones et al., 2013) and common soil bacteria. The association of such common soil bacteria with N_2O reduction speaks to the ubiquitous and environmentally relevant nature of N_2O reduction in soil, and further supports potential for the soil to act as an N_2O consumer even in semi-arid agricultural systems.

With the potential for N_2O consumption being observed in this study, N_2O emissions were measured throughout 2016 and 2017 (Figs. 4A,B). Negative fluxes of N_2O were determined during the fall/winter of both years of the study and are likely due to low levels of NO_3^--N present in the soil (Butterbach-Bahl et al., 1998; Kroeze et al., 2007; Minami, 1997; Rosenkranz et al., 2006; Ryden, 1981) and an abundant clade II

population. In the spring of 2016 (Fig. 2.4A) N_2O consumption was also determined for the MS treatment which had not yet received N fertilizer during the study period. Treatments with N fertilizer application increased N_2O emissions in Spring 2016. Increased emissions of N_2O are often associated with greater levels of NO_3^--N (Butterbach-Bahl et al., 1998; Kroeze et al., 2007; Mania, Heylen, van Spanning, & Frostegård, 2014, 2016; Minami, 1997) which can negatively impact the formation of the N_2O-reducing enzymes (Highton et al., 2020). Similar assumptions can be made regarding the negative and zero fluxes of N_2O-N in the fall/winter of 2016 and 2017 (Figs. 4A,B), where low levels of NO_3^--N would be present due to plant and microbial use of available soil N throughout the growing season. There is a significant increase in N_2O-N emissions during the summer season (Figs. 4A,B) which can be attributed to several factors including: increased temperature and moisture, increased plant and microbial activity, and the application of N fertilizer (Barnard, Leadley, & Hungate, 2005; Dobbie, McTaggart, & Smith, 1999; McSwiney & Robertson, 2005; Ryden, 1981; Shelton, Jacobsen, & McCulley, 2017). However, where N was not applied in the control, net consumption of N_2O-N was determined, further supporting the association of N_2O consumption in low inorganic N environments and providing evidence of an active N_2O-reducing community where clade II organisms play a significant role. After one year of treatment implementation, N_2O-N flux rates were reduced during the spring (Fig. 2.4B), which may be attributed to reduced NO_3^--N concentrations, and thus increased N_2OR activity. Monthly measurements were used for evaluation of treatment differences across the cotton growing season. Recent studies have reported the potential for under or

over estimation of total N_2O emissions with less frequent measurements (Su, Kang, Huang, & Fang, 2021), however our reported seasonal average emissions compare favorably with N_2O fluxes measured under similar climatic conditions (Domeignoz-Horta et al., 2018; Ryden, 1981; Shelton et al., 2017), although they were lower compared to emissions from wetter climates (Chantigny et al., 2010; Domeignoz-Horta et al., 2018). When the rate of N_2O consumption was compared, it was similar to previously reported rates in varied study areas indicating a functionally ubiquitous N_2O-consuming population in soil regardless of environment.

Cumulative emissions were calculated based upon monthly measurements of N_2O emissions and were positive for all treatments with N fertilizer application (Fig. 2.5). However, the control resulted in net negative emissions over the two-year study. The negative emissions recorded are likely the result of inorganic N loss (plant uptake, microbial use, leaching, etc.) from the system without significant replacement (Butterbach-Bahl et al., 1998; Kroeze et al., 2007; Rosenkranz et al., 2006) combined with selection for a N_2O-consuming population by both the soil chemical and physical characteristics. The calculation of cumulative emissions was conducted to estimate treatment effects on yearly N_2O emissions to determine potential best practices based on the data available. The total number of treatments evaluated for N_2O fluxes included 15 unique combinations of conservation systems and N treatments which required various field operations throughout the growing season to maintain production-level field conditions and thus installation of long-term chambers and stationary gas analysis was not feasible for a study of this size and scale. However, cumulative emissions produced

from this study were comparable to those from other studies in semi-arid and sub-humid regions (Dong, Kou, Yang, Chen, & Xu, 2018) although total emissions were potentially underestimated due to the inability to measure emissions following precipitation or irrigation events.

Although no definitive relationships can be observed between recorded N_2O emissions and the abundance of N_2O-reducing genes, it is clear that the treatments implemented here affect the pattern of N_2O emissions through alterations to the soil biological and chemical composition even within the first few years of implementation. It will thus be important to continue this research as the system matures to observe these expected changes and better elucidate how conservation system and N timing affect N_2O emissions in semi-arid agricultural systems. Understanding the mechanisms behind these changes in emissions, specifically the consumption of N_2O, will aid in the choosing of best management practices for reducing N_2O emissions in expanding semi-arid areas and potentially provide practices suited for net N_2O consumption.

2.6. Conclusions

Changes in N fertilizer management can alter the microbial community and change the rate at which N_2O gas is produced or consumed within semi-arid agricultural soils. The microbial community measured in this study contained an abundant *nosZ* clade II N_2O-reducing population that is likely the driver for N_2O consumption. The mechanisms behind the population shift are still being determined, but after two years of treatment implementation distinct communities appear to be forming which may further alter N_2O consumption and production. Based on the results from this study, it is likely

that a N treatment and conservation practice best suited for mitigating N_2O will emerge as the system matures.

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3. SOIL PORE SPACE GAS PROBES FOR USE IN AGRICULTURAL RESEARCH

3.1. Summary

Increasing agriculture intensification to meet global demand has sparked great interest in economic and environmentally sustainable cropping systems. One important measure of environmental sustainability is greenhouse gases production, which can be altered with soil health promoting practices such as cover cropping and no-tillage. Previously, the measurement of gas concentration and production in soils has been cost-prohibitive for factorial designs in agricultural research and may inhibit field operations. This study aimed to provide an affordable method for measuring below ground pore space concentrations of greenhouse gases. Testing was conducted in Lubbock, TX in 2019 in a cotton monoculture with gas sampling conducted about a month after fertilizer applications. Probes were installed at 7.5 and 15 cm depths and sampling was conducted with a portable Fourier-transform infrared gas analyzer. It was determined that consistent values of carbon dioxide (CO_2) concentration were measured with average CO_2 concentrations across tillage systems of 570 µL L^{-1} at 7.5 cm and 610 µL L^{-1} at 15 cm in June and 1050 µL L^{-1} at 7.5 cm and 1380 µL L^{-1} at 15 cm in August. Compared to pore space concentrations from similar soil moisture conditions, our measurements were highly comparable. Soil pore space probes used in this study provided comparable

measurement of soil gas concentrations at a low per-plot cost. In addition, our probes were able to be installed with minimal soil disturbance allowing for measurement in already established no-tillage systems and allowed for field operations to be conducted by placing probes within the crop row.

3.2. Introduction

Soil management practices such as no-tillage and cover cropping are associated with potential improvements in soil health and a reduction of environmental impacts of wind erosion in semi-arid environments (Lewis et al., 2018). As semi-arid environments spread with global climate change, understanding how soil conservation practices affect nutrient cycling, trace gas production, and productivity of cropping systems will be key to maintaining economic and environmental sustainability in semi-arid regions. Previous measures of trace gas production in agriculture have been focused on emissions from the soil surface (Martins et al., 2016; Zhang et al., 2018; McDonald et al., 2019) and in-ground concentrations of greenhouse gases (GHGs; Gao et al. 2014, DeSutter et al. 2008). Above ground concentrations are often measured with PVC collars covered with dynamic chambers integrated with portable gas analyzers such as Gasmet FTIR analyzers. However, pore space concentrations of gases are often measured using large static chambers or using semi-permeable tubing buried under crop production research

(Bekele et al. 2007, Flechard et al. 2007, Gao et al. 2014, Jassal et al. 2005, DeSutter et al. 2008). These measurements have provided vital information regarding the concentrations of trace gases in soil pores, but also introduce challenges for maintaining field operations and are cost prohibitive for research programs that use factorial research designs with large amounts of replication. In addition, these systems may be difficult to integrate into existing studies examining no-tillage and cover cropped systems where disturbance of the soil for implementation of these methods is not ideal.

Previous research conducted in the Arctic utilized steel pore space probes (PSPs) to measure gas concentrations of soil pores with minimal disturbance to the surrounding environment (Brummell and Siciliano, 2011). This concept is ideal for established no-tillage and cover cropped systems but is still cost-prohibitive for factorial designed agricultural research with many replications. Here we propose to use soil PSPs, constructed with more cost-effective materials, to measure soil pore space concentrations of trace gases in agricultural research systems evaluating soil conservation practice effects on crop productivity and soil health. By understanding GHG concentration at depth, we can begin to elucidate the effects of soil conservation practices on belowground cycling of GHGs and make better recommendations related to the environmental sustainability of these conservation practices.

3.3. Methods

3.3.1. Site and Study Information

Measurement of gas concentrations in the PSPs were conducted in June and August of 2019 in Lubbock, Texas (33.687°, −101.827°). The study used a split-plot design with the main plot being tillage system including no-till with a winter wheat (*Triticum aestivum)* cover crop (NTW), no-tillage winter fallow (NT), and conventional tillage winter fallow (CT). The split plot was nitrogen (N) treatment including 100% pre-plant N fertilizer application (PP), 100% mid-season N fertilizer application (MS), 40% PP and 60% MS application of N fertilizer (SPLIT), 100% PP with an N stabilizer product (STB), and a no added N control (McDonald et al., 2019). The N stabilizer product used was Limus® Nitrogen Management (N-butyl-thiophosphoric triamide and N-127 Propyl-thiophosphoric triamide, BASF Corporation, USA) a dual action urease inhibitor. Three replications of each tillage system and N treatment combination were used for this study and measurements were taken during vegetative and peak plant production, both about a month after fertilizer application, in a cotton monoculture. Plots were 4 rows wide (1 m row spacing) and 15 m long. The soil at the site is classified as an Acuff loam, described as fine-loamy, mixed, superactive, thermic Aridic Paleustolls (U.S. Department of Agriculture, 2016). Average soil pH in 2019 was 7.5. Conventional

tillage was in use at this research site for at least 60 year prior to no-tillage implementation in 2015. A second site located in Chillicothe, TX (34.194°, -99.530°) was also examined but due to issues related to probe installation, the results are not included.

3.3.2. Pore-Space Probe Construction

The PSPs for this study were constructed of 2.5 cm I.D. Schedule 40 PVC pipe with an end cap on the bottom (Fig. 1) with lengths of either 31 or 46 cm. For each PSP, twelve gas entry holes were concentrically drilled above the cap near the base and marks on the pipe were made to guide depth of insertion into the soil. Each PSP was installed by first creating a guide hole (5-10 cm from the plant row) by removing a soil core with a push hand probe (2.54 cm I.D., 2.97 cm O.D.; AMS Inc., American Falls, ID) to ease the force needed to insert the PSP and reduce horizontal compaction caused by displacement. Then the PSP was inserted into the soil to the desired depth, 7.5 cm for the shorter PSP and 15 cm for the longer PSP, using a 1.2 kg dead blow hammer. One of each probe length was placed in each plot with the difference in height above ground allowing for easy determination of measurement depth. The annular space around the PSP due to the larger cap size was backfilled with excavated soil from the guide hole and the PSPs were allowed to settle for at least 7 days prior to measurement. Once

installed, PSPs were capped with rubber stoppers (size 6) to prevent water and insect infiltration of the probes.

The Gasmet DX4040 FTIR (Gasmet Technologies, Helsinki, Finland) sample cell (0.5 L) was larger than the volume PSP alone and would create a vacuum on the soil pore space, selecting for gases in larger pores, and drawing gases from the soil surface in PSPs at shallower depths. The day prior to the sampling period PSPs were each connected to a separate reservoir bottle as outlined in Brummell and Siciliano (2011) to increase system volume above that of the sample cell. The equilibration time of the reservoir bottles is about 7 hrs and the system volume is increased to about 1.6 L. Reservoir bottles were made of high-density polyethylene (HDPE, VWR International, Radnor, PA, USA), which is not known to react with any of the gases expected in soil pores. The system is a closed loop system which would not create a vacuum on soil pores, but rather samples the equilibrated gases in the reservoir bottle and PSP. Tubes connecting the reservoir bottle to the probe were 6.4 mm o.d. and 3.2 mm i.d. Excelon Bev-A-Line IV tubing (Polyethylene liner with ethyl vinyl acetate shell, Thermoplastic Processes, Georgetown, DE) inserted through a rubber stopper (size 5) and sealed with weatherproof silicone on the bottom of the stopper (Fig. 3.1). Tubing was attached to the reservoir by inserting through a drilled hole in the cap of the reservoir bottle with rubber

grommets (Fig. 1, W. W. Grainger Inc., Lake Forest, IL, USA) acting as a seal. To aid in preventing water intrusion into the reservoir, and provide additional sealing of the reservoir cap, the rubber grommets were sealed with weatherproof silicone. A quick-connect was installed on one of the two tubes connecting the PSP and the reservoir bottle to allow for integration of a gas analyzer to the system, where the flow of the gas in the closed loop system would be out of the reservoir bottle, into the analyzer, and then back into the top of the PSP to complete the loop. With the smaller sample cell size, compared to the size of the whole PSP system, there would thus be no vacuum on the soil pore space, and thus no preferential selection. This does not allow for the determination of the area of pore space measured but provides an average concentration of gases from the pore space after equilibration.

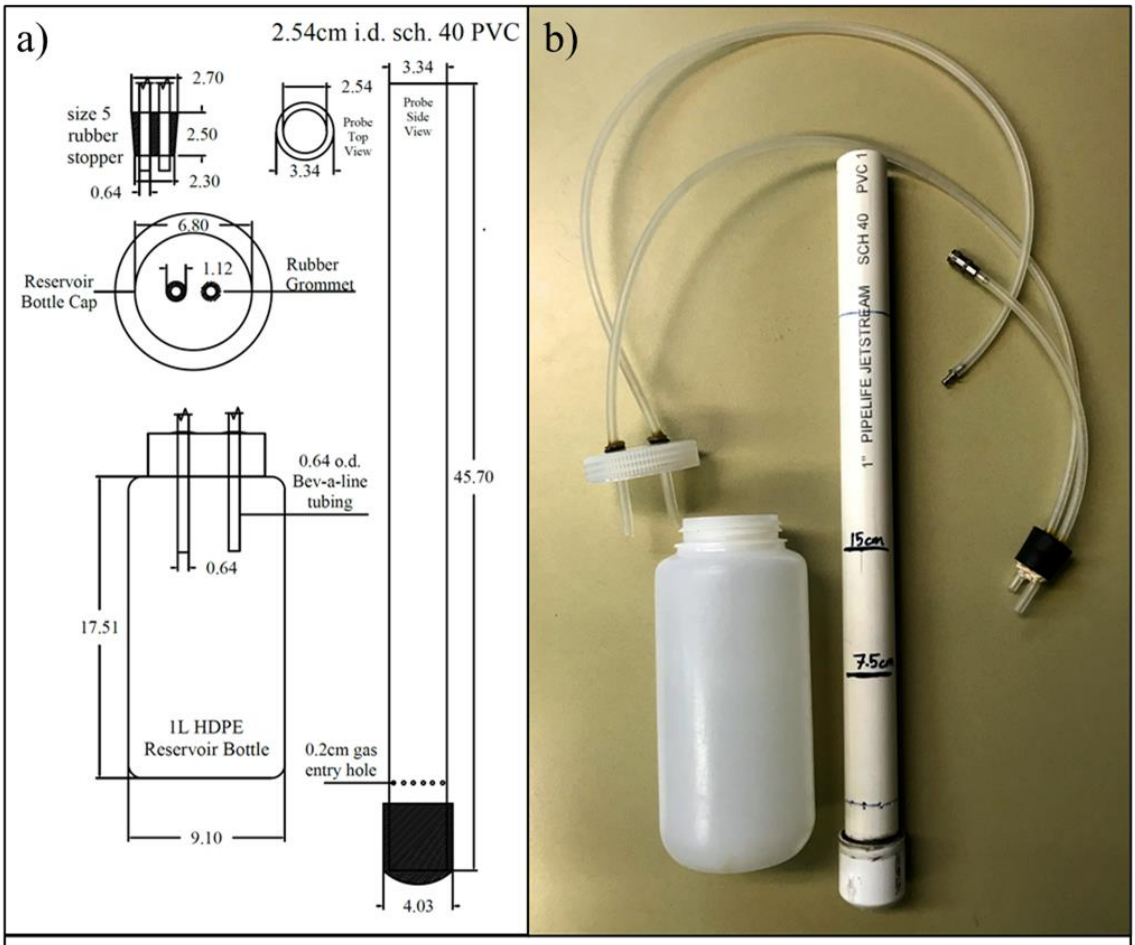

Figure 3.1 a) Schematic drawing of pore space probe (PSP) and reservoir bottle, all dimensions in cm. b) image of constructed PSP and reservoir bottle

3.3.3. Pore-Space Measurement

On the day of measurement, the Gasmet DX4040 analyzer was setup according to manufacturer protocol, with a zero-gas background taken using ultra-high purity di-nitrogen (N_2) gas. The CO_2 detection limit for this machine is < 20 µL L^{-1}. Multiple gas concentrations including nitrous oxide (N_2O), nitric oxide (NO), methane (CH_4), and CO_2 were collected, but only CO_2 was be analyzed for this manuscript due to the consistency and comparability to previous literature (Jassal et al., 2005; Bekele et al., 2007; DeSutter et al., 2008; Gao et al., 2014). Sampling of each PSP was conducted by attaching the Gasmet analyzer into the system at the metal quick connects. Gases were cycled into the sample cell (3 min sampling time) at a flow rate of 2 L min^{-1} and measured before being returned into the PSP in a closed loop system, with a 4-minute break between probes for the gas analyzer to return to atmospheric concentrations and allow for correction of PSP dilution due to gas in the sample cell being transferred into the attached PSP system. Sampling was conducted over an 8-12-hour period with soil surface temperature and moisture readings collected for each plot. On particularly high-temperature days (> 30°C max temperature), the readings were split over a two-day period, with sampling conducted in the morning, to reduce the temperature variation

which may cause changes in the soil microbial activity and the density of the gas present in the reservoir bottle. A hydrophobic filter and desiccator were used and were installed at a 90-degree angle from the gas inlet (Allan Bradley, Gasmet Technologies, personal communication, October 2019) to reduce water vapor intake by the FTIR which can stain the internal mirrors used in FTIR analysis.

3.3.4. Data Analysis and Gas Concentration Calculation

Data analysis was conducted in Excel where the volume of the PSP, reservoir bottle, and associated tubing was used to calculate the true gas concentration of the soil pore space. The calculation utilizes the atmospheric (or initial gas concentration) and the maximum reading to calculate the dilution of the PSP – reservoir bottle system with the connection of the Gasmet sample cell to the closed-loop system. The max reading (C_T) is a result of diluting the concentration of the sample probe (C_S, µL L^{-1}) with the concentration of the ambient air in the sample cell of the Gasmet FTIR (C_a) and their respective volumes (Brummell and Siciliano, 2011). The true concentration of the gases in the soil pore space can be calculated by:

$$C_S = \frac{C_T V_T - C_a V_{FTIR}}{V_S} \quad \textbf{Equation 3.1}$$

Where V_T is the volume of the whole system including the PSP, reservoir bottle, sample cell, and associated tubing, V_S is the volume of the PSP and reservoir bottle, and V_{FTIR} is

the volume of the sample cell within the gas analyzer (Brummell and Siciliano, 2011). Statistical analysis was conducted in SAS 9.4 using PROC GLIMMIX (SAS-Institute, 2017b) and PROC CORR (SAS-Institute, 2017a).

3.4. Results

Average CO_2 concentrations across tillage systems were 570 µL L^{-1} at the 7.5 cm depth and 610 µL L^{-1} at the 15 cm depth in June with no difference between depth at this early point in growing season (p=0.145). However, CO_2 concentrations in August differed with depth, averaging 1050 µL L^{-1} at the 7.5 cm depth and 1380 µL L^{-1} at the 15 cm depth (p=0.003). The implementation of tillage systems and N treatments and their interaction did not affect CO_2 concentrations at the 7.5 cm or 15 cm depth during the June measurements. (Table 3.1, Fig. 3.2). No N treatment or interaction effects were determined for either depth for the August sampling (Table 3.1, Fig. 3.2). Tillage system did affect CO_2 concentrations at the 15 cm depth in August, with the NT system having a greater concentration (1820 µL L^{-1}) of CO_2 than the NTW (1250 µL L^{-1} CO_2) and the CT (1060 µL L^{-1} CO_2) systems (Table 3.1). Although the August sampling was interrupted due to mechanical issues with the Gasmet DX4040 and only two replications were used for analysis, some patterns emerged within the NT system where applied N fertilizer

numerically increased CO_2 concentrations compared to the control for the PP, MS, and SPLIT treatments at the 15 cm depth (Fig. 3.2). No correlation between gas concentration and most recent CO_2 flux rate (CO_2 flux rate readings taken 1 day prior to soil concentration readings) were determined for this study (Table 3.2).

Table 3.1 ANOVA results for tillage system, nitrogen (N) treatment, and interaction effects on soil pore space CO2 concentrations in June and August 2019.

		Tillage System	N Treatment	Interaction
Month	Depth	ANOVA (*p*-value)		
June	7.5 cm	0.856[a]	0.641	0.788
	15 cm	0.915	0.791	0.591
August	7.5 cm	0.145	0.680	0.622
	15 cm	0.006	0.849	0.270

[a]$p<0.05$ considered significant

[b]June readings were conducted over 3 replications. August readings were conducted over 2 replications due to technical difficulties with the Gasmet analyzer.

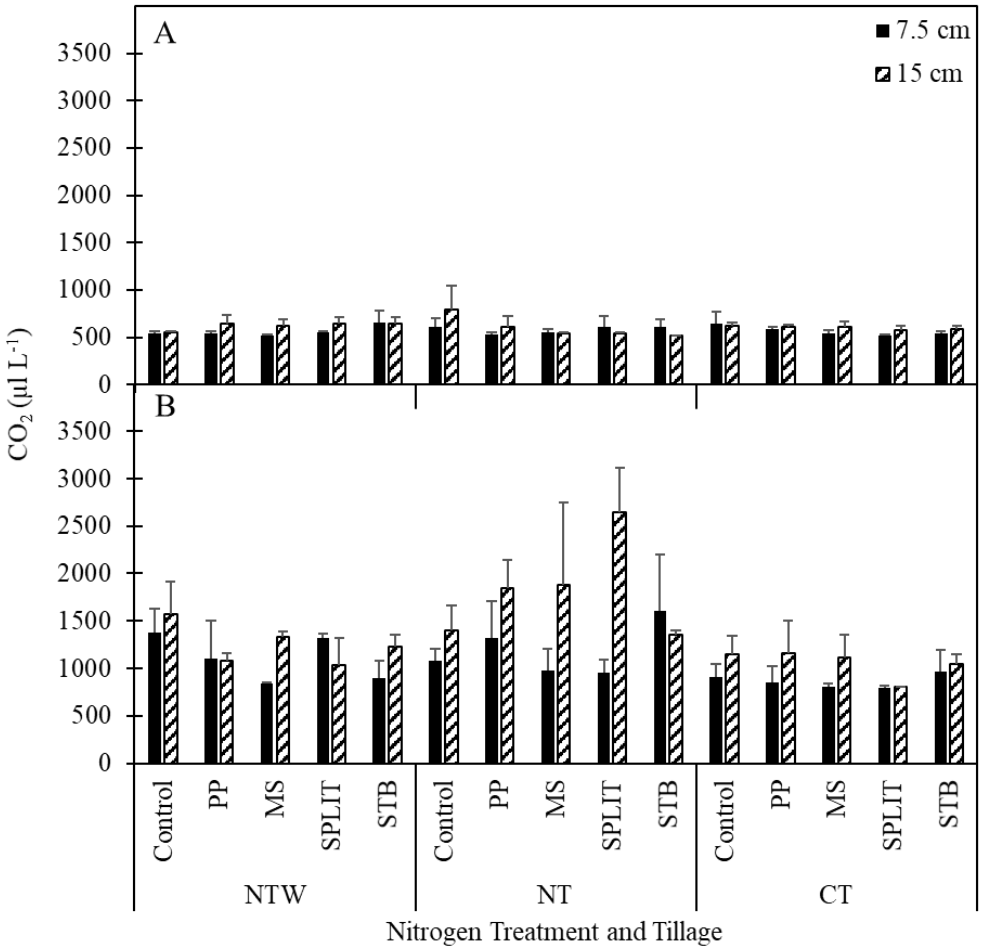

Figure 3.2 Soil pore space CO_2 concentrations in A) June 2019 and B) August 2019 at 7.5 and 15 cm depth. Control, no added nitrogen (N) fertilizer; PP, 100% pre-plant application of N fertilizer; SD, 100% side-dressed application of N fertilizer; SPLIT, 40% PP, 60% SD application of N fertilizer; STB, 100% PP application of N fertilizer with N stabilizer product. NTW, no-tillage with a winter wheat cover crop; NT, no-tillage winter fallow; CT, conventional tillage winter fallow. Error bars indicate standard error of the mean. June readings were conducted over 3 replications. August readings were conducted over 2 replications due to technical difficulties with the Gasmet analyzer.

Table 3.2 Pearson's correlation coefficients for carbon dioxide (CO2) emissions vs. pore space concentrations of CO_2 for June and August 2019 at depth.

Month	Depth	Pearson Correlation Coefficient (*p*-value)
June	7.5 cm	0.070[a]
	15 cm	0.121
August	7.5 cm	0.743
	15 cm	0.182

[a]$p<0.05$ considered significant
[b]June readings were conducted over 3 replications. August readings were conducted over 2 replications due to technical difficulties with the Gasmet analyzer.

3.5. Discussion

Carbon dioxide concentrations determined in this study were 3-10 times smaller than those reported in previous studies (Jassal et al., 2005; Bekele et al., 2007; DeSutter et al., 2008; Gao et al., 2014). However, the previous studies were conducted in natural and forest systems, and corn production fields in Iowa, where organic matter and microbial activity are expected to be greater than in semi-arid Southern High Plains soil (Major Land Resource Area 77C). Gravimetric water content (GWC) was determined for soil samples conducted in conjunction with the pore space measurements with average GWC at 0-10 cm of 0.12 g g^{-1} and 0.07 g g^{-1} for June and August, respectfully. These values are less than or equal to the drought conditions experienced in the Central Switzerland grassland soils of Flechard et al. (2007), and comparison of CO_2

concentrations during those conditions with our measurements is favorable with values ranging from 300-1200 μL L^{-1} for the grassland soils. With this favorable comparison, the measurement of soil-pore space concentrations of GHGs using this method appears to be representative of the actual pore space concentrations in an aridic soil. Other factors may affect GHG concentrations in the soil including bulk density, soil disturbance, and inputs of C and N from both natural and chemical sources which may cause the measurements from agricultural soils to be reduced compared to native grassland and forest soils.

No difference in pore space CO_2 concentration was determined between the NTW and CT systems in this study. However, NTW systems have previously been reported to increase gross CO_2 emissions compared to CT systems on the semi-arid Southern High Plains (McDonald et al., 2019). Previous research has indicated a strong relationship between CO_2 emissions and soil pore space concentrations of CO_2 in the Prairie Pothole region of Manitoba, Canada (Gao et al., 2014). This relationship was not apparent in this study where no correlation was determined between CO_2 emissions from the soil surface at the most recent measurement to the pore space concentrations reported here. However, it is likely that reduced carbon contents in conventionally managed soil increases carbon re-use within the soil profile. In contrast, a more carbon-rich

environment in the NTW system may increase emissions. Increased emissions from the NTW system and carbon re-use within the soil profile in the CT system, likely resulted in the lack of difference between the systems' CO_2 concentrations. There is a slight increase in CO_2 concentrations observed for the NT system compared to the NTW and CT systems at 15 cm in August, which is potentially the result of greater carbon due to the removal of tillage practice, but reduced permeability due to no use of a cover crop or tillage action. With data related to the physical and chemical characteristics of the soil, at the depths measured with our PSP design, one could thus better correlate soil health parameters to gas production and accumulation at depth in agricultural soil profiles. This correlation would help inform best practices for management as well as provide additional insight into the belowground production of GHGs in long-term systems while causing minimal disturbance.

The design of the probes allows manual insertion and removal from the soil which can be conducted throughout the year, including during crop growth periods where information may be lacking related to the cropping system effect on soil pore space gas concentrations. Our system fills this niche and can be integrated with any portable gas analyzer provided the analyzer uses an internal pump or could be used for static measurements should an analyzer be unavailable. The consistency and similarity

of our measurements with others of similar climate and soil types, would indicate that our modification of the design conceived by Brummell and Siciliano (2011) is valid for measuring pore space measurements in agricultural soils and could provide additional information related to the depth of production and cycling of soil nutrients with a gaseous form. With validation in loamy/sandy soils such as those on the Southern High Plains, and consistent and accurate performance the in semi-arid climate, the PSPs described here can provide interesting and relevant data regarding the gaseous content of expanding semi-arid soils and should aid in determining best practices for environmental sustainability within these systems.

3.5.1. Affordability and Implementation

We built 162 probes for about $2200 USD including the PVC pipe and fittings, bottles, tubing and connectors for an average of about $14 per PSP. Probe installation takes about 2 hours in a field with 45 plots (2 probes per plot, 90 probes total) with 3 people conducting the installation. Attaching the probe tops a day in advance only takes about 30 - 45 min with 4 people (90 probe tops), making the installation and preparation a quick process. Other gas analyzers could be integrated into the system described here; the Gasmet DX4040 was chosen to allow for the measurement of multiple gases

simultaneously including nitrous oxide, nitric oxide, ammonia, methane, and carbon monoxide.

3.5.2. Drawbacks

One major concern was the shrinkage of soil with water loss and the annular separation of the soil from the exterior of the probe during dry conditions, potentially allowing atmospheric intrusion into the closed loop system. However, with a more coarsely textured soil such as that present on the Southern High Plains in Lubbock, these issues were not noticed, and the probes stayed firmly in place unless physically disturbed. At a second location with clayey soils, probes were noticeably looser in the soil due to the separation of the soil from the probe surface and the concentration of CO_2 determined was much closer to atmospheric levels. The separation of the soil from the probe was due to improper installation of the probes following field operations, and thus would be avoided with careful installation.

3.6. Conclusions

The measurement of soil pore space gas concentrations has been conducted using various methods over several decades. Our pore space probe design modifies a previously verified method using a Gasmet FTIR analyzer for use in semi-arid

agricultural research fields where soil conservation management practices are being evaluated for economic and environmental sustainability. Our design is easy to produce and install, requiring basic hand tools and affordable parts, was minimally destructive upon installation and removal, and allowed most major field operations to be conducted without interference. Measurements conducted using this system resulted in consistent results at two sampling points during the cotton growing season, with distinct differences in concentration at depth. Carbon dioxide concentrations compared favorably to systems with similar soil characteristics. Thus, this design for soil pore space gas concentrations is a valid option for measurements to be conducted in systems where ease of installation and price are important.

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4. SOIL NUTRIENT MANAGEMENT AFFECTS CONTROLS FOR N TRACE GAS FLUXES

4.1. Summary

The production of nitrous oxide (N_2O), from agricultural soil is a major concern for researchers across the globe due to its contribution to global climate change and its function as a loss of plant-available nitrogen (N) from the soil. However, net consumption of N_2O has also been determined in a wide variety of ecosystems including agricultural soil. The objective of this study was to examine the changes in soil properties related to the implementation of soil conservation practices and N fertilizer management in a semi-arid agricultural soil known to produce and consume N_2O. Emissions of N_2O and pore-space concentrations of N_2O and nitric oxide (NO) were determined five years after conservation system and N management implementation and were evaluated for their relation to soil properties. It was determined that net emission of N_2O from the soil surface was greatest following N fertilizer application, but that the pore-space concentration was more consistent across the growing season. When evaluated for relation to soil parameters, the largest driver of N gas production in the soil pore-space was determined to be the application of N fertilizer and the associated increase in soil ammonium and nitrate. This confirms the potential production of N_2O and NO through both nitrification and denitrification due to the association with the appropriate substrates. In addition, consumption of N_2O was determined where N fertilizer was not applied as fertilizer (but was introduced through irrigation water), or several months after N fertilizer application. The consideration of irrigation water inputs

of inorganic-N into the total N application rate, may allow for the reduction of N_2O emissions specifically associated with N fertilizer application and may further initiate conditions favorable for N_2O consumption in semi-arid soil. However, the implementation of soil conservation practices did not significantly affect N gas emissions, consumption, or pore-space concentrations, despite altering soil chemical parameters known to be proximal controls over denitrification. 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 as indicated by the strong association with N gas production and soil N content.

4.2. Introduction

Producer decisions regarding soil tillage and nutrient management can have significant effects on chemical, physical, and biological properties of soil. Physical disturbances such as tillage can alter soil structure, introduce/bury organic inputs, increase water infiltration, and reduce microbial abundance (Peterson et al., 1998; van Groenigen et al., 2010). The application of fertilizer alters the soil chemical composition and can also impact the soil microbial community through selection and inhibition based on the fertilizer used (Geisseler and Scow, 2014). While all these changes can affect the agronomic productivity of the system, they can also alter the potential for production and consumption of greenhouse gases (GHGs) and affect their concentration in soil air (Malhi et al., 2006; Halvorson et al., 2008).

For fertilized and irrigated agricultural systems, nitrous oxide (N_2O) production is one of the major concerns 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, 300 times that of CO_2 (Ravishankara et al., 2009; IPCC, 2013). Nitrous oxide is produced through both nitrification and denitrification processes in the soil and is mediated by the microbial community (Bremner, 1997; Zumft, 1997; Barnard et al., 2005). The controls over these two N cycling processes can be broken down into proximal and distal effects based on their immediacy of impact on the process. Nitrogen gas cycling and N_2O production are controlled by the amount of soil carbon (C), soil nitrate (NO_3^-) and ammonium (NH_4^+), soil oxygen (O_2), and pH (Robertson, 1989;Wallenstein et al., 2006). Due to the requirement for anaerobic conditions for denitrification to occur, soil O_2 is often seen as the primary regulator between nitrification and denitrification conditions (Zumft, 1997), however other research suggests that the large C requirement (3 parts C for 1 part N) of denitrification may result in soil C content being a major limiting factor for whether denitrification can proceed (Shah and Coulman, 1978). In addition, greater amounts of soil C may increase soil respiration and reduce soil O_2 content, further promoting conditions favorable for denitrification even under bulk soil conditions favorable for nitrification (Wu et al., 2017). Nitrate concentrations in the soil are an obvious control over denitrification as NO_3^- is the primary substrate for the process, while the concentration of NH_4^+ often dictates the rate of nitrification. When concentrations of NO_3^- are low, there is more competition for labile N, and denitrification may be inhibited. However, in many

agricultural systems NO_3^- is present in high concentrations as fertilizer inputs of NH_4^+ are quickly oxidized to NO_3^- which can reduce soil pH and negatively impact the transcription and folding of N_2O-reducing enzymes, increasing N_2O emissions (Matson et al., 1999; Liu et al., 2014; Samad et al., 2016). Finally, NO_3^- can be reduced sequentially through denitrification, and when high concentrations are present, there may be a bottleneck for electrons at the reduction of nitrite (NO_2^-) to nitric oxide (NO), where the nitrite reductase (NIR) enzymes that catalyze this step will preferentially scavenge electrons over the N_2O reductase (N_2OR) enzymes for N_2O reduction to dinitrogen (N_2), increasing N_2O release from the soil (Highton et al., 2020). For nitrification, the high concentrations of NH_4^+ may create a bottleneck at the oxidation of NO to NO_2^-, which may thus induce NO release and/or reduction to N_2O (Caranto and Lancaster, 2017). Distal controls over N trace gas emissions mostly operate to select microbial community members capable of denitrification and nitrification. Distal controls can include disturbance (frequency and intensity), soil pH, soil C content, soil moisture and O_2 content, temperature, and predation (Robertson, 1989; Wallenstein et al., 2006). Not only do controls such as pH and soil C control process progression at a cellular level, but they also significantly impact the abundance and type of microbial community present, thus acting as a distal control.

Producer decisions thus would have both proximal and distal effects on N gas cycling in soil. However, like most decisions in agricultural production, there are complicated interactions and outcomes that must be considered beyond the effect on N_2O production. The decision to till can have beneficial effects for crop production

related to water infiltration, weed and erosion control, and preparing seed beds (Zobeck and Van Pelt, 2011). Tillage may also reduce the fungal population of the soil which has been shown to heavily contribute to N_2O emissions (McLain and Martens, 2006; van Groenigen et al., 2010; Higgins et al., 2016; Novinscak et al., 2016). The effects of no-tillage and cover crops, such as greater microbial diversity, water holding capacity, and soil C storage, may increase conditions favorable for denitrification (Peterson et al., 1998; Balota et al., 2003). However, implementation of no-tillage and cover crops can provide superior erosion control, and the gains in microbial diversity and soil C storage are likely more important for overall soil health and long-term sustainability of the production system. Most producers follow general rules for fertilizer application to maximize crop use and reduce excess N loss to the environment (Smith et al., 2008). These general rules include the right time and right amount of fertilizer applied to achieve the producer's goals while improper timing and amount may increase the activation and rate of denitrification (Meisinger and Delgado, 2002). The selection of application timing for maximum crop usage will reduce the labile N present in the soil and thus reduce the potential NO_3^- available for denitrification processes (Meisinger and Delgado, 2002; Shelton et al., 2017). The right amount of fertilizer can reduce excess N present in the soil in the same manner, as well as reduce downstream impacts of excess N such as eutrophication (Matson et al., 1999; Eghball et al., 2000). Choosing the right type of fertilizer for crop uptake can also impact N losses whereby selecting for more NH_4^+-based fertilizers for their reduced leaching potential. However, high NH_4^+ may result in greater NO and N_2O due to process bottlenecks (Caranto and Lancaster, 2017),

and can also be converted to NO_3^- through nitrification, contributing to the acidification of the soil, which can enhance N_2O production (Matson et al., 1999). Lastly, the placement of N fertilizer can impact crop N uptake, where placement outside the "reach" of crop roots may significantly increase the percentage of N fertilizer lost from the production system through pathways similar to those described above. It is thus imperative to understand how these management choices impact N_2O emissions in order to choose best practices for environmental and agronomic/economic sustainability for producers.

The objective of this study was to evaluate the inter-seasonal impacts of conservation practices and N fertilizer application timing on soil inorganic N, soil mineralizable C, and pH in a semi-arid cropping system in Texas. Nitrous oxide emissions from the soil surface and belowground N_2O and NO concentrations were evaluated in the final year to better understand the relationship between the implemented practices, soil chemical and physical parameters, and N gas production. It was hypothesized that the potential for bacterial denitrification, as indicated by increases in *nosZ* clade I denitrification gene abundance, would fluctuate during the growing season relating to the timing of N fertilizer application. In addition, it was hypothesized that seasonal variation in proximal controls over N gas cycling would result in variable levels of pore-space N gases.

4.3. Methods

4.3.1. Site Description

This research was conducted in continuous cotton (*Gossypium hiristum* L.) systems at the Texas A&M AgriLife Research Center in Lubbock, TX (33.687°, -101.827°) from 2018-2020. Annual precipitation at Lubbock is about 481 mm and the 30-year average temperature for Lubbock is 16.1°C (30-year average, 1991-2020; NOAA, 2021). Soil at the Lubbock location is an Acuff loam described as a fine-loamy, mixed, superactive, thermic Aridic Paleustoll (U.S. Department of Agriculture, 2016).

The study was designed as a split plot with the main plot of conservation practice (tillage system), and the split plot being N fertilizer application timing. 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 of 2015, with the field being under 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); 40% of N applied PP and 60% applied mid-season (SPLIT). The study consisted of 3 blocks (replicates) for a total of 27 plots measuring 15 m in length and four rows wide with 1 m row spacing. Nitrogen fertilizer was applied by knife injection using a coulter fertilizer applicator at a depth of 15 cm as urea-ammonium-nitrate (UAN-32, 32-0-0) at a rate of 168 kg N ha^{-1}. Furrow irrigation was conducted as needed for crop growth throughout the year with each event applying about 152 mm of water with four irrigations conducted in 208, two irrigation events in 2019, and four irrigation

events in 2020. The irrigation water had a moderate NO_3^--N content of 10.59 ppm in 2020.

Pre-plant N fertilizer was applied in May, or June of each year, depending on projected planting date. Mid-season N fertilizer applications were made in July at or near the first reproductive growth for the cotton crop. Application of N fertilizer was conducted using a knife and coulter fertilizer applicator mounted to a tractor. Nitrogen fertilizer was applied 10 – 15 cm from the cotton row and about 15 cm deep. Nitrate addition through the irrigation water amounted to about 47 kg N ha^{-1} in 2018 and 2020, and about 24 kg ha^{-1} in 2019, calculated from the concentration of nitrate-N in the irrigation water, the rate of irrigation applied, and the number of irrigation events in each year (DeLaune and Trostle, 2012).

4.3.2. Soil Sampling and Analysis

Soil sampling was conducted at three key cotton growth stages in each year: vegetative (Veg; 28 June 2018.14 June 2019, 24 June 2020), peak plant production (Peak; 24 August 2018, 14 August 2019, 05 August 2020), and reproductive (Repro; 1 November 2018, 24 October 2019, 20 October 2020). Soil samples were collected using 2.5 x 40 cm hand probes to a depth of 20 cm separated into 10 and 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 using flow injection spectrometry (FIAlab 2600, FIAlab Instruments Inc., Bellevue, WA) following cadmium reduction to NO_2^- and by the Berthelot reaction involving salicylate, respectively (Keeney and Nelson, 1982). In addition, soil samples were analyzed for mineralizable C by a 3-day rewetting incubation with 40 g of air-dried soil sieved at 4 mm (Franzluebbers, 2016). Soil pH was determined by a 1:2 dilution of 10 g soil with DI water (Schofield and Taylor, 1955). Samples were collected for analysis of bulk density after cotton harvest in the final year of study.

4.3.3. Microbial Analysis

Microbial analysis was conducted by quantitative polymerase chain reaction (qPCR) abundance of major denitrification genes (Table 4.1). 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 (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. (2005), where extracted DNA was added to a qPCR assay to quantify the abundance of *Vibrio alginolyticus* with *gyrB* as the gene target (Zhou, Hou, Li, & Qin, 2007). 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.

Table 4.1 Primer sequences and thermal profiles for bacterial N_2O consumption and bacterial and fungal abundance.

Target Group	Primers	Primer Sequence	PCR Cycling Profile	Reference
Total bacteria	Eub338	5'- ACTCCTACGGGAGGCAGCAG -3'	95°C/15 min; 95°C/60 s, 53°C/30s. 72°C/60s × 40 cycles	(Fierer et al., 2005)
	Eub518	5'- ATTACCGCGGCTGCTGG -3'		
Nitrous oxide reductase clade I	nosZ2F	5'- CGCRACGGCAASAAGGTSMSSGT -3'	95°C/5 min; 95°C/15s, 67-62°C/30S, 72°C/30s; 95°C/15s, 62°C/30S, 72°C/30s × 34 cycles	(Henry et al., 2006)
	nosZ2R	5'- CAKRTGCAKSGCRTGGCAGAA -3'		
Nitrous oxide reductase clade II	nosZIIF	5'- CTIGGICCIYTKCAYAC -3'	95°C/30 s; 95°C/15s, 54°C/30s, 72°C/45s, 78°C/10s × 40 cycles	(Jones et al., 2013)
	nosZIIR	5'- GCYTCGATVAGRTTRTGGTT -3'		

4.3.4. Greenhouse Gas Emissions and Pore-Space Concentrations

Nitrous oxide and carbon dioxide (CO_2) emissions and in-ground concentrations of N_2O, NO, and CO_2 were collected in real time during the 2020 growing season using a Gasmet DX4040 fully portable FTIR gas analyzer (Gasmet Technologies, Helsinki, Finland) integrated with a LiCor 8100A opaque chamber for soil surface emissions and connected to in-ground pore-space probes (PSPs) designed for this project. Soil N_2O emissions and pore-space samplings were conducted within 48 hours of soil sample collection. Pore-space concentration measurements were conducted according to

Brummell et al. (2012) and McDonald et al. (2021). Briefly, pore space probes (PSP) 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 Gasmet analyzer for soil N_2O and NO concentration determination. The reservoir bottle increased the system volume to greater than that of the sample cell within the Gasmet, thus eliminating any vacuum being placed on soil pores. Gas emissions and pore-space concentrations were also evaluated during the Veg sampling in 2018 and 2019 but were not included for analysis due to the lack of a complete data set for the year. In both 2018 and 2019, technical issues with the Gasmet DX4040 gas analyzer did not allow for gas sampling to be conducted at the Peak and Repro samplings.

4.3.5. Statistical Analysis

Statistical analysis was conducted in SAS 9.4 using PROC Glimmix (soil chemical characteristics, microbial abundance, gas emissions and pore-space concentrations in 2020) and PROC Corr (2020 data only) at a significance level of α=0.05 (SAS, 2013; SAS-Institute, 2017). Analyses were conducted to determine year effects, as well as evaluate the measured parameters within each sampling period over the three-year study. All analysis of variances were conducted with main-plot treatments and split-plot treatments treated as fixed effects, and replication was treated as a random effect. Means of significant effects were separated using Fisher's protected LSD at α=0.05. Non-metric multidimensional scaling (NMDS) analysis was conducted within

each sampling period at both the 0-10 cm and the 10-20 cm depth using the metamds function within the vegan package in R (Oksanen et al., 2020) and visualized using ggplot2 (Wickham, 2016). Natural log transformation was conducted for each gene abundance, and soil pore-space CO_2 concentration for NMDS analysis to eliminate effect bias for these parameters due to the large values naturally associated with these parameters in comparison to the rest of the data set.

4.4. Results

4.4.1. Soil Characteristics

4.4.1.1. Soil pH

Soil pH was affected by N treatment within each sampling period for nearly every year of the study at both the 0-10 cm and the 10-20 cm depth ($p<0.05$, Table S4.1). At the 0-10 cm depth within the Veg sampling, pH was reduced for the PP treatment compared to the control in 2018 ($p=0.006$) and 2019 ($p=0.012$, Table 4.2). Within the Veg sampling in 2020, pH was reduced for both the PP and SPLIT treatments compared to the control ($p=0.002$, Table 4.2). At the 10-20 cm depth within the Veg sampling, pH was reduced for both the PP and SPLIT treatments compared to the control in 2018 ($p=0.002$), 2019 ($p<0.001$), and 2020 ($p=0.032$, Table 4.2). The interaction of conservation system and N treatment also affected pH at the 10-20 cm depth in 2018 ($p=0.016$) and 2019 ($p<0.001$) within the Veg sampling (Table S4.2).

At the Peak sampling, N treatment affected pH at both the 0-10 cm and the 10-20 cm depth in 2018 (0-10 cm: $p=0.001$; 10-20 cm: $p=0.004$) and 2019 (0-10 cm: $p<0.001$; 10-20 cm: $p<0.001$), but not in 2020 (0-10 cm: $p=0.316$; 10-20 cm: $p=0.184$, Table 4.2),

with both the PP and SPLIT treatments having reduced pH compared to the control. Conservation system affected pH within the Peak sampling in 2019 at both the 0-10 cm (p=0.015) and the 10-20 cm depths (Table 4.2).

Nitrogen treatment affected pH at both the 0-10 cm and the 10-20 cm depth in 2018 (0-10 cm: p=0.004; 10-20 cm: p=0.006), 2019 (0-10 cm: p<0.001; 10-20 cm: p=0.001), and 2020 (0-10 cm: p=0.001; 10-20 cm: p=0.035) within the Repro sampling period. In all cases, the PP and SPLIT treatments had reduced pH compared to the control (Table 4.2). The interaction of conservation and N treatment only affected pH within the Repro sampling in 2018 at the 10-20 cm depth (p=0.022, Table S4.2).

Table 4.2 Main plot and split plot averages and LS means for conservation system and N treatment effects on soil pH within each depth and year for each sampling period

Depth	Year	Conservation System	N Treatment	Vegetative Growth		Peak Plant Production		Reproductive Growth	
				pH	LS Means (α=0.05)	pH	LS Means (α=0.05)	pH	LS Means (α=0.05)
0-10 cm	2018	NTW	-	7.6		7.8		8.1	
		NT	-	7.6		7.8		8.1	
		CT	-	7.7		7.7		8.3	
		-	Control	7.8	A	8.0	A	8.3	A
		-	PP	7.5	B	7.6	B	8.1	B
		-	SPLIT	7.6	AB	7.7	B	8.1	B
	2019	NTW	-	7.5		7.3	A	7.7	
		NT	-	7.3		7.3	A	7.6	
		CT	-	7.4		6.7	B	7.7	
		-	Control	7.7	A	7.8	A	8.2	A
		-	PP	7.1	B	6.6	B	7.4	B
		-	SPLIT	7.4	AB	6.9	B	7.5	B
	2020	NTW	-	7.5		8.0		7.4	
		NT	-	7.6		7.6		7.7	
		CT	-	7.4		7.8		7.6	
		-	Control	7.8	A	7.9		8.0	A
		-	PP	7.3	B	7.8		7.4	B
		-	SPLIT	7.4	B	7.7		7.4	B
10-20 cm	2018	NTW	-	7.8		7.9		8.3	
		NT	-	7.8		7.9		8.3	
		CT	-	7.7		7.8		8.2	
		-	Control	7.9	A	8.1	A	8.4	A
		-	PP	7.7	B	7.8	B	8.2	B
		-	SPLIT	7.6	B	7.8	B	8.1	B
	2019	NTW	-	7.7		7.7	A	7.9	
		NT	-	7.7		7.6	AB	7.9	
		CT	-	7.6		7.3	B	7.6	
		-	Control	7.8	A	8.0	A	8.1	A
		-	PP	7.5	B	7.4	B	7.7	B
		-	SPLIT	7.6	B	7.2	B	7.7	B
	2020	NTW	-	7.7		7.8		7.8	
		NT	-	7.8		7.6		7.8	
		CT	-	7.7		7.7		7.5	
		-	Control	8.0	A	7.9		8.0	A
		-	PP	7.6	B	7.6		7.6	B
		-	SPLIT	7.6	B	7.5		7.5	B

4.4.1.2. Gravimetric Water Content

At the 0-10 cm depth, conservation system affected GWC in 2020 within the Veg sampling (p=0.001, Table S4.1) with the NTW (13.52 g H_2O 100 g $soil^{-1}$) and NT (13.16 g H_2O 100 g $soil^{-1}$) system having greater GWC than the CT system (11.54 g H_2O 100 g $soil^{-1}$). No N treatment or conservation system affected GWC at the 10-20 cm depth within the Veg sampling in any year of the study, nor did the interaction of conservation system and N treatment affect GWC at either depth in any year of the study within the Veg sampling (Table S4.1). Main plot and split plot averages for GWC at 0-10 and the 10-20 cm depth, within each sampling period in each year of the study are reported in Table S4.3.

Within the Peak sampling, conservation system affected GWC at the 0-10 cm depth in 2020 (p=0.005, Table S4.1) with the NTW (13.33 g H_2O 100 g $soil^{-1}$) and the NT (13.15 g H_2O 100 g $soil^{-1}$) systems having greater GWC than the CT system (12.05 g H_2O 100 g $soil^{-1}$). At the 10-20 cm depth, conservation system affected GWC in 2018 within the Peak sampling (p=0.023) where the NTW (10.32 g H_2O 100 g $soil^{-1}$) and NT (10.31 g H_2O 100 g $soil^{-1}$) systems had greater GWC than the CT system (9.46 g H_2O 100 g $soil^{-1}$). In addition, N treatment affected GWC at the 10-20 cm depth within the Peak sampling in 2018 (p=0.001) where the PP treatment (10.1 g H_2O 100 g $soil^{-1}$) and the control (10.7 g H_2O 100 g $soil^{-1}$) had greater water content than the SPLIT treatment (9.28 g H_2O 100 g $soil^{-1}$).

Gravimetric water content was affected by conservation system within the Repro sampling at the 0-10 cm depth in 2020 (p=0.013, Table S4.1) with the NTW (5.06 g H_2O

100 g soil^{-1}) and CT (4.92 g H_2O 100 g soil^{-1}) systems having greater water content than the NT system (3.96 g H_2O 100 g soil^{-1}). At the 10-20 cm depth in 2020, GWC was also affected by conservation system (p=0.005) where the CT system (8.29 g H_2O 100 g soil^{-1}) had greater GWC than the NTW system (7.72 g H_2O 100 g soil^{-1}). At the 10-20 cm depth in 2020 within the Repro sampling, N treatment affected GWC (p=0.005) where the control (8.46 g H_2O 100 g soil^{-1}) had greater GWC than the PP (7.93 g H_2O 100 g soil^{-1}) and SPLIT (7.63 g H_2O 100 g soil^{-1}) treatments.

4.4.1.3. Mineralizable Carbon

Mineralizable carbon (Cmin) content was affected by conservation system at the 0-10 cm depth within the Veg sampling in 2020 (p=0.049, Table S4.1) where the NTW system (78.4 mg CO_2-C kg soil^{-1}) was determined to have greater Cmin content than the NT system (64.6 mg CO_2-C kg soil^{-1}) but not the CT system (68.4 mg CO_2-C kg soil^{-1}). Main plot and split plot averages for Cmin at 0-10 and the 10-20 cm depth, within each sampling period in each year of the study are reported in Table S4.3.

At the Peak sampling, conservation system affected Cmin in 2018 at the 0-10 cm depth (p=0.001, Table S4.1) and the 10-20 cm depth (p=0.018). At the 0-10 cm depth within the Peak sampling in 2018, the NTW (83.0 mg CO_2-C kg soil^{-1}) and NT (72.5 mg CO_2-C kg soil^{-1}) systems were determined to have greater Cmin content than the CT system (44.8 mg CO_2-C kg soil^{-1}), while at the 10-20 cm depth within the Peak sampling in 2018 the NT system (79.6 mg CO_2-C kg soil^{-1}) was determined to have greater Cmin content than the CT system (53.5 mg CO_2-C kg soil^{-1}) but not the NTW system (63.1 mg

CO_2-C kg $soil^{-1}$). The interaction of conservation system and N treatment was determined to affect Cmin at the 10-20 cm depth in 2018 (p=0.015, Table S4.4).

Conservation system was determined to affect Cmin content within the Repro sampling at the 0-10 cm depth in 2018 (p=0.006, Table S4.1) and 2020 (p=0.001). In 2018, the NTW treatment (133.1 mg CO_2-C kg $soil^{-1}$) was determined to have greater Cmin content than the NT (100.9 mg CO_2-C kg $soil^{-1}$) and CT (88.6 mg CO_2-C kg $soil^{-1}$) systems at the 0-10 cm depth. In 2020 at the 0-10 cm depth, the NTW system (104.4 mg CO_2-C kg $soil^{-1}$) was determined to have greater Cmin content than the NT (75.7 mg CO_2-C kg $soil^{-1}$) and CT (74.5 mg CO_2-C kg $soil^{-1}$) systems.

4.4.1.4. Soil Inorganic Nitrogen

Within the Veg sampling in 2019, soil NO_3^--N concentration was affected by conservation system at the 0-10 cm (p=0.011, Table S4.1) and the 10-20 cm depth (p=0.027), where the CT system (65.4 mg NO_3^--N kg $soil^{-1}$) had the greatest NO_3^- concentration compared to the NT (34.3 mg NO_3^--N kg $soil^{-1}$) and NTW (20.8 mg NO_3^--N kg $soil^{-1}$) systems at the 0-10 cm. At the 10-20 cm the CT system (37.6 mg NO_3^--N kg $soil^{-1}$) had greater NO_3^--N concentration than the NTW system (11.4 mg NO_3^--N kg $soil^{-1}$) but not the NT system (21.1 mg NO_3^--N kg $soil^{-1}$). Within the Veg sampling, N treatment affected soil NO_3^--N concentration in 2020 at the 0-10 cm depth (p=0.039) where the PP treatment (37.6 mg NO_3^--N kg $soil^{-1}$) was determined to have greater NO_3^--N concentration than the control (9.5 mg NO_3^--N^- kg $soil^{-1}$) but not the SPLIT treatment (30.9 mg NO_3^--N kg $soil^{-1}$). Soil NH_4^+-N concentration was affected by conservation

system (p=0.001) at the 0-10 cm depth in 2019 where the CT system (26.1 mg NH_4^+-N kg $soil^{-1}$) had the greatest concentration of soil NH_4^+-N compared to the NT (9.8 mg NH_4^+-N kg $soil^{-1}$) and NTW (8.4 mg NH_4^+-N kg $soil^{-1}$) systems. In addition, N treatment was determined to affect soil NH_4^+-N concentration at the 0-10 cm depth in 2019 (p<0.001) where the SPLIT treatment (29.2 mg NH_4^+-N kg $soil^{-1}$) was determined to have greater NH_4^+-N concentration than the PP treatment (7.7 mg NH_4^+-N kg $soil^{-1}$) and the control (7.4 mg NH_4^+-N kg $soil^{-1}$). The interaction of conservation system and N treatment was also determined to affect soil NH_4^+-N concentration within the Veg sampling at the 0-10 cm depth in 2019 (p<0.001, Table S4.5).

Conservation system affected soil NO_3^--N concentration at the 0-10 cm depth within the Peak sampling in 2018 (p=0.001, Table S4.1) where the CT system (42.6 mg NO_3^--N kg $soil^{-1}$) was determined to have greater soil NO_3^- than the NTW (21.4 mg NO_3^--N kg $soil^{-1}$) and NT (20.5 mg NO_3^--N kg $soil^{-1}$) systems. Within the Peak sampling in 2019, N treatment was determined to affect soil NO_3^--N at the 0-10 cm depth (p=0.001) where the SPLIT treatment (66.2 mg NO_3^--N kg $soil^{-1}$) was determined to have greater soil NO_3^--N than the PP treatment (17.2 mg NO_3^--N kg $soil^{-1}$) and the control (5.7 mg NO_3^--N kg $soil^{-1}$). Conservation system affected soil NO_3^--N at the 10-20 cm depth within the Peak sampling in 2019 (p=0.002) where the CT system (31.2 mg NO_3^--N kg $soil^{-1}$) was determined to have greater soil NO_3^--N than the NTW (11.8 mg NO_3^--N kg $soil^{-1}$) and NT (10.7 mg NO_3^--N kg $soil^{-1}$) systems. In addition, N treatment affected soil NO_3^--N within the Peak sampling at the 10-20 cm depth in 2019 (p<0.001) where the

SPLIT treatment (36.1 mg NO_3^--N kg $soil^{-1}$) had greater NO_3^--N concentration than the PP treatment (14.2 mg NO_3^--N $^-$ kg $soil^{-1}$) and the control (3.5 mg NO_3^--N kg $soil^{-1}$).

Within the Repro sampling, conservation system affected soil NO_3^--N concentration in 2019 at the 0-10 cm depth (p=0.009, Table S4.1) where the CT system (22.4 mg NO_3^--N kg $soil^{-1}$) was determined to have greater soil NO_3^--N than the NT (2.9 mg NO_3^--N kg $soil^{-1}$) and NTW (2.7 mg NO_3^--N kg $soil^{-1}$) systems. Conservation system also affected soil NO_3^--N at the 10-20 cm depth with the Repro sampling in 2019 (p<0.001) where the CT system (40.9 mg NO_3^--N kg $soil^{-1}$) was also determined to have greater soil NO_3^--N than the NT (3.8 mg NO_3^--N kg $soil^{-1}$) and NTW (1.2 mg NO_3^--N $^-$ kg $soil^{-1}$) systems. N treatment affected soil NH_4^+-N at the 0-10 cm depth in 2019 (p=0.042) and 2020 (p=0.010) within the Repro sampling. In 2019, the SPLIT treatment (3.8 mg NH_4^+-N kg $soil^{-1}$) had greater soil NH_4^+-N than the control (2.9 mg NH_4^+-N kg $soil^{-1}$) but not the PP treatment (3.4 mg NH_4^+-N kg $soil^{-1}$). In 2020, the PP (8.7 mg NH_4^+-N kg $soil^{-1}$) and SPLIT (8.7 mg NH_4^+-N kg $soil^{-1}$) treatments were determined to have greater soil NH_4^+-N than the control (7.0 mg NH_4^+-N kg $soil^{-1}$).

4.4.2. Microbial Abundance

4.4.2.1. 16S rRNA

The 16S rRNA gene abundance was not affected by conservation tillage, N treatment, or their interaction (conservation system × N treatment) within the Veg sampling in any year of the study at either the 0-10 cm or the 10-20 cm depth (Table S4.6). In general, 16S rRNA gene abundance was greater at the 0-10 cm depth compared

to the 10-20 cm depth within the Veg sampling in each year of the study Average main plot and split plot abundances for each gene measured are presented in Table S4.7. The 16S rRNA gene abundance was also not affected by the implementation of conservation tillage, N treatment, or their interaction (conservation system × N treatment) within the Peak sampling in any year of the study at either the 0-10 cm or the 10-20 cm depth (Table S4.6). Generally, 16S rRNA gene abundance was greater at the 0-10 cm depth compared to the 10-20 cm depth within the Peak sampling in each year of the study.

Within the Repro sampling, conservation system affected 16S rRNA gene abundance at the 10-20 cm depth (p=0.007, Table S4.6) with the NTW system having greater 16S abundance (3.35×10^7 gene copies g dry soil^{-1}) than the CT system (1.15×10^7 gene copies g dry soil^{-1}). The interaction of conservation system and N treatment affected 16S rRNA gene abundance at the 0-10 cm depth in 2019 (p=0.049, Table S4.8) No other conservation system, N treatment, or interaction (conservation system × N treatment) effects were determined within the Repro sampling at either the 0-10 cm or the 10-20 cm depth (Table S4.6). Within the Repro sampling, 16S rRNA gene abundances were generally greater at the 0-10 cm depth compared to the 10-20 cm depth for each year of the study.

4.4.2.2. *nosZ* clade I

The abundance of *nosZ* clade I N_2O-reducing bacteria was not affected by the implementation of conservation system, N treatment, or their interaction (conservation system × N treatment) within the Veg sampling in any year of the study (Table S4.6). In

general, average clade I abundance is greater at the 0-10 cm depth compared to average abundance of clade I at the 10-20 cm depth within the Veg sampling in each year of the study.

At Peak sampling, conservation system affected clade I abundance at the 0-10 cm depth in 2018 (p=0.024, Table S4.6) where the CT system (1.26×10^6 gene copies g dry soil^{-1}) had a greater abundance of clade I genes than the NT system (7.38×10^5 gene copies g dry soil^{-1}) but not the NTW system (1.08×10^6 gene copies g dry soil^{-1}). In general, clade I gene abundance within the Peak sampling was similar to that observed in the Veg sampling being greater within the 0-10 cm depth compared to the 10-20 cm depth in each year of the study.

Conservation system affected clade I gene abundance within the Repro sampling at the 0-10 cm depth in 2019 (p=0.030, Table S4.6) and 2020 (p<0.001). In 2019, the NTW system (1.28×10^6 gene copies g dry soil^{-1}) had greater clade I gene abundance than the NT system (5.90×10^5 gene copies g dry soil^{-1}) but not the CT system (8.59×10^5 gene copies g dry soil^{-1}). In 2020 within the 0-10 cm depth, the NTW system (1.49×10^6 gene copies g dry soil^{-1}) was greater than both the NT (1.08×10^6 gene copies g dry soil^{-1}) and CT (6.67×10^5 gene copies g dry soil^{-1}) systems, where the NT system also had greater clade I gene abundance than the CT system. The interaction of conservation system and N treatment also affected clade I gene abundance at the 0-10 cm depth in both 2019 (p=0.044) and 2020 (p=0.006, Table S4.9). At the 10-20 cm depth within the Repro sampling conservation system affected clade I gene abundance in 2020 (p=0.002), where the NTW system (8.32×10^5 gene copies g dry soil^{-1}) had greater clade I gene abundance

than the CT system (1.72×10^5 gene copies g dry soil^{-1}). At Repro, average clade I gene abundance was also generally greater at the 0-10 cm depth compared to the 10-20 cm depth in each of the three years of the study.

4.4.2.3. *nosZ* clade II

Within the Veg sampling, the abundance of *nosZ* clade II N_2O-reducing bacteria was not affected by conservation system, N treatment, or their interaction (conservation system × N treatment) at the 0-10 cm or the 10-20 cm depth in any year of the study (Table S4.6). In general, clade II gene abundance was greater at the 0-10 cm depth compared to the 10-20 cm depth within the Veg sampling in each year of the study.

The abundance of *nosZ* clade II N_2O-reducing bacteria was also not affected by conservation system, N treatment, or the interaction of conservation system and N treatment (conservation system × N treatment) in any year of the study at the 0-10 cm or the 10-20 cm depth (Table S4.6). Similar to the Veg sampling, clade II abundance was generally greater at the 0-10 cm depth compared to the 10-20 cm depth in each year of the study.

Nitrogen treatment affected clade II gene abundance within the Repro sampling at the 0-10 cm depth in 2018 (p=0.017) where the PP treatment (5.29×10^8 gene copies g dry soil^{-1}) had greater clade II abundance than the SPLIT treatment (1.09×10^8 gene copies g dry soil^{-1}) but not the control (3.48×10^8 gene copies g dry soil^{-1}). Conservation system affected clade II abundance at the 10-20 cm depth within the Repro sampling in 2020 (p=0.046) where the NTW system (2.94×10^8 gene copies g dry soil^{-1}) had greater

clade II abundance than the CT system (5.84×10^7 gene copies g dry soil^{-1}). The abundance of clade II genes was generally greater at the 0-10 cm depth compared to the 10-20 cm depth for the Peak and Repro samplings in each year of the study, while no discernable difference between depth was present within the Veg sampling.

4.4.2.4. Fungal Abundance (ITS)

Fungal abundance was not affected by conservation system, N treatment, or their interaction (conservation system × N treatment) within the Veg sampling in any year of the study at either the 0-10 cm or the 10-20 cm depth (Table S4.6). Fungal abundance was generally greater at the 0-10 cm depth compared to the 10-20 cm depth within the Veg sampling in each year of the study.

Nitrogen treatment affected fungal abundance at the 10-20 cm depth in 2018 within the Peak sampling (p<0.001 Table S4.6) where the SPLIT treatment (5.56×10^7 gene copies g dry soil^{-1}) had greater fungal abundance than the PP treatment (1.58×10^7 gene copies g dry soil^{-1}) and the control (1.42×10^7 gene copies g dry soil^{-1}). The interaction of conservation system and N treatment also affected fungal abundance at the 10-20 cm depth in 2018 (p<0.001, Table S4.10). Within the Peak sampling, fungal abundance was generally greater at the 0-10 cm depth compared to the 10-20 cm depth in each year of the study.

Conservation system affected fungal abundance within the Repro sampling at the 0-10 cm depth in 2019 (p=0.018, Table S4.6) and 2020 (p=0.047) and at the 10-20 cm depth in 2020 (p=0.020). In 2019 at the 0-10 cm depth, fungal abundance was greater in

the NTW system (8.91×10^6 gene copies g dry soil^{-1}) than in the NT system (4.44×10^6 gene copies g dry soil^{-1}) but not the CT system (6.97×10^6 gene copies g dry soil^{-1}). In 2020 at the 0-10 cm depth, fungal abundance was greatest in the NTW system (1.94×10^7 gene copies g dry soil^{-1}) compared to the NT system (8.46×10^6 gene copies g dry soil^{-1}) and the CT system (9.04×10^6 gene copies g dry soil^{-1}). At the 10-20 cm depth in 2020, fungal abundance was greater in the NTW system (8.38×10^6 gene copies g dry soil^{-1}) than in the CT system (2.95×10^6 gene copies g dry soil^{-1}). Nitrogen treatment also affected fungal abundance within the Repro sampling at the 0-10 cm depth in 2019 (p=0.004), with the PP treatment (9.98×10^6 gene copies g dry soil^{-1}) having greater fungal abundance than the SPLIT treatment (5.25×10^6 gene copies g dry soil^{-1}) and the control (5.09×10^6 gene copies g dry soil^{-1}). The interaction of conservation system and N treatment affected fungal abundance at the 0-10 cm depth in 2019 within the Repro sampling (p=0.010, Table S4.10). Fungal abundance was generally greater at the 0-10 cm depth compared to the 10-20 cm depth within the Repro sampling in each year of the study.

4.4.3. Nitrous Oxide Emissions

Nitrous oxide emissions were evaluated at each soil sampling event in the final year of the study to assess the impacts of conservation tillage and N treatment on production of N_2O from the soil-atmosphere interface. At the Veg sampling, N treatment was determined to affect N_2O emissions (p=0.002, Fig. 4.1) where emissions from the PP treatment (8071 µg N_2O-N m^{-2} d^{-1}) were greater than from the SPLIT treatment

(1697 µg N_2O-N m^{-2} d^{-1}) and the control (-218 µg N_2O-N m^{-2} d^{-1}). Conservation system (p=0.710) and the interaction of conservation system and N treatment (p=0.733) did not affect N_2O emissions at the Veg sampling (Tables S4.11,4.12).

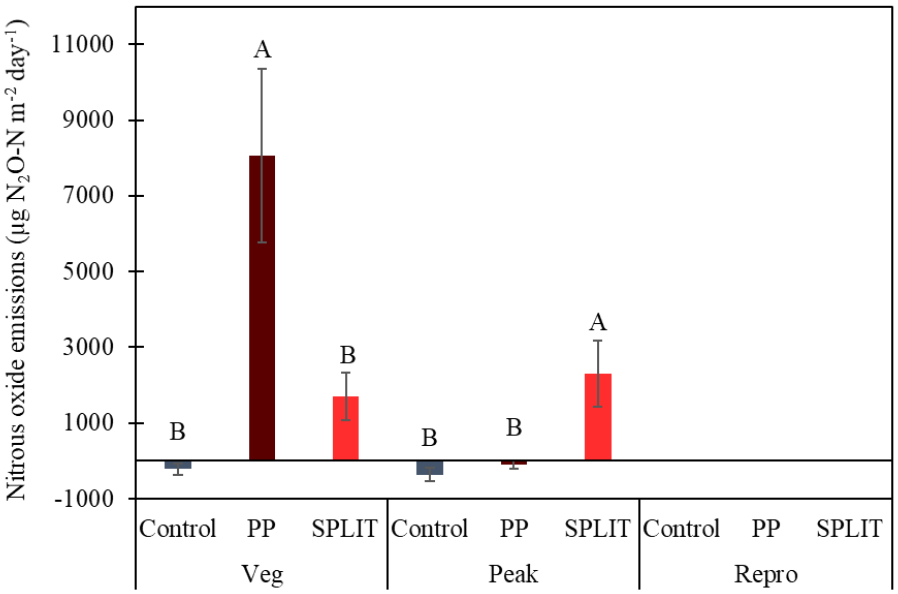

Figure 4.1 Nitrous oxide emissions (µg N2O-N m-2 day-1) measured at the soil-atmosphere interface in 2020 at the Vegetative Growth stage (Veg), Peak Plant Production (Peak), and Reproductive growth stage (Repro) of a cotton cropping system. Data was collected at a single timepoint within each growth 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. LSMEANS letters are compared within sampling period and are different at α=0.05.

At Peak sampling, N treatment affected N_2O emissions (p=0.001) where emissions from the SPLIT treatment (2307 µg N_2O-N m^{-2} d^{-1}) were greater than from the PP treatment (-106 µg N_2O-N m^{-2} d^{-1}) and the control (-362 µg N_2O-N m^{-2} d^{-1}). Conservation system (p=0.351) and the interaction of conservation system and N treatment (p=0.1516) did not affect N_2O emission within the Peak sampling (Fig. 4.1).

No net production or consumption of N_2O was determined at the Repro sampling in the final year of the study (Fig. 4.1, Table S4.11).

4.4.4. Soil Pore-Space Concentrations of Nitrogen Gases

Pore-space concentrations of N_2O and NO were analyzed at each sampling in 2020 at 7.5 cm and 15 cm depths. Within the Veg sampling at the 7.5 cm depth, pore-space concentration of N_2O was affected by N treatment (p=0.041, Table S4.11, Fig. 4.2) where the PP treatment had greater N_2O concentration (1.2 µL N_2O L^{-1}) than the control (0.88 µL N_2O L^{-1}), but not the SPLIT treatment (1.0 µL N_2O L^{-1}). Pore-space concentrations of NO were not affected by conservation system, N treatment, or their interaction (conservation system × N treatment) at either 7.5 cm or 15 cm depth (Fig. 4.2, Table S4.11,4.12). Within the both the Peak and Repro samplings, the pore-space concentration of N_2O was not affected by conservation system, N treatment, or their interaction (conservation system × N treatment) at either 7.5 cm or 15 cm depth (Fig. 4.2, Table S4.11,4.12).

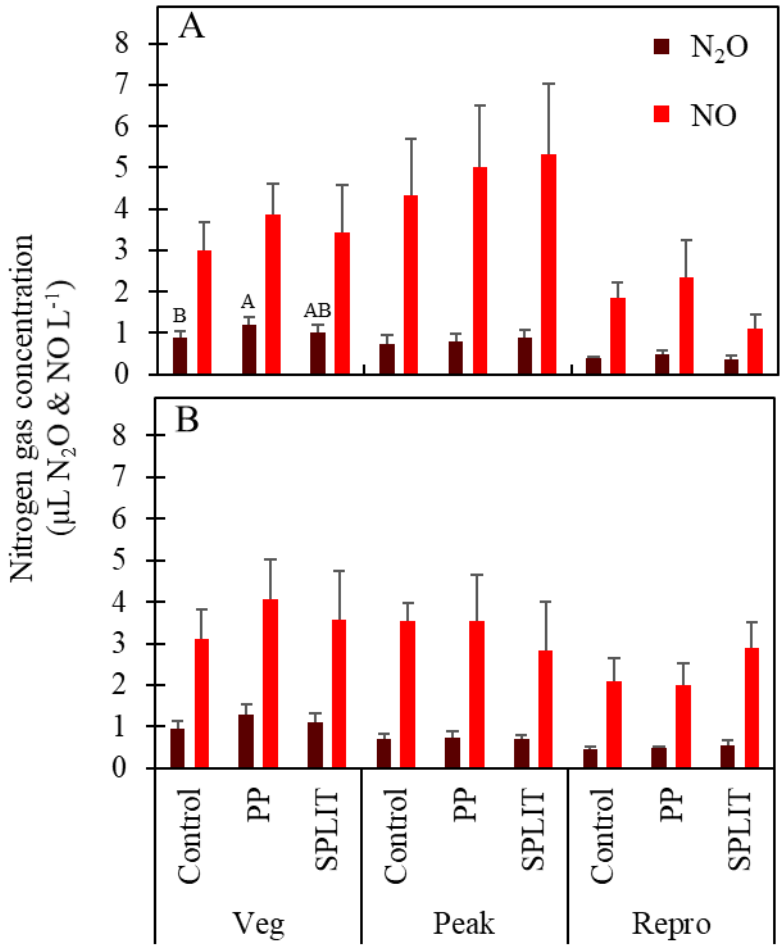

Figure 4.2 Nitrogen gas concentrations of soil pore-space N gases (N2O and NO, µL L^{-1}) measured at key growth stages for cotton cropping systems. A) 7.5 cm depth; B) 15 cm depth. Control, no-added N; PP; 100% pre-plant N fertilizer application; SPLIT 40% pre-plant 60% mid-season application of N fertilizer. LSM letters are different at p < 0.05, error bars represent standard error.

4.4.5. Correlation Analysis

Within each sampling event in 2020, a Pearson correlation coefficient was calculated between all measured soil parameters to determine significant (p<0.05) relationships between potential proximal and distal controls for N gas cycling, N_2O

emissions and pore-space concentrations, NO pore-space concentrations, and CO_2 emissions and pore-space concentrations. Correlations are presented within each sampling period for the 0-10 cm depth and the 10-20 cm depth in Tables 4.3 and 4.4, respectively. Briefly, Soil pH was negatively correlated with both NO_3^--N and NH_4^+-N concentration within the Veg sampling (*N*=27, *r*=-0.79 and *r*=-0.63, respectively). A negative correlation between soil pH and NO_3^--N (*N*=27, *r*=-0.70), NH_4^+-N (*N*=27, *r*=-0.49), ITS gene abundance (*N*=27, *r*=-0.44), and pore-space N_2O concentration (*N*=27, *r*=-0.50) was determined within the Veg sampling at the 10-20 cm depth. Soil pH was negatively correlated with pore-space N_2O (*N*=27, *r*=-0.48) at the 10-20 cm depth within the Peak sampling. At the 0-10 cm depth soil NO_3^--N was positively correlated with pore-space N_2O (*n*=27, *r*=0.44) and both NO_3^-N and NH_4^+-N were positively correlated with N_2O emissions (*N*=27, *r*=0.61 and *r*=0.61, respectively) and pore-space NO (*N*=27, *r*=0.40 and *r*=0.41, respectively) within the Peak sampling. At the 10-20 cm depth, soil NO_3^--N was positively correlated with pore-space N_2O and NO (*N*=27, *r*=0.49, *N*=27, *r*=0.47, respectively) within the Veg sampling. Soil NH_4^+-N was positively correlated with pore-space N_2O and NO (*N*=27, *r*=0.39, *N*=27, *r*=0.45, respectively) within the Veg sampling at the 10-20 cm depth. Within the Veg sampling, 16S rRNA gene abundance was positively correlated with *nosZ* clade I and ITS abundance (*N*=27, *r*=0.83 and *r*=0.55, respectively) at the 0-10 cm depth, with the same relationship occurring at the 0-10 cm depth within the Peak sampling (*N*=27, *nosZ* clade I *r*=0.38, ITS *r*=0.68).

Table 4.3 Pearson correlation coefficients for comparison of potential proximal and distal controls over N gas cycling in 2020 at 0-10 cm depth

Vegetative Growth

	Cmin	GWC	pH	NO_3^--N	NH_4^+-N	16S	clade I	clade II	ITS	N_2O Flux	CO_2 Flux	N_2O Conc	NO Conc	CO_2 Conc
Cmin	1													
GWC	0.13	1												
pH	-0.10	-0.15	1											
NO_3^--N	0.23	0.20	-0.77‡	1										
NH_4^+-N	0.06	0.31	-0.63‡	0.28	1									
16S	0.26	-0.07	-0.07	0.11	0.08	1								
clade I	0.04	0.06	-0.14	0.21	0.01	0.83‡	1							
clade II	-0.10	0.14	-0.11	0.11	0.07	0.30	0.45*	1						
ITS	0.00	0.12	-0.42*	0.34	-0.02	0.55†	0.54†	0.30	1					
N_2O Flux	-0.10	-0.13	-0.19	0.17	0.21	-0.02	0.11	-0.08	-0.11	1				
CO_2 Flux	0.06	0.28	0.02	0.07	-0.09	-0.08	0.11	0.23	-0.17	0.47*	1			
N_2O Conc.	0.10	0.35	-0.58†	0.44*	0.36	0.06	0.12	0.06	0.15	0.03	-0.12	1		
NO Conc.	0.12	0.01	-0.23	0.10	0.32	0.03	0.04	0.27	0.27	0.13	0.06	0.42*	1	
CO_2 Conc.	0.15	-0.17	-0.20	0.13	0.29	-0.10	-0.23	0.10	-0.11	-0.23	-0.33	0.33	0.08	1

Peak Plant Production

	Cmin	GWC	pH	NO_3^--N	NH_4^+-N	16S	clade I	clade II	ITS	N_2O Flux	CO_2 Flux	N_2O Conc.	NO Conc.	CO_2 Conc.
Cmin	1													
GWC	0.32	1												
pH	0.12	0.09	1											
NO_3^--N	0.23	0.05	0.02	1										
NH_4^+-N	0.33	0.18	-0.07	0.92‡	1									
16S	-0.11	0.09	0.33	-0.04	-0.08	1								
clade I	-0.08	-0.06	0.39*	-0.17	-0.21	0.68‡	1							
clade II	-0.13	0.02	0.24	-0.12	-0.21	0.03	-0.10	1						
ITS	-0.24	0.44	0.38	0.17	0.15	0.68‡	0.53†	0.18	1					
N_2O Flux	0.01	-0.15	-0.12	0.61‡	0.61‡	0.12	0.37	-0.19	0.25	1				
CO_2 Flux	0.09	0.20	0.07	0.36	0.35	0.29	0.46*	0.22	0.43*	0.63‡	1			
N_2O Conc.	0.15	-0.13	-0.12	0.23	0.28	-0.05	-0.10	-0.08	-0.15	0.12	-0.08	1		
NO Conc.	-0.06	-0.30	-0.02	0.40*	0.41*	0.04	-0.04	-0.14	0.08	0.25	-0.03	0.83‡	1	
CO_2 Conc.	0.02	0.35	-0.13	0.07	0.13	-0.03	0.01	0.29	-0.17	0.19	0.30	0.29	0.13	1

Reproductive Growth

	Cmin	GWC	pH	NO_3^--N	NH_4^+-N	16S	clade I	clade II	ITS	N_2O Flux	CO_2 Flux	N_2O Conc.	NO Conc.	CO_2 Conc.
Cmin	1													
GWC	0.15	1												
pH	-0.36	-0.01	1											
NO_3^--N	0.34	0.11	-0.53†	1										
NH_4^+-N	0.45*	0.08	-0.80‡	0.51†	1									
16S	-0.06	-0.05	0.42*	-0.34	-0.15	1								
clade I	0.36	-0.02	0.06	-0.29	0.07	0.66‡	1							
clade II	0.02	0.30	0.18	-0.12	0.00	0.51†	0.25	1						
ITS	0.30	0.53†	-0.07	0.06	0.27	0.27	0.13	0.74‡	1					
N_2O Flux	-	-	-	-	-	-	-	-	-	1				
CO_2 Flux	0.11	-0.13	0.13	-0.22	0.10	0.25	0.25	0.10	0.11	-	1			
N_2O Conc.	-0.17	-0.07	-0.03	-0.10	0.08	-0.01	-0.02	-0.06	-0.15	-	0.30	1		
NO Conc.	-0.33	-0.13	0.13	-0.08	-0.05	0.12	-0.05	-0.12	0.03	-	0.01	0.83‡	1	
CO_2 Conc.	-0.03	-0.01	0.14	-0.10	-0.11	-0.20	-0.26	0.07	-0.12	-	0.31	0.21	0.04	1

* significant at $p<0.05$
† significant at $p<0.01$
‡ significant at $p<0.001$

Table 4.4 Pearson correlation coefficients for comparison of potential proximal and distal controls over denitrification in 2020 at 10-20 cm depth

Vegetative Growth

	Cmin	GWC	pH	NO_3^--N	NH_4^+-N	16S	clade I	clade II	ITS	N_2O Flux	CO_2 Flux	N_2O Conc.	NO Conc.	CO_2 Conc.
Cmin	1													
GWC	0.16	1												
pH	0.16	0.14	1											
NO_3^--N	-0.21	0.03	-0.70‡	1										
NH_4^+-N	-0.24	0.06	-0.49†	0.95‡	1									
16S	-0.14	-0.14	-0.30	0.12	0.10	1								
clade I	-0.23	-0.16	-0.23	-0.01	-0.04	0.94‡	1							
clade II	-0.32	-0.24	0.04	0.07	0.13	0.27	0.33	1						
ITS	-0.16	-0.08	-0.44*	0.11	0.03	0.86‡	0.86‡	0.16	1					
N_2O Flux	0.04	-0.36	-0.17	0.15	0.11	0.24	0.08	-0.04	0.08	1				
CO_2 Flux	-0.25	-0.18	-0.01	-0.19	-0.22	0.16	0.13	0.06	0.17	0.47*	1			
N_2O Conc.	0.04	0.17	-0.50†	0.49†	0.39*	-0.07	-0.13	0.05	-0.13	-0.01	-0.23	1		
NO Conc.	-0.13	0.21	-0.23	0.47*	0.45*	-0.05	-0.05	-0.02	-0.16	0.07	-0.10	0.49†	1	
CO_2 Conc.	0.00	0.21	-0.27	0.25	0.19	-0.10	-0.13	-0.24	-0.04	-0.20	0.12	0.34	0.15	1

Peak Plant Production

	Cmin	GWC	pH	NO_3^--N	NH_4^+-N	16S	clade I	clade II	ITS	N_2O Flux	CO_2 Flux	N_2O Conc.	NO Conc.	CO_2 Conc.
Cmin	1													
GWC	0.14	1												
pH	0.62	0.04	1											
NO_3^--N	0.36	0.12	0.07	1										
NH_4^+-N	0.34	0.22	-0.07	0.62‡	1									
16S	0.08	-0.25	0.44*	-0.06	-0.12	1								
clade I	0.24	-0.63‡	0.24	0.21	0.13	0.67‡	1							
clade II	0.13	-0.29	0.05	0.43*	0.33	0.24	0.58†	1						
ITS	-0.12	-0.71‡	0.26	-0.01	-0.24	0.65‡	0.73‡	0.06	1					
N_2O Flux	0.16	-0.27	-0.16	0.60†	0.44*	-0.03	0.36	0.52†	0.11	1				
CO_2 Flux	0.44*	-0.01	-0.01	0.37	0.56†	0.07	0.29	0.33	-0.04	0.63‡	1			
N_2O Conc.	-0.14	-0.04	-0.48*	-0.08	0.06	-0.36	-0.28	-0.13	-0.18	0.04	-0.01	1		
NO Conc.	0.05	0.04	-0.22	-0.26	0.09	-0.05	-0.05	-0.13	-0.10	0.10	0.23	0.48*	1	
CO_2 Conc.	0.38	0.26	-0.08	0.18	0.22	-0.37	-0.28	-0.25	-0.35	-0.09	0.26	0.28	0.11	1

Reproductive Growth

	Cmin	GWC	pH	NO_3^--N	NH_4^+-N	16S	clade I	clade II	ITS	N_2O Flux	CO_2 Flux	N_2O Conc.	NO Conc.	CO_2 Conc.
Cmin	1													
GWC	-0.22	1												
pH	0.04	0.22	1											
NO_3^--N	0.25	-0.01	-0.02	1										
NH_4^+-N	0.14	-0.44	-0.36	0.05	1									
16S	0.57†	-0.45*	0.17	-0.07	0.02	1								
clade I	0.39	-0.52*	0.10	-0.12	0.06	0.87‡	1							
clade II	0.51*	-0.21	0.03	-0.10	-0.06	0.59†	0.53†	1						
ITS	0.51*	-0.26	0.06	0.17	-0.03	0.73‡	0.62‡	0.29	1					
N_2O Flux	-	-	-	-	-	-	-	-	-	1				
CO_2 Flux	0.37	-0.20	0.07	-0.31	0.28	0.21	0.11	0.12	0.17	-	1			
N_2O Conc.	0.26	0.04	-0.34	-0.19	0.22	-0.09	-0.07	0.05	-0.03	-	0.50†	1		
NO Conc.	0.19	0.11	-0.21	-0.27	0.03	-0.04	-0.10	-0.11	0.05	-	0.38	0.86‡	1	
CO_2 Conc.	0.13	-0.08	0.12	-0.04	-0.09	0.25	0.34	-0.02	0.11	-	0.09	0.01	-0.12	1

* significant at $p<0.05$
† significant at $p<0.01$
‡ significant at $p<0.001$

4.4.6. Ordination Analysis

Non-metric multidimensional scaling (NMDS) using Euclidean distance was conducted using the soil chemical, physical, and biological characteristics collected in the final year of the study (2020). Due to lack of convergence (at 500 iterations) and extremely low stress values determined when each sampling period was analyzed within

depth, the 0-10 cm and 10-20 depths were averaged for analysis. Soil pore-space CO_2 determined during each sampling period in 2020, as well as bulk density (BD) measured at the end of the study were also included in the analysis (Tables S4.13-15). Due to the large amount of zero-emissions measured for N_2O, N_2O emissions were converted to CO_2 equivalents and added to measured CO_2 emissions to yield a single CO_2 equivalent (CO2E) gas emission for each plot within each sampling in 2020.

Within each sampling period in 2020, no distinct clustering of conservation system was observed with NMDS analysis (Fig. 4.3). Some N treatment clustering was determined for the control at Veg; however, the control was not distinct from the PP or SPLIT treatment (Fig. 4.3). Sample scores within the Veg sampling were variable across conservation system and N treatment combinations, while within the Peak sampling, sample scored were more consistent except for the SPLIT treatment (Table S4.16). Sample scores within the Repro sampling were also more uniformly variable compared to the other two sampling periods (Table S4.16). Variable scores within the Veg sampling were similar across soil parameters for NMDS1 apart from CO2E, which was the only positive variable. Similarly, variable scores for NMDS2 were not highly variable with the exception of NO_3^--N and NH_4^+-N within the Veg sampling (Table S4.17). Similar to NMDS1 variable scores within the Veg sampling, CO2E had a positive variable score along with NO_3^--N and NH_4^+-N. In addition, NMDS1 and NMDS2 variable scores within the Peak sampling were most highly variable for NO_3^--N being highly positive and vegetive, respectively, compared to the reset of the variable scores. Both NMDS1 and NMDS2 variable scores were more similar for the Repro

sampling, although Cmin, NO_3^--N, and pore-space N_2O and NO were determined to have negative variable scores for NMDS1, and NO_3^--N and CO2E were varied for NMDS2 (Table S4.17). Due to similar variable scores across measured parameters, only those with distinct contribution across the three sampling periods were included within the NMDS plots.

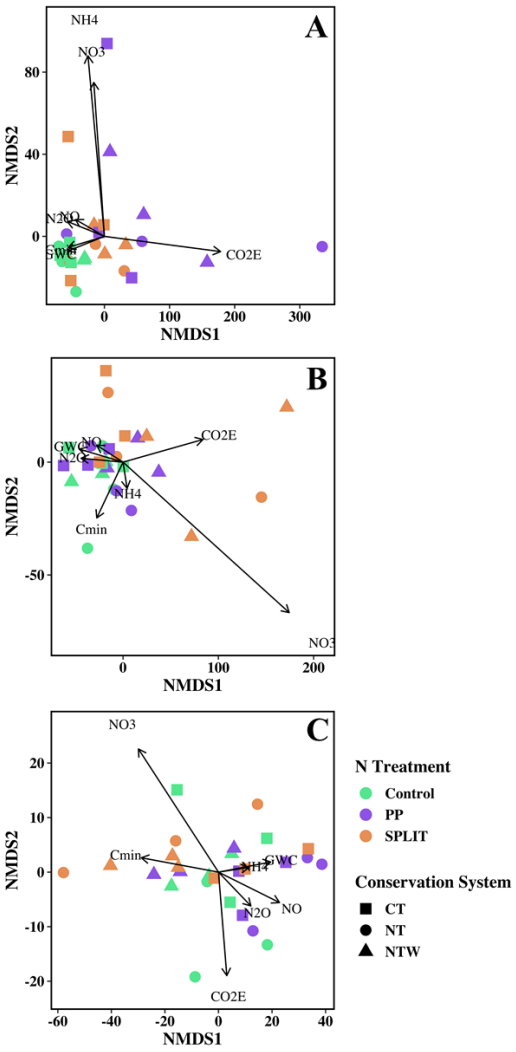

Figure 4.3 Non-metric multidimensional scaling (Euclidean distance) of soil chemical, biological, and physical characteristics within A) Vegetative Growth, B) Peak Plant Production, and C) Reproductive Growth in 2020. Stress for each NMDS is A) 0.024, B) 0.007, C) 0.008. Natural log transformation was conducted

for gene abundances and pore-space CO_2 concentrations (CO2) within each sampling period. Cmin, mineralizable carbon content; CO2E, CO_2 equivalent gas emissions ($N_2O + CO_2$); GWC, gravimetric water content; NH4, NH_4^+-N concentration; NO3, NO_3^--N concentration; N2O, pore-space N_2O concentration; NO, pore-space NO concentration. Not all parameters used for scaling are pictured as vectors, including 16S rRNA gene abundance, ITS gene abundance, *nosZ* clade I gene abundance, *nosZ* clade II gene abundance, pore-space CO_2 concentration, soil pH, and bulk density.

4.5. Discussion

This study demonstrates that the application of N fertilizer application introduces the greatest impact on N gas cycling in semi-arid agricultural soils with low soil C during periods of low GWC. As expected, soil NO_3^--N and NH_4^+-N concentrations increased with N fertilizer application, which also led to increased emissions of N_2O as well as increased pore-space concentration of N_2O and NO. Maximum N_2O emissions were recorded within the Veg sampling for the PP treatment, where fertilizer application occurred roughly one month prior to soil sampling. Pore-space N_2O stayed relatively consistent across N treatments, conservation systems, and time of year sampled, although pore-space N_2O was determined to have the same significance as N_2O emissions during the Veg sampling. However, pore-space NO stayed relatively consistent across sampling events with no effects of N treatment or conservation system observed. The differential response between NO in the pore-space across the year and emissions from the soil surface may be related to an abundant denitrifying community, which confers NO production deeper in the soil profile, but as the gas moves upward, it is reduced to N_2 when the N_2O-reducing population is active. The activity of the N_2O-reducing community was confirmed through the measurement of net consumption of

N_2O during both the Veg and Peak samplings, especially when inorganic N may be limiting (Butterbach-Bahl et al., 1998; Rosenkranz et al., 2006; Kroeze et al., 2007).

4.5.1. Agricultural production practices increase NO production in low-C content semi-arid soil profiles during periods of low GWC

High pore-space concentrations of NO compared to N_2O were determined across sampling events in 2020, with average concentrations at 7.5 cm reaching up to approximately 5.5 μL L^{-1}. An increased propensity for NO production via both nitrification and denitrification due to the application of UAN fertilizer containing significant amounts of both NH_4^+ and NO_3^-, may have conferred the production of NO under aerobic or semi-aerobic conditions, but not the further oxidation or reduction to NO_2^- and N_2O, respectively. Pore-space N_2O and NO were correlated with soil NH_4^+-N and NO_3^--N at 10-20 cm within the Veg sampling, indicating both nitrification and denitrification pathways contributing to N gas production as suggested above. Although greater NO production at depth was not specifically correlated with greater N_2O emission from the soil surface, due to low soil inorganic N in the top layer of the soil conferring net N_2O consumption from the atmosphere (Butterbach-Bahl et al., 1998; Rosenkranz et al., 2006; Kroeze et al., 2007), it would be feasible that this community also acts to significantly reduce the NO/N_2O moving upward in the soil profile, confounding the pore-space and emissions relationship. Compared to previous measurements in agricultural fields (Gut et al., 1999b) and forests (Dong et al., 2016), the concentration of NO in the soil pore-space was much higher for the semi-arid

agricultural soils of this study. The increase in NO compared to previous studies is likely the result of the combination of greater N fertilizer input compared to both previous studies. In addition, NO flux rates from this study, when determined to be significant (data not shown), were reduced compared to the fluxes determined by Gut et al. (1999a). It is likely that the inherent difference in soil type, measurement depth, rate and type of N fertilizer application, and average temperature during sampling would account for the difference in NO concentrations reported in each study. Seasonal variation in semi-arid soil pore-space NO was similar to the seasonal variation in soil NO observed in Dong et al. (2016), where high temperatures and the application of N fertilizer, and thus increased nitrification rates, were associated with the Veg and Peak samplings and account for the generally greater soil NO concentration compared to the Repro sampling.

Soil pore-space concentrations of N_2O differ with N treatment at the 7.5 cm depth within the Veg sampling, confirming the trend of N_2O production increasing following N fertilizer application (Liu et al., 2014; Samad et al., 2016; Highton et al., 2020). The effect of N treatment was not observed for pore-space NO, but the same trend is present at the Veg sampling. Although no other effects were seen for NO or N_2O pore-space concentrations, some general trends emerged at the 7.5 cm depth where during the Peak sampling, the SPLIT treatment had generally greater NO than the PP treatment or the control. This would follow along the same conclusion as at the Veg sampling, where the recent application of N fertilizer in the SPLIT treatment increased NO concentration. In the case of the Peak sampling, however, the trend did not continue with pore-space N_2O concentration but was reflected in greater N_2O emissions from the

SPLIT treatment. The presence of NO_3^- in the irrigation water at the research site likely complicated the determination of pore-space differences in N_2O and NO concentration within the N treatments and the control. However, the application of N with irrigation water did not likely translate to an effect on N_2O emissions as net N_2O-consumption was observed for the control, suggesting that the fate of the irrigation-N is either plant uptake, microbial sequestration, or general increase in microbial cycling of N within the system, conferring the lack of apparent differences between the control and the N treatments.

4.5.2. Under low soil GWC, soil inorganic N most consistently regulates N2O and NO production in low C content semi-arid agricultural soils

When examining the relationships between proximal controls over N_2O production and consumption within the Veg sampling period, there is a strong negative correlation between soil inorganic N (NO_3^--N and NH_4^+-N concentration) and soil pH. It is well known that the application of NH_4^+-N based N fertilizers, can induce acidification through nitrification of the applied NH_4^+. No significant relationship between N_2O emission and pH or inorganic N was determined at either depth within the Veg sampling, however the concentration of N_2O within the soil profile was negatively correlated with pH at both the 0-10 cm and the 10-20 cm depth and is likely related to the combined effect of inorganic N application and lower pH which has been shown to limit N_2O reduction to N_2 due to the pH sensitivity of the N_2O reductase enzyme (Liu et al., 2014; Samad et al., 2016).

Net consumption of N_2O was determined for the PP treatment and the control within the Peak sampling in 2020, where N_2O emission was also moderately correlated with soil inorganic N. Low concentrations of inorganic N were determined within the PP treatment and the control at Peak in 2020, but inorganic N was increased for the SPLIT treatment. It is likely that N applied within the SPLIT treatment fertilizer application two weeks prior to sampling increased N gas cycling processes, while in the PP treatment and control the low N with no additional fertilizer application supported an increase in net N_2O consumption (Butterbach-Bahl et al., 1998; Rosenkranz et al., 2006; Kroeze et al., 2007). Soil NO concentrations and N_2O emissions increased with NO_3^--N and NH_4^+-N at 0-10 cm depth within the Peak sampling in 2020, likely due to a preferential production of NO by soil microorganisms at or below 7.5 cm depth, and further reduction of the produced NO as it moves upward in the soil profile to the soil surface. Unlike previous determinations of a close relationship between soil NO concentrations and surface fluxes from forest soil (Medinets et al., 2019), no such distinct relationship was determined here. It is possible that there is an unmeasured factor more tightly regulating N gas emission from the soil surface within our system, but it may also relate to the low amounts of inorganic N, C resources and GWC present at the time of sampling compared to soils with greater organic matter content. Neither Cmin content, nor GWC were strongly correlated with N_2O or NO concentration in the soil pore-space, nor with N_2O emissions from the soil surface within any sampling point in 2020, although values were appreciably low for both parameters. Within both Peak and Veg, the treatment with the most recent application of N fertilizer (PP for Veg, SPLIT for

PEAK) induced more variability with ordination analysis than the other N treatment and control, further solidifying the strong effect of N fertilizer on the growth period specific variation observed for soil parameters and among correlations in this semi-arid system.

4.5.3. Genetic potential for traditional denitrifiers, but not clade II N_2O reducers, is related to soil characteristics

The abundance of bacteria and bacterial denitrifiers is expected to increase when pH increases from acidic to neutral or slightly alkaline conditions (Chen et al., 2015), and was observed in this study with the strong correlations between 16S rRNA abundance and clade I denitrifier abundance and soil pH. The lack of a significant relationship between clade II N_2O reducers and total bacterial abundance, within both the Veg and Peak sampling periods, is more interesting. According to the literature, *nosZ* clade II is highly ubiquitous in soil organisms (Jones et al., 2013). Unlike clade I organisms which co-occur with other denitrification genes about 83% of the time (Hallin et al., 2018), clade II organisms are suspected of utilizing the reduction of N_2O to N_2 as a means of energy production during fluctuating oxygen conditions (Lycus et al., 2018) and are less likely to be associated with other denitrification genes (Jones et al., 2014). With the lack of correlation between increasing bacterial abundance and clade II abundance, it may be that the highly abundant clade II gene is ubiquitous in the base microbial community, while increases in clade I abundance and total bacterial abundance together indicate a buildup of traditional denitrifiers within the soil around the time of the Veg and Peak sampling periods.

Gravimetric water content was moderate for this semi-arid soil within the Peak sampling in 2020 but was negatively correlated with clade I abundance, which may point to additional factors triggering N_2O production that may have also limited the reduction of N_2O such as brief periods of high soil respiration (Wu et al., 2017). The general increase in denitrifying populations was likely related to an increase in overall bacterial abundance, which was increased with NO_3^--N concentration and clade I abundance. Neither clade I abundance nor total bacterial abundance were negatively correlated with N_2O or NO concentration in the soil pore-space, which would be expected due to the association of the denitrifying population with greater soil NO_3^--N. It is possible that N_2O emission is tied to nitrifier denitrification, where the organisms may contain an inactive clade II gene under aerobic conditions. Nitrifier-led denitrification, with inactive clade II genes, may thus account for the correlation between N_2O emission and clade II abundance at the 10-20 cm depth and is supported by the strong relationship between NH_4^+-N and pore-space NO at the 7.5 cm depth at Peak which would suggest NO being sourced from nitrification. Estimated N_2O production potential through the association of clade I N_2O-reducing organisms with complete denitrification gene suites (Hallin et al., 2018) was not significantly correlated with any N gas measurement, but did increase with bacterial abundance. Interestingly, clade I abundance was also positively correlated with ITS abundance at the 0-10 cm and 10-20 cm depths within the Veg and Peak samplings. This association may point to community-based bacterial denitrification (Zumft, 1997) where clade I N_2O reducers are able to consume the N_2O produced at the termination of fungal denitrification (Shoun et al., 2012).

Fungal denitrification has been determined to be a significant contributor to N_2O production in soil (McLain and Martens, 2006; Novinscak et al., 2016), and may play a significant role in the production of N_2O within the semi-arid soils of this study. While fungal abundance was generally reduced compared to previously reported abundances it was determined to have a moderately negative correlation with soil pH within the Veg sampling where fungal denitrification may be more prominent under slightly acidic conditions (Chen et al., 2015). However, when ITS abundance was negatively correlated with pH, no significant relationship was determined between ITS and pore-space N_2O or NO or N_2O emissions, further veiling the microbial mechanisms driving NO buildup within the soil profile that does not necessarily confer greater emissions. Like NO produced at depth from bacterial denitrification, N_2O produced during fungal denitrification would likely be reduced to N_2 as it moves upward in the soil profile due to active N_2O-reducing populations in the N poor surface soils. An additional measure such as active microbial abundance or active N_2O reducer abundance within N-poor surface soils would likely confirm this theory, where spikes of N_2O are related to both an inactive N_2O reducer community and greater fertilizer N inputs.

While yearly variation in soil chemical and biological parameters make it difficult to relate the specific conclusions from the analyses conducted within 2020, inter-year variation between sampling events followed similar patterns, and it is likely that the relationships between soil chemical parameters would be similar in the initial two years of the study.

4.6. Conclusions

The application of N fertilizer elicits responses in controls over N gas cycling and the production of N gases both in the soil pore-space and at the soil-atmosphere interface. Soil GWC and C content were less responsive to conservation system and N fertilizer application timing and did not clearly relate to soil N gas cycling. The correlation and ordination analysis conducted in the final year of the study represents the cumulative changes among soil chemical, biological, and GWC 5 years after conservation management and altered N timing were implemented at this location. Timing of N fertilization has a strong control over the relationships between soil chemical and biological factors in addition to the activity changes associated with the soil parameters measured here. The variability in N_2O emission observed in the final year of the study highlights the difficulty in relating soil chemical and biological changes to N_2O release from the soil. However, the abundant relationships between soil parameters and pore-space concentrations of N gases observed within the Veg and Peak sampling events indicate that future research aimed at elucidating gas production dynamics within semi-arid agricultural systems will be enhanced with soil pore-space measurements in addition to quantifying surface emissions from these systems. For low N content, semi-arid agricultural systems with N fertilizer application, it is likely that the application of N fertilizer is the most important factor for increased NO and N_2O production within the soil profile, with a preferential production of NO at or below 7.5 cm depth, and subsequent reduction to N_2O and N_2 as the gas moves upward in the soil profile. In addition, with the predominant soil condition of low GWC, even during N_2O

emission from the soil surface, there is likely a combination of factors controlling N_2O and NO production and N_2O release from the soil surface.

4.7. References

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5. MICROBIAL RESPONSE TO CONSERVATION MANAGEMENT PRACTICES IN SEMI-ARID SOILS: A METATRANSCIPTOMIC ANALYSIS

5.1. Summary

The evaluation of the soil metatranscriptome can provide interesting and relevant information regarding active soil processes at the time of sampling. The initial evaluation of the soil metatranscriptome presented in this study revealed the presence of a large number of ammonia oxidation-associated transcripts taxonomically identified as both ammonia-oxidizing archaea and bacteria. Differential expression analysis revealed some clustering of a conventionally tilled system compared to two no-tillage systems, with and without winter cover crops. Although no specific determination of regulation differences for soil N cycling processes was made, it was determined that the soil organic carbon (SOC) and ammonium concentrations significantly contributed to the variation in gene expression between samples. Ultimately, this study represents an initial evaluation of the soil metatranscriptome of a semi-arid soil under different soil conservation systems and further evaluation will likely reveal specific differences in active soil processes between conservation systems.

5.2. Introduction

Conservation management practices such as no-tillage and cover cropping can improve soil health parameters such as soil carbon (C) storage, microbial community diversity, and microbial abundance and biomass (Mathew et al., 2012; Balota et al., 2014; Nivelle et al., 2016). Changes in soil characteristics through conservation

management can thus subsequently affect the cycling of C and soil nutrients, greenhouse gas (GHG) emissions, soil erosion, and plant growth and activity in agricultural production systems. However, in semi-arid environments the effects of conservation management may be delayed as evidenced by the lack of difference between conventional and conservation system organic C content after 19 years of conservation management (Lewis et al., 2018). In addition, the diversity of the *nosZ* clade II N_2O-reducing population, which is known to be ubiquitous in soil environments (Jones et al., 2013), was unaffected by conservation system implementation and altered nitrogen (N) fertilizer timing after two years (McDonald et al. 2021, *in review*). Indirect measurements of soil microbial activity (greenhouse gas flux) have been shown to be impacted by soil management practices in semi-arid environments including carbon dioxide (CO_2) and nitrous oxide (N_2O) emissions (Duval, 2020; McDonald et al. 2019; McDonald et al, 2021, *in review*). It is thus likely that measurement of the active diversity of microbial communities may reveal functional changes resulting from conservation management implementation, while holistic changes in semi-arid soils may be mitigated by other environmental factors.

Evaluating the functional diversity of microbial communities has been conducted using microarray technologies for decades (Bowtell, 1999), which allow for understanding the diversity of microbial transcripts on a limited basis (Parro et al., 2007; Gilbert et al., 2008). More recent developments have introduced the evaluation of the metatranscriptome allowing for functional diversity analysis of environments where gene-expression based microarrays may be limited by low quantity and quality of RNA

(Parro et al., 2007). Metatranscriptomic analysis has since been conducted in a variety of environments including grassland and forest soils (Geisen et al., 2015), marine environments (Gilbert et al., 2008), and has been reported to elucidate functional changes in agricultural soils under organic and conventional management (Sharma et al., 2019). Combining the evaluation of sensitive soil metatranscriptomic analysis with soil characteristics would thus likely better reveal the impacts of conservation systems on soil biochemistry than soil characteristics or metagenome analysis alone. Improvements in understanding the impacts of conservation system implementation on soil chemical, physical, and biological properties could impact future decision making for the environmental sustainability of agricultural production under conservation management.

The objective of this study was to conduct a metatranscriptomic analysis of semi-arid agricultural soils to determine the impacts of a 5-year conservation system related to microbial gene expression. In addition, evaluating the relationship between microbial functional changes, soil parameters known to control the production and consumption of greenhouse gases (GHGs), and the soil pore-space concentrations of GHGs was conducted to determine the role conservation system implementation has in altering soil biochemical processes in semi-arid systems. It is hypothesized that the implementation of no-tillage with a wheat cover crop will significantly increase overall gene expression of semi-arid agricultural soil compared to conventional management and that increases in functional gene expression will be related to differences in soil chemical and physical properties resulting from conservation system implementation.

5.3. Methods

5.3.1. Site Description and Soil Sampling

This metatranscriptomic analysis experiment was conducted in the semi-arid Southern High Plains at the Texas A&M AgriLife Research center in Lubbock, TX (33.687°, -101.827°) in a continuous cotton (*Gossypium hiristum* L.) research project. The overall objective of the research was to determine the impacts of soil conservation management system implementation on soil chemical, biological, and physical characteristics, and agronomic production. The study was designed as a split-plot with three replications where the main plot was levels of soil conservation system, and the split plot was the timing of N fertilizer application. Conservation systems included conventional tillage with the soil left fallow between cotton harvest and planting (CT), no-tillage with fallow soil between harvest and planting (NT), and no-tillage with a winter wheat cover crop planted following cotton harvest and terminated prior to planting cotton (NTW). Timing of N fertilizer application included a 100% preplant application of N fertilizer (PP) which was randomly placed within each conservation system. Nitrogen fertilizer was applied as urea-ammonium-nitrate (UAN, 32-0-0) at a total rate 168 kg N ha^{-1}. The NTW and NT systems were implemented in November 2015, with the field being under conventional tillage for at least 60 years prior to implementation. For this study, a total of 9 plots measuring 15 m in length and 4 m wide (4 crop rows with 1 m row spacing) were evaluated.

Soil at the research site is classified as an Acuff loam: fine-loamy, mixed, superactive, thermic Aridic Paleustoll (U.S. Department of Agriculture, 2016). Soil

samples were collected in August 2020 (5 years after conservation system implementation) to coincide with timing of greatest plant activity for the cropping system. Four soil samples were collected within 10 cm of the 2nd or 3rd crop row within each plot using 2.5 x 40 cm hand probes to a depth of 10 cm and homogenized. Soils samples were immediately (within 5 min of sampling) flash frozen using liquid N and stored under dry ice until transport to a -80ºC freezer for storage until RNA extraction. Soil samples were also collected for chemical analysis in conjunction with the RNA soil sampling. Chemical analyses were conducted to determine soil mineralizable C (Franzluebbers, 2016), soil pH (Schofield and Taylor, 1955), nitrate (NO_3^--N) and ammonium (NH_4^+-N; Keeney and Nelson, 1982), and gravimetric water content (drying for 7 days at 60°C). In addition, soil samples were collected at the end of the growing season in 2020 to determine the cumulative effects of conservation system on bulk density (BD) and soil organic carbon (SOC; McGeehan and Naylor, 1988; Storer, 1984).

5.3.2. Pore-space gas measurements

Greenhouse gas concentrations including nitrous oxide (N_2O), nitric oxide (NO), and carbon dioxide (CO_2) were measured from the soil pore-space according to the protocols described in McDonald et al. (2021) and (Brummell et al., 2012). Briefly, pore-space probes were installed at both the 7.5 cm depth and 15 cm depth, but only data from the 7.5 cm depth is presented here to align with the soil sample depth of 10 cm. The day before sampling, a reservoir bottle was attached to the probe to increase the volume of the probe system and reduce the vacuum placed on soil pores. On the day of

sampling, a Gasmet DX4040 FTIR analyzer was integrated into each probe system and gas concentrations were measured for 2 min per plot. Final concentration of pore-space gases was determined by calculating the dilution of the soil-probe gas concentration with atmospheric air from the gas analyzer (Brummell et al., 2012).

5.3.3. RNA Extraction

Total soil RNA was extracted from each homogenized sample using a Qiagen RNeasy PowerSoil Total RNA kit with slight modifications to increase RNA yield and decrease organic contamination. Specifically, Solution IRS volume was increased to 1 mL and Solution S3 was added at a volume of 2 mL (Qiagen LLC USA, Germantown, MD). Immediately following RNA extraction, eluted RNA was purified using a RNeasy PowerClean Pro CleanUP kit following manufacturer protocol, with a final elution volume of 75 µL (Qiagen LLC USA, Germantown, MD). Samples were then stored at -20ºC until analysis could be conducted.

5.3.4. DNA Extraction

Soil DNA was co-isolated from the RNA isolation described previously using a RNeasy PowerSoil DNA Elution Kit according to manufacturer protocol with a final elution volume of 100 µL (Qiagen LLC USA, Germantown, MD). DNA extractions were also stored at -20ºC until analysis.

5.3.5. Quantitative PCR Analysis

Microbial DNA isolated from the homogenized soil samples was evaluated for total bacterial abundance (16S rRNA gene abundance) by quantitative PCR analysis conducted on a Thermo Fisher Quant Studio 5 (Thermo Fisher Scientific Inc., Waltham Mass, US) where the standard curve was created from serial dilution of a gBlock gene fragment containing the 16S sequence of *Flavobacterium aquatile* (DSM 1132) produced by Integrated DNA Technologies, Inc. (Coralville, IA, USA). The primers used for this analysis were Eub338 (5'-ATCATGGTSCTGCCGCG-3') and Eub518 (5'-GCCTCGATCAGRTTGTGGTT-3') (Fierer et al., 2005). The qPCR thermal profile for the analysis included an initial denaturation at 95°C for 15 min followed by 40 cycles of 95°C for 1 min, 53°C for 30 s, and 72°C for 1 min. The efficiency of the qPCR reaction was determined to be 108% with an R^2 of 0.99.

5.3.6. Library Construction and Sequencing

Library preparation and sequencing were conducted at the Texas A&M Institute for Genome Sciences and Society. Ribosomal RNA depletion was not conducted for this experiment to aid in evaluation of the total active microbial population in addition to transcribed mRNA. Extracted RNA was quantified and evaluated for degradation with an Agilent Technologies 2200 TapeStation using the High Sensitivity RNA ScreenTape System protocol with 2 µL of RNA extract (Agilent Technologies Inc., CA, USA). Extracted RNA was determined to be in high enough quantity (< 200 pg μL^{-1}) from 8 of the 9 collected samples to conduct RNAseq analysis. The NT system within the first

replication had a concentration of 25 pg μL^{-1} with inconsistent banding and was not included for sequencing analysis. Library preparation for RNAseq was conducted according to the TruSeq Stranded Total RNA protocol (Illumina Inc., CA, USA) with slight modification. Library preparation began with adding 8.5 µL of extracted RNA to the DFP plate with no prior cleaning steps. No control was used for the library preparation and RSB was substituted where indicated. The final library was quantified with a Qubit 2.0 Fluorometer (ThermoFisher Scientific Inc., MA, USA) and with an Agilent 2200 TapeStation for bp size information. The nM concentration was calculated, and samples were normalized in a two-step process. Samples were first normalized to 10 nM, followed by normalization to 4 nM in low EDTA. Final 4 nM samples were pooled and sent to the North Texas Genome Center for preparation according to Protocol A of the Denature and Dilute Libraries Guide and were sequenced using an Illumina NovaSeq 6000 (Illumina Inc., CA, USA).

5.3.7. Transcript Sequence Processing

Bioinformatic analysis was conducted using the GRACE cluster maintained by Texas A&M High Performance Research Computing as well as with the web-based programs MetaGeneMark (Besemer and Borodovsky, 1999; Zhu et al., 2010) and GhostKOALA (Kanehisa et al., 2016). Raw sequences were evaluated for quality using the FASTQC tool (Andrews, 2010). Contigs were formed for each sample using MEGAHIT (Li et al., 2015). Coding sequences (nucleotide and translated amino acid sequences) were then predicted for assembled contigs from each sample using

MetaGeneMark version 3.25 (Besemer and Borodovsky, 1999; Zhu et al., 2010). Functional and taxonomic classification of predicted amino acid coding sequences was conducted using KEGG orthology (prokaryote + eukaryote + virus database) using GhostKOALA (Kanehisa et al., 2016). Contigs with assigned KEGG orthology numbers were used as the initial transcriptome for index building in Salmon (Patro et al., 2017) in order to restrict transcript quantification to identified genes.

Forward and reverse reads within each sample were then passed to Salmon for quantification against the index. Following initial quantification, the identity of highly abundant transcripts was evaluated. Three highly abundant transcripts (16000-89000 transcripts across the 8 samples) were determined to classify as specifically mammalian or plant sequences. The three non-microbial transcripts were removed from the indexing file and transcripts were re-quantified against the modified index. Transcript quantities were downloaded and converted for differential expression analysis using the DESeq2 pipeline in R (Love et al., 2014).

5.3.8. Statistical Analysis

Statistical analysis for soil chemical characteristics and pore-space gas concentrations was conducted in SAS 9.4 using PROC Glimmix at a significance level of $\alpha=0.05$ (SAS, 2013; SAS-Institute, 2017b). Analysis of variance was conducted with main-plot treatments and split-plot treatments treated as fixed effects and replication was treated as a random effect. Fisher's protected LSD was used to separate means of significant effects at $\alpha=0.05$. Differential expression analysis within the DESeq2

package in R including principal components analysis and gene clustering using regularized-logarithmic (rlog) transformation of transcript quantities and Euclidean distance (Love et al., 2014). Variation partitioning analysis was conducted using the varpart function within the vegan package in R (Oksanen et al., 2020), and tested for significance at α=0.1 using the anova function for redundancy analysis with complete permutation (40319 permutations) in R (R-Core-Team, 2019).

5.4. Results

5.4.1. Soil Characteristics and Pore-Space Concentrations

After 5 years of implementation, SOC was determined to be greater for the NTW system compared to the NT and CT systems (Table 5.1). No other differences were determined between conservation systems after 5 years of implementation. In addition, no difference in soil pore-space concentrations of GHGs between conservation system was determined. However, the same pattern in concentration was observed across measured GHGs where the NT system had generally greater pore-space concentration than the NTW system, followed by the CT system.

Table 5.1 Soil characteristics of the conservation systems.

Tillage	pH	Bulk Density g cm^{-3}	GWC g H_2O 100 g $soil^{-1}$	Cmin mg CO_2-C kg $soil^{-1}$	SOC Mg ha 10 cm^{-1}	NO_3^--N	NH_4^+-N	N_2O	NO	CO_2
						mg kg $soil^{-1}$		μL GHG L^{-1}		
NTW	7.95	1.33	13.74	75.74	3.97 A	6.50	8.12	0.79	5.86	1178.0
NT	7.68	1.34	13.16	67.00	3.53 B	1.85	6.60	1.23	9.53	1540.0
CT	7.77	1.23	11.92	69.99	3.19 B	4.47	6.03	0.70	3.49	881.1
p-value	0.387	0.247	0.115	0.741	0.004	0.351	0.533	0.181	0.188	0.137

5.4.2. Total Bacterial Abundance

Total bacterial abundance (16S rRNA gene abundance) was not affected by conservation system (p=0.285), although the NTW was determined to have generally greater total bacterial abundance (4.7×10^7 copies g dry soil^{-1}) compared to the NT (1.6×10^7 copies g dry soil^{-1}) and CT systems (1.9×10^7 copies g dry soil^{-1}).

5.4.3. Transcript Quantity and Gene Clustering

Transcript abundances averaged 709193 transcripts g soil^{-1} across all samples levels and were not significantly different between conservation systems (p=0.107). Principal components analysis of regularized-log transcript abundance revealed clustering of the CT system from the two conservation systems, NTW and NT (Fig. 5.1). The two principal components combined to explain 60% of the variation within the transcript quantity data, with the clustering of the CT system from the two no-tillage systems occurring due to a combination of both PC1 and PC2.

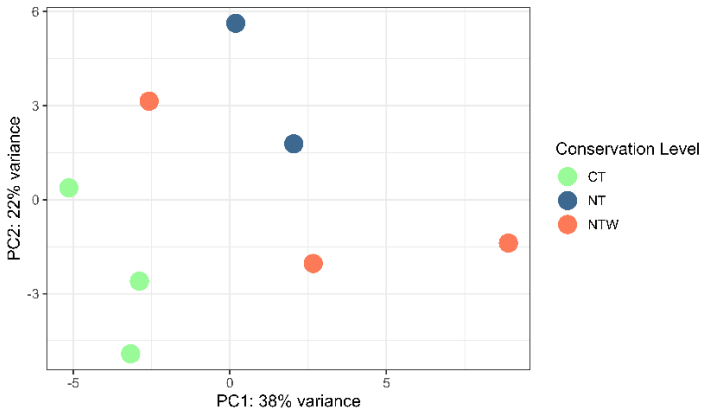

Figure 5.1 Principal components analysis of relativized-logarithmic transformed transcript quantities. CT, conventional tillage system with winter fallow soil; NT, no-tillage system with winter fallow soil; NTW, no-tillage with a winter wheat cover crop.

Gene clustering analysis was conducted for the 25 most variable transcripts (Fig. 2). Z-Score indicated by color represents the relative up-regulation and down-regulation for each gene across samples. Sample clustering by similar expression revealed patterns among conservation level, where the CT system clustered together, and variable transcripts were mostly downregulated (Fig. 5.2). As conservation system was expanded, more genes were upregulated, and clustering was observed for two of the NTW systems and one replication of the NT system. Functional identity of the variable transcripts is presented in Table S5.1.

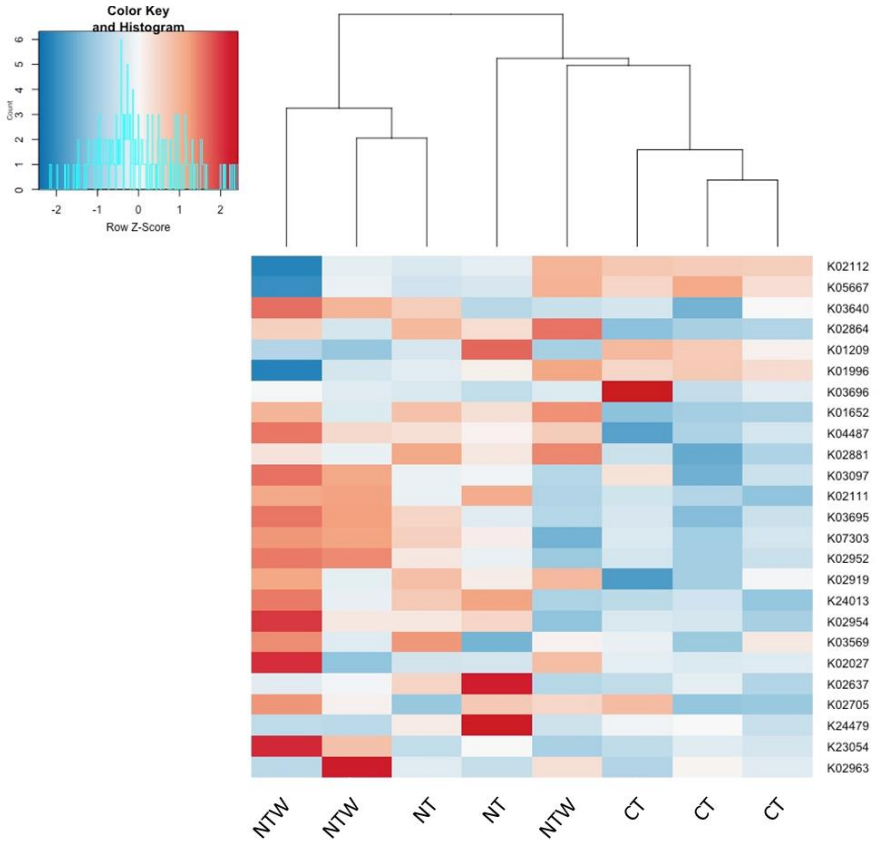

Figure 5.2 Heatmap and sample dendrogram of the 25 most variable transcripts. Z-score is represented by color with values ranging from -2 to 2 indicating downregulation and upregulation, respectively, of transcript within each sample compared across samples for each transcript. Samples with closer linkages within the dendrogram should be considered more similar in their expression of the variable transcripts presented here. Functional classification of transcripts is presented in Table S5.1.

5.4.4. Transcript Identification

Transcript identification was conducted for the 25 most expressed transcripts (>7500 total transcripts across all samples) and is presented in Table 5.2. Abundant transcript identification was dominated by *Actinobacteria* species, with classification resulting from both ribosomal and functional transcripts. In addition, three species of

ammonia-oxidizing bacteria (AOB) were identified via transcripts associated with N metabolism. However, the majority of taxonomic identifications were made from ribosomal, or ribosome-associated transcripts.

Table 5.2 Taxonomic classification and KEGG pathway/protein classification of the 25 most abundant transcripts

KEGG Orthology #	Domain	Classification		KEGG Pathway/Protein Classification
K09014	Archaea	*Thaumarchaeota*	*Nitrososphaera*	Fe-S cluster assembly protein SufB
K10945	Archaea	*Thaumarchaeota*	*Candidatus Nitrosocosmicus*	Methane metabolism; Nitrogen metabolism; Metabolic pathways; Microbial metabolism in diverse environments; Carbon metabolism
K10946	Archaea	*Thaumarchaeota*	*Candidatus Nitrosocosmicus, Nitrososphaera*	Methane metabolism; Nitrogen metabolism; Metabolic pathways; Microbial metabolism in diverse environments
K00962	Bacteria	*Actinobacteria*	*Iamia, Pimelobacter, Tetrasphaera*	RNA degradation
K00962	Bacteria	*Verrucomicrobia*	*Candidatus, Xiphinematobacter*	RNA degradation
K02703	Bacteria	*Cyanobacteria*	*Chamaesiphon, Chamaesiphon, Cyanobacterium, Dactylococcopsis, Fischerella, Geitlerinema, Gloeocapsa, Microcoleus, Moorea, Nostocales, cyanobacterium HT-58-2, Oscillatoria, Oxynema, Planktothrix, Stanieria, Synechococcus*	Photosystem II
K02836	Bacteria	*Actinobacteria*	*Nocardioides, Phytohabitans*	Genetic information processing (peptide chain release factor 2)
K02871	Bacteria	*Actinobacteria*	*Arthrobacter, Nocardioides, Pseudarthrobacter, Renibacterium*	Ribosome
K02871	Bacteria	*Betaproteobacteria*	*Caballeronia*	Ribosome
K02919	Bacteria	*Actinobacteria*	*Actinobaculum, Arthrobacter, Geodermatophilus, Mobiluncus, Modestobacter, Nocardioides, Pseudarthrobacter, Rhodoluna*	Ribosome
K02945	Bacteria	*Actinobacteria*	*Actinomadura, Actinoplanes, Amycolatopsis, Arthrobacter, Blastococcus, Catellatospora, Geodermatophilus, Kibdelosporangium, Microlunatus, Modestobacter, Mumia, Nocardioides, Paenarthrobacter, Phycicoccus, Pimelobacter, Polymorphospora, Pseudarthrobacter, Psychromicrobium, Thermomonospora, Verrucosispora, Xylanimicrobium*	Ribosome

Table continued from Table 5.2

KEGG Orthology #	Domain	Classification		KEGG Pathway/Protein Classification
K02945	Bacteria	*Alphaproteobacteria*	*Microvirga*	Ribosome
K02945	Bacteria	*Chloroflexi*	*Sphaerobacter*	Ribosome
K02945	Bacteria	*Gammaproteobacteria*	*Hydrogenovibrio*	Ribosome
K02946	Bacteria	*Actinobacteria*	*Amycolatopsis, Aquiluna, Arthrobacter, Dermatophilus, Mumia, Nocardioides, Paenarthrobacter, Phytohabitans, Pimelobacter, Pseudarthrobacter, Raineyella*	Ribosome
K02996	Bacteria	*Actinobacteria*	*Arthrobacter, Nocardioides, Pseudarthrobacter*	Ribosome
K03043	Bacteria	*Actinobacteria*	*Actinoplanes, Arsenicicoccus, Arthrobacter, Blastococcus, Catellatospora, Cellulomonas, Citricoccus, Geodermatophilus, Iamia, Microbacterium, Microlunatus, Micromonospora, Micropruina, Modestobacter, Nocardioides, Pimelobacter, Plantactinospora, Polymorphospora, Pseudarthrobacter, Streptomyces, Verrucosispora*	RNA polymerase
K03043	Bacteria	*Alphaproteobacteria*	*Candidatus Tokpelaia*	RNA polymerase
K03043	Bacteria	*Gemmatimonadetes*	*Gemmatirosa*	RNA polymerase
K03046	Bacteria	*Actinobacteria*	*Arthrobacter, Blastococcus, Conexibacter, Frankia, Geodermatophilus, Intrasporangium, Kocuria, Modestobacter, Nocardioides, Paenarthrobacter, Pseudarthrobacter, Raineyella, Yimella*	RNA polymerase
K03046	Bacteria	*Alphaproteobacteria*	*Azospirillum, Hypericibacter, Skermanella*	RNA polymerase
K03046	Bacteria	*Bacteroidetes*	*Flavobacteriaceae bacterium*	RNA polymerase
K03046	Bacteria	*Deltaproteobacteria*	*Labilithrix*	RNA polymerase

Table continued from Table 5.2

KEGG Orthology #	Domain	Classification		KEGG Pathway/Protein Classification
K03086	Bacteria	*Actinobacteria*	*Arthrobacter, Blastococcus, Conexibacter, Frankia, Kitasatospora, Pseudarthrobacter, Salinispora, Tetrasphaera*	Flagellar assembly
K03086	Bacteria	*Firmicutes*		Flagellar assembly
K03086	Bacteria	*Verrucomicrobia*	*Opitutaceae bacterium*	Flagellar assembly
K03183	Bacteria	*Acidobacteria*	*Candidatus Solibacter, Chloracidobacterium, Paludibaculum*	Ubiquinone and other terpenoid-quinone biosynthesis; Metabolic pathways; Biosynthesis of secondary metabolites; Biosynthesis of cofactors
K03885	Bacteria	*Actinobacteria*	*Arthrobacter, Pseudarthrobacter*	Oxidative phosphorylation
K03885	Bacteria	*Alphaproteobacteria*	*Tardiphaga*	Oxidative phosphorylation
K03885	Bacteria	*Betaproteobacteria*	*Herminiimonas*	Oxidative phosphorylation
K07305	Bacteria	*Alphaproteobacteria*	*Chelativorans*	Peptide-methionine (R)-S-oxide reductase
K09013	Bacteria	*Actinobacteria*	*Blastococcus, Geodermatophilus, Modestobacter, Paenarthrobacter, Plantibacter, Pseudarthrobacter*	Fe-S cluster assembly ATP-binding protein
K09014	Bacteria	*Actinobacteria*	*Actinomadura, Aeromicrobium, Arthrobacter, Blastococcus, Geodermatophilus, Kineococcus, Luteipulveratus, Micromonospora, Modestobacter, Paenarthrobacter, Polymorphospora, Pseudarthrobacter, Renibacterium, Sinomonas, Streptomyces, Verrucosispora*	Fe-S cluster assembly protein SufB

Table continued from Table 5.2

KEGG Orthology #	Domain	Classification		KEGG Pathway/Protein Classification
K10946	Bacteria	*Actinobacteria*	*Mycobacterium*	Methane metabolism; Nitrogen metabolism; Metabolic pathways; Microbial metabolism in diverse environments
K10946	Bacteria	*Betaproteobacteria*	*Nitrosospira*	Methane metabolism; Nitrogen metabolism; Metabolic pathways; Microbial metabolism in diverse environments
K13993	Bacteria	*Actinobacteria*	*Pseudarthrobacter*	Protein processing in endoplasmic reticulum
K13993	Bacteria	*Deltaproteobacteria*	*Cystobacter*	Protein processing in endoplasmic reticulum
K13993	Bacteria	*Planctomycetes*	*Aquisphaera, Candidatus Kuenenia*	Protein processing in endoplasmic reticulum
K15371	Bacteria	*Actinobacteria*	*Rhodococcus*	Arginine biosynthesis; Alanine, aspartate and glutamate metabolism; Taurine and hypotaurine metabolism; Nitrogen metabolism; Metabolic pathways; Microbial metabolism in diverse environments
K21147	Bacteria	*Actinobacteria*	*Blastococcus, Geodermatophilus, Modestobacter*	Sulfur relay system
K22769	Bacteria	*Actinobacteria*	*Arthrobacter, Blastococcus, Dermacoccus, Geodermatophilus, Nocardioides, Salinispora, Yimella*	Biosynthesis of unsaturated fatty acids; Metabolic pathways; Fatty acid metabolism

5.4.5. Variance Partitioning Analysis

Variance partitioning analysis was conducted for normalized transcript abundance variation across samples. Only the combination of SOC, NO_3^--N, and NH_4^+-N was determined to significantly affect the variation of gene abundance (p=0.060). When variance partitioning was conducted, SOC accounted for 4.6% of the variance, NO_3^--N accounted for 4.8% of the variance, and NH_4^+-N accounted for 8.5% of the variation (Fig. 5.3). Soil organic carbon and NH_4^+-N were the only soil parameters determined to individually affect variation in gene expression (p=0.090, p=0.048, respectively). No interactions between SOC, NH_4^+-N or NO_3^--N significantly affected variation.

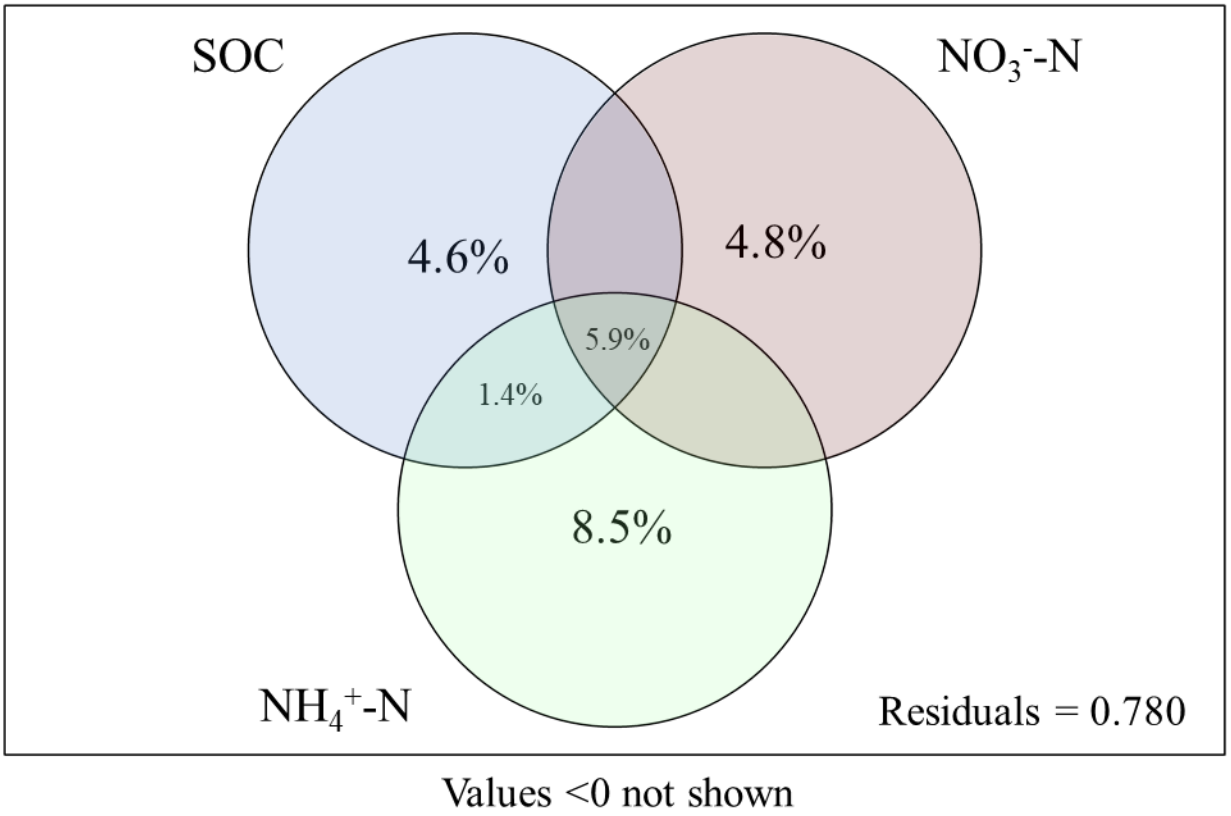

Figure 5.3 Partitioning of normalized transcript abundance variation by major soil parameters. SOC, soil organic carbon; NO_3^--N, nitrate concentration; NH_4^+-N ammonium concentration.

5.5. Discussion

From this initial characterization of the soil transcriptome under varying levels of conservation system, it is likely that the major factor inducing changes in the activity of soil microbes is the introduction of no-tillage regimes. Differential analysis of gene expression revealed clustering of the CT system compared to the two no-tillage systems, NTW and NT. The clustering of the CT system is likely related to the combined effect of soil parameters resulting from the implementation of no-tillage systems. Bulk density was generally greater for the NTW and NT systems compared to the CT system. Soil aeration has been reported to alter microbial activity (Li et al., 2016), especially when oxygen is introduced to previously anoxic environments such as during tillage disturbance of soil aggregates (Picek et al., 2000).

Gene clustering analysis of the 25 most highly variable transcripts revealed distinct separation of 2/3 of NTW system replications, with distinct upregulation of highly variable transcripts compared to the CT system. Despite generally reduced expression of highly variable transcripts, a strong upregulation of Clp protease (KO3696, responsible for stress tolerance in some microbial species) within the CT system supports the potential for climate stress on the microbial community, conferring the lack of transcript quantity differences. Of the highly variable transcripts identified, there were several related to ribosomal formation, and were specifically upregulated within the NTW system. The variation in ribosomal formation may be an additional result of climatic control, and soil conditions altered by the introduction of no-tillage, but the upregulation was not consistent across the NTW and NT system replicates.

Taxonomic classification of transcripts was carried out for the 25 most abundant transcripts across conservation systems. Identified abundant transcripts included three species of AOA. Ammonia oxidizing archaea are known to be highly abundant in soil where their

contribution to nitrification activities increases with temperature (Mukhtar et al., 2019). Thus, increased AOA activity would be expected with the climatic conditions present at the time of sampling, resulting in the production of AOA N metabolism transcripts identified in this study.

In addition, several relevant microbial species were identified from non-ribosomal transcripts including *Actinobacteria*, *Nitrosospira*, and *Mycobacterium*, which were both identified via a transcript associated with ammonium oxidation. Ribosomal RNA depletion was not conducted prior to sequencing for this study and several of the most highly abundant transcripts were related to ribosomal formation or were specifically identified as ribosomal transcripts. The ability to identify active microbes through RNAseq and transcriptomic analysis is highly beneficial for elucidating changes related to the activity and diversity of microbial populations under agricultural soil management practices.

Soil parameters expected to influence gene expression were evaluated for the contribution to the variance in normalized transcript abundance used for differential expression analysis. It was determined that the combination of SOC, NO_3^--N concentration, and NH_4^+-N concentration resulted in a combined significant effect on transcript abundance variation across samples. Specifically, it was determined that NH_4^+-N concentration had the greatest effect on variation out of the three soil parameters (8.5%), and significantly impacted the abundance of transcripts expressed in the soil. This further supported the high abundance of AOA and AOB genes taxonomically identified and indicates the activation of nitrification processes at the time of sampling.

The abundance of AOA and AOB genes at the time of sampling combined with the influence of NH_4^+-N concentration suggests that nitrification is the dominant N cycling process occurring in the soil at the time of sampling. A search of the identified transcripts only revealed a

single denitrification transcript present at the time of sampling (although the search is limited by the database used for functional annotation). It is known that a high rate of NH_4^+/NH_3 oxidation to hydroxylamine can cause a bottleneck at the oxidation of NO to NO_2^- (Caranto and Lancaster, 2017) and thus the release of NO into the soil pore-space. This process is likely causing the high concentration of NO in the soil pore-space, supporting the hypothesis that pore-space NO could be derived from both nitrification and denitrification.

5.6. Conclusion

This study represents an initial transcriptomic analysis across conservation system in semi-arid soils. This initial analysis revealed a trend toward clustering of the CT system compared to the two no-tillage systems and demonstrated the ability to identify unique regulation of transcripts through gene clustering analysis. The identification of microbial species through transcripts associated with microbial processes of interest provides support for continued evaluation of the soil transcriptome as a more sensitive measure of microbial contribution to soil processes. Finally, partitioning of the variance in gene expression supported the abundance of ammonia-oxidizing archaea and bacteria and likely indicates a large contribution of nitrification to N cycling processes at the time of sampling. Analysis of these transcripts will continue in conjunction with analysis of their relationship with soil parameters to determine the best pathway for analysis of this semi-arid agricultural soil transcriptome. Future analyses should reveal distinct relationships between the soil transcriptome and soil chemical characteristics measured at the time of sampling.

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6. CONCLUSIONS

The purpose of the studies reported within this dissertation was to elucidate the complex factors controlling N gas emissions from the soil surface in semi-arid agricultural systems. Through the studies presented here, it was determined that N gas production (emission rates at the soil-atmosphere interface and pore-space concentrations of N gases) is largely driven by the application of N fertilizer in semi-arid agricultural production systems. Despite abundant literature reporting soil carbon (C) resources and gravimetric water content (GWC) being strong controls for denitrification and N_2O emissions in agricultural systems, in semi-arid soils with N fertilizer application, as well as low C content and low GWC, this relationship was not determined. In fact, net nitrous oxide (N_2O) emissions from the soil surface were nearly absent except following N fertilizer application, and net N_2O consumption was determined when N fertilizer was not applied (no-added N control), or when N resources were depleted by the crop and microbial community or lost from the system.

Initial evaluations during the first stage of the study (first two years following implementation of conservation systems and altered N fertilizer timing) determined that an abundant N_2O-reducing population was present in the soil, likely facilitating the consumption of N_2O observed at the soil-atmosphere interface. Within the first two years of implementing a conservation system and altered timing of N fertilizer, only the timing of N fertilizer was determined to affect N_2O production or consumption. However, the combined effect of conservation system and N fertilizer timing may impact the abundance and diversity of the N_2O-reducing population in the future. During the initial evaluation of treatment effects, spikes of N_2O measured between 7 and 14 days following N fertilizer application were greatest where N fertilizer was applied. In addition, net N_2O consumption was determined where N was not

applied, and at 3-4 months following N fertilizer application. The results of this initial evaluation of N_2O cycling in semi-arid agricultural soils revealed that the timing of N fertilizer can alter the microbial community, which subsequently affects N_2O dynamics at the soil surface.

A lack of understanding N fertilizer timing effects on soil profile gas production, which may be more sensitive to N fertilizer application, lead to the development of pore-space probes (PSPs) for measurement of pore-space concentrations of N gases. The PSP method was developed from a more robust PSP design used for arctic measurements of pore-space gases to increase affordability and allow for less intense installation and removal. The PSP method was validated in the fourth year of the study (the second year of the second stage), where the pore-space concentrations of carbon dioxide (CO_2) were consistent across conservation system and N treatment replications and were in-line with pore-space CO_2 concentrations measured by alternative methods in other systems.

The second stage of the study sought to expand upon the determination of N fertilizer timing effects on the denitrifying population by increasing the frequency of soil sampling to key crop growth stages including: vegetative growth, peak plant production, and reproductive growth. Soil parameters known to be controls for denitrification processes were evaluated along with the abundance of denitrifying organisms, and the abundant clade II N_2O-reducing population to better elucidate how these parameters may interact. Due to technical issues related to the sampling of gas flux within the soil pore-space and at the soil-atmosphere interface, the final year of the study was the only year where full evaluation of soil parameters, gene abundances, N_2O emissions, and pore-space N_2O and NO were evaluated. This final year analysis represents the cumulative effects of the implemented conservation systems and N fertilizer timing alterations over a 5-year period. Interestingly, it was determined that the

application of N fertilizer increases NO concentration at depth compared to N_2O concentration and is likely related to the type of fertilizer applied (urea-ammonium-nitrate) which contains the precursors for both nitrification and denitrification. Significant relationships between pore-space N_2O/NO and soil parameters were determined within the final year but differed with the timing of sampling related to when N fertilizer was recently applied. Through ordination analysis it was confirmed that the application of N fertilizer (increasing the concentration of inorganic-N in the soil), introduced a large amount of variability into the system. Although a strong relationship between N fertilizer application, pore-space N_2O/NO concentrations, and N_2O emissions could be concluded from the results of the second stage of the study, there was still a missing link that likely further controls N gas dynamics in these systems. It is hypothesized that this missing link may be related to the overall activity of the microbial community at the time of sampling, specifically the activated processes at the various depths measured which would likely further explain the abundant NO concentration in the soil, but little relation between pore-space concentrations and emissions from the soil surface.

A final evaluation of the effect of the implemented conservation systems on microbial gene expression was conducted in the final year of the study (year 5). The expression of rRNA and mRNA transcripts was evaluated via the RNA seq protocol to elucidate any effects the implementation of conservation system may have on the active microbial population during the peak plant production growth stage. In the literature, the implementation of no-tillage with cover crops is often associated with greater microbial activity and abundance, but this is less well documented in semi-arid agricultural systems. In addition, the abundance and diversity of N cycle microbial transcripts may be more strongly related to the cycling of N gases. Conservation system effects on the microbial community was suggested to provide insight into the lack of

significant effects of N fertilizer timing, but large production of NO in the soil pore-space. This initial transcriptomic analysis revealed a trend toward clustering of the CT system compared to the two no-tillage systems. Identification of nitrifier transcripts associated with microbial processes of interest, including N metabolism, supports the assumption that N gas cycling is controlled by both nitrification and denitrification within this semi-arid system. This was an initial analysis of the soil transcriptome for this study. Analysis of the transcriptome will be expanded to the final two years of the study, which will provide greater replication and may reveal more distinct patterns of gene expression between conservation systems.

Overall, it is clear that the implementation of conservation practices and altered N fertilizer timing can have significant effects on the soil biochemical properties of semi-arid systems. The ability to significantly alter N gas cycling and microbial gene expression with conservation system and N fertilizer timing will strongly support the development of best management practices for environmental sustainability of agricultural production on the semi-arid Southern High Plains of Texas. From the results of this study, it would be recommended that the introduction of a no-tillage system with a winter cover crop and a split application of N fertilizer would increase soil C content while reducing N loss as N_2O, thus increasing the potential N use efficiency and promoting a more healthy soil.

1. APPENDIX A

SUPPLEMENTAL MATERIAL: NITROUS OXIDE CONSUMPTION POTENTIAL IN A SEMI-ARID AGRICULTURAL SYSTEM: EFFECTS OF CONSERVATION SOIL MANAGEMENT AND NITROGEN TIMING ON NOSZ MEDIATED N_2O CONSUMPTION

Tables S2.1 Soil characterization of samples collected at a depth of 0–15 cm following cover crop termination in April 2016 (original data reported in McDonald et al., 2019).

Tillage System[a]	pH	OC[b]	TN[c]	NO_3^--N	P	K	Ca	Mg	S	Na
		g kg^{-1}			mg kg^{-1}					
NTW	7.4	5.3	0.692	0.4b	42	423	1859	823	13	29
NT	7.4	5.4	0.745	6.9a	49	463	1993	809	14	36
CT	7.5	5.1	0.690	6.8a	46	419	1931	852	11	32
p-value	0.901	0.264	0.305	0.028	0.604	0.188	0.519	0.337	0.528	0.217

[a] NTW, no-till with winter wheat cover; NT, No-till winter fallow; CT, conventional tillage winter fallow.
[b] OC, organic carbon.
[c] TN, total nitrogen.

Tables S2.2 ANOVA results for conservation system, nitrogen (N) treatment, and interaction effects on nitrate (NO_3^--N) and ammonium (NH_4^+-N) levels of samples collected at 0-15 cm prior to mid-season application of N fertilizer in 2016 and 2017

Year	Effect	NO_3^--N	NH_4^+-N	N_{inorg}[a]
		ANOVA (*p*-values<0.1)		
2016	Tillage	0.517	0.329	0.661
	N Treatment	0.002	0.139	0.005
	Interaction	0.74	0.964	0.812
2017	Tillage	0.089	0.833	0.135
	N Treatment	0.002	0.218	0.003
	Interaction	0.021	0.078	0.013

[a] N_{inorg}, NO_3^--N + NH_4^+-N (total inorganic N)

Tables S2.3 ANOVA results for 16S, *nosZ* clade I, and *nosZ* clade II abundance in 2016 and 2017.

Year	Effect	16S	Clade I	Clade II
		ANOVA (*p*-values<0.1)		
2016	Tillage	0.258	0.708	0.991
	N Treatment	0.536	0.696	0.174
	Interaction	0.470	0.789	0.183
2017	Tillage	0.852	0.787	0.743
	N Treatment	0.004	0.201	0.217
	Interaction	0.118	0.790	0.081

Tables S2.4 Correlation Analysis of bacterial (16S) and N_2O-reducing populations with nitrate (NO_3^--N), ammonium (NH_4^+-N) and inorganic N (N_{inorg}, NO_3^--N + NH_4^+-N)

	16S	Clade I	Clade II
	Pearson's Correlation (p-values<0.05)		
	2016		
NO_3^--N	0.318*	0.112	0.242
NH_4^+-N	0.476	0.276	0.304
N_{inorg}	0.297	0.099	0.195
	2017		
NO_3^--N	0.335	0.289	0.254
NH_4^+-N	0.564	0.981	0.488
N_{inorg}	0.341	0.374	0.258

*p<0.05 significant

Tables S2.5 Complete DNA sequences of the 8 most abundant Amplicon Sequence Variants (ASV)

#	ASV Sequence
1	AGAGTTCGACGGAAACGGCCACGCGTACACGTCGGTCTTCATCTCCTCTGAGATCGTGAAGTACACGCTCCCTGGATGCGAAGTCGTCG ATCGCGTGCCCACGTATTACTCCATCGGGCACCTGATGGTACCGGGTGGCGATACGCGCAAACCGTATGGCAAGTACGTCATCGCACTC AACAAAATCACGAAGGACCGCTATCTCCCGACGGGGCCCGAGCTCACGCAGTCAGCTCAGTTGTATGACATCACGGGCGACAAGATGA AGCTCCTGCTCGACTTCCCCACGATCGGCGAGCCGCACTACGCGCAGGCGATCGATGCCAAACTCGTGAAGGATCGACAGACCAAGTTC TATAAGCTCGCGGAGAACAGGCATCCGTACGTGGCGAAATCGGAGAAGGAGACCAACGTCACCCGGCAGGGGAAGACGGTGCACGTGA AGATGACGGCGATCCGCAGTCACTTCGCGCCGGACAACATCGAAGGCATTCAGGTCGGCGACACCGTGTACTTCCACGTCACCAACCTC GAACAGGATTGGGACGTGCCCCATGGCATGGCGACGATCGGTTCCGCGCATGACTCCGAGTTGCTGATCATGCCTGGCGAAACACGCAC GCTGAAGTGGGTGGCCAAGTTCCCGGGTGTGTTCCCGTTCTACT
2	CGTGTTCGACGACAAGGGTTTCGCATACACCTCGGTGTTCATCGAGAGCAAGGTGGCCAAGTGGTCGCTCAAAGACATGAAGCTCGTCG AGAAGCTCTCGGCCCACTACAACATCGGACACATCCTCTCGGCGGAGGGCGATACGGTGAGCCCCGACGGCAAGTACGTGGTCGCCATG AACAAGATGTCGATCGATCGCTTCGATCCGGTCGGCCCGCTCTACCCCCAAAACTTCCAACTGGTCGACATCTCCGGCGAAAAGATGCG CCTCCTGTACGACATGCCGATCGGCATCGGCGAGCCGCACTATTCGCAGATGATCAAGGCGGACAAGCTGAAGCCGATCAAGTTCTACC CAGCCGGGCACAATCTCTACACGGGCAAGGAGGATCCCGAAGCCGTGACGGGTGGGAAAGAGCGGATCGTGCGTAATGGCAACGTGGT GGACGTGTACATGACCGCCGTACGCAGTCACTTCACCCCCGATCGCATCGAAGTCAATCAGGGCGACACGGTGAATCTGCACATCACGA ACCTCGAGCAGGCCGAAGATCAGACGCACGGCTTCACGCTCAACATGCACAACATCAACCTGAGTCTCGAGCCCGGAAAGCACGAGAA CGTGACGTTCAAGGCGGACGTGGCCGGTGTTTACCCCATGTTTT
3	TGAATTCGATGGAAAGGGTAATGCGTACACGTCTATGTTTGTATCCTCGGAAATCGTAAAATGGAATGTAAAAACATTGGAAATACTGG ACCGGGTGCCAACCTATTATTCTATTGGTCACCTTAGTGTGCCCGGCGGCCCAACGAAGACACCACACGGCAAATATGTGATCGCCTAC AATAAGATTACCAAGGACCGCTATCTTCCAACAGGTCCGGAGTTAACACAGTCGGCACAACTGTACGATATCTCAGGCGATAAAATGCG TTTGCTCCTCGACTTTCCCACAGCGGGCGAGCCACACTACGCTGAAGCGATACCAGCCAGTATGATTCAAGCCAACTCGCTTAAGTTTTT TAAGATCGAAGAAAATGAGCATCCCTTTGCTGCAAAAGGCGAAGGGCAGGCCCGGGTAGAACGCAAAGGCAAAGAAGTCCATGTTTAT ATGACTGCCATCCGTTCACATCTAACCCCTGATAATATTGAAGGCGTAAATGTCGGAGACGATGTATATTTCCACGTTACGAATCTTGAA CAGGATTGGGATGTTCCTCATGGTTTTGCGATAAAGGGAGCTAATAACGCCGAAATATTAATTATGCCAGGAGAAACACAAACCTTTCT CTGGAAGCCACTCAGCACGGGTGTGTTCCCATTCTATT
4	AGAGTTTGACGGAAATGGTAATGCATATACTTCATTCTTTGTTTCATCTGAAATTGTAAAGTGGAGTGTAAAAGACCTGAAAGTACTGGA CAGAGTTCCTACATATTATTCCATCGGTCACTTATGTGTTCCCGGTGGTCCCACGAAAAAGCCATGGGGTAAATATGTGATCGCTTATAA CAAAATAACGAAAGATCGGTACCTGCCTACGGGTCCAGAGCTTGCCCAGAGTGCACAATTGTATTCTATTGATGGTGATAAAATGAAAC TCTTACTTGACTTCCCCACAATTGGTGAACCGCACTATGCTGAAGCGATCCCGGCAGACCTGATCATGAAGAATTCTCAGAAGATCTATA AGATCGAGGAAAATAAGAACCCTTATGCAACACTGGGAGATAACAATTCAAAAGTGGAAAGAAAAGGTAACGAGGTACATGTGTATAT GACATCAATTCGTTCACATTTTACACCTGATAATATAGAAGGTGTAAAAATGGGTGATGTTGTCTATTTCCATGTAACAAATCTTGAACA GGATTGGGATGTGCCGCATGGTTTTGCGATCAAAGGCGCAAACAATGCTGAGTTATTGATCATGCCCGGTGAAACTCAAACCTTATCCTG GAAACCTGAACGCACCGGGATCTTTCCGTTTTATT

* Table continued from Table S2.5

#	ASV Sequence
5	AGAGTTTGACGGAAATGGTAATGCATATACTTCATTTTTTGTTTCATCTGAAATTGTAAAGTGGAGTGTAAAAGACCTGAAAGTACTGGA CAGAGTTCCTACATATTATTCCATCGGTCACTTATGTGTTCCCGGTGGTCCCACGAAAAAGCCATGGGGTAAATATGTGATCGCTTATAA CAAAATAACGAAAGATCGGTACCTGCCTACGGGTCCAGAGCTTGCCCAGAGTGCACAATTGTATTCTATTGATGGTGATAAAATGAAAC TCTTACTTGACTTCCCCACAATTGGTGAACCGCACTATGCTGAAGCGATCCCGGCAGACCTGATCATGAAGAATTCTCAGAAGATCTATA AGATCGAGGAAAATAAGAACCCTTATGCAACACTGGGAGATAACAATTCAAAAGTGGAAAGAAAAGGTAACGAGGTACATGTGTATAT GACATCAATTCGTTCACATTTTACACCTGATAATATAGAAGGTGTAAAAATGGGTGATGTTGTCTATTTCCATGTAACAAATCTTGAACA GGATTGGGATGTGCCGCATGGTTTTGCGATCAAAGGCGCAAACAATGGTGAGTTATTGATCATGCCCGGTGAAACTCAAACCTTATCCT GGAAACCTGAACGCACCGGGATCTTTCCGTTTTATT
6	TGAATTTGATGGCAACGGATATGCGTACACGTCAATGTTCATCTCGTCCGAAGTTGTGAAGTGGAAACTGGGCACCTGGGAAGTGGTCG ATCGGGCGCCGACGTTCTATTCCGTCGGTCACATCATGATTCCAGGTGGCGATTCCAAGAAGCCGTTTGGCAAGTACCTGGTCGCGATGA ACAAGATCACCAAGGATCGCTATCTGCCGACCGGACCAGAATTGTTCCAGTCCGCGCAGTTGTACGACATCTCGGGGGATCGCATGAAG TTGCTGCTCGACTTCCCGACCATCGGTGAGCCGCACTACGCGCAGGCGCTCCCCGCAGAACTGATCAAGGATAAACAGGTCAAGTTCTA CAAACTTTCCGAAAGCACACATCCCGACAAGATCATGGCGGAAAGCGAGGCGGGAATCACTCGCAAGGGGCGTCGCGTCGACATCAAA ATGATCGCAGTGCGCAGTCACTTTGCTCCAGACAACATCGAAGGTGTTGCACTCGGTGATACGGTGTACTTCCACGTCACGAACATCGA ACAGGATTGGGATATTCTGCATGGATTCGCCATTCTTGGTGCGCAAAACTCAGAGTTGATTCTCAATCCAGGGGAAACGAGAACACTCA AGTGGGTACCAACCAGCACCGGAGTCTATCCGTTCTATT
7	CGAGTTCGATAATGACGGCAATGCCTACACTTCGATGTTCGTCTCATCCGAAATTGTGAAATGGAACGTCAAATCACTCGAGATCCTTGA TCGAATACCGACTTACTACTCGATCGGTCACCTGAGTGTGATGGGCGGGCCCACACGGAAGCCGTACGGAAAATACATGATTGCTTATA ACAAGATCACTAAAGACCGTTATCTGCCTACGGGTCCGGAACTGGCTCAATCGGCACAGCTGTATGACATCTCGGGAGAAAAAATGCGT CTGCTGCTCGACTTCCCTACCGTGGGAGAGCCGCATTACGCCGAAGCACTGCCTGCAAGCAAGATTCAGGAAGCCTCTCTCAAGTTCTTC AAGCTGGAGGAAAATGAACACCCTTACGCCACGAAAGGTGAAGGTCAGACAAAGGTCGAGCGCAAAGGCAACCAGGTGCATGTCTGGA TGACGGCCATTCGCTCCCACCTCACACCCGATAATATTGAAGGTGTGAAGGTCGGCGATGATGTGTATTTCCATGTGACCAACCTCGAGC AGGATTGGGACGTCCCCCACGGCTTCGCTATCAAAGGTGCGAACAATGCCGAGATACTGATCATGCCTGGCGAAACGCAAACACTGAAA TGGAAAGCAACGACGGCGGGAGTGATCCCTTATTACT
8	AGAGTTCGACGGAAACGGCCACGCGTACACGTCGGTCTTCATCTCCTCTGAGATCGTGAAGTACACGCTCCCTGGATGCGAAGTCGTCG ATCGCGTGCCCACGTATTACTCCATCGGGCACCTGATGGTACCGGGTGGCGATACGCGCAAACCGTATGGGAAGTACGTCATCGCACTC AACAAAATCACGAAGGACCGCTATCTCCCGACGGGGCCCGAGCTCACGCAGTCAGCTCAGTTGTATGACATCACGGGCGACAAGATGA AGCTCCTGCTCGACTTCCCCACGATCGGCGAGCCGCACTACGCGCAGGCGATCGATGCCAAACTCGTGAAGGATCGACAGACCAAGTTC TATAAGCTCGCGGAGAACAGGCATCCGTACGTGGCGAAATCGGAGAAGGAGACCAACGTCACCCGGCAGGGGAAGACGGTGCACGTGA AGATGACGGCGATCCGCAGTCACTTCGCGCCGGACAACATCGAAGGCATTCAGGTCGGCGACACCGTGTACTTCCACGTCACCAACCTC GAACAGGATTGGGACGTGCCGCATGGCATGGCGACGATCGGTTCCGCGCATGACTCCGAGTTGCTGATCATGCCTGGCGAAACACGCAC GCTGAAGTGGGTGGCCAAGTTCCCGGGTGTGTTCCCGTTCTACT

Tables S2.6 ANOVA results for conservation system, nitrogen (N) treatment, and interaction effects on N_2O-N flux rate in 2016 and 2017

Year	Season	Conservation system	N Treatment	Interaction
		ANOVA (*p*-values<0.1)		
2016	Spring	0.488	0.118	0.779
	Summer	0.998	0.013	0.231
	Fall	0.133	0.736	0.965
2017	Spring	0.392	0.397	0.371
	Summer	0.598	0.076	0.368
	Fall	0.706	0.116	0.696

Tables S2.7 ANOVA results for conservation system, nitrogen (N) treatment and interaction effects on cumulative N_2O-N emissions in 2016 and 2017

Year	Conservation system	N Treatment	Interaction
	ANOVA (*p*-values<0.1)		
2016	0.644	0.027	0.485
2017	0.551	0.104	0.310

2. APPENDIX B

SUPPLEMENTAL MATERIAL: SOIL NUTRIENT MANAGEMENT AFFECTS PROXIMAL CONTROLS FOR N GAS CYCLING

Table S4.1 Analysis of Variance (ANOVA) values for soil chemical and physical characteristics

Sampling Period	Depth	Year	pH			Gravimetric Water Content			Mineralizable C			Soil NO_3^- Concentration			Soil NH_4^+ Concentration		
			Conservation System	N treatment	Interaction	Conservation System	N treatment	Interaction	Conservation System	N treatment	Interaction	Conservation System	N treatment	Interaction	Conservation System	N treatment	Interaction
			ANOVA (p-values<0.05)														
Vegetative Growth	0-10 cm	2018	0.901	0.006	0.798	0.418	0.178	0.646	0.905	0.470	0.669	0.450	0.134	0.610	0.901	0.331	0.723
		2019	0.624	0.012	0.546	0.384	0.181	0.641	0.974	0.070	0.302	0.011	0.144	0.148	0.001	<0.001	<0.001
		2020	0.465	0.002	0.531	0.001	0.376	0.308	0.049	0.849	0.733	0.479	0.039	0.130	0.522	0.122	0.776
	10-20 cm	2018	0.356	0.002	0.016	0.429	0.548	0.570	0.093	0.363	0.422	0.785	0.066	0.554	0.807	0.105	0.926
		2019	0.087	<0.001	<0.001	0.470	0.568	0.595	0.398	0.275	0.180	0.027	0.114	0.421	0.199	0.567	0.604
		2020	0.665	0.032	0.521	0.149	0.661	0.722	0.246	0.689	0.084	0.342	0.146	0.724	0.328	0.392	0.449
Peak Plant Production	0-10 cm	2018	0.703	0.001	0.687	0.209	0.143	0.374	0.001	0.626	0.530	0.001	0.919	0.644	0.896	0.405	0.530
		2019	0.015	<0.001	0.990	0.055	0.421	0.390	0.573	0.336	0.280	0.072	0.001	0.752	0.424	0.545	0.173
		2020	0.078	0.316	0.398	0.005	0.478	0.470	0.610	0.526	0.507	0.866	0.088	0.882	0.541	0.250	0.853
	10-20 cm	2018	0.970	0.004	0.252	0.023	0.001	0.622	0.018	0.629	0.015	0.847	0.646	0.519	0.479	0.754	0.444
		2019	0.036	<0.001	0.061	0.052	0.412	0.619	0.252	0.951	0.741	0.002	<0.001	0.339	0.205	0.613	0.589
		2020	0.623	0.184	0.660	0.889	0.489	0.738	0.077	0.554	0.053	0.754	0.077	0.874	0.290	0.337	0.723
Reproductive Growth	0-10 cm	2018	0.064	0.004	0.454	0.043	0.945	0.885	0.006	0.172	0.907	0.916	0.063	0.054	0.409	0.424	0.265
		2019	0.497	<0.001	0.511	0.913	0.436	0.485	0.230	0.127	0.157	0.009	0.137	0.105	0.132	0.042	0.132
		2020	0.278	0.001	0.176	0.013	0.641	0.830	0.001	0.107	0.074	0.432	0.890	0.260	0.463	0.010	0.645
	10-20 cm	2018	0.799	0.006	0.022	0.412	0.164	0.397	0.445	0.420	0.681	0.644	0.513	0.565	0.606	0.238	0.672
		2019	0.011	0.001	0.163	0.055	0.475	0.599	0.088	0.526	0.936	<0.001	0.197	0.335	0.358	0.906	0.786
		2020	0.134	0.035	0.087	0.005	0.005	0.316	0.131	0.689	0.456	0.993	0.514	0.317	0.984	0.237	0.506

Table S4.2 pH values and LS Means letters for significant Conservation System and N Treatment interactions

Sampling Period	Year	Depth	Conservation System	N Treatment	pH	LS Means (α=0.05)
Vegetative Growth	2018	10-20 cm	NTW	Control	7.99	A
				PP	7.61	C
				SPLIT	7.72	BC
			NT	Control	7.85	ABC
				PP	7.64	C
				SPLIT	7.82	ABC
			CT	Control	7.93	AB
				PP	7.77	ABC
				SPLIT	7.34	D
	2019	10-20 cm	NTW	Control	7.92	A
				PP	7.37	DE
				SPLIT	7.87	A
			NT	Control	7.77	AB
				PP	7.55	CD
				SPLIT	7.72	ABC
			CT	Control	7.83	AB
				PP	7.62	BC
				SPLIT	7.31	E
Reproductive Growth	2018	10-20 cm	NTW	Control	8.44	AB
				PP	8.19	AB
				SPLIT	8.25	AB
			NT	Control	8.39	AB
				PP	8.16	BC
				SPLIT	8.32	AB
			CT	Control	8.47	A
				PP	8.39	AB
				SPLIT	7.88	C

Table S4.3 Soil chemical and physical characteristic averages

Sampling Period	Depth	Year	Conservation System	N Treatment	Gravimetric Water Content	Mineralizable C	NO_3^--N Concentration	NH_4^+-N Concentration
					g water 100 g $soil^{-1}$	mg CO_2-C kg $soil^{-1}$	mg $kg\ soil^{-1}$	mg $kg\ soil^{-1}$
Vegetative Growth	0-10 cm	2018	NTW	-	13.82	135.3	9.7	13.7
			NT	-	14.30	124.9	5.7	14.8
			CT	-	12.82	134.7	8.1	10.8
			-	Control	12.66	137.9	5.5	19.7
			-	PP	13.48	113.0	11.6	6.2
			-	SPLIT	14.81	144.1	6.4	13.4
		2019	NTW	-	13.84	35.0	20.8	8.4
			NT	-	14.32	33.7	34.3	9.8
			CT	-	12.77	32.7	65.4	26.1
			-	Control	12.64	23.9	24.4	7.4
			-	PP	13.48	29.4	50.2	7.7
			-	SPLIT	14.80	48.1	45.8	29.2
		2020	NTW	-	13.52	78.4	29.2	12.7
			NT	-	13.16	64.6	18.6	8.9
			CT	-	11.54	68.4	30.2	18.0
			-	Control	12.66	69.0	9.5	7.1
			-	PP	12.48	72.1	37.6	23.0
			-	SPLIT	13.09	70.3	30.9	9.5
	10-20 cm	2018	NTW	-	15.67	102.8	6.7	1.7
			NT	-	15.32	98.1	4.9	4.7
			CT	-	14.08	70.9	5.8	2.7
			-	Control	14.22	85.8	2.6	0.0
			-	PP	15.49	83.0	9.1	9.1
			-	SPLIT	15.37	103.0	5.7	0.0
		2019	NTW	-	15.66	44.7	11.4	3.4
			NT	-	15.31	52.1	21.1	3.9
			CT	-	14.13	25.3	37.6	7.1
			-	Control	14.24	24.2	13.1	3.5
			-	PP	15.49	57.3	32.6	5.7
			-	SPLIT	15.36	40.6	24.5	5.2
		2020	NTW	-	15.11	51.3	8.4	7.2
			NT	-	13.78	60.9	7.2	7.5
			CT	-	14.51	56.0	22.2	17.7
			-	Control	14.13	55.9	2.4	7.3
			-	PP	14.68	58.6	25.0	17.0
			-	SPLIT	14.58	53.8	10.4	8.0
Peak Plant Production	0-10 cm	2018	NTW	-	9.22	83.0	21.4	9.4
			NT	-	8.99	72.5	20.5	9.2
			CT	-	8.68	44.8	42.6	8.4
			-	Control	8.98	63.7	29.4	9.6
			-	PP	9.26	65.6	27.6	7.2
			-	SPLIT	8.65	71.0	27.5	10.2
		2019	NTW	-	8.12	55.8	22.7	27.9
			NT	-	7.75	56.7	17.2	12.5
			CT	-	8.66	49.7	49.2	31.4
			-	Control	8.04	47.9	5.7	16.1
			-	PP	8.04	56.5	17.2	23.0
			-	SPLIT	8.45	57.8	66.2	32.8
		2020	NTW	-	13.33	74.1	8.8	8.4
			NT	-	13.15	74.0	6.5	7.6
			CT	-	12.05	63.8	6.7	6.1
			-	Control	13.07	77.7	3.8	6.0
			-	PP	12.84	70.1	4.4	6.8
			-	SPLIT	12.62	64.2	13.9	9.3

* Table Continued from Table S4.3

Sampling Period	Depth	Year	Conservation System	N Treatment	Gravimetric Water Content	Mineralizable C	NO_3^--N Concentration	NH_4^+-N Concentration

					g water 100 g soil^{-1}	mg CO_2-C kg soil^{-1}	mg kg soil^{-1}	mg kg soil^{-1}
Peak Plant Production	10-20 cm	2018	NTW	-	10.32	63.1	18.5	5.9
			NT	-	10.31	79.6	19.5	5.9
			CT	-	9.46	53.5	16.9	5.0
			-	Control	10.71	66.7	17.2	5.3
			-	PP	10.10	68.6	16.9	5.6
			-	SPLIT	9.28	60.9	20.7	5.9
		2019	NTW	-	10.42	33.9	11.8	3.2
			NT	-	9.62	49.9	10.7	2.4
			CT	-	9.76	40.9	31.2	9.6
			-	Control	10.15	43.1	3.5	7.4
			-	PP	9.71	41.5	14.2	3.2
			-	SPLIT	9.94	40.2	36.1	4.7
		2020	NTW	-	13.53	60.8	5.3	6.3
			NT	-	13.50	65.4	4.5	6.4
			CT	-	13.31	48.1	2.2	5.8
			-	Control	13.74	59.0	0.8	5.9
			-	PP	13.47	61.6	1.0	6.1
			-	SPLIT	13.13	53.7	10.1	6.5
Reproductive Growth	0-10 cm	2018	NTW	-	17.13	139.1	3.2	1.9
			NT	-	15.99	100.9	3.1	2.0
			CT	-	15.62	88.6	3.3	2.2
			-	Control	16.20	94.1	2.9	1.8
			-	PP	16.18	113.5	3.8	2.1
			-	SPLIT	16.36	121.0	2.9	2.2
		2019	NTW	-	4.87	52.5	2.7	3.4
			NT	-	4.54	64.1	2.9	3.6
			CT	-	5.03	49.2	22.4	2.9
			-	Control	4.76	61.0	2.6	2.9
			-	PP	5.61	44.4	16.1	3.4
			-	SPLIT	4.08	60.4	9.3	3.8
		2020	NTW	-	5.06	104.4	0.9	8.5
			NT	-	3.96	75.7	1.0	8.1
			CT	-	4.92	74.5	1.5	7.8
			-	Control	4.83	81.5	1.0	7.0
			-	PP	4.61	79.0	1.3	8.7
			-	SPLIT	4.49	94.2	1.2	8.7
	10-20 cm	2018	NTW	-	15.89	104.0	2.8	2.0
			NT	-	16.17	118.8	2.8	2.0
			CT	-	15.49	105.1	3.2	2.2
			-	Control	15.28	105.1	2.8	2.2
			-	PP	16.07	103.7	3.3	1.8
			-	SPLIT	16.20	119.1	2.8	2.2
		2019	NTW	-	7.74	53.5	1.2	3.1
			NT	-	11.01	36.1	3.8	2.8
			CT	-	6.28	41.0	40.9	2.9
			-	Control	8.23	47.3	5.7	2.9
			-	PP	9.54	38.8	20.3	3.0
			-	SPLIT	7.26	44.6	19.9	2.9
		2020	NTW	-	7.72	87.2	0.8	8.2
			NT	-	-	-	-	-
			CT	-	8.29	70.6	0.8	8.2
			-	Control	8.24	83.6	0.5	7.1
			-	PP	7.83	63.8	0.6	8.2
			-	SPLIT	5.30	79.7	0.9	9.4

Table S4.4 Mineralizable Carbon (Cmin, mg CO_2-C kg soil^{-1}) content and LS Means letters for significant conservation system and N treatment interaction effects on Cmin within the Peak sampling in 2018

Sampling Period	Year	Depth	Conservation System	N Treatment	Cmin (mg CO_2-C kg $soil^{-1}$)	LS Means (α=0.05)
Peak Plant Production	2018	10-20 cm	NTW	Control	57.00	C
				PP	60.50	C
				SPLIT	71.75	ABC
			NT	Control	92.00	AB
				PP	99.00	A
				SPLIT	47.75	C
			CT	Control	51.00	C
				PP	46.25	C
				SPLIT	63.25	BC

Table S4.5 Ammonium (NH_4^+-N) concentration and LS Means letters for significant conservation system and N treatment interactions

Sampling Period	Year	Depth	Conservation System	N Treatment	NH_4^+-N (mg kg $soil^{-1}$)	LS Means (α=0.05)
Vegetative Growth	2019	0-10 cm	NTW	Control	8.39	BC
				PP	13.79	BC
				SPLIT	2.92	C
			NT	Control	7.46	BC
				PP	4.41	BC
				SPLIT	17.56	B
			CT	Control	6.33	BC
				PP	4.90	BC
				SPLIT	67.17	A

Table S4.6 Analysis of Variance (ANOVA) values for gene abundances

Sampling Period	Depth	Year	16S rRNA			ITS			*nosZ* Clade I			*nosZ* Clade II		
			Conservation System	N treatment	Interaction	Conservation System	N treatment	Interaction	Conservation System	N treatment	Interaction	Conservation System	N treatment	Interaction
			ANOVA (*p*-values<0.05)			ANOVA (*p*-values<0.1)			ANOVA (*p*-values<0.1)			ANOVA (*p*-values<0.1)		
Vegetative Growth	0-10 cm	2018	0.342	0.445	0.364	0.992	0.379	0.948	0.071	0.679	0.513	0.074	0.533	0.558
		2019	0.661	0.100	0.455	0.591	0.319	0.585	0.665	0.582	0.147	0.261	0.513	0.373
		2020	0.507	0.374	0.621	0.857	0.970	0.982	0.944	0.254	0.426	0.452	0.621	0.182
	10-20 cm	2018	0.367	0.389	0.436	0.699	0.122	0.486	0.116	0.458	0.884	0.250	0.816	0.670
		2019	0.446	0.085	0.716	0.183	0.431	0.476	0.194	0.258	0.258	0.330	0.230	0.671
		2020	0.896	0.615	0.199	0.942	0.993	0.218	0.648	0.575	0.176	0.068	0.078	0.082
Peak Plant Production	0-10 cm	2018	0.553	0.471	0.361	0.299	0.974	0.909	0.024	0.893	0.282	0.315	0.164	0.532
		2019	0.144	0.249	0.843	0.826	0.963	0.675	0.389	0.313	0.459	0.445	0.806	0.182
		2020	0.880	0.961	0.315	0.451	0.499	0.187	0.338	0.822	0.307	0.950	0.143	0.583
	10-20 cm	2018	0.253	0.964	0.879	0.083	<0.001	<0.001	0.343	0.145	0.379	0.059	0.190	0.552
		2019	0.154	0.115	0.151	0.901	0.900	0.346	0.529	0.127	0.408	0.642	0.287	0.364
		2020	0.457	0.657	0.905	0.445	0.775	0.492	0.890	0.783	0.767	0.595	0.224	0.835
Reproductive Growth	0-10 cm	2018	0.127	0.447	0.408	0.435	0.399	0.694	0.286	0.947	0.644	0.274	0.017	0.241
		2019	0.471	0.614	0.049	0.018	0.004	0.010	0.030	0.188	0.044	0.991	0.245	0.152
		2020	0.143	0.126	0.256	0.047	0.510	0.521	<0.001	0.564	0.006	0.230	0.669	0.926
	10-20 cm	2018	0.358	0.484	0.875	0.242	0.616	0.324	0.113	0.605	0.347	0.291	0.580	0.502
		2019	0.987	0.596	0.373	0.912	0.354	0.454	0.800	0.322	0.551	0.168	0.143	0.208
		2020	0.007	0.710	0.422	0.020	0.828	0.951	0.002	0.156	0.083	0.046	0.129	0.302

Table S4.7 Total bacterial, fungal, and denitrification gene abundances

Sampling Period	Depth	Year	Conservation System	N Treatment	16S rRNA	ITS	*nosZ* Clade I	*nosZ* Clade II
					count g dry soil^{-1}	count g dry soil^{-1}	count g dry soil^{-1}	count g dry soil^{-1}
Vegeative Growth	0-10 cm	2018	NTW	-	8.61E+07	7.69E+07	9.01E+05	1.36E+09
			NT	-	3.14E+07	7.49E+07	3.64E+05	3.92E+08
			CT	-	4.13E+08	7.83E+07	1.01E+06	1.27E+09
			-	Control	8.99E+07	9.28E+07	8.73E+05	1.22E+09
			-	PP	3.81E+08	5.38E+07	6.28E+05	7.33E+08
			-	SPLIT	5.95E+07	8.35E+07	7.78E+05	1.07E+09
		2019	NTW	-	6.25E+07	8.64E+06	9.48E+05	2.94E+08
			NT	-	6.54E+07	7.57E+06	8.15E+05	3.06E+08
			CT	-	7.40E+07	1.20E+07	9.86E+05	8.41E+07
			-	Control	6.05E+07	6.13E+06	9.72E+05	3.14E+08
			-	PP	5.70E+07	1.30E+07	7.97E+05	1.41E+08
			-	SPLIT	8.44E+07	9.07E+06	9.81E+05	2.29E+08
		2020	NTW	-	4.77E+07	5.79E+06	1.05E+06	3.59E+08
			NT	-	4.44E+07	5.69E+06	1.06E+06	2.12E+08
			CT	-	5.92E+07	6.81E+06	9.74E+05	1.59E+08
			-	Control	5.87E+07	5.80E+06	1.17E+06	3.09E+08
			-	PP	5.24E+07	6.15E+06	1.16E+06	2.66E+08
			-	SPLIT	4.01E+07	6.34E+06	7.56E+05	1.55E+08
	10-20 cm	2018	NTW	-	2.24E+07	2.72E+07	1.41E+05	3.67E+08
			NT	-	2.36E+07	2.72E+07	8.92E+04	5.66E+08
			CT	-	1.60E+08	3.67E+07	2.14E+05	1.65E+09
			-	Control	2.58E+07	1.96E+07	1.10E+05	1.06E+09
			-	PP	1.57E+08	4.64E+07	1.51E+05	9.48E+08
			-	SPLIT	2.30E+07	2.52E+07	1.83E+05	5.74E+08
		2019	NTW	-	2.64E+07	4.03E+06	2.32E+05	7.33E+07
			NT	-	2.62E+07	1.59E+06	1.44E+05	2.88E+08
			CT	-	1.81E+07	1.21E+06	1.24E+05	9.60E+07
			-	Control	2.32E+07	1.15E+06	1.45E+05	8.31E+07
			-	PP	1.51E+07	3.23E+06	1.30E+05	6.37E+07
			-	SPLIT	3.24E+07	2.46E+06	2.26E+05	3.10E+08
		2020	NTW	-	2.22E+07	2.02E+06	3.96E+05	5.35E+07
			NT	-	2.52E+07	1.65E+06	5.23E+05	2.56E+08
			CT	-	2.00E+07	1.84E+06	2.54E+05	5.41E+07
			-	Control	2.66E+07	1.79E+06	5.62E+05	2.47E+08
			-	PP	2.46E+07	1.80E+06	3.42E+05	8.87E+07
			-	SPLIT	1.61E+07	1.91E+06	2.69E+05	2.78E+07
Peak Plant Production	0-10 cm	2018	NTW	-	3.14E+08	7.36E+07	1.08E+06	1.29E+09
			NT	-	1.25E+08	3.54E+07	7.38E+05	2.66E+09
			CT	-	2.62E+08	5.73E+07	1.26E+06	9.76E+08
			-	Control	2.35E+08	5.29E+07	1.01E+06	2.92E+09
			-	PP	1.22E+08	5.83E+07	1.08E+06	1.28E+09
			-	SPLIT	3.44E+08	5.52E+07	9.99E+05	7.24E+08
		2019	NTW	-	4.94E+07	6.73E+06	8.33E+05	5.03E+08
			NT	-	5.81E+07	7.78E+06	7.62E+05	3.33E+08
			CT	-	4.30E+07	7.33E+06	6.38E+05	2.19E+08
			-	Control	5.61E+07	7.02E+06	8.64E+05	3.51E+08
			-	PP	4.35E+07	7.46E+06	6.47E+05	4.24E+08
			-	SPLIT	5.08E+07	7.36E+06	7.22E+05	2.79E+08
		2020	NTW	-	6.56E+07	9.16E+06	1.19E+06	4.65E+08
			NT	-	6.18E+07	7.91E+06	8.62E+05	4.01E+08
			CT	-	6.93E+07	7.99E+06	1.00E+06	3.87E+08
			-	Control	6.67E+07	7.61E+06	1.01E+06	2.60E+08
			-	PP	6.31E+07	8.63E+06	9.54E+05	7.32E+08
			-	SPLIT	6.69E+07	8.82E+06	1.09E+06	2.61E+08

* Table continued from Table S4.7

	Depth	Year			16S rRNA	ITS	*nosZ* Clade I	*nosZ* Clade II

Sampling Period			Conservation System	N Treatment	count g dry soil^{-1}	count g dry soil^{-1}	count g dry soil^{-1}	count g dry soil^{-1}
Peak Plant Production	10-20 cm	2018	NTW	-	5.26E+07	3.79E+07	3.36E+05	3.54E+08
			NT	-	8.53E+07	2.20E+07	2.80E+05	6.13E+08
			CT	-	1.30E+08	2.56E+07	5.41E+05	1.28E+09
			-	Control	9.18E+07	1.42E+07	1.71E+05	4.52E+08
			-	PP	8.24E+07	1.58E+07	4.57E+05	6.54E+08
			-	SPLIT	9.38E+07	5.56E+07	5.29E+05	1.14E+09
		2019	NTW	-	1.63E+07	1.78E+06	1.67E+05	1.25E+08
			NT	-	2.44E+07	1.66E+06	2.15E+05	7.68E+07
			CT	-	1.72E+07	1.96E+06	1.61E+05	1.54E+08
			-	Control	2.47E+07	1.66E+06	2.44E+05	1.01E+08
			-	PP	1.55E+07	1.96E+06	1.45E+05	1.93E+08
			-	SPLIT	1.77E+07	1.78E+06	1.54E+05	6.20E+07
		2020	NTW	-	1.29E+07	1.56E+06	1.43E+05	5.58E+07
			NT	-	1.29E+07	1.06E+06	1.81E+05	1.21E+08
			CT	-	1.84E+07	2.40E+06	1.75E+05	1.14E+08
			-	Control	1.73E+07	1.30E+06	1.32E+05	8.52E+07
			-	PP	1.41E+07	2.05E+06	1.87E+05	4.10E+07
			-	SPLIT	1.28E+07	1.66E+06	1.80E+05	1.64E+08
Reproductive Growth	0-10 cm	2018	NTW	-	6.91E+07	1.59E+07	8.46E+05	3.80E+08
			NT	-	1.11E+08	2.80E+07	1.44E+06	4.02E+08
			CT	-	6.35E+07	1.89E+07	1.38E+06	2.04E+08
			-	Control	9.72E+07	1.70E+07	1.29E+06	3.48E+08
			-	PP	6.57E+07	2.86E+07	1.16E+06	5.29E+08
			-	SPLIT	8.10E+07	1.72E+07	1.21E+06	1.09E+08
		2019	NTW	-	6.13E+07	8.91E+06	1.28E+06	7.62E+08
			NT	-	4.64E+07	4.44E+06	5.90E+05	7.31E+08
			CT	-	5.29E+07	6.97E+06	8.59E+05	7.85E+08
			-	Control	4.67E+07	5.09E+06	7.11E+05	7.54E+08
			-	PP	5.62E+07	9.98E+06	1.15E+06	4.17E+08
			-	SPLIT	5.78E+07	5.25E+06	8.63E+05	1.11E+09
		2020	NTW	-	5.48E+07	1.94E+07	1.49E+06	4.71E+08
			NT	-	5.38E+07	8.46E+06	1.08E+06	2.19E+08
			CT	-	3.96E+07	9.04E+06	6.67E+05	1.71E+08
			-	Control	5.93E+07	1.48E+07	1.12E+06	3.78E+08
			-	PP	4.23E+07	9.47E+06	1.13E+06	2.65E+08
			-	SPLIT	4.66E+07	1.26E+07	9.87E+05	2.19E+08
	10-20 cm	2018	NTW	-	2.19E+07	2.23E+06	1.00E+05	9.56E+07
			NT	-	3.30E+07	2.28E+06	1.22E+05	6.58E+08
			CT	-	2.57E+07	4.43E+06	2.32E+05	8.58E+07
			-	Control	2.15E+07	2.25E+06	1.21E+05	1.40E+08
			-	PP	3.00E+07	3.02E+06	1.48E+05	1.74E+08
			-	SPLIT	2.92E+07	3.67E+06	1.85E+05	5.25E+08
		2019	NTW	-	2.81E+07	3.04E+06	5.06E+05	1.95E+08
			NT	-	2.90E+07	2.84E+06	4.93E+05	7.20E+07
			CT	-	2.68E+07	2.32E+06	2.95E+05	3.64E+08
			-	Control	2.01E+07	1.27E+06	1.71E+05	3.32E+08
			-	PP	3.02E+07	3.22E+06	4.07E+05	2.63E+08
			-	SPLIT	3.35E+07	3.70E+06	7.16E+05	3.68E+07
		2020	NTW	-	3.35E+07	8.38E+06	8.32E+05	2.94E+08
			NT	-	-	-	-	-
			CT	-	1.15E+07	2.95E+06	1.72E+05	5.84E+07
			-	Control	1.90E+07	5.61E+06	3.19E+05	3.67E+07
			-	PP	2.57E+07	4.95E+06	7.21E+05	3.23E+08
			-	SPLIT	2.27E+07	6.44E+06	4.66E+05	1.69E+08

Table S4.816S rRNA gene abundance and LS Means letters for significant conservation system and N treatment interaction within the Repro sampling in 2019 at 0-10 cm depth

Sampling Period	Year	Depth	Conservation System	N Treatment	16S rRNA gene abundance (copies g dry soil soil^{-1})	LS Means (α=0.05)
Reproductive Growth	2019	0-10 cm	NTW	Control	3.48E+07	BC
				PP	6.83E+07	ABC
				SPLIT	8.09E+07	A
			NT	Control	3.19E+07	BC
				PP	4.21E+07	ABC
				SPLIT	6.50E+07	ABC
			CT	Control	7.33E+07	AB
				PP	5.82E+07	ABC
				SPLIT	2.73E+07	C

Table S4.9 nosZ clade I gene abundances and LS Means letters for significant conservation system and N treatment interaction within the Repro sampling in 2019 and 2020 at 0-10 cm depth

Sampling Period	Year	Depth	Conservation System	N Treatment	*nosZ* clade I gene abundance (copies g dry soil soil^{-1})	LS Means (α=0.05)
Reproductive Growth	2019	0-10 cm	NTW	Control	4.84E+05	B
				PP	1.62E+06	A
				SPLIT	1.73E+06	A
			NT	Control	5.68E+05	B
				PP	7.10E+05	B
				SPLIT	4.91E+05	B
			CT	Control	1.08E+06	AB
				PP	1.13E+06	AB
				SPLIT	3.69E+05	B
	2020	0-10 cm	NTW	Control	1.32E+06	B
				PP	2.09E+06	A
				SPLIT	1.07E+06	BC
			NT	Control	1.29E+06	BC
				PP	9.05E+05	BCD
				SPLIT	1.05E+06	BC
			CT	Control	7.64E+05	CD
				PP	3.98E+05	D
				SPLIT	8.38E+05	BCD

Table S4.10 ITS gene abundances and LS Means letters for significant conservation system and N treatment interaction within the Peak sampling in 2018 at 10-20 cm depth and the Repro sampling in 2019 at 0-10 cm depth

Sampling Period	Year	Depth	Conservation System	N Treatment	ITS gene abundance (copies g dry soil soil^{-1})	LS Means (α=0.05)
Peak Plant Production	2018	10-20 cm	NTW	Control	1.21E+07	CD
				PP	2.55E+06	D
				SPLIT	9.91E+07	A
			NT	Control	1.89E+07	BCD
				PP	1.86E+07	BCD
				SPLIT	2.86E+07	BC
			CT	Control	1.16E+07	CD
				PP	2.63E+07	BCD
				SPLIT	3.90E+07	B
Reproductive Growth	2019	0-10 cm	NTW	Control	2.75E+06	C
				PP	1.41E+07	A
				SPLIT	9.90E+06	AB
			NT	Control	3.46E+06	C
				PP	6.62E+06	BC
				SPLIT	3.24E+06	C
			CT	Control	9.07E+06	AB
				PP	9.23E+06	AB
				SPLIT	2.60E+06	C

Table S4.11 Analysis of variance (ANOVA) for nitrous oxide (N_2O) emissions, pore-space N_2O concentration and pore-space NO concentration within each sampling period in 2020

Depth	Gas Type	Sampling Period	Conservation System	N treatment	Interaction
			ANOVA (*p*-values<0.05)		
Surface	N_2O Emissions	Vegetative Growth	0.002	0.508	0.768
		Peak Plant Production	0.002	0.351	0.152
		Reproductive Growth	-	-	-
7.5 cm	N_2O Pore-Space Concentrations	Vegetative Growth	0.545	0.041	0.122
		Peak Plant Production	0.569	0.426	0.312
		Reproductive Growth	0.370	0.100	0.868
15 cm	N_2O Pore-Space Concentrations	Vegetative Growth	0.775	0.099	0.143
		Peak Plant Production	0.727	0.844	0.463
		Reproductive Growth	0.560	0.190	0.308
7.5 cm	NO Pore-Space Concentrations	Vegetative Growth	0.928	0.328	0.301
		Peak Plant Production	0.710	0.751	0.384
		Reproductive Growth	0.303	0.098	0.841
15 cm	NO Pore-Space Concentrations	Vegetative Growth	0.989	0.350	0.225
		Peak Plant Production	0.625	0.629	0.741
		Reproductive Growth	0.471	0.111	0.489

Table S4.12 Nitrous oxide (N_2O) emissions collected at Vegetative Growth, Peak Plant Production, and Reproductive Growth stages in 2020

Sampling Period	N Treatment	Conservation System	N_2O-N Emissions ($\mu g\ N_2O\text{-}N\ m^{-2}\ h^{-1}$)	N_2O-N Emissions Standard Error	N_2O Pore-space Concentration 7.5 cm ($\mu L\ N_2O\ L^{-1}$)	N_2O Pore-space Concentration 7.5 cm Standard Error	N_2O Pore-space Concentration 15 cm ($\mu L\ N_2O\ L^{-1}$)	N_2O Pore-space Concentration 15 cm Standard Error	NO Pore-space Concentration 7.5 cm ($\mu L\ NO\ L^{-1}$)	NO Pore-space Concentration 7.5 cm Standard Error	NO Pore-space Concentration 15 cm ($\mu L\ NO\ L^{-1}$)	NO Pore-space Concentration 15 cm Standard Error
Vegetative Growth	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
Peak Plant Production	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
Reproductive Growth	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

Table S4.13 Analysis of variance (ANOVA) values for carbon dioxide emissions and pore-space concentrations

Depth	Gas Type	Sampling Period	Conservation System	N treatment	Interaction
			ANOVA (p-values<0.05)		
Surface	CO_2 Emissions	Vegetative Growth	<0.001	0.112	0.297
		Peak Plant Production	0.005	0.002	0.065
		Reproductive Growth	0.352	0.243	0.071
7.5 cm	CO_2 Pore-Space Concentrations	Vegetative Growth	0.612	0.559	0.988
		Peak Plant Production	0.055	0.410	0.455
		Reproductive Growth	0.605	0.398	0.467
15 cm	CO_2 Pore-Space Concentrations	Vegetative Growth	0.432	0.471	0.995
		Peak Plant Production	0.001	0.928	0.904
		Reproductive Growth	0.781	0.342	0.057

Table S4.14 Carbon dioxide (CO_2-C) emissions and pore-space concentration collected within each sampling period in 2020

Sampling Period	N Treatment	Conservation System	CO_2-C Emissions (mg CO_2-C m^{-2} h^{-1})	CO_2-C Emissions Standard Error	CO_2 Pore-space Concentration 7.5 cm (μL CO_2 L^{-1})	CO_2 Pore-space Concentration 7.5 cm Standard Error	CO_2 Pore-space Concentration 15 cm (μL CO_2 L^{-1})	CO_2 Pore-space Concentration 15 cm Standard Error
Vegetative Growth	NTW	Control	52.53	9.79	956.77	133.88	1021.45	143.82
		PP	89.21	15.52	984.77	128.57	1143.20	222.44
		SPLIT	90.90	10.80	913.10	73.71	974.79	78.83
	NT	Control	38.34	12.17	892.54	98.87	952.74	104.65
		PP	60.43	33.21	984.22	210.33	1049.81	220.19
		SPLIT	44.40	9.80	925.88	65.73	985.51	68.06
	CT	Control	31.44	5.86	965.34	135.14	1084.52	134.07
		PP	26.43	4.20	1100.58	62.12	1230.10	103.36
		SPLIT	38.64	6.79	986.91	22.04	1107.10	62.61
Peak Plant Production	NTW	Control	74.10	15.43	1072.24	138.43	1303.26	85.89
		PP	113.47	16.07	1178.03	254.59	1413.09	143.27
		SPLIT	142.64	26.88	1073.55	39.21	1407.28	146.08
	NT	Control	85.30	5.59	977.71	32.29	1454.63	51.72
		PP	93.78	15.40	1341.77	212.40	1436.08	121.47
		SPLIT	104.01	28.61	1244.68	105.06	1387.88	271.31
	CT	Control	74.70	16.97	957.31	110.46	978.92	49.45
		PP	62.55	14.34	881.12	46.41	937.15	8.63
		SPLIT	84.80	8.96	963.70	91.76	1057.63	69.87
Reproductive Growth	NTW	Control	23.73	2.25	734.65	14.07	942.04	28.91
		PP	22.96	2.33	762.99	109.21	1015.42	89.99
		SPLIT	23.95	1.34	770.61	63.84	852.15	50.37
	NT	Control	34.34	5.27	776.44	11.20	963.38	81.26
		PP	22.71	4.80	803.61	62.88	956.61	47.94
		SPLIT	19.16	5.76	800.89	106.21	817.48	22.61
	CT	Control	17.80	5.14	820.78	33.49	934.42	39.45
		PP	23.55	3.35	1013.34	239.89	874.38	4.38
		SPLIT	20.58	2.83	682.14	34.34	1009.45	32.10

Table S4.15 Average bulk density within each conservation system after 5 years of implementation

Depth	Conservation System[a]	Bulk Density	Standard Error	LSMeans (α=0.05)
0-10 cm	NTW	1.316112	0.042858	AB
	NT	1.383852	0.035604	A
	CT	1.247377	0.023805	B
10-20 cm	NTW	1.627359	0.075817	
	NT	1.540023	0.083144	
	CT	1.393501	0.041926	

Table S4.16 Sample Scores from NMDS analysis using Euclidean distance

		Vegetative Growth		Peak Plant Production		Reproductive Growth	
Conservation System	N Treatment	NMDS1	NMDS2	NMDS1	NMDS2	NMDS1	NMDS2
NTW	Control	-31.501	-11.604	-17.985	0.228	-3.528	-0.734
		-57.118	-9.902	-21.567	-5.107	-17.556	-2.570
		-30.305	-10.680	-54.124	-8.675	4.969	3.384
	PP	157.756	-12.646	37.464	-4.474	5.807	4.390
		8.431	41.208	15.417	10.671	-24.128	-0.437
		60.249	10.604	-16.662	-2.567	-14.286	0.080
	SPLIT	0.532	-8.593	171.806	24.356	-15.009	0.865
		-15.916	5.514	71.796	-32.964	-40.333	1.208
		32.824	-4.118	24.804	11.418	-17.194	2.975
NT	Control	-43.615	-26.944	-21.625	7.222	18.253	-13.317
		-65.039	-12.252	-37.336	-38.159	-8.743	-19.195
		-69.982	-4.819	-9.746	-12.037	-4.235	-1.739
	PP	334.370	-5.000	8.728	-21.409	38.614	1.447
		-58.043	1.156	-6.787	-12.736	12.882	-10.769
		57.657	-2.453	-33.553	7.232	33.197	2.652
	SPLIT	30.430	-16.812	145.227	-15.504	14.582	12.459
		-53.656	-12.258	-6.875	2.459	-57.889	-0.080
		-13.790	-3.799	-15.846	30.860	-15.982	5.743
CT	Control	-51.396	-12.753	0.198	-1.933	18.075	6.196
		-67.875	-9.242	-21.929	-0.456	4.329	-5.516
		-53.203	-2.949	-56.772	6.352	-15.481	15.090
	PP	41.826	-20.191	-14.587	5.914	8.993	-7.909
		3.943	93.942	-62.415	-1.509	25.126	1.808
		-8.370	1.936	-36.909	-1.311	7.602	0.182
	SPLIT	-51.707	-21.568	1.938	11.629	33.541	4.292
		-55.663	48.608	-18.010	40.495	9.933	0.585
		-0.839	5.617	-24.654	0.003	-1.539	-1.091

Table S4.17 Variable scores from NMDS analysis using Euclidean distance

Variable	Vegetative Growth		Peak Plant Production		Reproductive Growth	
	MDS1	MDS2	MDS1	MDS2	MDS1	MDS2
Cmin	-55.652	-5.414	-27.581	-24.722	-28.824	2.635
GWC	-56.819	-7.215	-45.933	5.784	19.497	1.855
pH	-54.026	-10.988	-45.944	6.033	22.666	1.395
NO3	-16.306	74.975	174.336	-66.663	-29.895	22.580
NH4	-25.269	87.944	4.215	-11.605	11.330	0.851
X16S	-50.681	-6.285	-43.998	7.939	19.129	1.711
cladeI	-49.642	-6.243	-42.197	8.473	17.134	1.632
cladeII	-48.920	-7.610	-42.993	7.977	17.533	2.394
ITS	-51.804	-6.048	-43.330	8.260	17.822	2.062
N2O	-57.113	7.287	-44.027	1.706	12.095	-6.200
NO	-44.413	8.200	-27.615	7.509	22.797	-5.578
CO2	-54.029	-6.474	-43.553	6.755	20.767	1.725
CO2E	178.371	-7.413	83.854	10.032	3.107	-18.974
BD	-41.522	-9.279	-41.436	6.163	21.710	1.465

3. APPENDIX C

SUPPLEMENTAL MATERIAL: MICROBIAL RESPONSE TO CONSERVATION MANAGEMENT PRACTICES IN SEMI-ARID SOILS: A METATRANSCIPTOMIC ANALYSIS

Table S5.1 KEGG classification for the 25 most variable transcripts

KEGG Orthlogy #	KEGG Pathway/Protein Classification
K02112	Oxidative phosphorylation; Photosynthesis; Metabolic pathways
K05667	ATP-binding cassette
K03640	Peptidoglycan-associated lipoprotein
K02864	Ribosome
K01209	Amino sugar and nucleotide sugar metabolism
K01996	ABC transporters; Quorum sensing
K03696	ATP-dependent Clp protease ATP-binding subunit ClpC
K01652	Valine, leucine and isoleucine biosynthesis; Butanoate metabolism; C5-Branched dibasic acid metabolism
K04487	Thiamine metabolism; Metabolic pathways; Biosynthesis of cofactors; Sulfur relay system
K02881	Ribosome
K03097	Ribosome biogenesis in eukaryotes
K02111	Oxidative phosphorylation; Photosynthesis; Metabolic pathways
K03695	ATP-dependent Clp protease ATP-binding subunit ClpB
K07303	Isoquinoline 1-oxidoreductase subunit beta
K02952	Ribosome
K02919	Ribosome
K24013	Trimeric intracellular cation channel
K02954	Ribosome
K03569	Rod shape-determining protein MreB and related proteins
K02027	Multiple sugar transport system substrate-binding protein
K02637	Photosynthesis; Metabolic pathways
K02705	Photosynthesis; Metabolic pathways
K24479	Astrotactin
K23054	SN-1 stearoyl-lipid 9-desaturase
K02963	Ribosome