MICROBIAL SOURCES OF NITROUS OXIDE EMISSIONS FROM DIVERSE CROPPING SYSTEMS

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ABSTRACT

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Nitrous oxide (N_2O) is a potent greenhouse gas with a global warming potential ~300 times higher than CO_2. As the primary source of reactive nitrogen oxides (NO_x) in the stratosphere, N_2O also depletes stratospheric ozone. N_2O concentrations in the atmosphere are increasing rapidly, primarily due to agricultural activity. Nitrification, an autotrophic process that converts ammonia (NH_3) into nitrite (NO_2^-) and nitrate (NO_3^-), and denitrification, a heterotrophic process that reduces NO_3^- into NO, N_2O and N_2, are the two major processes leading to N_2O emissions. Nitrification has been reported to dominate N_2O emissions from agricultural soils under aerobic conditions.

Ammonia oxidizing bacteria (AOB) and ammonia oxidizing archaea (AOA) are the two main taxa involved in nitrification. Both AOA and AOB are capable of producing N_2O, but their relative importance in nitrification is still largely unknown. In this dissertation I address three nitrification knowledge gaps: 1) Importance: what is the contribution of nitrification versus other microbial processes for producing N_2O in systems under different management intensities (Chapter 2)? 2) Ecology: can high NH_4^+ inputs induce niche differentiation between AOA and AOB (Chapter 3)? 3) Complexity: how do plants mediate N_2O emissions from AOA and AOB *in situ* in annual and perennial bioenergy cropping systems (Chapter 4)?

In Chapters 2 and 3, I sampled soils from ecosystems under a management intensity gradient ranging from heavily-managed row crop agriculture to unmanaged deciduous forest. Results in chapter 2 show that soil nitrification is unlikely to be the dominant source of N_2O in

annual row crop systems, as the 25th – 75th percentile of the maximum potential contribution ranged only between 13-42% of total N_2O. In contrast, a maximum potential contribution of 52-63% of total N_2O emissions could be attributed to nitrification in perennial or successional systems. In Chapter 3, I found high NH_4^+ inputs could inhibit nitrification of AOB but not AOA, especially in perennial and successional systems. Moreover, long-term N fertilization significantly promoted nitrification potentials of both AOA and AOB in the early succession but not in the deciduous forest systems. In summary, results from these two chapters suggest 1) nitrification is a minor source of N_2O, especially in row crop systems, and 2) NH_4^+ inhibition of AOB could be another mechanism leading to niche differentiation between AOA and AOB in terrestrial environments.

In Chapter 4, I examined nitrifier N_2O emissions from annual (corn) and perennial (switchgrass) bioenergy cropping systems during different seasons that differ in plant nutrient demands. Both AOA and AOB responded to N fertilizer applications *in situ* but N fertilizer-induced N_2O emissions were mainly observed in corn but not in switchgrass system. Because plants can compete with soil nitrifiers for NH_4^+ during the growing season, competition for NH_4^+ appeared to reduce N_2O emissions from nitrification. Thus, synchronizing fertilizer application with plant nutrient uptake can be an important strategy for mitigating nitrification-derived N_2O. Overall, results from this dissertation suggest that nitrifier-derived N_2O in terrestrial ecosystems is significant but not a dominant source of N_2O, and although AOB are more responsive to added N than are AOA, AOB can also be inhibited by high NH_4^+ concentrations in soil.

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Chapter 1: Introduction

Overview

N_2O is the third most important anthropogenic greenhouse gas in the atmosphere, after CO_2 and CH_4, for contributing to radiative forcing (Myhre et al., 2013). As a long-lived greenhouse gas, N_2O stays in the atmosphere for about 114 years. Atmospheric N_2O abundance has increased from a preindustrial baseline of 270 to 328 ppb_v in 2016 (Blasing, 2016), with a 0.73 ± 0.03 ppb/yr increase over the past three decades (IPCC, 2014). N_2O is also a dominant ozone depleting substance because it reacts with electronically excited oxygen atoms O (^1D) in the stratosphere to form reactive nitrogen oxides (NO_x), which participate in ozone-depleting cycles (Ravishankara et al., 2009; Revell et al., 2012). Soil N_2O is mainly produced via enzymatic process such as nitrification and denitrification, including nitrifier denitrification. As agricultural soils contribute the majority of anthropogenic N_2O to the atmosphere, understanding sources of N_2O from agricultural soils is critically important for developing N_2O mitigation practices (Paustian et al., 2016).

Sources of N_2O

Nitrification

Nitrification is the process that converts ammonia (NH_3) into hydroxylamine (NH_2OH), which is further oxidized into nitrite (NO_2^-) and nitrate (NO_3^-). N_2O from autotrophs is mainly produced during NH_3 oxidation, the rate limiting step of nitrification. For over 100 years, NH_3 oxidation was thought to be solely facilitated by ammonia oxidizing bacteria (AOB) (Frankland and Frankland, 1890). It was not until the late 1990s that a different and novel group was suspected

to also contribute to nitrification. The first line of evidence for this was from a failure to find bacterial 16S rRNA genes related to known AOB in nitrifying reactors at the Shedd Aquarium in Chicago, Illinois (Stahl and Torre, 2012). Years later, when PCR primers were expanded to include archaeal 16S rRNA, abundant sequences associated with marine group 1 Crenarchaeota was found in estuary sediment enrichments and in Seattle Aquarium's nitrifying filtration systems, which eventually led to the first pure culture of ammonia oxidizing archaea (AOA), *N. maritimus* SCM1 (Könneke et al., 2005).

Both AOA and AOB have been experimentally confirmed to be able to produce N_2O (Hooper and Terry, 1979; Santoro et al., 2011). While AOB nitrification leading to N_2O is mostly associated with enzymatic pathways, it is not entirely clear if AOA-derived N_2O is enzymatically or abiotically produced, although evidence suggests a hybrid formation (Stieglmeier et al., 2014). In addition, heterotrophic nitrifiers (Papen et al., 1989) and Comammox (Complete Ammonia Oxidation to Nitrate) bacteria (Kits et al., 2019) are also able to produce N_2O, but their ecological importance is largely unknown.

Partitioning microbial sources of N_2O has been extensively studied with short-term lab incubations (Stevens et al., 1997; Bateman and Baggs, 2005; Liu et al., 2016). However, little is known *in situ* about the relative contribution of AOA and AOB to nitrification-derived N_2O or the overall importance of nitrification versus other microbial processes that produce N_2O. The ecology of AOA in particular is not well understood, especially regarding the potential for niche differentiation between AOA and AOB as induced by high N inputs. Additionally, most studies on separating AOA from AOB have been based on lab microcosms without including plants

(Giguere et al., 2015; Lu et al., 2015). Thus, the *in situ* responses of AOA and AOB to N fertilizer mediated by plant nutrient acquisition are still largely unknown.

Denitrification

Denitrification sequentially reduces NO_3^- into NO_2^-, NO, N_2O and N_2 under oxygen-limited conditions (Robertson and Groffman, 2015). Each step of denitrification is catalyzed by different enzymes: NO_3^-reductase (Nar and Nap), NO_2^- reductase (NirK and NirS), NO reductase (cNor and qNor) and N_2O reductase (NosZ) (Philippot et al., 2007). Unlike nitrification, which is mainly limited to autotrophs, most of the denitrifiers are heterotrophic bacteria belonging to *Proteobacteria* (Zumft, 1997) although some archaea such as *Halobacterium marismortui* have also been reported (Oren et al., 1990; Ichiki et al., 2001). Moreover, emerging evidence suggests denitrifying fungi can play an important but overlooked role in N_2O emissions (Laughlin and Stevens, 2002). For example, summarizing published data from 1972-2014, 119 fungal species that represent 60 genera were found capable of producing N_2O via denitrification (Mothapo et al., 2015). Similarly, Higgins et al. (2016) reported that of 214 fugal isolates cultivated from Midwest agricultural soils, 151 of them can produce N_2O from NO_2^-. Denitrifying fungi reduce NO to N_2O via cytochrome P450 nitric oxide reductase (P450nor), which is different from bacterial denitrifiers that catalyze NO reduction with cNor or qNor. Furthermore, most of the fungal denitrifiers do not have genes encoding for Nar or nosZ, thus fungal denitrification commonly starts with NO_2^- and ends with N_2O (Mothapo et al., 2015).

Nitrifier denitrification

Nitrifier denitrification refers to the reduction of NO_2^- derived from NH_3 oxidation into NO and N_2O by nitrifiers under low oxygen conditions (Wrage et al., 2001). The reasons nitrifiers perform denitrification have not been fully resolved but removing NO_2^- might be a detoxification mechanism for AOB, and advantageous for AOB to conserve O_2 for NH_3 oxidation (Poth and Focht, 1985; Hu et al., 2015). Although N_2O produced by nitrifier denitrification has been observed for decades from pure cultures of *Nitrosomonas europaea* (Ritchie and Nicholas, 1972; Poth and Focht, 1985), the ecological importance of nitrifier denitrification is still under considerable debate. For example, Webster and Hopkins (1996) reported nitrifier denitrification contributed less than 3% of total N_2O in wet sandy-loam soils when using combinations of air, C_2H_2, and O_2 as inhibitors to differentiate N_2O generating processes. In comparison, with ^{15}N and ^{18}O tracer techniques, Zhu et al. (2013) found nitrifier denitrification contributed 34-66% of total N_2O when O_2 levels decreased from 21 to 0.5% in fertilized clay loam soils. These conflicting results might be partially due to problems of inhibitor methods (Wrage et al., 2001): O_2 cannot fully inhibit nitrifier denitrification, which was confirmed by further research showing AOB cultures can produce N_2O aerobically via nitrifier denitrification (Shaw et al., 2006). In addition, current research seems to support AOA's being incapable of producing N_2O from nitrifier denitrification due to the absence of genes encoding for Nor (Stieglmeier et al., 2014).

Chapter organization

In chapter 2 of this dissertation, I focus on evaluating the relative importance of nitrification versus other microbial processes in contributing to N_2O emissions by combining lab incubations and long-term field N_2O observations. I first established kinetics models of nitrification-derived

N_2O for diverse ecosystems under a management intensity gradient, and then used these models to calculate how much field-based N_2O might possibly be attributed to soil nitrification using a Bayesian approach. In chapter 3, I test the hypothesis that niche differentiation between AOA and AOB can be induced by high NH_4^+ concentrations. I conducted a nitrification kinetics assay for AOA and AOB separately in ecosystems varying in management intensities and fertilization regimes. I also explored the link between ecological process such as maximum nitrification rate (V_{max}) and microbial community structure (amoA gene abundance) or environmental variables (soil pH). In chapter 4, I investigate the *in situ* responses of AOA and AOB to N fertilization in annual (corn) and perennial (switchgrass) cropping systems. To test the hypothesis that plants can mediate N_2O emissions by competing for soil NH_4^+ with nitrifiers, I compared nitrification-derived N_2O emissions of AOA and AOB among seasons that differ in plant nutrient demands. Additionally, I compare the efficiency of AOA and AOB for producing N_2O via nitrification in both corn and switchgrass systems.

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Chapter 2: Nitrification is a minor source of N_2O along a management intensity gradient in the US Midwest

Abstract

The relative contribution of nitrification to nitrous oxide (N_2O) emissions from terrestrial ecosystems is poorly known and therefore poorly constrained in biogeochemical models. I tested the relative importance of nitrification-derived N_2O in six cropped and unmanaged ecosystems along a management intensity gradient in the U.S. Midwest by using Bayesian inference to couple site-specific Michaelis-Menten kinetics of nitrification-derived N_2O with >20 years of *in situ* N_2O flux measurements. Ammonia oxidizing bacteria (AOB) dominated nitrification-derived N_2O in all of the ecosystems but a mown grassland, where the contribution from ammonia oxidizing archaea (AOA) was about 70% as compared to 11-36% in all other systems. AOB and AOA together contributed a maximum potential of 13-17% of field-based N_2O fluxes in a conventionally fertilized annual crop and 27-42% in a similar but cover-cropped system. In perennial systems, a median of 52-63% of total N_2O could be attributed to nitrification. Because the *in vitro* methods calculate the potential maximum contribution from nitrification assuming all available ammonium is oxidized and no nitrification-derived N_2O is further reduced, percentages *in situ* are likely far smaller. I thus conclude nitrification is a minor source of N_2O in these systems, and especially in row crops.

Introduction

Nitrous oxide (N_2O) is a potent greenhouse gas with a global warming potential ~300 times higher than CO_2. N_2O has the third largest radiative forcing (0.17 ± 0.03 W m^{-2}, from 1750 to

2011) among anthropogenic gases (Myhre et al., 2013) and is also a primary source of the reactive N that contributes to stratospheric ozone depletion (Revell et al., 2012). Globally, soils are the dominant sources of both anthropogenic and natural emissions of N_2O, with 1.7-4.8 Tg N_2O-N yr^{-1} emitted by agricultural soils and 3.3-9.0 Tg N_2O-N yr^{-1} from soils under natural vegetation (Ciais et al., 2013). Nitrification, performed in soil mainly by aerobic ammonia oxidizing bacteria (AOB) and archaea (AOA), releases N_2O during conversion of ammonia (NH_3) to nitrite (NO_2^-) and nitrate (NO_3^-), and denitrification, performed in soil mainly by heterotrophic bacteria, releases N_2O during the reduction of NO_3^- to N_2O and dinitrogen (N_2) when O_2 is limiting (Robertson and Groffman, 2015). Nitrification and denitrification, including nitrifier denitrification, occur in many soils, and understanding the relative contributions of each is important for informing future N_2O mitigation potentials and strategies, and as well, for allowing biogeochemical models to constrain uncertainties associated with simulating N_2O emissions.

Partitioning N_2O emission pathways between nitrification and denitrification *in situ* is challenging. Both aerobic and anaerobic soil microsites occur within the same soil volume, such that nitrification and denitrification often occur simultaneously (Smith, 1980; Kuenen and Robertson, 1994). In general, three types of approaches have been used to attribute N_2O emission sources: specific inhibitors, stable isotope enrichment, and isotopomer analysis. Specific inhibitors have mainly been used in short-term laboratory incubations, where acetylene (C_2H_2) is used to selectively inhibit NH_3 oxidation at 10 Pa and N_2O reduction at 10 kPa (Robertson and Tiedje, 1987), and 1-octyne is used to selectively inhibit AOB ammonia monooxygenase (AMO) (Taylor et al., 2013; Taylor et al., 2015). Isotope enrichment approaches typically use either ^{15}N-

NH_4^+ or ^{15}N-NO_3^- to differentiate nitrification and denitrification-derived N_2O in short-term laboratory experiments (Stevens et al., 1997). Isotopomers of N_2O reflect the differential intramolecular distribution (site preference, SP) of ^{15}N at α and β positions of the N_2O molecule (N^{β}-N^{α}-O) and can differentiate N_2O production of nitrification from denitrification in both the lab (Sutka et al., 2006) and field (Opdyke et al., 2009; Ostrom et al., 2010; Buchen et al., 2018).

The use and interpretation of inhibitors and isotope enrichment approaches suffer from the difficulty of achieving homogenous distributions of added compounds in intact soils where microsites are usually heterogeneously distributed (Groffman et al., 2006). The use of C_2H_2 includes further concerns of microbial C_2H_2 consumption (Terry and Duxbury, 1985; Topp and Germon, 1986), and as well some nitrifiers are resistant to C_2H_2 (Hynes and Knowles, 1982; Schimel et al., 1984). ^{15}N enrichment adds additional N to soils, potentially leading to overestimated rates of nitrification and denitrification especially in non-agricultural soils (Baggs, 2008). And isotopomer approaches can be confounded by the overlap of SP values among different microbial processes. For example, N_2O from fungal denitrification has an SP of 37‰, which is within the range of nitrifier nitrification (hydroxylamine oxidation) (Sutka et al., 2008). Additionally, SP analysis is currently hampered by the lack of standard isotopic reference materials (Mohn et al., 2014; Ostrom and Ostrom, 2017). An additional limitation of all three techniques is their short-term nature in light of highly dynamic microbial processes known to exhibit substantial temporal variation (Boone et al., 1999) and either directly or indirectly affect N_2O production.

An alternative method for assessing the potential *in situ* importance of nitrification versus other N_2O generating processes in soil is to combine the kinetics of nitrification-derived N_2O with

long-term field N_2O flux measurements. Nitrification kinetics provide a measure of a soil's *in situ* potential to nitrify NH_4^+ to N_2O and NO_3^- under conditions unconstrained by resource limitations (Stark and Firestone, 1996; Norton and Stark, 2011), so that the maximum potentials for nitrification-derived N_2O emissions can be predicted. Such potentials might then be combined with field-based measurements of N_2O fluxes to allow estimation of the likely maximum percentage of nitrification-derived N_2O in relation to all other N_2O sources.

Here I combine measured site-specific nitrification kinetics with 20+ years of field-based N_2O fluxes to estimate the maximum potential contribution of nitrification to N_2O emissions along a long-term management intensity gradient in the upper U.S. Midwest. Ecosystems range from intensively managed annual cropping systems to an unmanaged late successional deciduous forest. I first use short-term laboratory incubations to build Michaelis-Menten kinetics models of N_2O-NH_4^+ relationships, shown to be seasonally stable. Then I predict the potential maximum nitrification-derived N_2O of each ecosystem assuming all microbially available (soil solution phase) NH_4^+ is nitrified to N_2O. I then use a Bayesian approach to calculate the maximum relative importance of nitrification for N_2O emissions from each ecosystem based on long-term field-based N_2O fluxes.

Methods

Study site

This study was conducted in the Main Cropping System Experiment (MCSE) of the Kellogg Biological Station (KBS) LTER site located in southwest Michigan (42° 24'N, 85° 23'W). The MCSE was established in 1988 and includes, on the same soil series, ecosystems that form a management intensity gradient: annual cropping systems, perennial cropping systems, and

unmanaged systems at different stages of ecological succession (Robertson and Hamilton, 2015). Most of the ecosystems are replicated in blocks as 1 ha (90 m × 110 m) plots. KBS features a temperate climate with an average of 1005 mm annual precipitation distributed evenly throughout the year and 10.1 °C mean annual temperature (30-year mean from 1981). Soils are well drained Alfisol loams (co-mingled Kalamazoo and Oshtemo series Typic Hapludalfs), formed from glacial till and outwash with some intermixed loess (Crum and Collins, 1995, Luehmann et al., 2016). Average sand and clay contents in surface soils are 43% and 17%, respectively (Robertson and Hamilton, 2015).

I studied two annual cropping systems: conventionally managed (Conventional) and biologically managed (Biologically-based) corn-soybean-winter wheat rotations; a hybrid poplar system (Poplar); and three successional systems of different stages: an early successional system (Early successional), a never-tilled annually mown grassland system (Grassland), and a late successional deciduous forest (Deciduous forest). The two annual cropping systems and the Poplar and Early successional systems are replicated in each of the six randomized blocks; four were selected for this study. The Grassland system is replicated four times and the Deciduous forest system is replicated three times.

The Conventional system received standard rates of N fertilizer: 137 ± 20 kg N ha^{-1} yr^{-1} for corn and 77 ± 17 kg N ha^{-1} yr^{-1} for wheat (Gelfand et al., 2016). Soybeans received less than 5 kg N ha^{-1} yr^{1}. N fertilizer was mostly applied as urea-ammonium nitrate (28-0-0). The Biologically-based system received no N fertilizer; instead, winter cover crops included red clover (*Trifolium pratense* L.) during the wheat phase prior to corn, and rye grass (*Lolium multiflorum* L.)

following corn harvest before soybean. Red clover is a perennial legume that fixes N_2 and both red clover and ryegrass scavenge soil N otherwise leached or denitrified. Tillage for both systems included chisel plowing followed by secondary tillage. Herbicides were used to suppress weeds in the Conventional system and additional tillage provided weed control in the Biologically-based system.

The Poplar system was planted in 1989 to *Populus* × *canadensis* Moench "Eugenei". Fertilizer was applied as 123 kg N ha^{-1} ammonium nitrate in the establishment year and the first harvest was in 1999. After the second harvest in 2008 and one fallow year, *Populus nigra* × *P. maximowiczii* "NM6" was planted in 2009. Fertilizer was then applied once in 2011 at 157 kg N ha^{-1} as ammonium nitrate.

The Early successional system was abandoned from agriculture in 1989 and has been burned every spring since 1997 to exclude woody plants. Canada goldenrod (*Solidago canadensis* L.), Kentucky bluegrass (*Poa pratensis* L.), arrow leaved aster (*Aster sagittifolius*), and timothy grass (*Phleum pratense* L.) were the current dominants https://lter.kbs.msu.edu/datatables/237). The Grassland system was established on a cleared woodlot ca. 1959 and has never been plowed. Grass is mown annually to inhibit woody species. Current dominants include smooth brome grass (*Bromus inermis* Leyss.), Canada goldenrod (*Solidago canadensis* L.), tall oatgrass (*Arrhenatherum elatius* L.), blackberry (*Rubus allegheniensis* Porter), sassafras (*Sassafras albidum*) and Kentucky bluegrass (*Poa pratensis* L.) (https://lter.kbs.msu.edu/datatables/237). The late successional Deciduous forest is unmanaged and has never been cleared or plowed. Overstory dominant species include red oak (*Quercus rubra* L.), pignut hickory (*Carya glabra*

Mill.), white oak (*Q. alba* L.) and sugar maple (*Acer saccharum* Marsh.) (https://lter.kbs.msu.edu/datatables/238).

Soil sampling

Soils were sampled seasonally for testing nitrification-derived N_2O potentials, and once for nitrification-derived N_2O kinetics, and once for solution phase NH_4^+ partitioning. For nitrification-derived N_2O potentials, soils from all systems but the Grassland were sampled in summer (late June 2016), winter (early December 2016) and spring (early May 2017). Grassland soils were sampled when determining the kinetics of nitrification-derived N_2O, for which samples were collected in 2017 from all systems in the period of late September to early December, after having first established no seasonal patterns for nitrification-derived N_2O potentials. For determining solution phase NH_4^+ partitioning, soil samples were collected in June 2019 in all systems. For all experiments, five random samples were taken at either 0-15 cm (N_2O potentials and N_2O kinetics experiments) or 0-25 cm (solution phase NH_4^+ partitioning) depths and composited by field replicate. Soils were passed through a 4 mm mesh immediately and sieved soils were stored at 4°C before analysis within four days.

Nitrification potentials

To evaluate potentials for nitrification-derived N_2O, 5 g of fresh sieved soil were placed into a 155 mL Wheaton bottle amended with 50 mL nanopure water containing 10 mM NH_4Cl. I used 1-octyne, a recently developed and tested chemical inhibitor of AOB ammonia monooxygenase (AMO) to distinguish the relative contribution from AOA and AOB (Taylor et al., 2013; Taylor et al., 2015). I used a gradient of octyne concentrations ranging from 0-10 µM aqueous

concentration (C_{aq}) to test for optimal inhibition and I found 4 μM C_{aq} sufficient to inhibit AOB in all soils (Figure 2.6), which is in agreement with previous studies (Taylor et al., 2013). Capped bottles with or without 4 μM C_{aq} octyne were immediately placed on a shaker table and shaken for 24 hours at a constant speed of 200 rpm at room temperature (25 °C). This method inhibits denitrification-derived N_2O as soil slurries are continuously aerated by high speed shaking, confirmed by treating slurries with 10 or 10 kPa of C_2H_2 with no N_2O emissions detected.

Samples for N_2O and NH_4^+ were taken at 2 and 24 hours and N_2O emission rates were calculated as N_2O accumulations over 22 hours. Slurry pH was buffered naturally as no apparent pH change was detected during the incubation. N_2O emission in the presence of octyne is attributed to AOA. N_2O emission from AOB is calculated as the difference between N_2O without octyne (total nitrification-derived N_2O) minus N_2O from AOA. N_2O samples were stored over-pressurized in 6 mL N_2-flushed glass vials (Exetainers, Labco Ltd, High Wycombe, UK). N_2O was measured with a gas chromatograph (Agilent 7890A, Santa Clara, CA) coupled to an autosampler (Gerstel MPS2XL, Mülheim An Der Ruhr, Germany) and equipped with a ^{63}Ni electron detector at 350 °C and a Porapak Q column (1.8 m, 80/100 mesh) at 80° C (https://lter.kbs.msu.edu/protocols/159). NH_4^+ concentrations were measured by a Lachat QuikChem 8500 flow injection analyzer (Hach, Loveland, CO).

Nitrification kinetics

For soils from each ecosystem, I placed 5 g of fresh sieved soil into a 155 mL Wheaton bottle. Then I added $(NH_4)_2SO_4$ to make eight different NH_4^+ concentrations ranging from 0.01 to 15.0

mM (0.01, 0.05, 0.1, 0.5, 1, 5, 10 and 15 mM NH_4^+) with a final liquid volume of 50 mL. Bottles were capped and placed on a shaker table at a constant speed of 200 rpm at room temperature (25 °C) and shaken for 24 hours. Initial N_2O samples were taken after 2 hours, and I then added either 2.8 mL of octyne stock gas (see Taylor et al. (2013) for octyne stock gas preparation) to create 4 μM C_{aq} concentrations or 2.8 mL of air without octyne. Another set of N_2O samples were taken at 24 hours. N_2O emissions rates were calculated as N_2O accumulations over 22 hours, with N_2O emission in the presence of octyne attributed to AOA. Nitrification kinetics were based on measured NH_4^+ concentrations, so included any NH_4^+ produced from net N mineralization during the incubation.

Kinetics of nitrification-derived N_2O emissions were fit to Michaelis-Menten models using the equation (Eq. 1):

$$V = \frac{V_{max} S}{K_m + S} \quad (1)$$

where V is the N_2O emission rate from nitrification, V_{max} is the maximum N_2O emission rate from nitrification under conditions of unlimited substrate (NH_4^+), S is the NH_4^+ concentration, and K_m is the half-saturation constant that represents the NH_4^+ concentration when the N_2O emission rate from nitrification is ½ V_{max}. V_{max} reflects the maximum capacity of a soil to oxidize NH_4^+ and produce nitrification-derived N_2O, and K_m reflects the NH_4^+ affinity of soil AMO.

In addition, because nitrification can be inhibited at very high NH_4^+ concentrations (Suwa, 1994), I also fitted data with Haldane models when appropriate (Stark and Firestone, 1996; Koper et al., 2010) (Eq. 2):

$$V = \frac{V_{max}S}{K_m + S + S^2/K_i} \quad (2)$$

The Haldane model introduces a third parameter K_i that reflects the maximum NH_4^+ concentration at which nitrification-derived N_2O emissions rates are ½ V_{max}. I performed a model comparison to determine which model provides a better fit (Table 2.1).

In situ N_2O flux, soil NH_4^+, and soil bulk density

I used *in situ* N_2O flux data from 1991 to 2016 to calculate the relative contribution of nitrification to N_2O emissions within each system, except for the Grassland and Deciduous forest systems for which N_2O fluxes were measured from 1992-2016 and 1993-2016, respectively. Most of these data have been previously published (Robertson et al., 2000; Gelfand et al., 2016). From 1991 to 2012, emissions were sampled every two weeks from March/April to November/December with the static chamber method (Holland et al., 1999). Additional winter samples were taken monthly starting from 2013. Square chambers (29 cm × 29 cm × 14 cm high) were placed on aluminum bases (28 cm × 28 cm × 10 cm high) semi-permanently installed about 3 cm into soil. Gas samples were taken at approximately 20 minute intervals during a one hour sampling period. N_2O fluxes were calculated by regressing headspace N_2O concentrations over time (g N_2O-N ha^{-1} day^{-1}).

Soil cores for inorganic N determinations were taken approximately biweekly after the soils thawed in the spring, usually in March or April, and discontinued before soils froze, usually in November. Soils were sampled to 25 cm depth from 1989-2016 except from 1993-2016 for the Deciduous forest system. Soil was sieved through a 4 mm sieve and 10 g of fresh soil was extracted with 100 mL 1M KCl to determine NH_4^+ concentrations. Soil bulk density (0-10 cm

depth) was measured in 2013 when collecting deep core soil samples to a depth of 1 meter with a hydraulic probe (Salina, KS, USA). Soil was sieved through a 4 mm sieve and then oven-dried at 60 °C for 48 hours; gravel-free bulk density was calculated as the dry mass divided by the volume of the core.

Microbially available (solution phase) soil NH_4^+

I partitioned long-term KCl extracted soil NH_4^+ pools into sorbed-phase ($srNH_4^+$) and solution-phase ($slNH_4^+$) pools by performing an NH_4^+ sorption capacity assay modified from Venterea et al. (2015). I assume only $slNH_4^+$ is available to soil nitrifiers. Briefly, for each ecosystem, I added 10 g of sieved fresh soils into 100 mL of water containing an NH_4^+ gradient ranging from 0 to 50 mg NH_4^+-N/L (0, 5, 10, 20, 30, 40 and 50 mg NH_4^+-N/L generated by $(NH_4)_2SO_4$ addition). Mixtures were shaken on an orbital shaker table at a constant speed of 100 rpm at room temperature (25 °C) for 18 hours. I centrifuged 10 mL aliquots at 10,000 g at room temperature (25 °C) for 15 minutes. NH_4^+-N was then analyzed by flow injection analyzer as above after filtering aliquots through a 1 mm glass fiber syringe. I calculated $srNH_4^+$ as the difference between added NH_4^+ ($addNH_4^+$) and the $slNH_4^+$ (measured as above) accounting for soil NH_4^+ contents ($soilNH_4^+$) (Eq. 3-5):

$$soilNH_4{}^+ = NH_4{}^+{}_{KCl} - NH_4{}^+{}_0 \quad (3)$$

$$srNH_4{}^+ = addNH_4{}^+ - slNH_4{}^+ + soilNH_4{}^+ \left(\text{when } addNH_4{}^+ > 0\right) \quad (4)$$

$$srNH_4{}^+ = soilNH_4{}^+ \left(\text{when } addNH_4{}^+ = 0\right) \quad (5)$$

where $NH_4^+{}_{KCl}$ is the 1M KCl extractable NH_4^+ concentrations and $NH_4^+{}_0$ is the water extractable NH_4^+ concentrations at 0 NH_4^+-N/L addition. The relationship between $srNH_4^+$ (mg N kg^{-1}) and $slNH_4^+$ (mM) is usually described by a Langmuir model (Eq. 6):

$$srNH_4^+ = \frac{\mu \times slNH_4^+}{K + slNH_4^+} \quad (6)$$

where μ (mg N kg^{-1}) is the maximum NH_4^+ content adsorbed by soil and K (mM) is the NH_4^+ concentration in solution phase at which $srNH_4^+$ is ½ μ. I modelled and plotted $srNH_4^+$ against $slNH_4^+$ (Figure 2.7), which allows me to convert total KCl-based soil NH_4^+ values into $slNH_4^+$ for every NH_4^+ soil measurement taken between 1989 and 2016.

Statistical analysis

ANOVA for seasonal nitrification-derived N_2O. I converted gravimetric N_2O emissions from the nitrification potential experiment into areal N_2O emissions based on soil depth (15 cm) and bulk density (Eq. 7):

$$N_2O_{area} = N_2O_{mass} \times DP \times \frac{BD}{10} \quad (7)$$

where N_2O_{area} is expressed as g N_2O-N ha^{-1} day^{-1} and N_2O_{mass} is expressed as ng N_2O-N g^{-1} dry soil day^{-1}, DP is soil depth in cm, and BD (0-10 cm depth) is bulk density expressed as g cm^{-3}.

Potentials for nitrification-derived N_2O were analyzed with PROC GLIMMIX of SAS 9.4 (SAS Institute, Cary, NC, USA). The statistical model included 5 ecosystem types × 3 seasons × 2 sources of nitrification-derived N_2O, and the interaction among them were considered fixed factors. Field replicates nested within ecosystem types and the interaction between field replicates and seasons nested within ecosystem types were considered random factors. Analysis of variance (ANOVA) was performed by considering ecosystem types as a whole plot factor and season and sources of nitrification-derived N_2O as subplot and sub-subplot factors. Homogeneity

of variance assumptions were checked by Levene's test and normality of residuals was visually inspected. No violations of assumptions were detected.

Distribution for field N_2O fluxes. In situ N_2O fluxes typically show a highly skewed distribution with a long tail of high values, which makes constraining the range of the mean fluxes challenging (Cowan et al., 2017). N_2O emissions can be assumed proportional to the product of the interactions of multiple biological and environmental variables such as population sizes and activities of soil nitrifiers and denitrifiers, soil moisture, soil temperature, soil inorganic N contents, and soil oxygen status. Thus, I consider multiplicative processes to influence N_2O emissions, which follow lognormal distributions (Limpert et al., 2001) (Eq. 8):

$$F_{N_2O} \sim lognorm\ (\bar{x},\ s^2) \tag{8}$$

where $\bar{x}$ and s are the mean and standard deviation of log-transformed N_2O emissions.
The mean of a lognormal distribution (without log-transformation) is usually described as (Eq. 9):

$$\mu = \exp\left(\bar{x}\ + \frac{s^2}{2}\right) \tag{9}$$

Here, I estimated lognormal means of N_2O fluxes using a Bayesian approach by evaluating the parameters in Equation (9). I chose vague prior probability distributions to reduce their impact on the inference. Although fitting lognormal distributions for N_2O fluxes makes biological and theoretical sense, there are other distributions that describe continuous positive data with large variances well. Thus, I also fit N_2O data with other candidate distributions including Gamma and Weibull distributions using the 'fitdistrplus' package for R (Delignette-Muller and Dutang, 2015) (Table 2.2). Because biases can be introduced when estimating lognormal means and there are no agreed upon best practices (Finney, 1941; Zellner, 1971; Parkin et al., 1988; Longford, 2009), I

also fit *in situ* N_2O fluxes with normal distributions and calculated the unbiased arithmetic means of N_2O fluxes, which is the most commonly used method to estimate mean N_2O fluxes and serves as a benchmark for comparing with other methods.

Estimation of contributions from nitrification. Similar to N_2O emissions from nitrification potentials, before fitting Michaelis-Menten models I converted gravimetric N_2O emissions from each nitrification kinetics experiment into areal N_2O emissions (Eq. 7) based on soil depth (15 cm) and bulk density. I then used the 'nls' function in R (version 3.5.0, R Core Team, 2018) to estimate V_{max} and K_m and their associated standard errors, which were then specified as prior information when I conducted a Markov Chain Monte Carlo (MCMC) simulation to sample posterior parameter distributions with the 'jagsUI' package (Kellner, 2017) for R. I ran three chains of 15000 iterations with 2000 burn-in iterations with a thinning rate of three, which yielded 13002 total samples for posterior distribution. Based on the Michaelis-Menten model I developed for each ecosystem, long-term solution phase NH_4^+ data were applied to predict maximum potential N_2O emissions from nitrification. The potential maximum contribution of nitrification to total N_2O was estimated with the mean of the predicted nitrification-derived N_2O divided by either the lognormal mean or arithmetic mean of field N_2O measurements for Conventional, Biologically-based, Poplar, Grassland, and Deciduous forest systems. Because the contribution from nitrification cannot be greater than 100%, I constrained my analysis with contributions ranging between 0 and 1. Overall, over 96% of the posterior distributions for contributions from total nitrification and over 99% of the posterior distributions for contributions from AOB-derived nitrification were included.

Results and discussion

Seasonal N_2O emissions from nitrification potential

Across all three seasons, soils from the Conventional and Biologically-based systems emitted the highest nitrification-derived N_2O potentials (Figure 2.1), ranging from 17.6 to 24.8 and from 13.1 to 24.6 g N_2O-N ha^{-1} day^{-1}, respectively. In comparison, Deciduous forest soils exhibited the lowest total and AOB-derived N_2O potentials: 2.39 ± 0.67 (standard error of the mean) and 2.98 ± 1.28 g N_2O-N ha^{-1} day^{-1}, respectively, for spring, and 1.56 ± 0.60 and 2.93 ± 0.60 g N_2O-N ha^{-1} day^{-1} for winter. Although seasonal nitrification-derived N_2O potentials from the Conventional and Biologically-based systems were significantly higher than from the Early successional or Deciduous forest ($P < 0.05$) systems, the differences between the two agricultural systems were not significant ($P > 0.30$) for two out of three seasons. Similarly, N_2O potentials via nitrification were generally indistinguishable among Poplar, Early successional and Deciduous forest systems ($P > 0.15$) every season.

Seasonal nitrification-derived N_2O potentials from AOB were 5.3 to 26.5 times higher than from AOA in Conventional and Biologically-based systems (Figure 2.8), suggesting a greater capacity of AOB for emitting N_2O from agricultural soils. Others have also reported the dominance of AOB over AOA for N_2O produced in soils amended with inorganic ammonium fertilizer (Wang et al., 2016; Hink et al., 2017; Hink et al., 2018) although these studies were conducted in static microcosm systems rather than on shaker tables, so results could have been confounded by nitrifier denitrification since anoxic conditions can develop in soil aggregates during incubation (Lu et al., 2018). Notwithstanding, I found no consistently significant differences between AOA and AOB-derived N_2O in non-cropping systems (Figure 2.8). Similar N_2O yields (%, defined as

N_2O-N per NO_2^- + NO_3^--N accumulated) between AOA and AOB have been reported previously for Oregon forest soils (Giguere et al., 2017).

Taken together, these results suggest that low ammonium supply, mainly derived from mineralization in unfertilized systems, promotes a greater relative contribution of AOA to nitrification-derived N_2O. Alternatively, nitrifier community compositions in unfertilized systems could be very different from row crop systems, which in turn could affect relative N_2O production. For example, most long-term fertilization studies have identified *Nitrosospira* spp. as the dominant AOB genera, with less consistent results for AOA (Phillips et al., 2000; Wu et al., 2011; Bertagnolli et al., 2016; Xue et al., 2016; Kong et al., 2019). In addition, soil *Nitrosospira* spp. have been shown to positively respond to urea and correlate with N_2O emissions (Cassman et al., 2019).

No significant overall seasonal differences of nitrification-derived N_2O potentials were observed ($P = 0.30$, Figure 2.1). There were also no significant interaction effects between sources of N_2O and seasons ($P = 0.76$) nor interactions among ecosystem types, sources of N_2O, and seasons ($P = 0.73$). The lack of seasonal effects suggests that the composition and capacity for soil nitrifiers to produce N_2O remain constant throughout years.

Kinetics of nitrification-derived N_2O

Michaelis-Menten models fit nitrification-derived N_2O data well (Figure 2.2, Table 2.1). Conventional and Biologically-based systems were associated with the highest V_{max} (Table 2.3), ranging from 12.7 to 15.1 g N_2O-N ha^{-1} day^{-1} for total nitrification-derived N_2O, and 11.4 to 13.8 g N_2O-N ha^{-1} day^{-1} for AOB-derived N_2O, suggesting a greater capacity of row crop systems to

emit N_2O from nitrification. The Grassland system had the lowest V_{max}, 1.59 ± 0.08 N_2O-N ha^{-1} day^{-1} and 0.49 ± 0.09 g N_2O-N ha^{-1} day^{-1} for total and AOB-derived N_2O, respectively, followed by Poplar but with a V_{max} 2-6 times higher than the Grassland system. V_{max} for Early successional and Deciduous forest systems were similar, ranging from 3.01 to 3.31 and 4.12 to 4.54 g N_2O-N ha^{-1} day^{-1} for AOB and total nitrification-derived N_2O, respectively. The exceptionally low V_{max} for the Grassland system indicates a very low capacity to produce nitrifier-derived N_2O even under substrate-unlimited conditions.

K_m values indicate how quickly NH_4^+ saturates nitrification-derived N_2O (Table 2.3). The Conventional system had the highest K_m for both total and AOB-derived N_2O, reaching 0.20 ± 0.06 and 0.24 ± 0.06 mM NH_4^+, respectively, which was about 2.5 times higher than the Biologically-based agricultural system, and 5-20 times higher than for all other systems. This suggests that the long-term application of chemical fertilizers in the Conventional system has cultivated a nitrifier community that is tolerant to high NH_4^+ pools. In comparison, the nitrifier communities of Biologically-based and other unfertilized systems appear not to have adapted to such high NH_4^+ inputs. In addition, the fact that the Biologically-based system has a similar V_{max} but lower K_m compared with the Conventional system may indicate that nitrifier phylotypes associated with high NH_4^+ affinity were selected by the slower-paced release of NH_4^+ from decomposing cover crop inputs in the Biologically-based system. The low V_{max} and K_m in Early successional, Grassland, and Deciduous forest systems may reflect their histories of no fertilizer inputs.

Existing studies of nitrification kinetics have mainly focused on the effects of NH_4^+ on NO_2^- + NO_3^- accumulation. Koper et al. (2010) reported the V_{max} of soils receiving ammonium sulfate at 200 kg N per hectare for 6 years was about twice higher than the V_{max} of non-fertilized soils, but no significant differences in K_m were detected. It is possible that substrate affinity responds to fertilizer more slowly than maximum nitrification rate. In addition, although V_{max} and K_m of AOB and total nitrification could be boosted significantly by fertilization within a month's time, they can also show a rapid decline within three months of fertilizer application (Ouyang et al., 2017). Together, these results suggest that long-term management practices in the MCSE shaped differences in V_{max} and K_m responses among ecosystems varying in management intensity.

The relative importance of AOA and AOB in nitrification-derived N_2O

I used a Bayesian approach to calculate the relative contributions of AOA vs. AOB to nitrification-derived N_2O based on posterior distributions of V_{max} for each ecosystem, which is different from the traditional method of separating AOA from AOB based on 1 mM NH_4^+ addition (Taylor et al., 2010; Lu et al., 2015; Ouyang et al., 2016). As noted earlier, 1 mM NH_4^+ additions did not always yield the highest N_2O emissions in MCSE systems (Figure 2.2), especially for agricultural soils. Thus, partitioning sources of nitrification-derived N_2O with V_{max} derived from substrate kinetics aligns with the concept of nitrification potential assays, which reflect the maximum nitrification-derived N_2O from nitrifier communities (Norton and Stark, 2011).

Compared to AOA, AOB were the major contributors to nitrification-derived N_2O in most systems, accounting for more than 70% (Figure 2.3) in all but the Grassland system, where the contribution from AOB averaged only 31 ± 4% of the total nitrification-derived N_2O. In addition,

there was a decreasing trend of AOB's contribution to N_2O along the management gradient: about 90% of the nitrification-derived N_2O was from AOB in row crop agricultural systems, whereas in the Early successional and Deciduous forest systems, AOB's contribution decreased to about 70% of total N_2O.

The declining importance of AOB to N_2O production along the management intensity gradient likely reflects different strategies of soil nitrifiers' responding to different agronomic practices. First, the Conventional system constantly received high N inputs, which favors nitrification activity or population size of AOB in agricultural soils (Shen et al., 2008; Jia and Conrad, 2009; Taylor et al., 2010; Habteselassie et al., 2013; Taylor et al., 2013). In contrast, AOA's contribution is more important in systems where the major NH_4^+ source is via decomposition of soil organic matter. Thus, the speed of NH_4^+ supply to soil seems important for shaping the dynamics of AOA vs. AOB N_2O-generating activities. Indeed, Hink et al. (2018) observed the dominance of AOA in nitrification-derived N_2O from soils receiving slow-release fertilizer instead of free urea.

A second major difference between row crop and unfertilized systems is the history of tillage. Both the Conventional and Biologically-based systems have been either moldboard or chisel-plowed since well before 1988. In contrast, the Early successional and Poplar systems have been free of tillage since 1989 and the Deciduous forest and Grassland systems have never been plowed. Tillage accelerates soil organic matter turnover, which results in more pulse-like releases of NH_4^+ in soil compared with non-tilled systems. As a result, AOB likely outcompetes AOA following tillage-induced pulses of NH_4^+.

The dominance of AOA for nitrification-derived N_2O in the Grassland system seems anomalous and might be attributed to the differential inhibition of AOB vs. AOA induced by root-released nitrification inhibitors by some grasses. While I have no direct evidence of inhibitors produced by grasses in the study sites, in one recent three-year field study (Subbarao et al., 2009), brachialactone, a root exudate isolated from forage grass *Brachiaria* spp., has been shown to inhibit 90% of *in situ* NH_4^+ oxidation and over 90% of cumulative N_2O emissions in pastures. Moreover, the inhibition seemed to be specific to AOB rather than AOA. Historically, among all ecosystems in MCSE, the Grassland system has always had the highest monthly soil NH_4^+ concentrations and exhibited low relative nitrification potentials (Millar and Robertson, 2015). Since root exudates of *Bromus* spp., a dominant species in the Grassland system, have been reported to significantly inhibit nitrification in both AOB culture and in whole soils (O'Sullivan et al., 2017), I suspect AOB inhibition in the Grassland system.

Contribution of nitrification to long-term N_2O emissions

I used solution phase concentrations of soil NH_4^+ with Michaelis-Menten kinetics to predict the proportion of N_2O fluxes that could be produced by nitrifiers *in situ*. Soil solution phase NH_4^+ concentrations ($slNH_4^+$) were estimated from the long-term KCl-extracted NH_4^+ measurements (https://lter.kbs.msu.edu/datatables/55). The relationship between sorbed and solution-phase NH_4^+ as revealed by Langmuir models (Figure 2.7) was similar among ecosystems except for Deciduous forest soils, where there was a lower proportion of sorbed NH_4^+ ions (i.e., for any given sample date, a greater proportion of total KCl-based NH_4^+ concentrations were in solution). This difference most likely reflects differences in cation exchange capacity (CEC) as affected by soil pH. Venterea et al. (2015) showed that the ratio of slopes (the linear portion of the Langmuir models in Figure 2.7) among different soils is almost identical to the ratio of their

CEC values. Because all MCSE soils have the same mineralogy, Deciduous forest differences likely reflect the effects of this site's low pH (Figure 3.3A in Chapter 3) on its soil organic matter's contribution to CEC (Sollins et al., 1988).

Seasonally stable nitrification-derived N_2O fluxes allow me to apply kinetics models to predict potential maximum N_2O emissions from nitrification and, subsequently, the theoretical maximum relative contribution of nitrification to field-based N_2O. Since the kinetics results are based on aerobic incubations of shaken soil slurries that largely eliminate N_2O reduction and N_2O from nitrifier denitrification (Wrage et al., 2001; Wrage-Mönnig et al., 2018), nitrification-derived N_2O rates can be considered nitrifier nitrification rather than nitrifier denitrification, and when applied to historical solution phase *in situ* NH_4^+ pools, maximum potential nitrifier-derived N_2O *in situ*.

Among all ecosystems, row crop systems appear to have the lowest total N_2O contributed from nitrification. The percentage of 25^{th}-75^{th} posterior intervals from nitrification ranged between 13.1 - 16.7% for the Conventional system and 27.4 - 41.6% for the Biologically-based system (Figure 2.4). For the Poplar and Grassland systems, a median of 52.0% and 54.8% of field-based N_2O fluxes can be attributed to nitrification. The Deciduous forest system was associated with the highest contribution from nitrification, with the percentage of 25^{th}-75^{th} posterior intervals ranging between 51.2 - 76.9% for total nitrification-derived N_2O and 27.2 - 49.6% for AOB-derived N_2O (Figure 2.4 and Figure 2.5). For all ecosystems, the median contributions of AOB to N_2O were below 40%, ranging from 11.4 - 36.4% (Figure 2.5).

Although for all ecosystems studied, the lognormal distribution had the smallest AIC value compared with other distributions (Table 2.2), I also estimated the relative contribution of nitrification to N_2O based on unbiased arithmetic means of long-term N_2O fluxes (Figure 2.9 and Figure 2.10) because normal distribution is most often assumed to describe N_2O fluxes and therefore serves as a well-known benchmark. For most of the systems, results are similar compared with fitting N_2O fluxes with lognormal distributions. The only exception is for the Poplar system, where the 25^{th}–75^{th} posterior percentage of contribution from total and AOB-derived nitrification were 31.1 - 48.2% and 20.4 - 33.3%, respectively, much lower percentage than for estimates based on lognormal distributions.

The finding that total nitrification contributed a theoretical maximum of 13 - 17% of field-based N_2O fluxes in the Conventional system suggests that nitrification is unlikely to be a significant source of N_2O in long-fertilized systems. That a theoretical maximum of only 27 - 42% of field-based fluxes were nitrifier-derived in the Biologically-based system suggests that nitrification is likewise unlikely to be a dominant N_2O source in even unfertilized annual cropping systems. Using SP, Opdyke et al. (2009) and Zou et al. (2014) reported a small role for nitrification in N_2O produced by agricultural soils, although these studies lasted less than one year. Similarly, with the median AOB contributions for all ecosystems ranging between 11 - 36% of total N_2O fluxes, results show that AOB-derived nitrification is unlikely to be the major process leading to N_2O production in any of ecosystems regardless of management. These results are also consistent with Buchen et al. (2018), who also used SP *in situ* to show that over 80% of N_2O can be attributed to denitrification (whether heterotrophic or nitrifier-derived) in managed grasslands.

Since the Michaelis-Menten models were necessarily developed under laboratory conditions that favored nitrification, the calculated contributions of nitrification to N_2O reflect maximum *in situ* potentials that assume all solution phase NH_4^+ is oxidized and no nitrification-derived N_2O is further reduced to N_2. Neither of these assumptions are realistic when extrapolated to field conditions. Soils are rarely completely aerobic, and even if *in situ* nitrification emitted N_2O equivalent to the amount from shaken soil slurries, some of the N_2O will be captured by denitrifiers and reduced to N_2 before being emitted to the atmosphere (Decock and Six, 2013; Lewicka-Szczebak et al., 2017). Malhi and McGill (1982) estimated that the daily maximum NH_4^+-N oxidation rate is less than 10% of added NH_4^+-N (100 μg N g^{-1}) based on lab incubation. Process-based ecosystem models such as DAYCENT typically assume that only 2% of nitrified N lost as N_2O during nitrification (Parton et al., 2001). Goodroad and Keeney (1984) reported only 0.1 – 0.3% of the nitrified NH_4^+ was lost as N_2O from a fertile soil in Wisconsin, USA. In comparison, the potential maximum rates of this study assume 100% of daily NH_4^+ is nitrified and consequently eligible to be transformed to N_2O. Thus, the actual contributions of nitrification to measured N_2O fluxes *in situ* are likely to be far below the potential maximum rates estimated here.

The least-constrained nitrifier contribution to N_2O fluxes was measured in Early successional and Deciduous forest soils where over 95% of the predicted nitrification-derived N_2O was higher than the field fluxes in the Early successional system, and 51 - 77% of total N_2O fluxes might potentially derive from nitrification in the Deciduous forest system (Figure 2.4). But here, perhaps especially, the extrapolation assumptions seem severe. The Early successional and Deciduous forest soils contain, among all of soils, the most macroaggregates (2000 - 8000 μm)

(Grandy and Robertson, 2007) and thus a larger volume fraction of anoxic centers (Schlüter et al., 2018), which result in high measured denitrification rates (Robertson and Tiedje, 1984). I suspect that especially in Early successional and Deciduous forest soils, the estimates for nitrifier-derived N_2O are substantially over estimated. Overall, then, I conclude that nitrification is not a dominant process leading to N_2O emissions in any of systems.

Conclusions

1. Nitrification-derived N_2O emissions exhibited Michaelis-Menten kinetics and were seasonally stable.
2. AOB dominated nitrification-derived N_2O emissions in all ecosystems but a mown grassland where AOA contributed about 70%.
3. Nitrification is a minor source of N_2O, especially in row crop systems.

APPENDIX

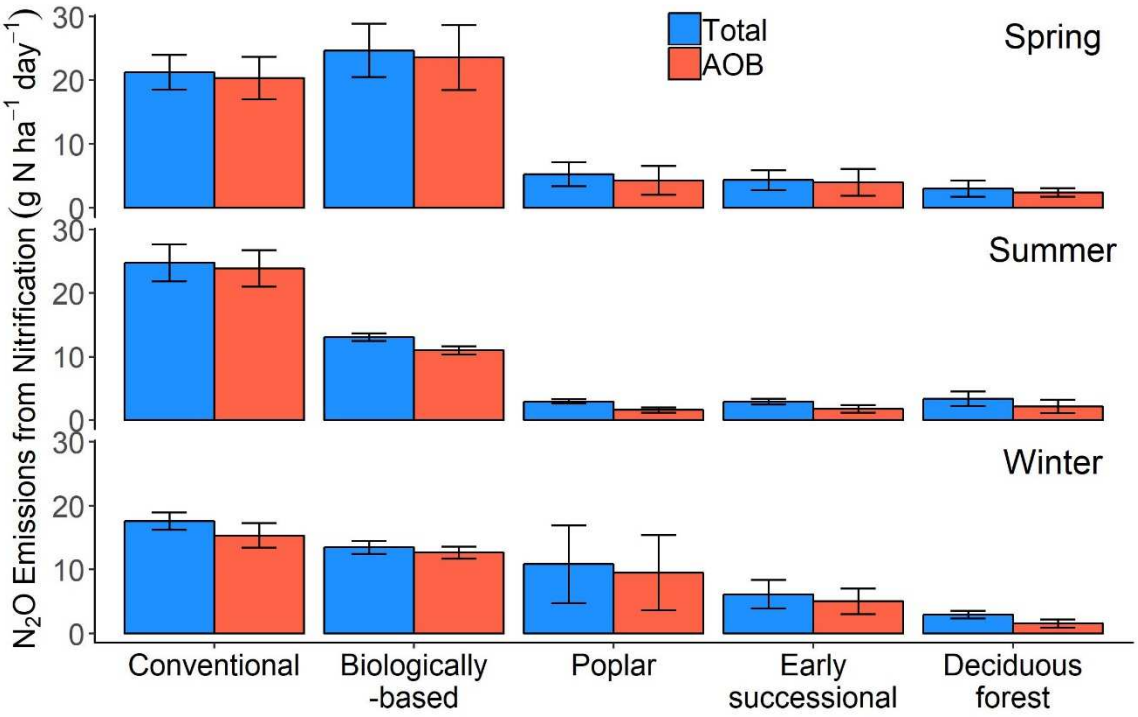

Figure 2.1 Seasonal potential N_2O production from nitrification (total or AOB-derived) across a management intensity gradient; bars represent standard errors (for total, n = 4 except deciduous forest n = 3; for AOB, n = 3-4 except deciduous forest n = 2-3). No significant differences among seasons were detected ($P = 0.30$).

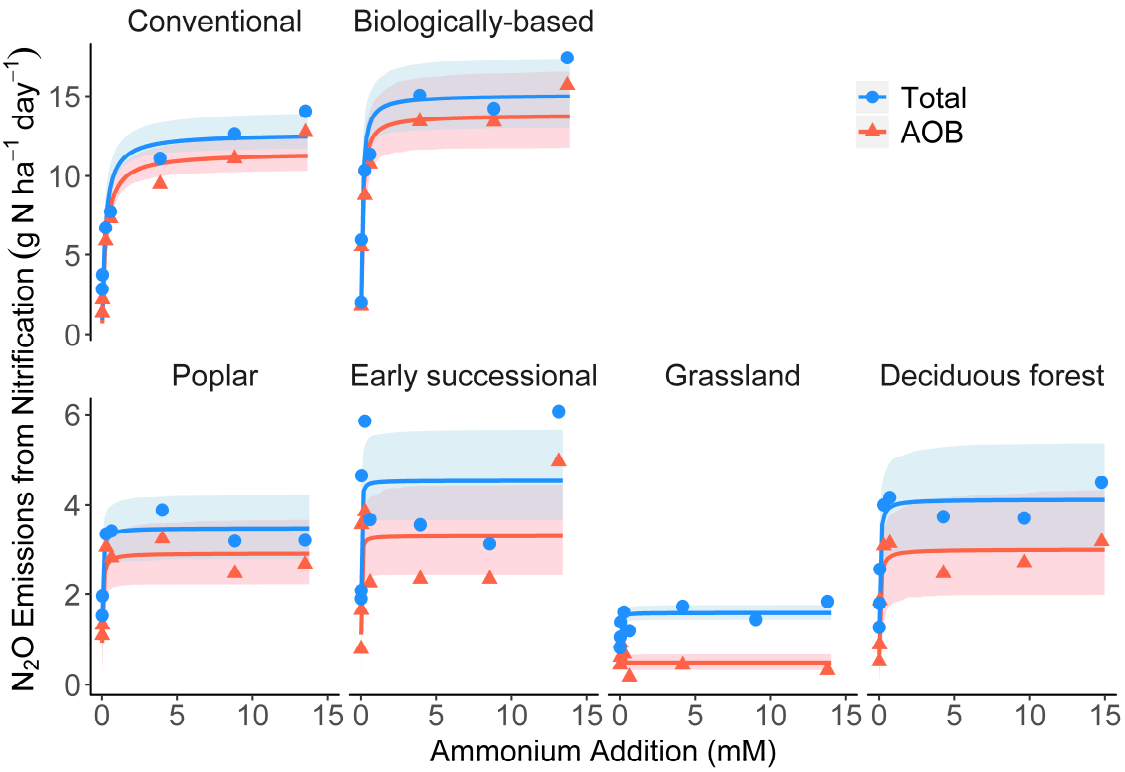

Figure 2.2 The kinetics of nitrification-derived N_2O in soils from different systems varying in management intensities. Michaelis-Menten models were fit to total nitrification-derived N_2O emissions (blue lines) and AOB-derived N_2O emissions (orange lines). Blue circles and orange triangles are the mean N_2O emissions from total and AOB-derived nitrification at each ammonium addition. Note y-axis scale differs by system. Shaded bands represent 95% confidence intervals.

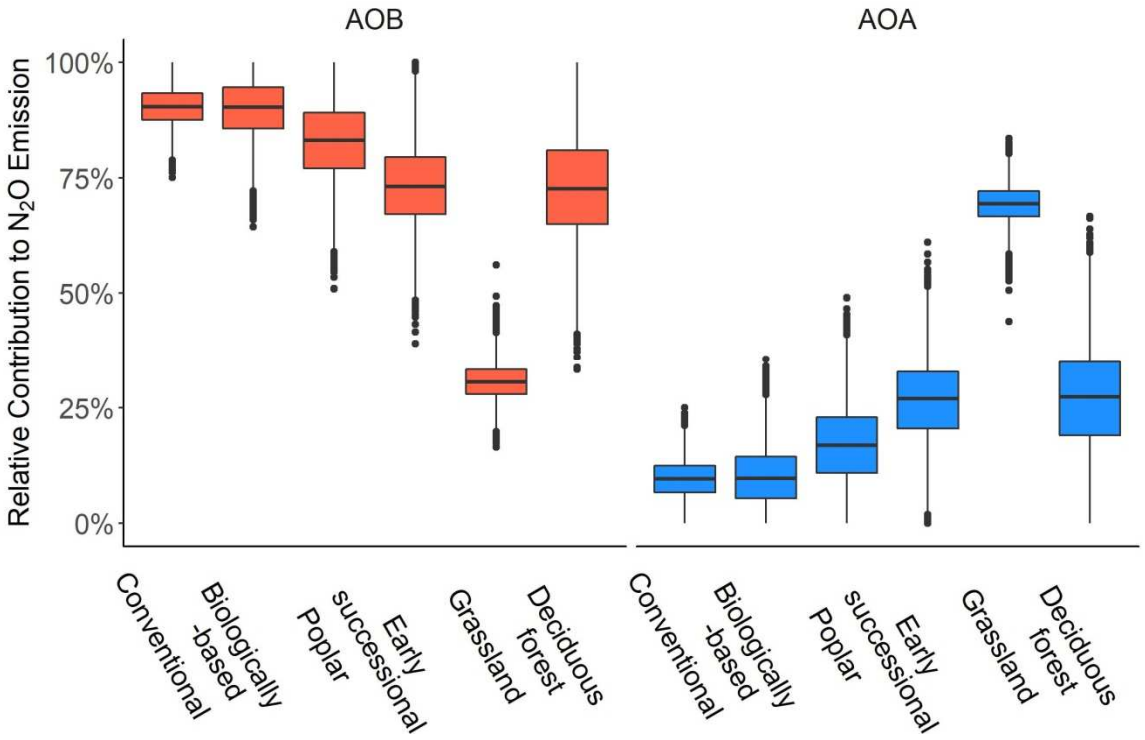

Figure 2.3 Relative contributions of AOA and AOB to nitrification-derived N_2O emissions in systems that differ in management intensities. Contributions from AOB (%, orange) were calculated with posterior distributions of V_{max} derived from Michaelis-Menten models for AOB and total nitrification-derived N_2O kinetics. Contributions from AOA (%, blue) were calculated as 1 - AOB (%).

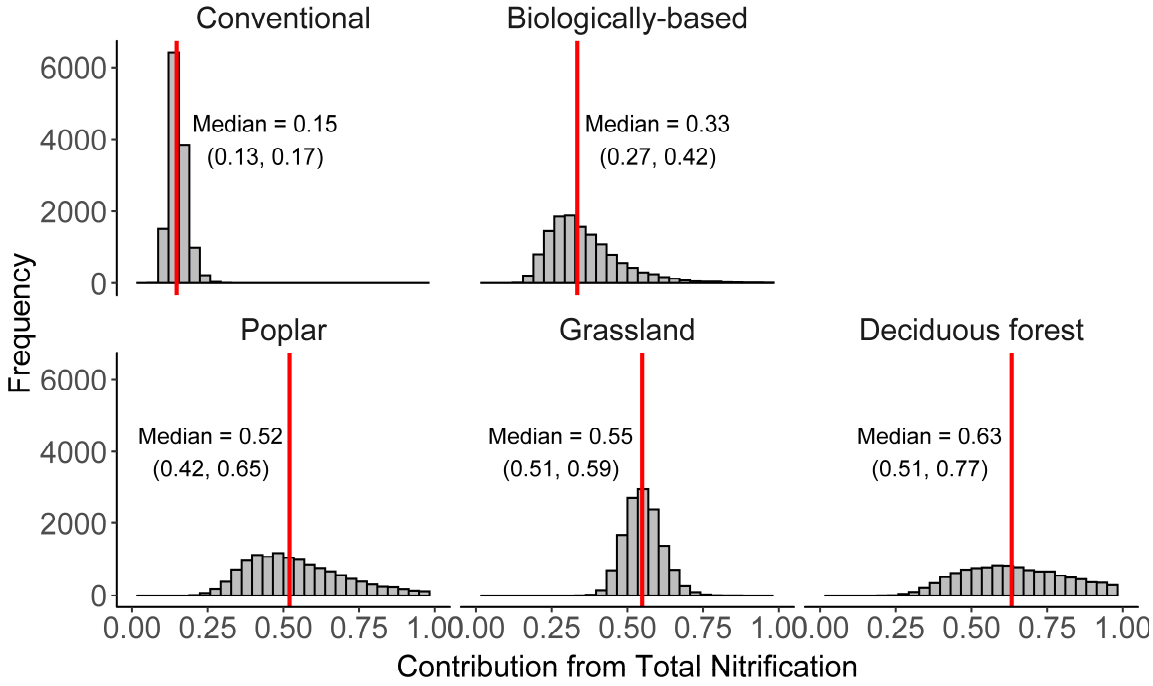

Figure 2.4 Maximum relative contributions of total nitrification to long-term field N_2O emissions in systems that differ in management intensities assuming all solution phase *in situ* ammonium is oxidized and no nitrification-derived N_2O is reduced. Field-based N_2O fluxes were estimated assuming log-normal distributions. Red vertical lines indicate the median contribution for each system. Values in parentheses indicate the 25^{th}-75^{th} posterior intervals, respectively. Note Early successional system is not included as 95% of the posterior nitrification-derived N_2O was higher than the field fluxes.

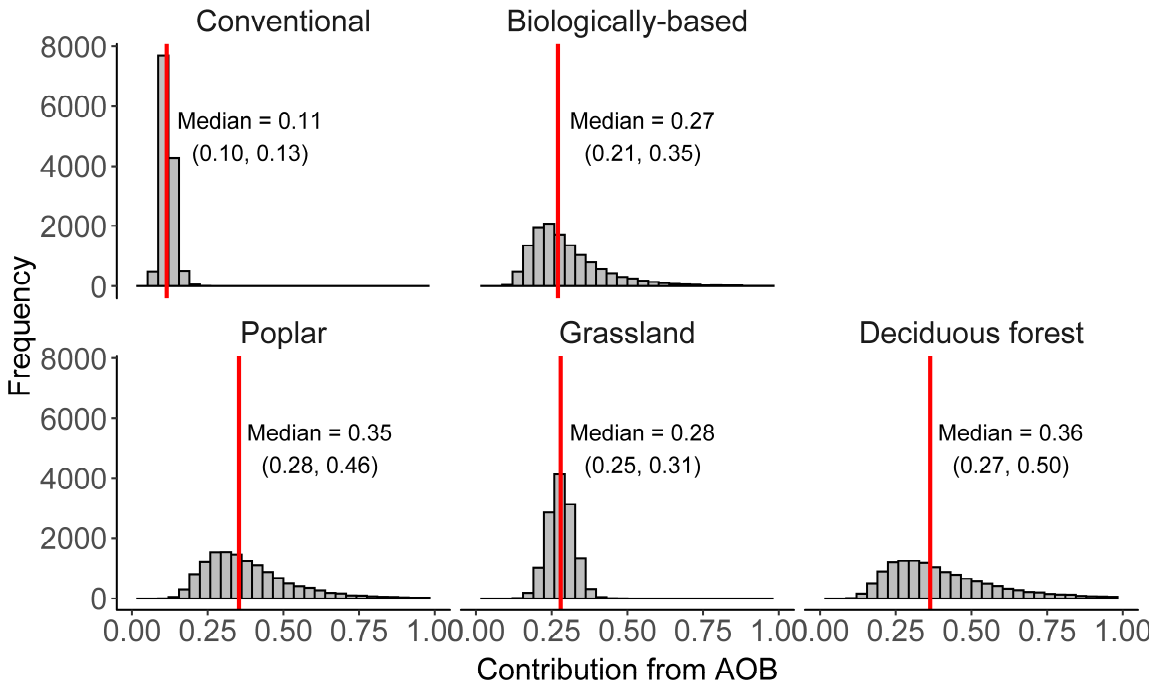

Figure 2.5 Maximum relative contributions of nitrification by AOB to long-term field N_2O emissions in systems that vary in management intensities assuming all solution phase *in situ* ammonium is oxidized and no nitrification-derived N_2O is reduced; see Figure 2.4 legend for further details.

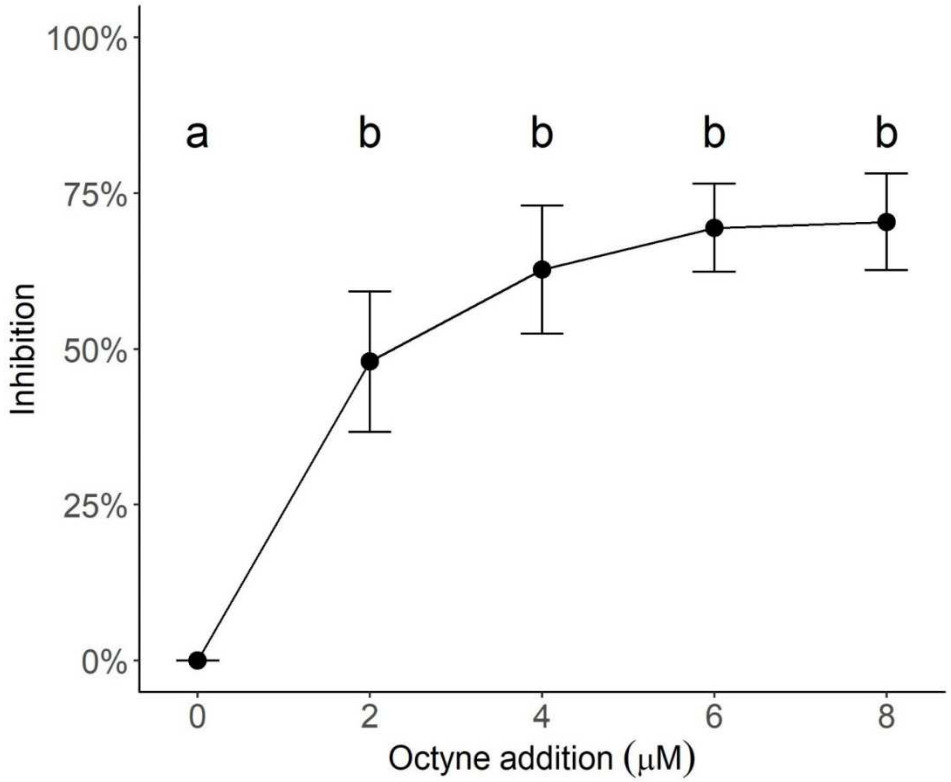

Figure 2.6 Inhibition of AOB-derived NO_2^- + NO_3^- production by octyne in all systems; bars represent standard errors (n=6, including Conventional, Biologically-based, Poplar, Early successional, Grassland and Deciduous forest systems). Inhibition (%) is calculated as the percentage reduction of accumulated NO_2^- + NO_3^- compared with no octyne added. Different lowercase letters indicate significantly different inhibition effects among soils treated with different amount of octyne ($P < 0.05$).

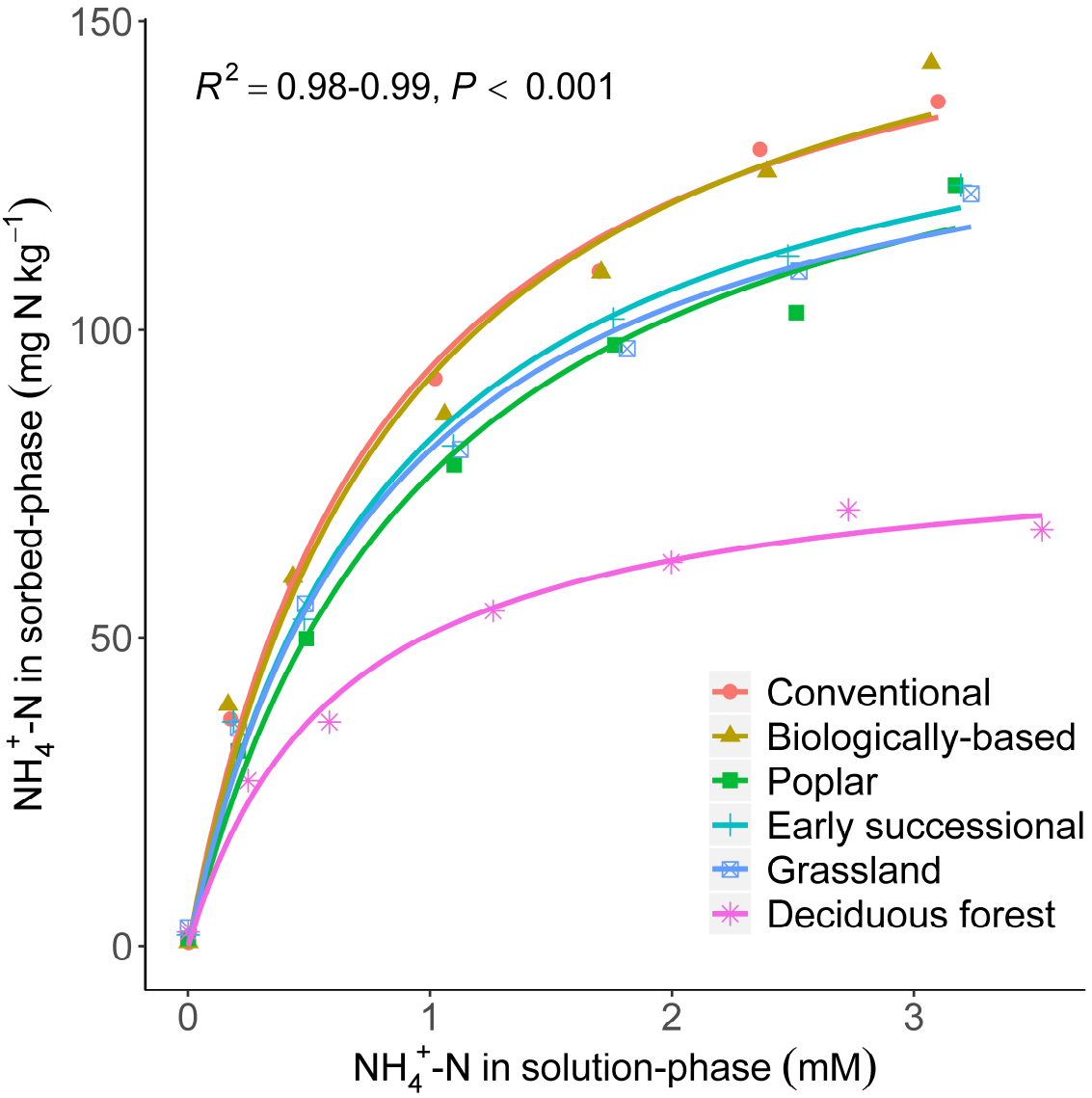

Figure 2.7 The relationship between NH_4^+-N in solution-phase (mM) vs. NH_4^+-N in sorbed-phase (based on dry soil mass) among different ecosystems varying in management intensities; a Langmuir model was used to fit relationships.

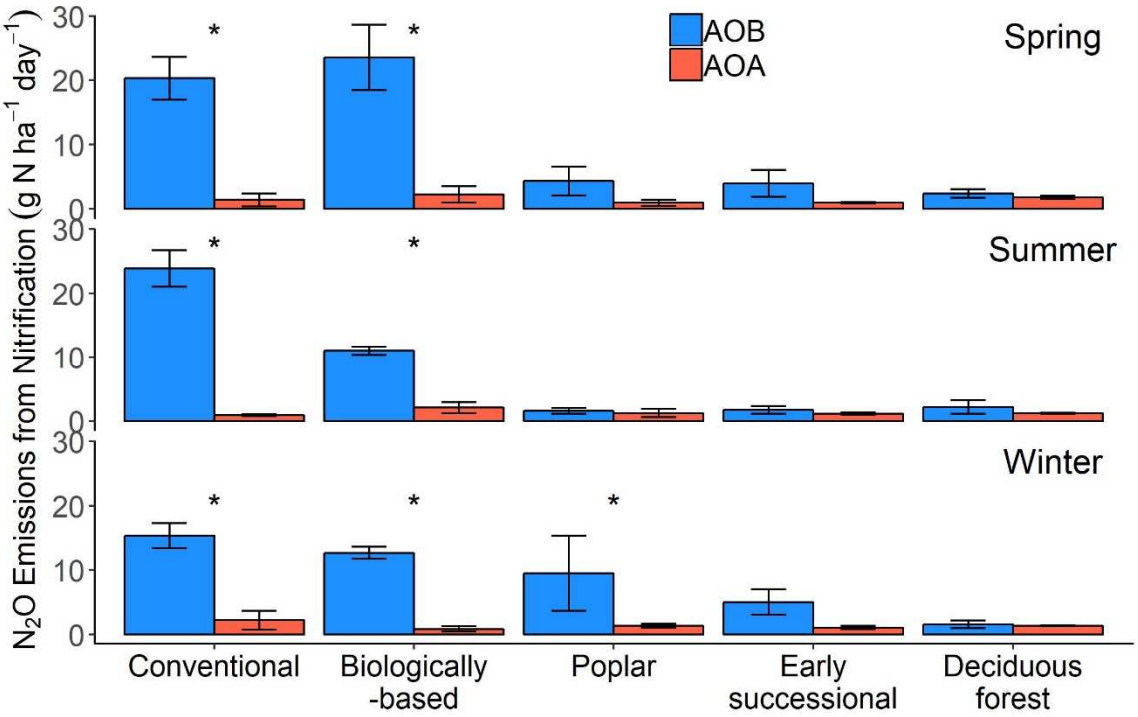

Figure 2.8 Seasonal N_2O potentials from nitrification (AOB or AOA-derived) across a management intensity gradient; bars represent standard errors (n = 3-4 except deciduous forest n = 2-3). No significant differences among seasons were detected ($P = 0.28$). "*" indicates significant differences between AOA and AOB-derived N_2O potentials.

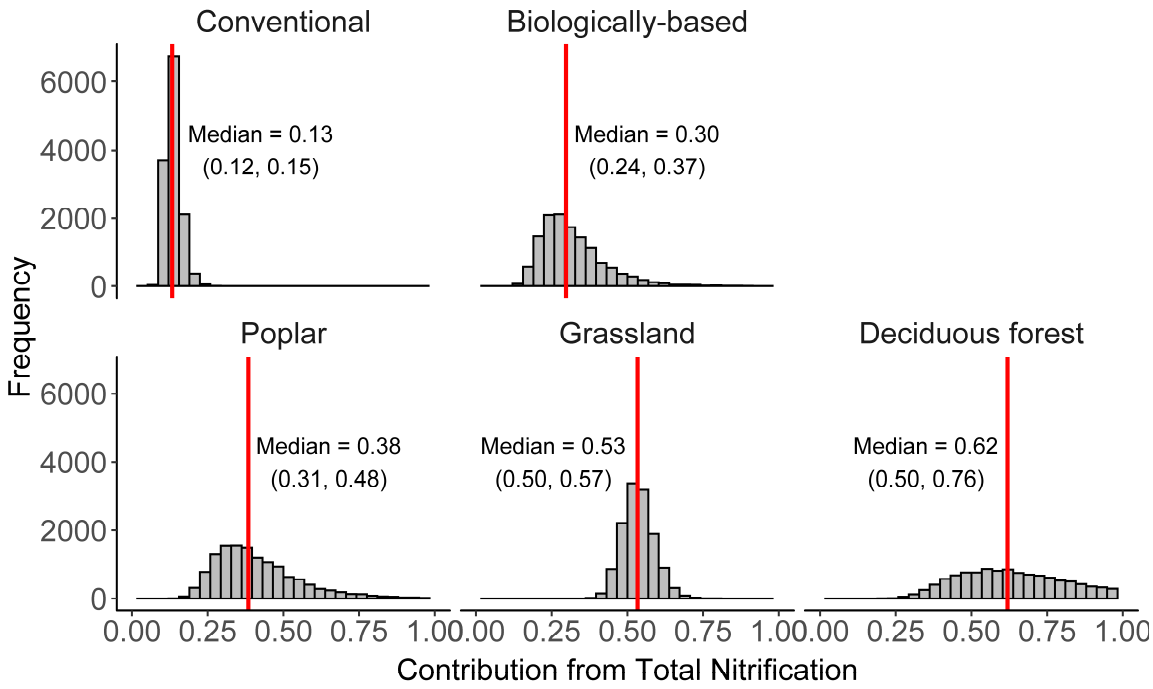

Figure 2.9 Maximum relative contributions of total nitrification to long-term field N_2O emissions in different systems varying in management intensities assuming all solution phase *in situ* ammonium is oxidized and no nitrification-derived N_2O is reduced; field-based N_2O fluxes were estimated assuming normal distributions. Red vertical line indicates the median contribution for each system. Numbers in brackets indicate the 25^{th}-75^{th} posterior intervals, respectively. Note Early successional system is not included as 95% of the posterior nitrification-derived N_2O was higher than the field fluxes.

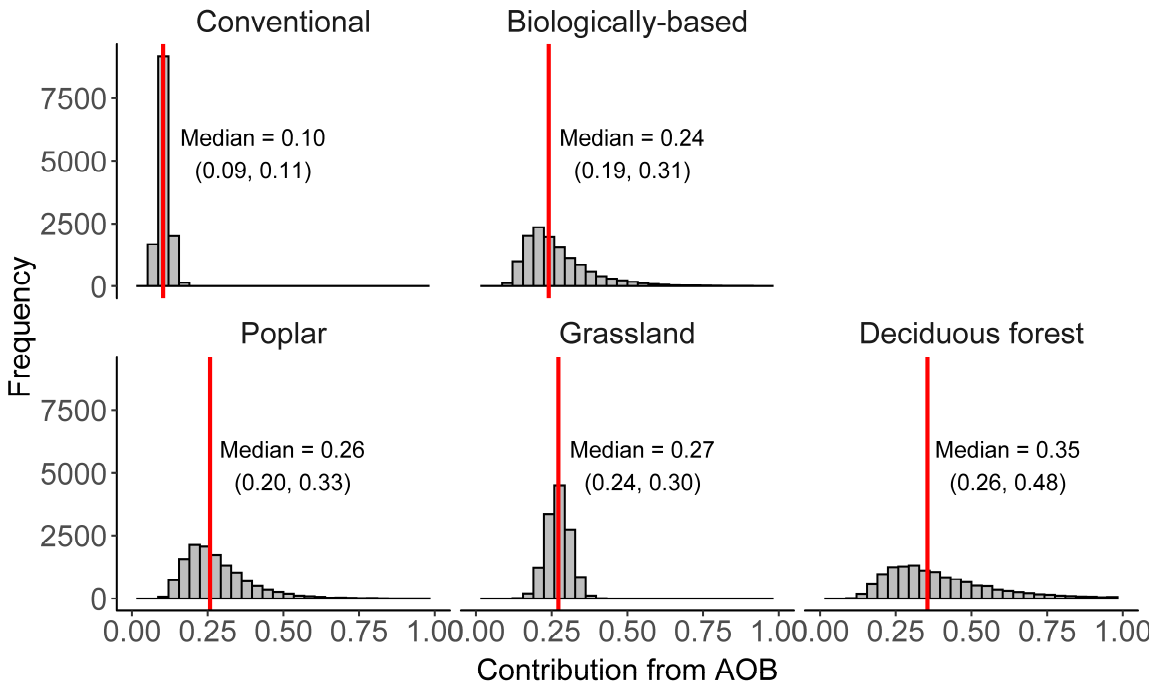

Figure 2.10 Maximum relative contributions of AOB to long-term field N_2O emissions in different systems varying in management intensities assuming all solution phase *in situ* ammonium is oxidized and no nitrification-derived N_2O is reduced; estimates based on normal distributions. See Figure 2.9 legend for further details.

Table 2.1 Comparisons between Michaelis-Menten and Haldane kinetics models for total or AOB-derived N_2O emissions from nitrification; AIC represents Akaike information criterion.

Ecosystem [a]	Nitrification	AIC [b] (Michaelis-Menten)	AIC [b] (Haldane)	*F*-value [c]	*P*-value [c]
Poplar	Total	111	113	0.188	0.668
	AOB	105	106	0.488	0.491
Early successional	Total	143	144	0.134	0.718
	AOB	130	131	1.13	0.298
Grassland	Total	27.9	28.1	1.70	0.202
	AOB	30.2	30.6	1.50	0.233
Deciduous forest	Total	109	111	0.001	0.980
	AOB	106	108	0.049	0.827

[a] Data from Conventional and Biologically-based systems were not fit to Haldane models because no signs of inhibition of nitrification-derived N_2O were found.

[b] Models with lower AIC were considered superior.

[c] Models were also compared based on F-test. A *P*-value > 0.05 supports the minimal model as the adequate model.

Table 2.2 AIC of field-based N_2O fluxes from different ecosystems fitted with different distributions; AIC represents Akaike information criterion.

	Distribution			
Ecosystem	Lognormal	Gamma	Weibull	Normal
Conventional agriculture	4602	5038	4915	7922
Biologically-based agriculture	5030	5489	5344	8629
Poplar	2303	2881	2659	6378
Early successional	2591	2804	2808	4392
Grassland	1733	1872	1865	3106
Deciduous forest	2452	2690	2648	4687

Table 2.3 Michaelis-Menten kinetic parameters of total or AOB-derived N_2O emissions from nitrification; V_{max} represents maximum nitrification-derived N_2O emissions (g N_2O-N ha^{-1} day^{-1}) and K_m represents half saturation constant (mM).

Ecosystem	Nitrification	V_{max}	K_m
Conventional agriculture	Total	12.7 (0.6)	0.20 (0.06)
	AOB	11.4 (0.6)	0.24 (0.06)
Biologically-based agriculture	Total	15.1 (1.2)	0.079 (0.042)
	AOB	13.8 (1.3)	0.088 (0.056)
Poplar	Total	3.48 (0.40)	0.025 (0.019)
	AOB	2.92 (0.36)	0.033 (0.026)
Early successional	Total	4.54 (0.52)	0.009 (0.008)
	AOB	3.31 (0.47)	0.012 (0.011)
Grassland	Total	1.59 (0.08)	0.012 (0.004)
	AOB	0.49 (0.09)	-0.002 (0.004)
Deciduous forest	Total	4.12 (0.61)	0.031 (0.026)
	AOB	3.01 (0.58)	0.042 (0.045)

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Chapter 3: Niche differentiation of bacterial versus archaeal soil nitrifiers induced by ammonium inhibition

Abstract

Soil nitrification, mediated mainly by ammonia oxidizing archaea (AOA) and ammonia oxidizing bacteria (AOB), is the process by which ammonium (NH_4^+) is converted into nitrite (NO_2^-) and nitrate (NO_3^-). Previous studies have extensively examined the differential responses of AOA and AOB to N fertilization, but the potential niche differentiation induced by NH_4^+ inhibition has not yet been addressed. I investigated the nitrification kinetics of AOA and AOB under eight replicated cropped and unmanaged ecosystems (including two fertilized successional systems) along a long-term management intensity gradient in the upper U.S. Midwest. For five of eight ecosystems, AOB exhibited Haldane kinetics (inhibited by high NH_4^+ additions), especially for perennial and successional systems. In contrast, AOA predominantly exhibited Michaelis-Menten kinetics, suggesting greater resistance to high N inputs than AOB. Additionally, long-term fertilization significantly enhanced maximum nitrification rates (V_{max}) in the Early successional systems for both AOA and AOB but not in the Deciduous forests systems, likely due to the suppression of nitrification in the acidic forest soils, further corroborated by a positive correlation of V_{max} with soil pH but not with amoA gene abundance. Results also demonstrate that soil nitrifier communities are relatively stable, as there were no seasonal differences in nitrification potentials. Overall, this study provides evidence that 1) NH_4^+ inhibition of AOB but not AOA can be another factor contributing to niche differentiation between AOA and AOB, and 2) nitrification by both AOA and AOB can be significantly promoted by long-term N inputs.

Introduction

Soil nitrification is the microbial process that oxidizes ammonium (NH_4^+) into nitrite (NO_2^-) and nitrate (NO_3^-), and is central to the global nitrogen cycle as it influences ecosystem nitrogen retention (Kowalchuk and Stephen, 2001) and can regulate the forms of reactive N that enters the environment (Robertson and Groffman, 2015). Unlike NH_4^+ that binds with cation-exchange sites on soil organic matter and mineral surfaces in soil, both NO_2^- and NO_3^- are mobile anions, and can thereby be easily leached from soil by precipitation to result in water pollution (Robertson and Vitousek, 2009). In addition, nitrification is an important biological process leading to emissions of N_2O (Davidson et al., 1986), which is a potent greenhouse gas with a global warming potential ~300 times higher than CO_2 and N_2O as well destroys stratospheric ozone (Ravishankara et al., 2009). Since NH_4^+ can enter ecosystems via multiple processes such as mineralization, N_2 fixation, deposition, and fertilizer application, understanding the factors regulating soil nitrification is crucial for improving N use efficiency and reducing the negative environmental impacts of reactive N.

For over 100 years, ammonia oxidizing bacteria (AOB), first cultivated in 1890 (Frankland and Frankland, 1890), were believed to be the sole agents of autotrophic nitrification. This view was transformed by the discovery of the ammonia oxidizing archaea (AOA) *Nitrosopumilus maritimus* SCM1 in 2005 (Könneke et al., 2005). Since then the numerical dominance of AOA over AOB in diverse ecosystems including terrestrial (Leininger et al., 2006), marine (Wuchter et al., 2006), and hot springs (Hatzenpichler et al., 2008) have been widely reported, indicating a potentially more versatile energy metabolism employed by AOA (Stahl and de la Torre, 2012). In addition, because *Nitrosopumilus maritimus* SCM1 has greater NH_4^+ sensitivity and a much

higher substrate affinity than most of the known AOB cultures (Martens-Habbena et al., 2009), NH_4^+ affinity has been suggested as an important factor leading to niche differentiation between AOA and AOB.

The view that AOA prefer oligotrophic environments while AOB dominate nutrient-enriched environments (Schleper, 2010) was challenged by the discovery of soil AOA '*Ca. Nitrososocosmicus franklandus*', which can tolerate NH_3 at higher concentrations than most AOB strains (Lehtovirta-Morley et al., 2016a). This finding also revealed the lack of knowledge about niche differentiation induced by NH_3 inhibition (Verhamme et al., 2011) and the overlooked role AOA may play in fertilized soils. It seems clear that our understanding of NH_3 sensitivity for soil AOA is constrained by the limited numbers of pure cultures and enrichments available (Jung et al., 2011; Lehtovirta-Morley et al., 2011; Tourna et al., 2011; Jung et al., 2014; Lehtovirta-Morley et al., 2014; Lehtovirta-Morley et al., 2016a). In addition, as phylogenetic studies have revealed an extensive diversity of soil nitrifiers, it is unknown if the existing cultures and enrichments for AOA and AOB are environmentally representative (Prosser and Nicol, 2012), especially given the vast difference between culture and natural soil environments.

NH_3 tolerance can also be investigated by nitrification kinetics, which eliminate the inherent biases of isolation techniques and directly measure the responses of *in situ* nitrifier communities to NH_4^+ additions (Norton and Stark, 2011). Several studies have examined the effects of NH_4^+ additions on soil nitrification in general (Lu et al., 2015; Mushinski et al., 2017), but the potentially different responses of AOA and AOB to a gradient of NH_4^+ have not yet been fully articulated (Ouyang et al., 2017). Existing soil nitrification kinetic studies have mainly focused

on a single management type and without differentiating between AOA and AOB (Stark and Firestone, 1996; Koper et al., 2010; Auyeung et al., 2015). Thus, it is unclear how kinetics of AOA and AOB might be differentially affected in ecosystems with varying management intensities.

Here I investigate the nitrification kinetics of AOA and AOB separately along a long-term management intensity gradient in the upper U.S. Midwest. I use the recently developed inhibitor 1-octyne (Taylor et al., 2013; Taylor et al., 2015) to separate AOA from AOB nitrification in whole soil. I selected ecosystems ranging from intensively managed annual row crops to unmanaged late successional deciduous forest (including long-term fertilized successional ecosystems), which allow me to test the hypotheses that 1) both nitrification potentials and nitrification kinetics of AOA and AOB will respond to management intensities and long-term N fertilization; 2) high NH_4^+ can lead to niche differentiation between AOA and AOB, especially in perennial and successional ecosystems and 3) environmental variables such as soil pH can be a strong predictor of nitrification kinetics parameters for both AOA and AOB.

Methods

Study site

This study was conducted in the Main Cropping System Experiment (MCSE) of the Kellogg Biological Station (KBS) LTER site located in southwest Michigan (42° 24'N, 85° 23'W). The MCSE was established in 1988 and includes 11 ecosystems that form a management intensity gradient on the same soil series: annual crops, perennial crops, and unmanaged systems at different stages of ecological succession (Robertson and Hamilton, 2015). The KBS climate is humid continental with 1005 mm annual precipitation spread evenly throughout the year and a

10.1 °C mean annual temperature (30-year mean from 1981). Soils are well drained Alfisol loams (co-mingled Kalamazoo and Oshtemo series Typic Hapludalfs), formed from glacial till and outwash with some intermixed loess (Crum and Collins, 1995, Luehmann et al., 2016). Average sand and clay contents in surface soils are 43% and 17%, respectively (Robertson and Hamilton, 2015).

I chose to study: 1) two annual cropping systems: a conventionally managed corn-soybean-winter wheat rotation (Conventional) and a biologically managed corn-soybean-winter wheat rotation (Biologically-based); 2) a hybrid poplar system (Poplar); and 3) three successional systems of different ages: an early successional system (Early successional), a never-tilled annually mown grassland system (Grassland), and a late successional deciduous forest (Deciduous forest). The two annual cropping systems and the Poplar and Early successional systems as 1 ha plots (90 × 110 m) are replicated in each of six randomized blocks; four were selected for this study. The Grassland system is replicated four times and the Deciduous forest system is replicated three times at nearby locations on the same soil series (Robertson and Hamilton, 2015). In addition, subplots received long-term N fertilizer addition in the Early successional (Fertilized Early successional) (since 1990) and the Deciduous forest (Fertilized Deciduous forest) (since 2007) and were also sampled.

The Conventional system has received standard rates of N fertilizer since establishment in 1988: 137 ± 20 kg N ha^{-1} yr^{-1} for corn and 77 ± 17 kg N ha^{-1} yr^{-1} for wheat (Gelfand et al., 2016). Soybeans received less than 5 kg N ha^{-1} yr^{1}. N fertilizer was mostly applied as urea-ammonium nitrate (28-0-0). The Biologically-based system has received no N fertilizer; instead, winter

cover crops, including red clover (*Trifolium pratense* L.) during the wheat phase prior to corn, and rye grass (*Lolium multiflorum* L.) following corn harvest before soybean, provide additional N. Red clover is a legume that fixes N_2 and both red clover and ryegrass scavenge soil N otherwise leached or denitrified during non-crop seasons. Tillage for both systems included chisel plowing followed by secondary tillage. Herbicides were used to suppress weeds in the Conventional system and additional tillage provided weed control in the Biologically-based system.

The Poplar system was planted in 1989 to *Populus* × *canadensis* Moench "Eugenei". Fertilizer was applied as 123 kg N ha^{-1} ammonium nitrate in the establishment year and the first harvest was in 1999. After the second harvest in 2008 and one fallow year, *Populus nigra* × *P. maximowiczii* "NM6" was planted in 2009. Fertilizer was then applied once in 2011 at 157 kg N ha^{-1} as ammonium nitrate.

The Early successional system was abandoned from agriculture in 1989 and has been burned every spring since 1997 to exclude woody plants. Canada goldenrod (*Solidago canadensis* L.), Kentucky bluegrass (*Poa pratensis* L.), arrow leaved aster (*Aster sagittifolius*), and timothy grass (*Phleum pratense* L.) were the dominants during the current study (https://lter.kbs.msu.edu/datatables/237). Since 1990, a 5m × 5m subplot located at the northwest corner of each main plot was fertilized annually with 120 kg N ha^{-1} ammonium nitrate pellets in early July (Huberty et al., 1998; Grman et al., 2010). The Grassland system was established on a cleared woodlot ca. 1959 and has never been plowed. Grass is mown annually to inhibit woody species. Current dominants include smooth brome grass (*Bromus inermis* Leyss.), Canada

goldenrod (*Solidago canadensis* L.), tall oatgrass (*Arrhenatherum elatius* L.), blackberry (*Rubus allegheniensis* Porter), sassafras (*Sassafras albidum*), and Kentucky bluegrass (*Poa pratensis* L.) (https://lter.kbs.msu.edu/datatables/237). The late successional Deciduous forest stands are unmanaged and have never been plowed. Overstory dominants include red oak (*Quercus rubra* L.), pignut hickory (*Carya glabra* Mill.), white oak (*Q. alba* L.) and sugar maple (*Acer saccharum* Marsh.) (https://lter.kbs.msu.edu/datatables/238). Since 2007, a 2m × 2m subplot in each of the Deciduous forest stands was fertilized with 100 kg N ha^{-1} ammonium nitrate or urea applied as a 4-L solution annually.

Soil sampling

Soils were sampled seasonally for testing nitrification potentials, soil pH, and amoA gene abundance and once for nitrification kinetics. For testing nitrification potentials, soil pH, and amoA gene abundance, soils from all systems but the Grassland were sampled in summer (late June 2016), winter (early December 2016), and spring (early May 2017). Grassland soils were sampled when determining nitrification kinetics, for which samples were collected in 2017 from all systems in the period of late September to early December, after first having established no seasonal patterns for nitrification potentials. For all experiments, five random soils samples per plot (0-15 cm depth) were composited by plot and then passed through a 4 mm mesh sieve immediately on return to the lab. About 15 g of sieved soil were then transferred to a -80°C freezer for future DNA extraction and the remaining soil was stored at 4°C before analysis, which occurred within four days.

Nitrification potential and soil pH

To evaluate seasonal patterns of nitrification potentials, 5 g of fresh sieved soil were placed in 155 mL Wheaton bottles amended with 50 mL nanopure water containing 10 mM NH_4Cl. I used 1-octyne, a recently developed and tested chemical inhibitor of AOB ammonia monooxygenase (AMO), the enzyme involved in NH_3 oxidation, to distinguish the relative contribution of AOA and AOB (Taylor et al., 2013; Taylor et al., 2015). I used a gradient of octyne concentrations ranging from 0-10 μM aqueous concentration (C_{aq}) to test for optimal inhibition and I found 4 μM C_{aq} sufficient to inhibit AOB in all soils (see Chapter 2), which is in agreement with previous studies (Taylor et al., 2013).

Capped bottles with or without 4 μM C_{aq} octyne were immediately placed on a shaker table and shaken for 24 hours at a constant speed of 200 rpm at room temperature (25 °C). Samples for $NO_2^- + NO_3^-$ and NH_4^+ were taken at 2 and 24 hours and nitrification rates were calculated as $NO_2^- + NO_3^-$ accumulations over 22 hours. Slurry pH was buffered naturally as no pH change was detected during the incubation. Nitrification in the presence of octyne is attributed to AOA. Nitrification from AOB is calculated as the difference between total nitrification (without octyne) minus AOA nitrification. $NO_2^- + NO_3^-$ and NH_4^+ were measured by a Lachat QuikChem 8500 flow injection analyzer (Hach, Loveland, CO).

To test soil pH, I placed 15 g of sieved soil into extraction cups containing 30 mL nanopure water; cups were subsequently capped and shaken to form slurries. I then removed the caps and let slurries stand for at least 30 minutes before measuring soil pH. Soil moisture was determined by oven drying sieved soil at 60 □ for 48 hours until constant mass.

Nitrification kinetics

For soils from each ecosystem, I placed 5 g of fresh sieved soil into a 155 mL Wheaton bottle. Then I added $(NH_4)_2SO_4$ to make eight different NH_4^+ concentrations ranging from 0.01 to 15.0 mM (0.01, 0.05, 0.1, 0.5, 1, 5, 10 and 15 mM NH_4^+) with a final liquid volume of 50 mL. Bottles were capped and placed on a shaker table at a constant speed of 200 rpm at room temperature (25 °C) for 24 hours. Samples for initial $NO_2^- + NO_3^-$ and NH_4^+ were taken after 2 hours, at which time I then added either 2.8 mL octyne stock gas (see Taylor et al. (2013) for octyne stock gas preparation) to create a 4 μM C_{aq} concentration, or 2.8 mL of air without octyne. Another set of $NO_2^- + NO_3^-$ and NH_4^+ samples were taken at 24 hours. Nitrification rates (expressed as mg N kg^{-1} dry soil day^{-1}) were calculated as $NO_2^- + NO_3^-$ accumulation over 22 hours, with nitrification rate in the presence of octyne attributed to AOA. Nitrification kinetics were based on measured NH_4^+ concentrations, so included any NH_4^+ produced from net N mineralization during the incubation.

Nitrification kinetics were fit to Michaelis-Menten models using the equation (Eq. 1):

$$V = \frac{V_{max} S}{K_m + S} \quad (1)$$

where V is the nitrification rate, V_{max} is the maximum nitrification rate under conditions of unlimited substrate (NH_4^+), S is the NH_4^+ concentration, and K_m is the half-saturation constant, which represents the NH_4^+ concentration when the nitrification rate is ½ V_{max}. V_{max} reflects the maximum capacity of a soil to oxidize NH_4^+, and K_m reflects the NH_4^+ affinity of soil AMO. In addition, because nitrification can be inhibited at very high NH_4^+ concentrations (Suwa, 1994), I also fitted data with Haldane models when appropriate (Stark and Firestone, 1996; Koper et al., 2010) (Eq. 2):

$$V = \frac{V_{max}S}{K_m + S + S^2/K_i} \quad (2)$$

The Haldane model introduces a third parameter K_i that reflects the maximum NH_4^+ concentration at which nitrification rates are ½ V_{max}.

DNA extraction and quantification of amoA gene abundance

Soil DNA was extracted with the Qiagen DNeasy PowerSoil Kit (Qiagen, Germantown, MD) using 0.30 g field-moist soil. The abundance of soil AOB and AOA was quantified by targeting the amoA gene with primer amoA-1F/ amoA-2R (Rotthauwe et al., 1997) and Arch-amoAF/ Arch-amoAR (Francis et al., 2005), respectively. Quantitative PCR was performed on QuantStudio 7 Flex (Genomic Core, Michigan State University) with Power SYBR Green Master Mix (Applied Biosystems, Foster City, CA). The 25 µL reaction mixture including 12.5 µL Power SYBR Green Master Mix, 1.25 µL forward and reverse primers, 9 µL molecular grade water, and 1 µL soil DNA extract (10-fold diluted) was first mixed in 96-well plates and then 20 µL aliquots were transferred to a 384-well plate before analysis. The thermal cycling conditions for quantitative PCR were as follows: initial denaturation at 95 □ for 10 minutes, followed by 40 cycles of denaturation at 95 □ for 45 seconds, annealing at 60 □ (AOB) or 58 □ (AOA) for 1 minute and a final extension at 72 □ for 45 seconds. Amplification efficiencies ranged between 76-98%, with standard curve R^2 for both AOA and AOB > 0.99.

Statistical analysis

Analysis of variance (ANOVA) for seasonal nitrification potentials. Seasonal nitrification potentials were analyzed with PROC GLIMMIX of SAS 9.4 (SAS Institute, Cary, NC, USA). The statistical model included 8 ecosystem types × 3 seasons × 2 sources of nitrification

potentials and interactions among them were considered fixed factors. Field replicates nested within ecosystem types and the interaction between field replicates and seasons nested within ecosystem types were considered random factors. ANOVA was performed by considering ecosystem types as a whole plot factor and season and sources of nitrification as subplot and sub-subplot factors. Homogeneity of variance assumptions were checked by Levene's test and because AOA and AOB had significantly different residual variability ($P < 0.05$), heterogeneous variance for sources of nitrification potentials was included in the statistical model by a random _residual_/group = source statement. Normality of residuals was visually inspected, and no violations of assumptions were detected.

Comparison of kinetic parameters for AOA and AOB. For model comparisons, I first used the 'nls' function in R (version 3.5.0, R Core Team, 2018) to obtain AIC values for Michaelis-Menten and Haldane kinetic models for AOA and AOB in every ecosystem. Then an F-test was conducted using the 'anova' function in R to further determine model superiority. The more complicated Haldane kinetic model was selected (Table 3.1) only when smaller AIC values and a statistically better fit than for the Michaelis–Menten model were observed ($P < 0.1$).

Once appropriate models were chosen, I performed a Bayesian F-test (Kéry, 2010a) to investigate how management intensities affected kinetic parameters in the managed systems (Conventional, Biologically-based, Poplar, and Grassland), and a Bayesian T-test (Kéry, 2010b) to explore fertilization effects on kinetic parameters for unmanaged systems (Early successional and Deciduous forest) (Table 3.2). I modelled each of the kinetic parameters of different ecosystems following a normal distribution (Eq. 3-4):

$$Parameter_i \sim Normal\ (\mu_i, {\tau_i}^2) \quad (3)$$

$$\mu_i \sim Normal\ \left(mean_i, {SD_i}^2\right) \quad (4)$$

where $Parameter_i$ represents V_{max}, K_m or K_i, and μ_i and ${\tau_i}^2$ represent mean and variation of the parameter, respectively. $mean_i$ and SD_i were the estimated kinetic parameters and their standard errors obtained by 'nls' function, which were then specified as prior information when I conducted Markov Chain Monte Carlo (MCMC) simulations to sample posterior parameter distributions with JAGS (Plummer, 2003) and the 'jagsUI' package for R (Kellner, 2017). I assumed a vague prior for ${\tau_i}^2$. I ran three chains of 15000 iterations with 2000 burn-in iterations with a thinning rate of three, which yielded 13002 total samples for posterior distribution. All the posterior distributions for kinetic parameters were used for analysis.

amoA gene abundance, soil pH, contribution of AOA and AOB and correlational analysis. Soil pH and log-transformed amoA gene abundance were first averaged across three seasons, which were then correlated with V_{max} using the 'lm' function in R. Constancy of variance and residual normality were checked by plotting residuals against predicted values, with no apparent violation of assumptions observed. To compare the relative contributions of AOA and AOB to nitrification of every ecosystem, a T-test was conducted at each NH_4^+ addition using the 't.test' function in R.

I performed one-way ANOVA to investigate the effects of management intensities on averaged soil pH. In addition, to study how amoA gene abundance of AOA and AOB was affected by different ecosystems, a hierarchical model including 8 ecosystem types, 2 taxa, and their interactions were established. I considered field replicates nested within ecosystem types as a random factor. Thus, ecosystem type and taxa were the whole plot and subplot factors, respectively. Statistical analyses were conducted with PROC MIXED and PROC GLIMMIX of

SAS 9.4 and heterogeneous variance for ecosystem types was included by a Repeated/group = ecosystem statement based on Levene's test. Normality of residuals was visually inspected, and no violations of assumptions were detected. Pairwise comparisons among different ecosystems were conducted and we refer to $P < 0.05$ as significantly different throughout the paper.

Results

Seasonal nitrification potentials

Conventional and Biologically-based systems had the highest AOB-derived potential nitrification rates across three seasons, ranging between 4.16 to 5.66 mg N kg^{-1} day^{-1} (Figure 3.1). In comparison, Deciduous forest and its fertilized subplots were associated with the lowest seasonal AOB-derived nitrification potentials, 0.26-0.51 and 0.27-0.35 mg N kg^{-1} day^{-1}, respectively. In general, AOB-derived nitrification potentials in cropping systems were significantly higher than in perennial and successional systems ($P < 0.05$), although Conventional and Biologically-based systems did not significantly differ from each other for two out of three seasons. For AOA, the Biologically-based system had a seasonal nitrification potential of 1.40-2.04 mg N kg^{-1} day^{-1}, which was significantly higher than other systems ($P < 0.05$). No seasonal differences for AOA-derived nitrification potentials were detected among Poplar, Early successional, Deciduous forest nor their fertilized subplots except for the spring sampling period, for which the mean nitrification potential for the Fertilized Early successional system was significantly higher than Poplar and Fertilized Deciduous forest systems ($P < 0.05$). In addition, although not significant, long-term fertilization resulted in 1.4-1.9 times as high as nitrification potentials in Early successional but not in Deciduous forest systems compared with their non-fertilized main plots for both AOA and AOB.

No overall seasonal differences in nitrification potentials were detected ($P = 0.25$). Similarly, there were no detectable two-way interaction effects between season and ecosystem types ($P = 0.95$) or season and nitrifier taxa ($P = 0.48$), nor were there detectable three-way interaction effects among season, ecosystem types, and nitrifier taxa ($P = 0.50$). AOB overall had significantly higher nitrification potentials than AOA ($P < 0.05$); AOB nitrification potentials were consistently higher ($P < 0.05$) than AOA nitrification potentials for Conventional, Biologically-based, and Fertilized Early successional systems in every season but were similar ($P > 0.05$) in Deciduous forest and its fertilized subplots (all three seasons) and in Poplar and Early successional systems (two out of three seasons) (Figure 3.1).

Kinetics of AOA and AOB

I conducted an AIC-based model comparison to choose kinetics parameters for AOA and AOB (Table 3.1). For AOA, Michaelis-Menten kinetic models provided significantly better fits ($P < 0.1$) in all but the Deciduous forest system, whereas for AOB, Haldane kinetic models fit best ($P < 0.1$) except for Conventional and Grassland systems. Nitrification of AOB in the Early successional system showed slight inhibition at high NH_4^+ concentrations (Figure 3.2B), but overall Michaelis-Menten kinetic model fit the data better (Table 3.1).

Among managed ecosystems, Conventional and Biologically-based soils were associated with the highest V_{max} for AOB, reaching 4.80 ± 0.29 and 5.29 ± 0.36 mg N kg^{-1} day^{-1}, respectively (Figure 3.2A). In comparison, significantly lower V_{max} for AOB were found in Poplar and Grassland systems ($P < 0.05$), 1.90 ± 0.27 and 0.38 ± 0.05 mg N kg^{-1} day^{-1}, respectively. In addition, the Conventional system had the highest AOB-derived K_m of 22.8 ± 9.6 μM NH_4^+

(Table 3.2), which was significantly higher than that in Grassland ($P < 0.05$) but not in Biologically-based or Poplar systems. For AOA, V_{max} was significantly lower than AOB ($P < 0.05$) within each ecosystem. The highest V_{max} for AOA was 2.82 ± 0.11 mg N kg^{-1} day^{-1} in Biologically-based soils, which was significantly higher than in all other systems (Figure 3.2A, $P < 0.05$). Additionally, V_{max} for AOA in the Conventional system was significantly higher than that in either Poplar or Grassland systems ($P < 0.05$). No significant differences for K_m of AOA were detected among managed ecosystems (Table 3.2). Moreover, both Biologically-based and Poplar systems exhibited nitrification inhibition for AOB at high NH_4^+ concentrations (> 1 mM), with K_i of 52.2 ± 32.1 and 14.8 ± 9.5 mM NH_4^+, respectively.

Long-term N fertilization resulted in a significant increase ($P < 0.05$) in V_{max} for both AOA (2.11 ± 0.21 vs. 0.89 ± 0.08 mg N kg^{-1} day^{-1}) and AOB (3.15 ± 0.45 vs. 1.30 ± 0.14 mg N kg^{-1} day^{-1}) in the Early successional system (Figure 3.2B); V_{max} in the fertilized Early successional subplots was about 2.4 times higher than that in the main plots. Similarly, although not significant, K_m for both AOA and AOB were enhanced by long-term fertilization. For the Deciduous forest system, no fertilization-induced increases for V_{max} and K_m for either AOA or AOB were detected. However, the AOB-derived K_i in the fertilized subplots was significantly lower than that in the unfertilized main plots ($P < 0.05$). In addition, regardless of fertilization, V_{max} of AOB was significantly higher than AOA in the Early successional ($P < 0.05$) but not in the Deciduous forest system; K_m or K_i were not significantly different between AOA and AOB in either the Early successional or the Deciduous forest system.

Soil pH, amoA gene abundance, and relationship between maximum nitrification rate (V_{max}) and Soil pH

Conventional and Biologically-based systems had the highest soil pH, 6.74 ± 0.04 and 6.72 ± 0.08, respectively, significantly higher than other systems ($P < 0.05$, Figure 3.3A). Soil pH was lowest in the Deciduous forest system and its fertilized subplots, 5.61 ± 0.16 and 4.95 ± 0.03, respectively. In addition, long-term fertilization significantly reduced soil pH in both Early successional and Deciduous forest systems ($P < 0.05$).

The abundance of amoA genes was significantly higher for AOA than for AOB in each ecosystem ($P < 0.05$, Figure 3.3B). In addition, for both AOA and AOB, Poplar and Fertilized Deciduous forest systems were associated with the lowest and highest amoA gene abundance, respectively. Long-term N fertilization led to significantly more abundant amoA genes in Deciduous forest ($P < 0.05$) but not in Early successional systems for both AOA and AOB. Moreover, soil pH was significantly correlated with both V_{max} for AOA ($P < 0.05$) and AOB ($P < 0.01$) and explained 13.1% and 28.5% of total variance, respectively (Figure 3.3C). In comparison, no significant relationship was found between amoA gene abundance and V_{max} for AOA or AOB (Figure 3.3D).

Relative contributions of AOA and AOB to nitrification

Among managed ecosystems, the relative contributions of AOB to nitrification were significantly higher than AOA ($P < 0.05$) at most of the NH_4^+ concentrations tested (Figure 3.4A). The contribution of AOB increased from 66.2% at 0.05 mM NH_4^+ to 78.2% at 15 mM NH_4^+ in the Conventional system and from 55.4% at 0.05 mM NH_4^+ to 65.9% at 5 mM NH_4^+ in

the Biologically-based system. However, due to inhibition, AOB's contribution decreased to about 60% at 10 and 15 mM NH_4^+ in the Biologically-based system. Similarly, the contribution of AOB to nitrification generally increased in the Poplar system along the NH_4^+ gradient from 0.05 to 5 mM, reaching 69.7%, but reduced to 56.7% and 54.0% at 10 and 15 mM NH_4^+, respectively, which were not significantly different from the contributions of AOA. Different from the cropping and Poplar systems, in the Grassland system, there was generally a lack of response of AOA and AOB along the NH_4^+ gradient although the contribution of AOA to nitrification was significantly higher than AOB at each NH_4^+ concentration ($P < 0.05$).

For the Early successional system, the contribution of AOB to nitrification increased along the NH_4^+ gradient from 45.2% at 0.01 mM NH_4^+ to 61.4% at 1 mM NH_4^+, and then decreased to 56.2% at 10 and 15 mM NH_4^+ (Figure 3.4B). AOB's contribution to nitrification was also consistently higher than AOA's across the 0.05-15 mM NH_4^+ gradient, although significant differences were only detected at 5 and 10 mM NH_4^+. Long-term fertilization resulted in a similar initial increase in the contribution of AOB, from 42.9% at 0.01 mM NH_4^+ to 59.0% at 1 mM NH_4^+, but there was a greater reduction to about 50% at 5-15 mM NH_4^+. For the Deciduous forest system, the relative contribution of AOA and AOB to nitrification was similar and relatively stable across the NH_4^+ gradient. Long-term fertilization resulted in a reduction of AOB's contribution from 49.0% at 0.05 mM NH_4^+ to 21.9% at 15 mM NH_4^+. AOA's contribution was consistently higher than AOB along the NH_4^+ gradient and significant differences ($P < 0.05$) were detected at 0.01, 5, and 10 mM NH_4^+.

Discussion

To test the hypothesis of niche differentiation between AOA and AOB as a result of NH_4^+ inhibition, I first surveyed the seasonal contribution of AOA and AOB to nitrification potentials in ecosystems varying in management intensity and fertilization history. After confirming soil nitrifier communities were stable across seasons, I tested and modeled nitrification kinetics of AOA and AOB from all ecosystems. Observations reveal that nitrification of both AOA and AOB responded to long-term agronomic management and N fertilization, and the inhibition of AOB but not AOA by high NH_4^+ inputs suggest NH_4^+ inhibition could be another mechanism leading to niche differentiation between AOA and AOB.

The responses of nitrification potentials and V_{max} of AOA and AOB to management intensities and long-term N fertilization

Consistent with the first hypothesis, management intensities significantly affect nitrification by both AOA and AOB ($P < 0.05$). For AOB, nitrification potentials in Conventional and Biologically-based systems were significantly higher than in Poplar, Early successional, or Deciduous forest systems for all three seasons (Figure 3.1, $P < 0.05$). Similarly, V_{max} of AOB in annual cropping systems was significantly higher than in Poplar and Grassland systems (Figure 3.2A, Table 3.2, $P < 0.05$). These results may reflect the differential N input received by various systems. Compared with perennial or successional systems that have never or rarely been fertilized, row crop soils have been receiving chemical N fertilizer (Conventional) or relying on cover crop N (Biologically-based) annually since 1988. Thus, my results are in agreement with previous studies reporting elevated AOB-derived nitrification in fertilized agricultural soils

(Taylor et al., 2010; Zeglin et al., 2011; Taylor et al., 2012; Ouyang et al., 2016, Chapter 2 of this dissertation).

For AOA, I found a significantly higher nitrification potential in the Biologically-based agricultural soil ($P < 0.05$) compared with all other systems, but no significant differences were detected among Conventional, Poplar, Early successional and Deciduous forest soils for each season (Figure 3.1). Similarly, AOA V_{max} in the Biologically-based system was about 2 - 3.5 times as high as in other managed systems ($P < 0.05$, Figure 3.2A, Table 3.2). These results seem to suggest that cover crops, rather than chemical fertilizers, facilitated AOA nitrification. Previous studies have shown nitrification activities of AOA were stimulated when NH_4^+ is mainly derived from organic matter (Gubry-Rangin et al., 2010; Stopnišek et al., 2010; Levičnik-Höfferle et al., 2012; Hink et al., 2017; Hink et al., 2018). Thus, it seems likely that the decomposition of cover crop-derived organic matter promoted AOA nitrification in the Biologically-based system.

I also hypothesized that both AOA and AOB respond to long-term N fertilization. Support for this hypothesis comes from the evidence that in the Early successional system, long-term N fertilization resulted in a 40-90% increase in nitrification potentials (Figure 3.1), and as well a significantly higher V_{max} in fertilized subplots than in the unfertilized main plots (Figure 3.2B, Table 3.2) ($P < 0.05$). The positive responses in the Early successional system were expected because fertilizer as ammonium nitrate (120 kg N ha^{-1}) has been applied annually for over 30 years. It is also worth noting that 30 years of continuous fertilizer application in the Early successional system resulted in an even higher V_{max} for AOA (2.11 ± 0.21 mg N kg^{-1} day^{-1}) than

in the Conventional system (1.44 ± 0.092 mg N kg^{-1} day^{-1}) where chemical fertilizer was only applied in corn and wheat phase. Thus, I present evidence that AOA nitrification activities could be significantly stimulated by long-term inorganic fertilizer application, which contrasts with most of the studies reporting either no response (Ouyang et al., 2016; Ouyang et al., 2017) or negative responses (Giguere et al., 2015) of AOA nitrification to N fertilization. The discrepancy between this study and previous ones could be explained by the slower growth of AOA than AOB under high NH_4^+ conditions (Prosser and Nicol, 2012), such that a consistent and long-term fertilization regime (e.g, 30 yrs.) is necessary to observe the positive responses of AOA nitrification.

Contrary to the first hypothesis, no fertilization effects were observed in the Deciduous forest system (Figure 3.1 and Figure 3.2B). This lack of response might be explained by the low soil pH. Previous research has demonstrated that NH_3, rather than NH_4^+, was the most likely substrate for ammonia monooxygenase (AMO) (Suzuki et al., 1974; Herbold et al., 2017), and NH_3 concentrations are significantly reduced in acidic environments because NH_3 is mainly in its protonated form ($NH_4^+ \rightleftarrows NH_3$, $pK_a = 9.25$). Fertilized Deciduous forest soils had a pH of 4.95 ± 0.03 (Figure 3.3A), which was significantly lower than its unfertilized counterpart (pH 5.61 ± 0.16, $P < 0.05$) and, as well the Early successional system and its fertilized subplots (pH 5.87 - 6.42, $P < 0.05$). Despite the low nitrification potentials in Fertilized Deciduous forest soils, these soils had the highest nitrifier abundance: the amoA gene for AOB was significantly more abundant here than in all other systems ($P < 0.05$, Figure 3.3B). Overall, results suggest that low pH inhibited nitrification activities in fertilized forest soils despite the fact that AOB population size was stimulated by long-term N fertilization.

Results from the seasonal nitrification potential survey also show soil nitrifier communities have been established and stabilized after 30 years of consistent management because I found: 1) no significant seasonal differences in nitrification potentials and 2) no significant two-way or three-way interactions effects among season, ecosystem types, and nitrifier taxa (Figure 3.1). This study therefore provides evidence that for both AOA and AOB, soil nitrification potentials can be very stable across years despite temporal variations in soil temperature, moisture, oxygen, and inorganic nitrogen contents (Boone et al., 1999). O'Sullivan et al. (2013) also demonstrated a lack of correlation between total nitrification potential and sampling season in southern Australian agricultural soils but it is unknown if their conclusions apply to AOA and AOB separately.

The influences of management intensities on K_m of AOA and AOB

The NH_4^+ affinities (K_m) of AOA were consistently higher than AOB in all ecosystems but Grassland (Table 3.2), which agrees with most of the enrichment and pure culture studies (Lehtovirta-Morley, 2018). The different K_m's between AOA and AOB have been suggested to be a key explanation leading to niche differentiation (Martens-Habbena et al., 2009; Verhamme et al., 2011). Compared with AOB, the lower K_m of AOA seem to reflect their smaller genome and cell volume, lower specific cell activities, and lower maintenance energy, which may have provided evolutionary advantages for AOA to thrive in nutrient-depleted environments (Könneke et al., 2005; Tourna et al., 2011; Urakawa et al., 2011; Hatzenpichler, 2012). While I do not have direct evidence, I suspect the bacterial nitrification inhibition by root exudates of *Bromus* spp. (O'Sullivan et al., 2017), a dominant species in the Grassland system, have resulted in the very low K_m of AOB observed in the Grassland soils.

Niche differentiation between AOA and AOB induced by high NH_4^+ inputs

For five out of eight ecosystems (including fertilized subplots), Haldane models provided significantly better fits than Michaelis-Menten models for AOB-derived nitrification kinetics (Figure 3.2, $P < 0.05$). In contrast, AOA-derived nitrification exhibited Michaelis-Menten kinetics in all ecosystems but one (Deciduous forest). This suggests that AOA are more resistant to high N inputs than are AOB, which challenge the conventional view that AOA are less tolerant to NH_3 (Könneke et al., 2005; Martens-Habbena et al., 2009). Lack of support for inhibition of AOA by high NH_4^+ is corroborated by Lehtovirta-Morley et al. (2016a), who discovered soil AOA isolate, '*Ca. Nitrosocosmicus franklandus*', capable of tolerating more than 100 mM of NH_4^+, which is even higher than that for most AOB strains who are typically inhibited at 7-50 mM NH_4^+ concentrations (Prosser and Nicol, 2012). AOA's tolerance for high NH_4^+ concentrations may be due to AOA, especially acidophilic AOA, possess genes encoding cation (NH_4^+) transporters. In contrast, AOB appear to lack cation transporters and thus can transport only NH_3 (Morley et al., 2016b). Together, consistent with the second hypothesis, these results suggest that in addition to substrate affinity, soil pH, and mixotrophy as reviewed by Prosser and Nicol (2012), NH_4^+ inhibition of AOB in contrast to AOA may be another important factor leading to niche differentiation between AOA and AOB in terrestrial environments.

Soil pH as a predictor of V_{max}

Existing studies typically examined correlations between potential nitrification rates and amoA copy numbers for AOA and AOB (He et al., 2007; Shen et al., 2008; Bernhard et al., 2010; Zeglin et al., 2011; Lu et al., 2015; Ouyang et al., 2016). However, no theoretical basis has been proposed to support a similar response of nitrifier abundance and activities to environmental

disturbance. In addition, amoA gene abundance may not be a reliable surrogate for nitrification activities (Prosser and Nicol, 2012). It is well known that the presence of functional genes does not necessarily indicate active microbial communities (Levy-Booth et al., 2014; Bier et al., 2015). Not surprisingly, no significant correlations between V_{max} and amoA copy numbers across a management gradient were found in this study for either AOA or AOB (Figure 3.3D).

Consistent with the third hypothesis, I show soil pH as a strong environmental factor for explaining maximum nitrification rate (V_{max}) for both AOA and AOB (Figure 3.3C). Soil pH has been shown to be a strong selection force for shaping the community compositions of soil nitrifiers (Nicol et al., 2008; Hu et al., 2013; Baolan et al., 2014; Stempfhuber et al., 2015) although not necessarily activities (Robertson and Vitousek, 1981; Robertson 1982). Changes of community compositions could nevertheless affect maximum nitrification rates because V_{max} measures the average nitrification of active nitrifiers under optimal conditions (Prosser and Nicol, 2012). Results also show that nitrification activities of AOB were positively influenced by soil pH twice as much as of AOA (28.5% vs. 13.1%, Figure 3.3C). This difference seems to coincide with the different pH ranges reported for AOB and AOA, both in pure culture and in terrestrial environments. Compared with AOB cultures that typically cannot grow below pH of 6.5 (Allison and Prosser, 1993; Jiang and Bakken, 1999), AOA appear to be widely distributed from very acidic (pH = 2.5) to alkaline (pH = 8-9) conditions (Erguder et al., 2009). Additionally, in acidic soils where AOA have been found to actively dominate nitrification (Gubry-Rangin et al., 2010; Jiang et al., 2015; Li et al., 2019), AOB were more often in low abundance or even absent (Stopnišek et al., 2010; Yao et al., 2011).

Implications for modelling and soil nitrification management

Overall, results show that while AOB nitrification can be inhibited by high NH_4^+ inputs and exhibits Haldane kinetics, AOA nitrification better fits Michaelis-Menten kinetics in general. Thus, results reveal the need for careful model selection in future studies as most of kinetics and modelling studies consider only Michaelis-Menten models (Malhi and McGill, 1982; Müller et al., 2007; Palmer et al., 2012; Nowka et al., 2015). In addition, since AOB generally have higher nitrification potentials and V_{max} than AOA, the inhibition of AOB by high NH_4^+ could be a potential strategy reducing N_2O emissions. For example, Deppe et al. (2017) used a controlled uptake long-term ammonium nutrition (CULTAN) fertilization strategy to substantially reduce of soil nitrification rates and concurrent N_2O emissions by creating fertilizer microsites with extremely high NH_4^+ concentrations (> 2000 mg N kg^{-1} soil, equivalent to > 15 mM NH_4^+), although it is unclear if AOB rather than AOA were inhibited. Nevertheless, the overall N use efficiency and N budget implications of CULTAN are largely unknown. Although CULTAN has shown benefits in grain yields and crop N uptake compared with surface N fertilizer application, eventually nitrifier inhibition will be relieved by dilution of NH_4^+ from plant uptake, precipitation, and diffusion away from microsites, and both N_2O and nitrate loss will result (Deppe et al., 2016). Future studies might explore the inhibition of AOB vs. AOA in soil NH_4^+ 'hotspots' created by certain fertilizer placement techniques such as injection of anhydrous NH_3 or fertilizer banding, and resulting N_2O suppression.

I also demonstrate that the relative importance of AOB vs. AOA in contributing to nitrification is ecosystem dependent (Figure 3.4). Compared with annual row crop systems where AOB dominated soil nitrification, AOA's contribution was also important in Early successional,

Grassland and Deciduous forest systems. Thus, management practices to control nitrification should target either or both of AOB and AOA.

Conclusions

1. AOB nitrification exhibited Haldane kinetics, especially in perennial and successional ecosystems.
2. AOA nitrification exhibited Michaelis-Menten kinetics in most ecosystems.
3. NH_4^+ inhibition of AOB but not AOA, as indicated by kinetics models, can be another factor contributing to niche differentiation between AOA and AOB.
4. Both AOA and AOB nitrification were significantly promoted by long-term N inputs in the Early successional but not in the Deciduous forest systems.
5. Soil pH is a strong predictor of nitrification kinetic parameters for both AOA and AOB.

APPENDIX

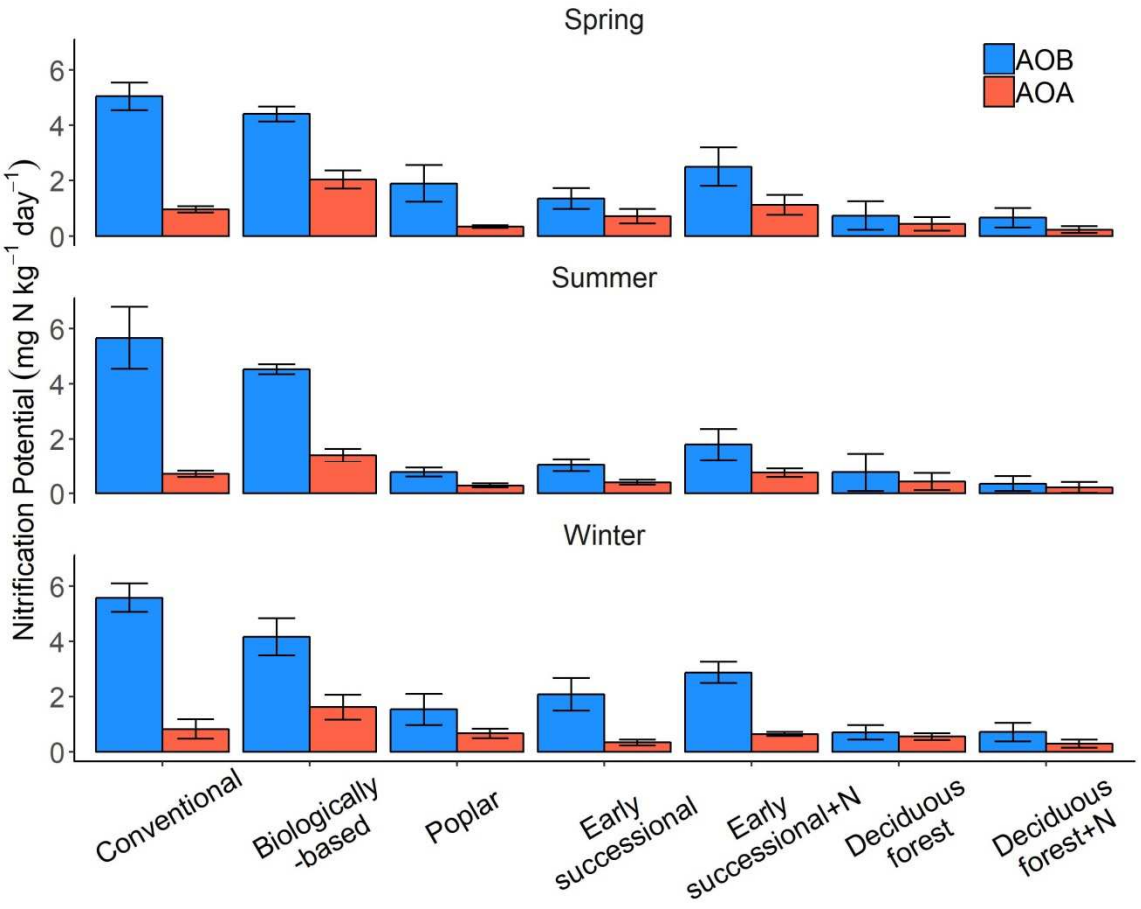

Figure 3.1 AOA (orange) and AOB (blue)-derived seasonal nitrification potentials in systems varying in management intensities; bars represent standard error (n = 4 field replicates except for deciduous forest n = 2-3). "+N" indicates subplots receiving N fertilizer. No significant differences among seasons were detected (P = 0.25).

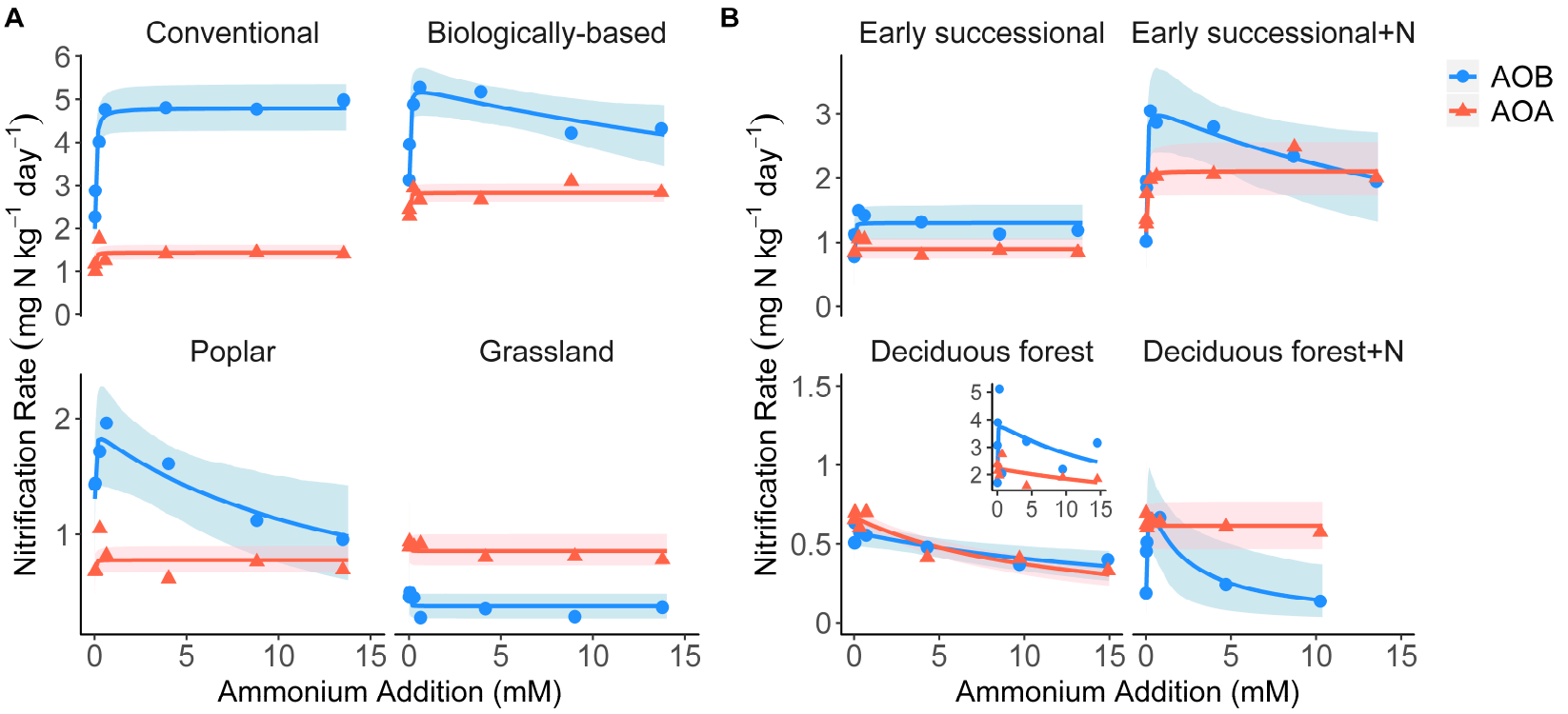

Figure 3.2 Nitrification kinetics of (A) systems varying in management intensities and (B) unmanaged systems with (+N) or without long-term N fertilization; Michaelis-Menten or Haldane models were fit to AOB-derived (blue line) and AOA-derived (orange line) nitrification rates. Blue circles and orange triangles are the mean nitrification rates at each ammonium addition. Note y-axis scale differs by systems. Shading represents 95% bootstrap confidence intervals based on n = 3-4 field replicates except for Deciduous forest and its fertilized subplots where n = 2-3. Inset shows one removed replicate from Deciduous forest adjacent to a dairy farm. Ammonium addition ranged between 0.05-15 mM for Poplar and annual cropping systems because NO_3^- + NO_2^- accumulation at 0.01 mM cannot be reliably estimated; ammonium addition ranged between 0.01-10 mM for Fertilized Deciduous forest because NO_3^- + NO_2^- accumulation at 15 mM was too low to be detected. For all other systems, ammonium additions ranged between 0.01-15 mM.

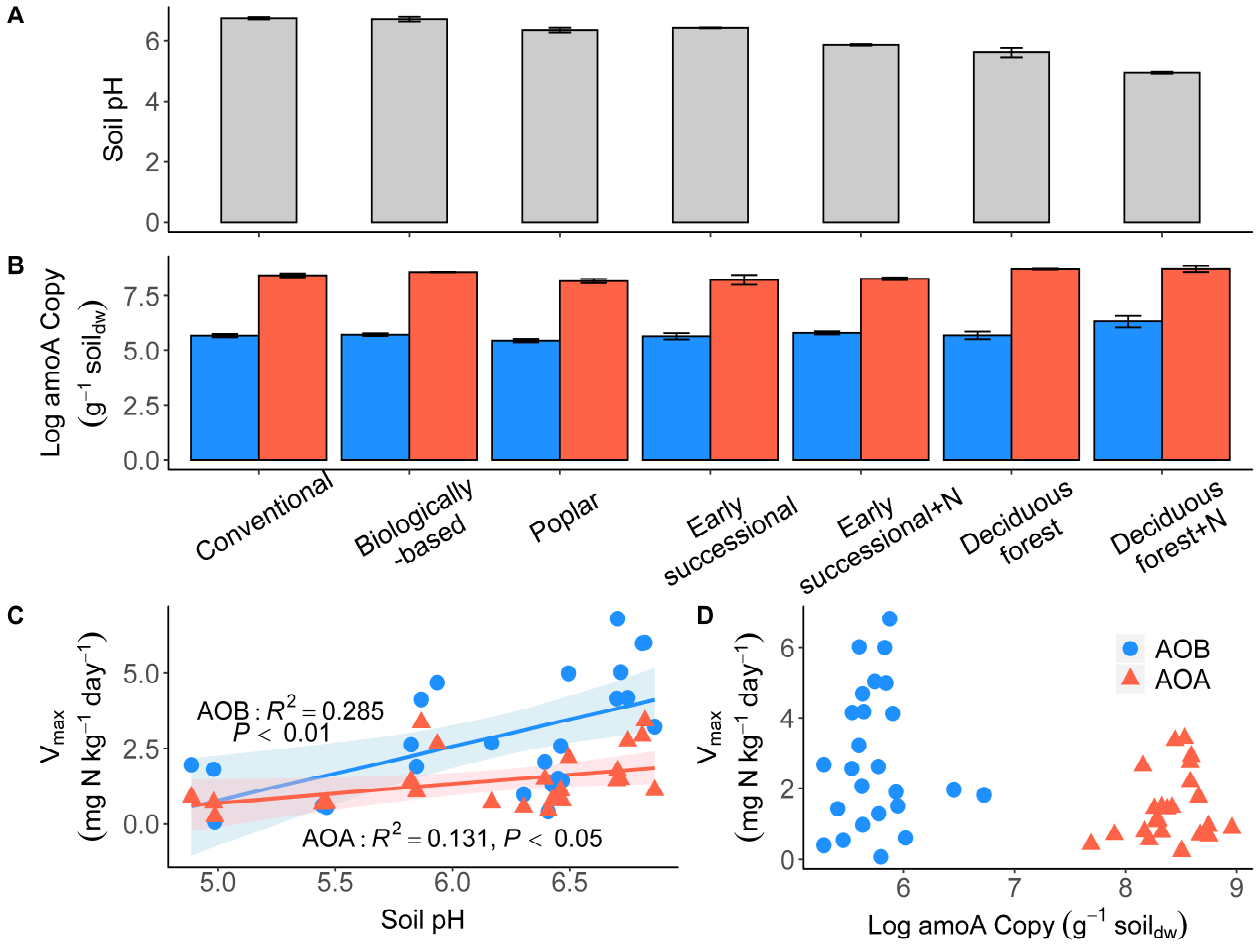

Figure 3.3 Soil pH (A) and log-transformed amoA copy numbers (B) in systems along a management intensity gradient; "+N" indicates subplots receiving long-term N fertilizer. Bars represent standard errors based on n = 4 field replicates except for Deciduous forest and its fertilized subplots where n = 3. Correlations between maximum nitrification rate (V_{max}) with soil pH (C) or log-transformed amoA gene abundance (D) are shown for all systems. A Deciduous forest site adjacent to a dairy farm is not included. Blue dots/bars represent AOB and orange triangles/bars represent AOA. Insignificant *P* values are not shown for Figure 3.3 (D).

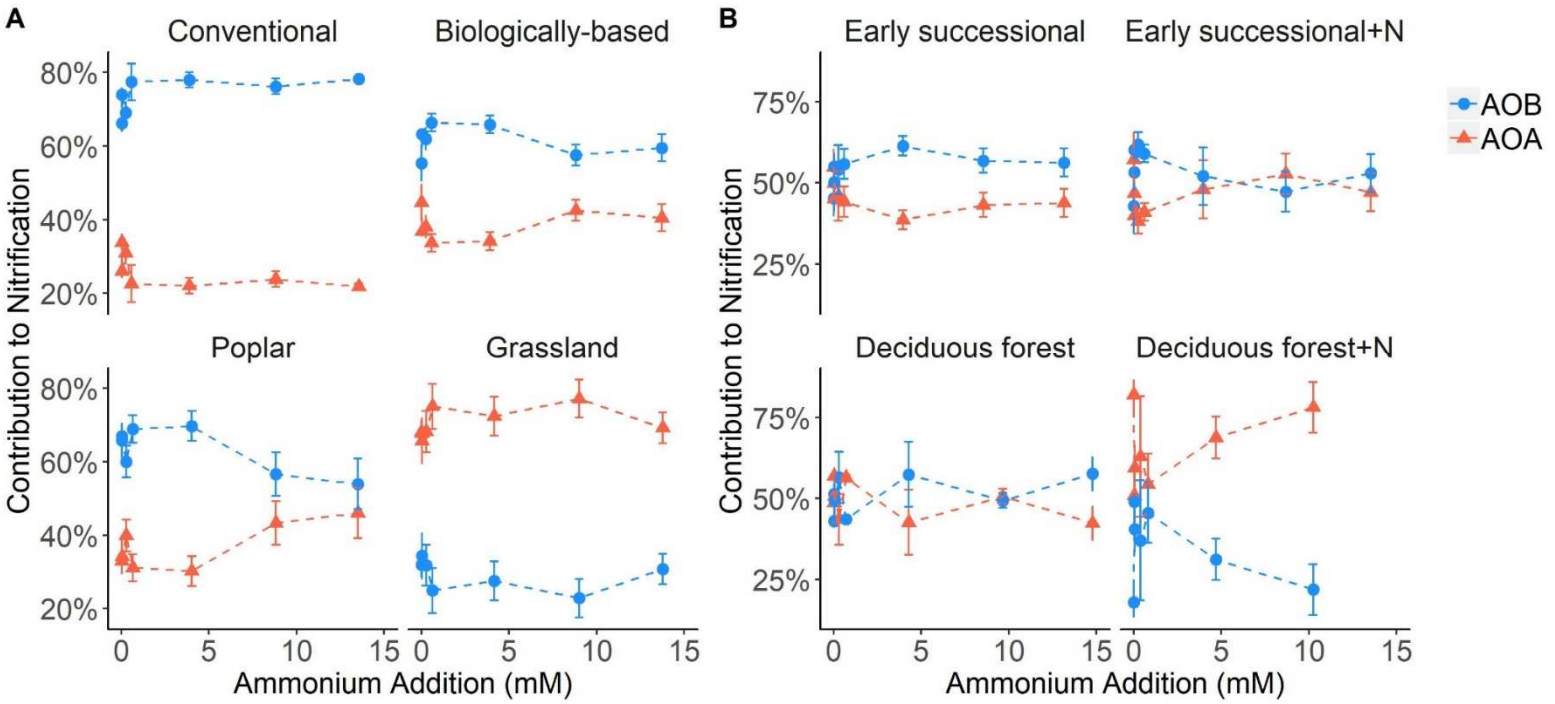

Figure 3.4 Relative contributions of AOA (orange triangles) and AOB (blue dots) to soil nitrification in (A) systems varying in management intensities and (B) unmanaged systems with ("+N") or without long-term N fertilization; bars represent standard errors based on n = 3-4 field replicates except for Deciduous forest and its fertilized subplots where n = 2-3. A Deciduous forest site adjacent to a dairy farm is not included. See Figure 3.2 legend for further details.

Table 3.1 Comparisons between Michaelis-Menten and Haldane kinetics models for AOB-derived or AOA-derived nitrification rates among different ecosystems; ecosystems followed by "+N" indicate subplots receiving long-term N fertilizer. AIC represents Akaike information criterion.

Ecosystem	Taxon	Model AIC		Model comparison		Model selection
		Michaelis-Menten	Haldane	F-value	P-value	
Conventional	AOB	90.8*	92.7	0.10	0.76	Michaelis-Menten
	AOA	31.1*	32.9	0.10	0.76	Michaelis-Menten
Biologically -based	AOB	83.6	82.0*	3.45	0.075**	Haldane
	AOA	42.4*	44.0	0.42	0.52	Michaelis-Menten
Poplar	AOB	64.9	61.6*	5.23	0.031**	Haldane
	AOA	5.20	5.17*	1.88	0.18	Michaelis-Menten
Early successional	AOB	66.3*	67.5	0.78	0.38	Michaelis-Menten
	AOA	37.0*	38.7	0.29	0.60	Michaelis-Menten
Early successional+N	AOB	93.8	92.7*	2.94	0.098**	Haldane
	AOA	90.3*	92.2	0.072	0.79	Michaelis-Menten
Grassland	AOB	8.79*	10.2	0.53	0.47	Michaelis-Menten
	AOA	23.4*	24.5	0.77	0.39	Michaelis-Menten
Deciduous forest	AOB	-18.2	-23.2*	7.52	0.019**	Haldane
	AOA	-12.5	-31.3*	34.8	<0.01**	Haldane
Deciduous forest+N	AOB	21.0	16.0*	7.23	0.016**	Haldane
	AOA	14.3*	16.2	0.088	0.77	Michaelis-Menten

*Model with lower AIC.
**Haldane model provides statistically better fit than Michaelis-Menten model ($P < 0.1$).

Table 3.2 Kinetics parameters of managed systems and unmanaged systems for AOA and AOB nitrification; "+N" indicates subplots receiving long-term N fertilizer. Parameters are estimated with either Michaelis-Menten or Haldane kinetics models based on the model selection results in Table 3.1. For each ecosystem, a "–" indicates Michaelis-Menten model is applied and thus K_i does not exist. Numbers within the parentheses represent standard errors. Lower case letters indicate significantly different kinetics parameters among ecosystems ($P < 0.05$).

			AOA			AOB			AOA vs. AOB		
			V_{max}	K_m (μM)	K_i (mM)	V_{max}	K_m (μM)	K_i (mM)	V_{max}	K_m	K_i
Managed	Conventional		1.44 (0.09)^{b}	5.67 (4.34)^{a}	–	4.80 (0.29)^{c}	22.8 (9.6)^{b}	–	√	NS	–
	Biologically-based		2.82 (0.11)^{c}	2.84 (1.64)^{a}	–	5.29 (0.36)^{c}	9.18 (3.46)^{b}	52.2 (32.1)^{a}	√	NS	–
	Poplar		0.78 (0.06)^{a}	2.26 (3.51)^{a}	–	1.90 (0.27)^{b}	6.91 (6.79)^{ab}	14.8 (9.5)^{a}	√	NS	–
	Grassland		0.86 (0.07)^{a}	-0.58 (2.49)^{a}	–	0.38 (0.05)^{a}	-2.42 (3.41)^{a}	–	√	NS	–
Unmanaged	Early successional	0N	0.89 (0.08)	0.02 (1.86)	–	1.30 (0.14)	4.32 (4.26)	–	√	NS	–
		+N	2.11 (0.21)*	9.14 (6.82)	–	3.15 (0.45)*	21.5 (12.4)	23.6 (18.2)	√	NS	–
	Deciduous forest	0N	0.67 (0.04)	0.14 (1.46)	12.5 (3.3)	0.57 (0.05)	1.18 (2.42)	24.5 (12.0)	NS	NS	NS
		+N	0.61 (0.08)	-0.79 (2.45)	–	0.87 (0.35)	33.6 (37.3)	2.01 (2.38)*	NS	NS	–

√: kinetics parameters of AOA are significantly different from AOB within the same ecosystem ($P < 0.05$).
NS: kinetics parameters of AOA are not significantly different from AOB within the same ecosystem.
*kinetics parameters of +N treatments are significantly different from 0N ($P < 0.05$).

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Chapter 4: *In situ* N_2O responses of ammonia oxidizing archaea (AOA) and bacteria (AOB) to N fertilization in annual and perennial agricultural systems

Abstract

Soil nitrification, performed mainly by ammonia oxidizing bacteria (AOB) and ammonia oxidizing archaea (AOA), is a significant source of N_2O, a potent greenhouse gas. Understanding how AOA and AOB respond to nitrogen (N) fertilizer in agricultural systems is critical for developing future greenhouse gas mitigation strategies. I conducted a field study to investigate *in situ* responses of AOA and AOB to N fertilizer in corn and switchgrass cropping systems. For both AOA and AOB, nitrification potentials and nitrification-derived N_2O emissions were significantly stimulated by N fertilizer in the corn but not in the switchgrass system. Fertilization effects were mainly observed within 13 days of fertilizer application and disappeared quickly. Plants appeared to directly compete with soil nitrifiers for NH_4^+ during the peak growing season, which diminished fertilizer-induced N_2O emissions from nitrification. Moreover, AOB had a higher yield of N_2O (N_2O-N per NO_2^- + NO_3^--N produced) than AOA and contributed more to nitrification in both systems. Findings highlight the importance of including plants in assessments of nitrifier contributions to N_2O and reveal potential strategies for mitigating N_2O from nitrification.

Introduction

Soil nitrification, the microbial process that sequentially oxidizes ammonia (NH_3) into hydroxylamine (NH_2OH), nitric oxide (NO), nitrite (NO_2^-) and further into nitrate (NO_3^-) (Caranto and Lancaster, 2017), is an important source of N_2O emissions. As a long-lived greenhouse gas with a global warming potential ~300 times higher than CO_2, N_2O stays in the

atmosphere for about 114 years. There is some evidence that nitrification can dominate N_2O emissions, especially under aerobic (Khalil et al., 2004) or unsaturated soil moisture conditions (Mathieu et al., 2006). Since agriculture is the largest source of anthropogenic N_2O (Ciais et al., 2013), understanding how agronomic practices affect soil nitrification-derived N_2O emissions can inform future greenhouse gas mitigation development.

Ammonia oxidizing archaea (AOA) and ammonia oxidizing bacteria (AOB) are the two main taxa involved in NH_3 oxidation (Könneke et al., 2005), the rate-limiting step of soil nitrification and catalyzed by ammonia monooxygenase (AMO). Both AOA and AOB can produce N_2O via nitrification but the mechanisms are different. AOB-derived N_2O is mainly formed indirectly from NH_2OH (Kozlowski et al., 2014) either by 1) aerobic NH_2OH oxidation, whereby NH_2OH is first oxidized to NO by hydroxylamine oxidoreductase (HAO) and NO is then further reduced to N_2O by nitric oxide reductase (NorB); or 2) nitrifier denitrification, in which NO_2^- formed from NH_2OH is reduced to NO and N_2O catalyzed by nitrite reductase (NirK) and NorB, respectively, under oxygen limited conditions (Wrage et al., 2001). In addition, N_2O can be generated abiotically by chemical reactions among nitrification intermediates such as NO_2^-, NO and NH_2OH (Zhu-Barker et al., 2015; Heil et al., 2016). The mechanisms by which AOA produce N_2O are less clear. Current studies mostly suggest AOA cannot generate N_2O through NH_3 oxidation or the nitrifier denitrification pathway because they lack the genes encoding HAO and NorB (Stieglmeier et al., 2014; Hink et al., 2017a). Instead, evidence seems to support the AOA N_2O formation through abiotic reactions of nitrification intermediates (Kozlowski et al., 2016; Stein, 2019).

Current research on N_2O emissions from AOA and AOB have mostly been based on *in vitro* microcosm experiments. Few, if any, studies have directly examined *in situ* responses of AOA and AOB to N fertilizer. So far, results seem to suggest NH_4^+-based fertilizers promote AOB-derived N_2O emissions instead of AOA in agricultural soils (Wang et al., 2016; Meinhardt et al., 2018). Similarly, N_2O in fertilized soils have been found to be positively correlated with the abundance of the amoA gene of AOB rather than AOA (Krauss et al., 2016; Pannu et al., 2019). In contrast, AOA seem to dominate nitrification-derived N_2O emissions in acidic forest soils (Tzanakakis et al., 2019) and in unfertilized agricultural soils or soils treated with slow-release fertilizer (Hink et al., 2017b; Hink et al., 2018). However, due to the exclusion of plant-microbe interactions, huge differences in spatial and temporal scales, and the predetermined nature of microcosm experiments (Kampichler et al., 2001), it is not known the extent to which results from lab microcosms can be extended to field conditions.

I conducted a field experiment to explore how N fertilizer might differentially affect N_2O emissions of AOA and AOB in soils from corn (*Zea mays* L.) and switchgrass (*Panicum virgatum* L.) cropping systems located in the upper U.S. Midwest. Switchgrass is a perennial grass native to North America that is a promising feedstock for producing cellulosic biofuels (Mitchell et al., 2012). My objectives are to test the hypotheses that 1) both AOA and AOB respond positively to added N fertilizer *in situ*; 2) plants can mediate N_2O emissions from nitrification by competing with soil nitrifiers for NH_4^+; and 3) AOB have a higher N_2O yield (N_2O-N per NO_2^- + NO_3^--N produced) because AOB harbor more versatile mechanisms for generating N_2O (both biotic and abiotic) than do AOA (abiotic) in nitrification.

Methods

Site description

I conducted this study in the Biofuel Cropping System Experiment (BCSE) of the Great Lakes Bioenergy Research Center (GLBRC), located at the Kellogg Biological Station (KBS) Long-term Ecological Research site in southwest Michigan (42° 24'N, 85° 23'W). I chose continuous corn and switchgrass systems among others in the BCSE (https://lter.kbs.msu.edu/research/long-term-experiments/glbrc-intensive-experiment/) for this study. Prior to the establishment of the BCSE, fields were mainly used for grain crop production and alfalfa (*Medicago sativa* L.) until spring 2008, when soils were chisel plowed and followed by a secondary tillage before planting the various cropping systems, all of which have been maintained as no-till since then (Sanford et al., 2016; Hussain et al., 2019). Each cropping system is replicated in five blocks as a 27 m × 43 m plot; four blocks were selected for this study.

Corn was planted in the first two weeks of May each year and switchgrass (Cave-in-Rock variety) was planted in late June of 2008. Corn planting density was about 80,000 seeds ha^{-1} with a 76 cm row spacing; the seeding rate for switchgrass was 7.5 kg ha^{-1}. Annual N application rates were 150-170 and 56 kg N ha^{-1} urea-ammonium nitrate for corn and switchgrass, respectively. Both crops were grown without irrigation and harvested annually in the fall.

KBS has an average of 1005 mm annual precipitation spread evenly throughout the year and mean annual temperature is 10.1 °C (30-year mean from 1981). Soils are well drained Alfisol loams (co-mingled Kalamazoo and Oshtemo series, both Typic Hapludalfs), formed from glacial till and outwash with some intermixed loess (Crum and Collins, 1995, Luehmann et al., 2016).

Sand, silt and clay content in BCSE soils are 65%, 27%, and 8% on average, respectively (Kravchenko et al., 2019).

Experimental design and soil sampling

This experiment was a randomized complete block design with split plots. For each field replicate of corn and switchgrass, N fertilizer was applied at two levels: unfertilized vs. fertilized. Fertilized subplots (0.66 m × 2.50 m) received either 137 or 56 kg N ha^{-1} as urea for corn and switchgrass, respectively. Fertilizer was applied on April 19 and July 12 of 2016 in a 2.5 mm simulated rainfall (4.19 L) added slowly to avoid runoff; unfertilized subplots (0.66 m × 2.50 m) received de-ionized water only.

Soil samples were taken during spring (April 14 - May 31) and summer (June 30 - August 23) in 2016. In each season, five soil samples were randomly taken from each fertilized or unfertilized subplot before and 1, 5, 13, 26 (spring) or 21 (summer), and 42 days after fertilization. The five soil samples were composited by subplot. Thus, on each sample date, there were four composite samples (n=4) from experimental or control subplots for both switchgrass and corn systems. Soils were passed through a 4 mm mesh in the field and the mesh was wiped with 70% ethanol between subplot samples. Sieved soils were immediately brought back to the lab and stored at 4°C before analysis within five days.

Nitrification potentials, nitrification-derived N_2O, and soil properties

To determine soil nitrification potentials and nitrification-derived N_2O emissions, 5 g of fresh sieved soil were placed into a 155 mL Wheaton bottle amended with 50 mL nanopure water

containing 10 mM NH_4Cl. I used 1-octyne, a recently developed and tested chemical inhibitor of AOB ammonia monooxygenase (AMO) (Taylor et al., 2013; Taylor et al., 2015) to distinguish the relative contribution of AOA and AOB. I used a gradient of octyne concentrations ranging from 0-10 μM aqueous concentration (C_{aq}) to test for optimal inhibition and found 4 μM C_{aq} sufficient to inhibit AOB in all soils (Chapter 2). Thus, I added either 2.8 mL of octyne stock gas (see Taylor et al. (2013) for octyne stock gas preparation) to create a 4 μM C_{aq} concentration, or 2.8 mL of air without octyne, into capped bottles. Bottles were immediately placed on a shaker table and shaken for 24 hours at a constant speed of 200 rpm at room temperature (25 °C). Samples for N_2O, $NO_2^- + NO_3^-$, and NH_4^+ were taken from each bottle at 2 and 24 hours, and nitrification potentials and nitrification-derived N_2O emissions were respectively calculated as the accumulation of $NO_2^- + NO_3^-$ or N_2O over 22 hours. Slurry pH was buffered naturally; no apparent pH change was detected during the incubation.

Nitrification in the presence of octyne is attributed to AOA. Nitrification from AOB is calculated as the difference between total nitrification minus AOA. $NO_2^- + NO_3^-$ and NH_4^+ were measured by a Lachat QuikChem 8500 flow injection analyzer (Hach, Loveland, CO). N_2O samples were stored over-pressurized in 6 mL N_2-flushed glass vials (Exetainers, Labco Ltd, High Wycombe, UK). N_2O was measured with a gas chromatograph (Agilent 7890A, Santa Clara, CA) coupled to an autosampler (Gerstel MPS2XL, Mülheim An Der Ruhr, Germany) and equipped with a ^{63}Ni electron detector at 350 °C and a Porapak Q column (1.8 m, 80/100 mesh) at 80° C (https://lter.kbs.msu.edu/protocols/159).

Soil NH_4^+ and $NO_2^- + NO_3^-$ concentrations were determined from 10 grams of sieved soil extracted with 100 mL 1M KCl in triplicate. Soil moisture was determined by oven drying sieved soil at 60 °C for 48 hours until constant mass.

Statistical analysis

Seasonal nitrification potentials and nitrification-derived N_2O emissions were analyzed with PROC GLIMMIX of SAS 9.4 (SAS Institute, Cary, NC, USA). I analyzed nitrification of AOA and AOB in corn and switchgrass systems separately. The statistical model included 2 fertilizer levels × 2 seasons × 6 sampling dates and the interactions among them were considered fixed factors. Blocks, fertilizer levels nested within blocks, and seasons nested within blocks and fertilizer levels were considered random factors. Analysis of variance (ANOVA) was performed by considering fertilizer levels as a whole plot factor and seasons and sampling dates as subplot and sub-subplot factors. Homogeneity of variance assumptions were checked, and a heterogeneous variance model was chosen when Levene's test showed significant residual variability ($P < 0.05$). I addressed repeated measures among sampling dates by modelling covariance structure as described by Littell et al. (2006). Normality of residuals was visually inspected, and no violations of assumptions were detected.

To determine if N fertilization resulted in higher nitrification potentials and more nitrification-derived N_2O in fertilized plots than in unfertilized plots, a one-tailed T-test was conducted for each sampling date using the 't.test' function in R (version 3.5.0, R Core Team, 2018). In addition, to compare AOA and AOB yields of N_2O (N_2O-N per $NO_2^- + NO_3^-$-N produced) in corn and switchgrass systems, a two-tailed T-test was performed for each system. I combined

data from all sampling dates for both fertilizer levels and seasons for each taxon after confirming 1) N_2O yield was generally not affected by fertilizer levels or seasons; and 2) there were no significant interaction effects between seasons and fertilizer levels or among seasons, fertilizer levels, and sampling dates.

Results

Soil properties

N fertilizer resulted in a significant increase in soil NH_4^+ concentrations one day after application ($P < 0.05$), increasing from 1.4 ± 0.07 to 38.5 ± 10.7 mg N kg^{-1} (standard error of the mean) in spring, and from 1.6 ± 0.3 to 61.8 ± 7.4 mg N kg^{-1} in summer, respectively (Figure 4.1A). In comparison, soil NH_4^+ in the unfertilized treatment was mostly below 1 mg N kg^{-1} throughout the experiment for both seasons. Soil NH_4^+ in the fertilized treatment was thereafter quickly consumed and was below 3 mg N kg^{-1} 42 days after fertilization. For $NO_2^- + NO_3^-$, a constant increase from 2.1 ± 0.08 to 27.3 ± 3.5 mg N kg^{-1} was observed in the fertilized treatment in spring (Figure 4.1B), which was consistently higher than the unfertilized treatment throughout the experiment ($P < 0.05$). In summer, this difference was not observed until 13 days after fertilization (29.1 ± 2.2 vs. 12.2 ± 2.3 mg N kg^{-1}, $P < 0.05$), but was followed by an abrupt decline to 11.5 ± 1.1 and 3.4 ± 0.3 mg N kg^{-1} in fertilized and unfertilized treatments at 42 days, respectively.

In the switchgrass system, patterns of NH_4^+ concentrations following fertilizer were similar to those in the corn system (Figure 4.2A); NH_4^+ concentrations in the fertilized treatment were significantly higher than in the unfertilized treatment one day after application in spring (17.2 ±

3.3 vs. 2.4 ± 0.4 mg N kg^{-1}), and one to five days after application in summer (5.3-14 vs. 1.1-1.3 mg N kg^{-1}), respectively ($P < 0.05$). Unlike the constant increase of NO_2^- + NO_3^- observed in the corn system, soil NO_2^- + NO_3^- concentrations in the switchgrass soil were highest 13 days after N fertilization in spring (Figure 4.2B), reaching 3.2 ± 0.3 mg N kg^{-1} (1.8 ± 0.4 mg N kg^{-1} for unfertilized treatment, $P < 0.05$) and then quickly dropped to below 1 mg N kg^{-1} at 42 days. In summer, soil NO_2^- + NO_3^- concentrations were significantly higher ($P < 0.05$) than in the unfertilized treatment one and five days after fertilization and peaked at 3.1 ± 0.7 mg N kg^{-1}, and eventually decreased to 1.4-2.0 mg N kg^{-1} by the end of the experiment.

Nitrification potentials of AOA and AOB

In the corn system, the nitrification potentials of AOB in the fertilized treatment were not significantly different from the unfertilized treatment before the application of N fertilizer (2.6 ± 0.2 vs. 2.3 ± 0.6 mg N kg^{-1} day^{-1}) in spring. However, one and five days after fertilization, a significant ($P < 0.05$) increase in nitrification potentials was observed, to 3.5 ± 0.3 and 4.0 ± 0.8 mg N kg^{-1} day^{-1}, respectively, which eventually declined to below 2 mg N kg^{-1} day^{-1} at 42 days (Figure 4.3A). In summer, compared to the unfertilized treatment, the only significant increase in AOB-derived nitrification potentials was observed 13 days after N fertilization (3.6 ± 0.8 vs. 1.7 ± 0.4 mg N kg^{-1} day^{-1}, $P < 0.05$). For AOA, nitrification potentials responded to N fertilizer at one day (1.4 ± 0.1 vs. 1.0 ± 0.1 mg N kg^{-1} day^{-1}, $P < 0.05$) and 13 days (2.4 ± 0.4 vs. 1.6 ± 0.2 mg N kg^{-1} day^{-1}, $P = 0.05$) after application in spring but not in summer (Figure 4.3B). Both AOA and AOB-derived nitrification potentials exhibited significant temporal variation ($P < 0.05$) and the temporal variation was season-dependent ($P < 0.05$). An overall significant seasonal effect was observed for AOB ($P < 0.05$) but not AOA. Except for day13 in both

seasons, AOB contributed more than 50% to the total nitrification potential, and ranged between 54.9-77.3% in the unfertilized treatment and 56.1-83.1% in the fertilized treatment (Figure 4.3C). There were no overall seasonal or fertilization effects on the relative contribution of AOB to nitrification potentials and the only exception occurred five days after N fertilizations in spring.

No fertilization effects for either AOA or AOB-derived nitrification potentials were observed in the switchgrass system (Figure 4.4A, B). AOB-derived nitrification potentials exhibited significant seasonal differences ($P < 0.05$), ranging between 1.9 ± 0.6 to 2.8 ± 0.6 mg N kg^{-1} day^{-1} in spring and 0.9 ± 0.4 to 2.6 ± 0.7 mg N kg^{-1} day^{-1} in summer. There were no seasonal effects on AOA nitrification potentials, but significant temporal variation was observed ($P < 0.05$) that was season-dependent ($P < 0.05$). The AOA-derived nitrification potentials were mostly below 1 mg N kg^{-1} day^{-1} throughout the experiment, and were 2-6 times lower than AOB potentials. Similar to the patterns in the corn system, the relative contribution of AOB to total nitrification potentials in the switchgrass system were > 50% for both seasons (Figure 4.4C). Moreover, in spring, AOB's contribution did not respond to N fertilization and ranged between 69.3-85.6% of total nitrification potentials. In comparison, a significantly higher contribution of AOB compared to the unfertilized treatment in summer was observed five and 21 days after N fertilization ($P < 0.05$). AOB contributed 58.8-77.4% and 68.5-86.5% to the total nitrification potentials of unfertilized and fertilized treatments in summer, respectively.

Nitrification-derived N_2O emissions from AOA and AOB

For nitrification-derived N_2O in the corn system, the responses of AOB to N fertilizer were similar to the patterns of AOB-derived nitrification potentials (Figure 4.3A and Figure 4.5A). In

spring, AOB-derived N_2O emissions from the fertilized treatment (5.4 ± 0.2 μg N kg^{-1} day^{-1}) were significantly higher than from the unfertilized treatment (3.0 ± 0.7 μg N kg^{-1} day^{-1}) one day after fertilization ($P < 0.05$). At day 5 and 26, AOB-derived N_2O emissions from the fertilized treatment were also 1.7-2.4 times as high as emission from the unfertilized treatment ($P = 0.06$). In summer, AOB-derived N_2O emissions in fertilized treatment were only significantly higher than in the unfertilized treatment 13 days after fertilization ($P < 0.05$). For AOA, no significant differences in N_2O emissions between fertilized and unfertilized treatments were observed in spring (Figure 4.5B). However, in summer, N fertilizer resulted in about twice the N_2O emissions as the unfertilized treatment one day after fertilization (1.8 ± 0.7 vs. 0.8 ± 0.03 μg N kg^{-1} day^{-1}) but these differences were not statistically different ($P = 0.14$) and gradually decreased from day five ($P < 0.05$) to day 42 ($P > 0.8$). AOB's contribution to nitrification-derived N_2O did not differ between the unfertilized and fertilized treatments and no seasonal effects were detected (Figure 4.5C). AOB dominated nitrification-derived N_2O emissions, contributing 77.6-88.3% in spring and 54.5-91.3% in summer.

No significant N fertilization effects were observed for the nitrification-derived N_2O for either AOA or AOB in the switchgrass system (Figure 4.6A, B). However, AOB did exhibit significant seasonal differences in N_2O emissions ($P < 0.05$): 2.3-6.8 and 0.4-3.2 μg N kg^{-1} day^{-1} in spring and summer, respectively. In comparison, no significant seasonal effects were apparent for AOA-derived N_2O, and emissions were mostly below 1.5 μg N kg^{-1} day^{-1} in spring and 1 μg N kg^{-1} day^{-1} in summer. The relative contribution of AOB to total nitrification-derived N_2O did not appear to respond to N fertilizer (Figure 4.6C) and there was a weak overall seasonal effect ($P = 0.07$). In spring, except for five days after fertilization, AOB's contribution in the fertilized

treatment was consistently lower than in the unfertilized treatment (62.9-84.6% vs. 82.4-91.8%), although not significantly so. In summer, AOB's contribution to nitrification-derived N_2O spanned 50.2-90.2% and 52.7-82.3% in unfertilized and fertilized treatments, respectively.

N_2O yields for AOA and AOB

For both corn and switchgrass systems, no significant fertilization effects were observed for yield of N_2O for either AOA or AOB. In addition, except for AOB in the switchgrass system, there were no overall significant seasonal effects. There were also no significant 2-way interaction effects between season and fertilization or 3-way interaction effects among season, fertilization and temporal variation. Thus, to investigate the differences between AOA and AOB-derived N_2O yields for the corn and switchgrass systems, I grouped data from all sampling dates in both seasons from both unfertilized and fertilized treatments (Figure 4.7). The 25^{th}-75^{th} percentile range of AOB-derived N_2O yield was similar between the corn and switchgrass systems, ranging between 0.10 to 0.18% for corn and 0.097 to 0.19% for switchgrass, respectively. In comparison, N_2O yield of AOA ranged between 0.034-0.085% in the corn system and 0.042-0.12% in the switchgrass system. Significant N_2O yield differences between AOA and AOB were observed in the corn system ($P < 0.05$) but not in the switchgrass system ($P = 0.11$).

Discussion

I found support for all three hypotheses: first, that both AOA and AOB will respond to N fertilizer *in situ*, and second that plants can mediate nitrification-derived N_2O emissions by competing with soil nitrifiers for NH_4^+, and third that AOB will have a higher N_2O yield than AOA. Results reveal the fertilizer-stimulated nitrification potentials and nitrification-derived

N_2O emissions were from both AOA and AOB. In addition, fertilization effects were mainly observed in spring but not in summer, consistent with a higher nutrient demand by plants during the peak growing season, evidenced by more than twice as high NH_4^+ consumption rates in summer as in spring (Figure 4.1A), leading to diminished N_2O emissions from nitrification in summer. Finally, N_2O yield of AOB was higher than AOA for both corn and switchgrass systems.

Temporal dynamics of soil inorganic nitrogen

Soil nitrifiers in both the corn and switchgrass systems quickly converted added NH_4^+ into NO_2^- + NO_3^- *in situ*. After 42 days, NH_4^+ dropped back to almost pre-fertilization levels (Figure 4.1 and Figure 4.2). Thus, this experiment allowed me to comprehensively investigate of the responses of AOA and AOB to N fertilizer since the temporal dynamics of NH_4^+ encompassed a full addition and consumption cycle. Hink et al. (2018) also compared temporal patterns of nitrification-derived N_2O from AOA and AOB following urea versus slow-release N fertilizer in a microcosm system, and found AOB dominated urea treated soils and AOA dominated soils receiving slow-release fertilizer, but the interpretation of their results may be cofounded by the controlled lab conditions and the exclusions of plants.

In the corn system, the overall increase of NO_2^- + NO_3^- in spring versus the initial increase followed by a quick consumption 13 days after fertilization (Figure 4.1B) in summer may have reflected differences in the N requirement of corn during various stages of development. Corn was planted on May 9th of 2016 and most of the plants were still at the V2 or V3 stage (second and third leaf collar visible) by the end of the spring phase of the experiment

(https://phenocam.sr.unh.edu/webcam/browse/kelloggcorn2/2016/05/31/). Thus, the N demand in spring was likely much lower than in summer; in the Midwest, rapid N uptake does not begin until about 4-6 weeks after corn planting (Fortin and Pierce, 1990; Stute and Posner, 1995). For switchgrass, the similar initial increase of $NO_2^- + NO_3^-$ followed by a reduction in both spring and summer seem to suggest soil nitrification gradually fell behind N uptake by switchgrass as the growing season progressed (Figure 4.2B). Additionally, compared to summer, the lower $NO_2^- + NO_3^-$ concentrations after 42 days of fertilizer application in spring could be potentially explained by a higher leaching rate of $NO_2^- + NO_3$ because potential evapotranspiration (PET) exceeds precipitation from June to August (summer), which in turn could reduce soil water drainage (Syswerda et al., 2012; Hamilton, 2015).

The in situ response of AOA and AOB to N fertilizer

The hypothesis that both AOA and AOB respond to N fertilizer *in situ* is supported by evidence that significantly higher nitrification potentials and nitrification-derived N_2O emissions were observed in fertilized treatments than in unfertilized treatments for both AOA and AOB (Figure 4.3 and Figure 4.5). In addition, fertilization effects were more evident in corn than switchgrass. Previous studies have demonstrated that both AOA and AOB-derived nitrification positively respond to an NH_4^+ gradient (Mushinski et al., 2017; Ouyang et al., 2017), so the different fertilization responses between corn and switchgrass in this study likely reflect the different amounts of N fertilizer applied, 137 and 56 kg N ha^{-1}, respectively. Results also show that nitrification by AOB was more strongly stimulated by N fertilizer than was nitrification by AOA. This could be explained by the difference in NH_4^+ affinities between AOA and AOB. Expressed in terms of half saturation constants (K_m), AOA have a much higher NH_4^+ affinity than AOB in

both pure cultures (0.34-1.27 vs. 50-1780 μM NH_4^+) (Koper et al., 2010; Straka et al., 2019) and soil slurry incubation studies (2.9-6.0 vs. 14-161 μM NH_4^+) (Ouyang et al., 2017, Chapter 3 of this dissertation). Therefore, AOA is more likely to be saturated by N fertilizer whereas AOB can oxidize higher concentrations of fertilizer NH_4^+.

I also hypothesized that crops would mediate the *in situ* responses of AOA and AOB to N fertilizer. Evidence in support of this hypothesis comes from the fact that in the corn system, fertilizer stimulated nitrification potentials or nitrification-derived N_2O mostly in spring rather than in summer (Figure 4.3 and Figure 4.5). Corn removes both NH_4^+ and NO_3^- from the soil solution (Schrader et al., 1972; Teyker and Hobbs, 1992; Colmer and Bloom, 1998; George et al., 2016; Zhang et al., 2019), and while NH_4^+ can bind to soil cation exchange sites and thereby be less readily available to plants as compared to NO_3^- (Bloom, 1997), NH_4^+ that is encountered by roots is taken up more rapidly than NO_3^-(Miller and Cramer, 2005). Thus, it seems likely that in the fertilized corn soils, plants competed with soil nitrifiers for NH_4^+ during the peak growing season, which consequently diminished fertilization effects on AOA and AOB.

Indeed, regression of soil NH_4^+ against days after fertilization in the corn system indicates that the NH_4^+ consumption rate in summer was more than twice as high as in spring (-2.77 vs. -1.34, $P < 0.05$, Figure 4.1A). Although I cannot rule out the possibility that the different fertilization effects between spring and summer were caused by season instead of by plants, this possibility appears low because I brought soils back to the lab for incubation under constant lab conditions. Admittedly, the faster NH_4^+ consumption rate in summer than in spring could also be attributed to differences in soil temperature, as previous studies have shown a positive response of AOA

and AOB to incubation temperature increases between 10-20 °C in cropped soils (Ouyang et al., 2017; Taylor et al., 2017). However, that soil NO_2^- + NO_3^- was consumed quickly in summer (Figure 4.1B) but not in spring, and that potentially higher nitrification rates (NH_4^+ consumption rates) in summer than in spring did not result in more significant N fertilization effects, supports the hypothesis that plants' competing with soil nitrifiers during peak growing season reduces nitrifier N_2O emissions. It also seems unlikely that the nitrifier populations were much different in spring than in summer because nitrifiers are mostly slow growing (Habteselassie et al., 2013); soil AOA populations are typically unaffected by chemical fertilizer (Shen et al., 2008; Ai et al., 2013, Chapter 3 of this dissertation) and for AOB, previous studies have shown it was not until the second fertilization or later that growth responds to N addition under field conditions (Ouyang et al., 2016).

N_2O yields of AOA and AOB

N_2O yield is typically calculated as the ratio between the production of N_2O to the production of NO_2^- or NO_3^- (Anderson et al., 1993). Consistent with my hypothesis, the 25^{th}-75^{th} percentile of N_2O yield of AOB ranges between 0.097-0.19%, which was higher than that of AOA ranging between 0.034-0.12%, for both corn and switchgrass systems (Figure 4.7). Thus, results agree with previous pure culture and incubation studies reporting N_2O yield of 0.1-8% for AOB and 0.04-0.3% for AOA (Prosser et al., 2019). The higher N_2O yield of AOB than AOA seems to reflect that AOB harbor more versatile pathways to generate N_2O in comparison to AOA (Hink et al., 2017b), who produce N_2O mainly through abiotic reactions. The more N_2O-efficient AOB, together with the dominance of AOB over AOA in contributing to nitrification-derived N_2O

(Figure 4.5C and Figure 4.6C) suggest AOB plays a more important role than AOA in N_2O emissions in both corn and switchgrass systems.

Implications for N_2O mitigation practices

Overall, results show plants can mediate N_2O emissions from AOA and AOB by competing with soil nitrifiers for NH_4^+. Additionally, there was no significant response of nitrification-derived N_2O to N in the switchgrass system. Together, these results suggest that both timing and quantities of N added to soils are important for curbing nitrification-derived N_2O emissions. First, for both AOA and AOB, the fertilizer-stimulated increase of N_2O emissions in corn mostly happened within 13 days of fertilizer application, which was followed by a rapid reduction of N_2O and a concurrent decrease in soil NH_4^+ concentrations. These episodic N_2O emissions from nitrification appear to be mediated by plants competing with soil nitrifiers for NH_4^+, which highlights the importance of synchronizing N fertilizer application with plant N demand (Millar et al., 2010). Second, in the switchgrass system, N fertilizer did not significantly stimulate nitrification-derived N_2O at 56 kg N ha^{-1}. Thus, results corroborate the findings of Ruan et al. (2016) showing low N input allows optimum yield of switchgrass for biomass production while preserving the mitigation benefits.

Conclusions

1. Both AOA and AOB nitrification responded to N fertilizer *in situ*.
2. Plants appeared to compete with soil nitrifiers for NH_4^+, resulting in diminished N_2O emissions from nitrification for 13 days after fertilizer application.
3. AOB have higher N_2O yields than AOA.

4. AOB dominated nitrification-derived N_2O emissions in both corn and switchgrass systems.

APPENDIX

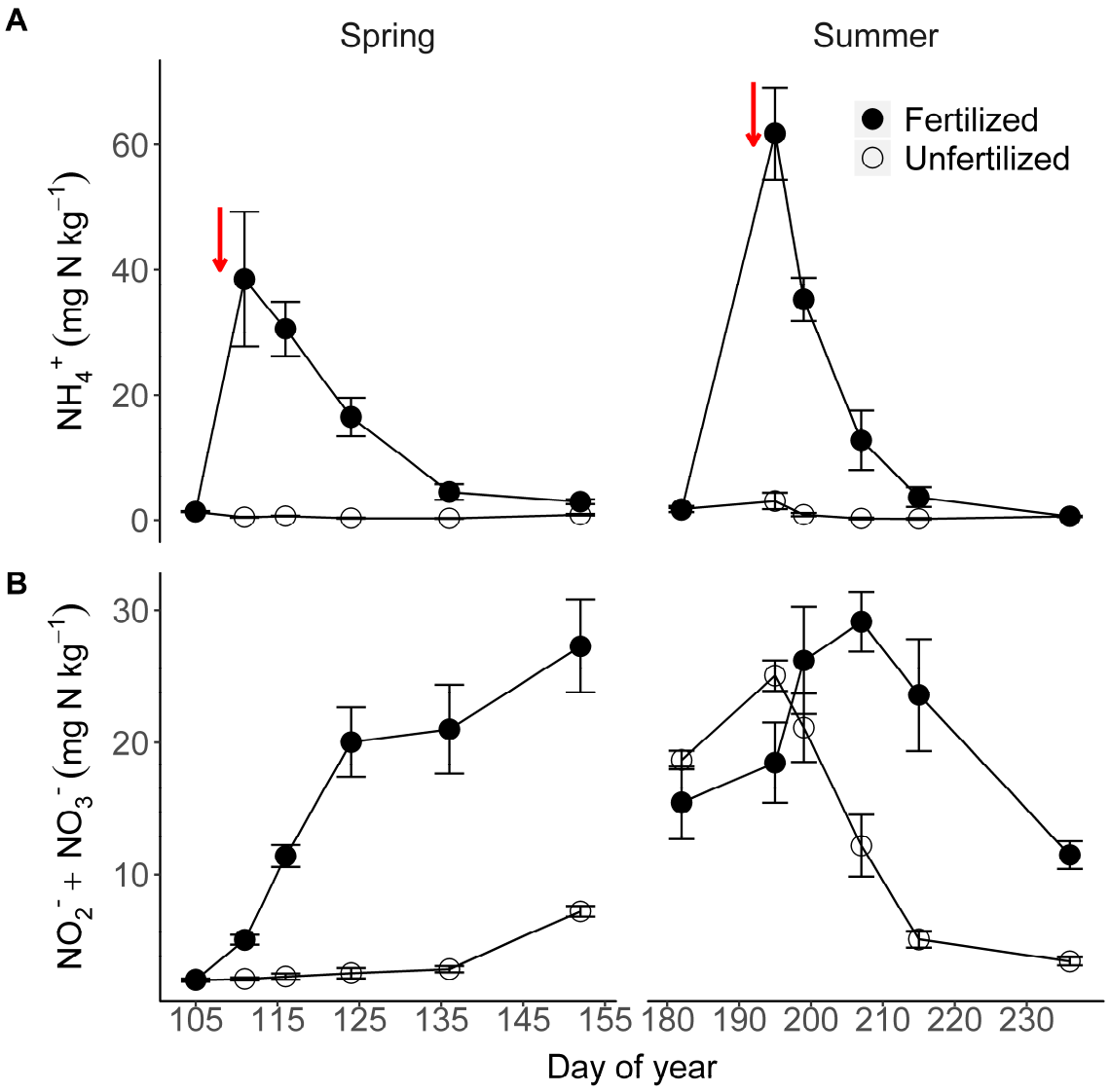

Figure 4.1 Seasonal dynamics of soil NH_4^+ (A) and NO_2^- + NO_3^- (B) in unfertilized (open circles) and fertilized (solid circles) treatments in the corn system; red arrows indicate the dates when N fertilizer was applied. Each column represents a different season. Error bars are based on n=4 field replicates.

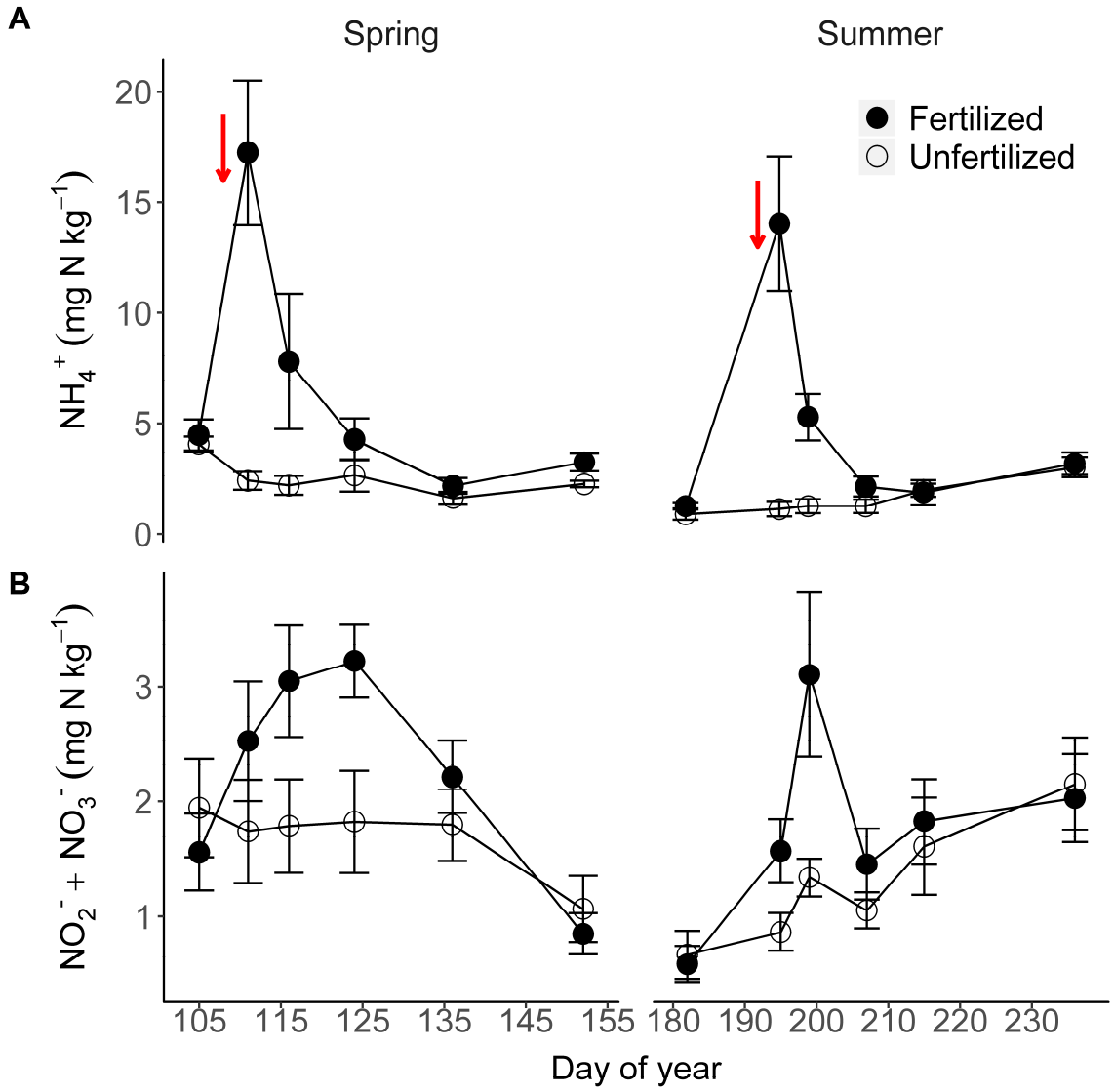

Figure 4.2 Seasonal dynamics of soil NH_4^+ (A) and NO_2^- + NO_3^- (B) in unfertilized (open circles) and fertilized (solid circles) treatments in the switchgrass system; red arrows indicate the dates when N fertilizer was applied. Each column represents a different season. Error bars are based on n=4 field replicates.

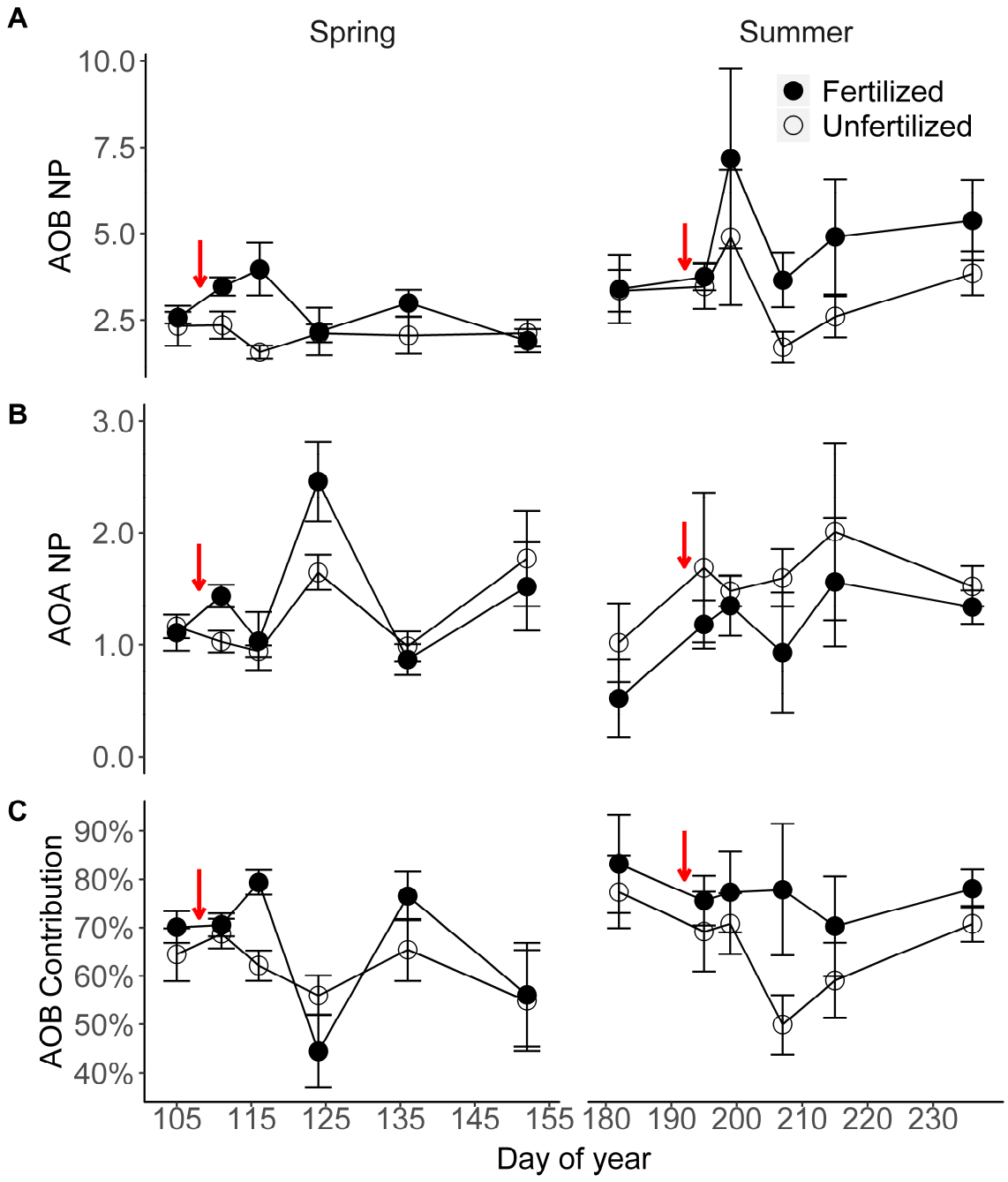

Figure 4.3 Seasonal nitrification potentials (NP, mg N kg^{-1} day^{-1}) of (A) AOB and (B) AOA and the relative contribution of AOB to total nitrification (C) in unfertilized (open circles) and fertilized (solid circles) treatments in the corn system; red arrows indicate the dates when N fertilizer was applied. Each column represents a different season. Bars represent standard errors based on n=4 field replicates.

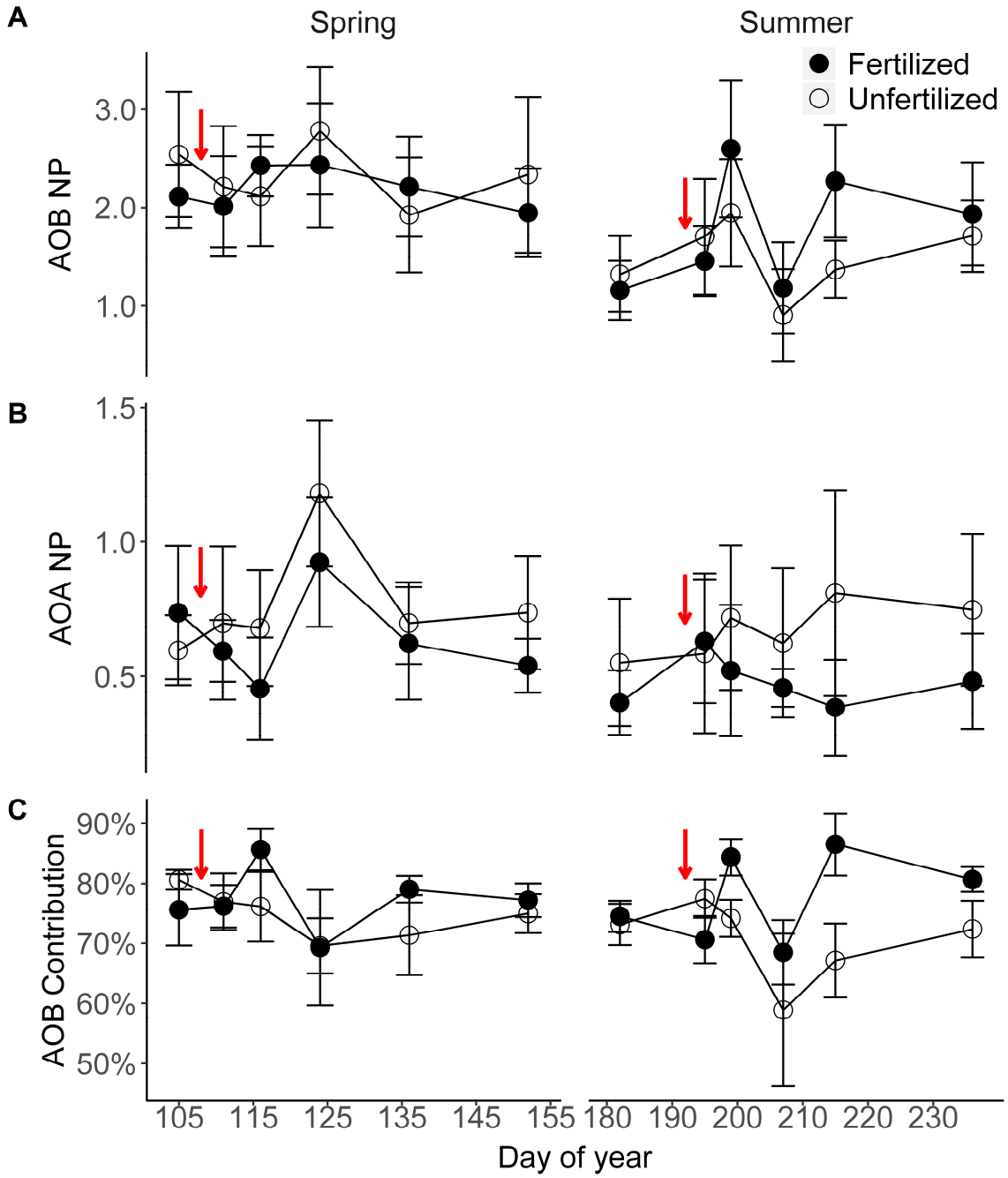

Figure 4.4 Seasonal nitrification potentials (NP, mg N kg^{-1} day^{-1}) of (A) AOB and (B) AOA and the relative contribution of AOB to total nitrification (C) in unfertilized (open circles) and fertilized (solid circles) treatments in the switchgrass system; red arrows indicate the dates when N fertilizer was applied. Each column represents a different season. Bars represent standard errors based on n=4 field replicates.

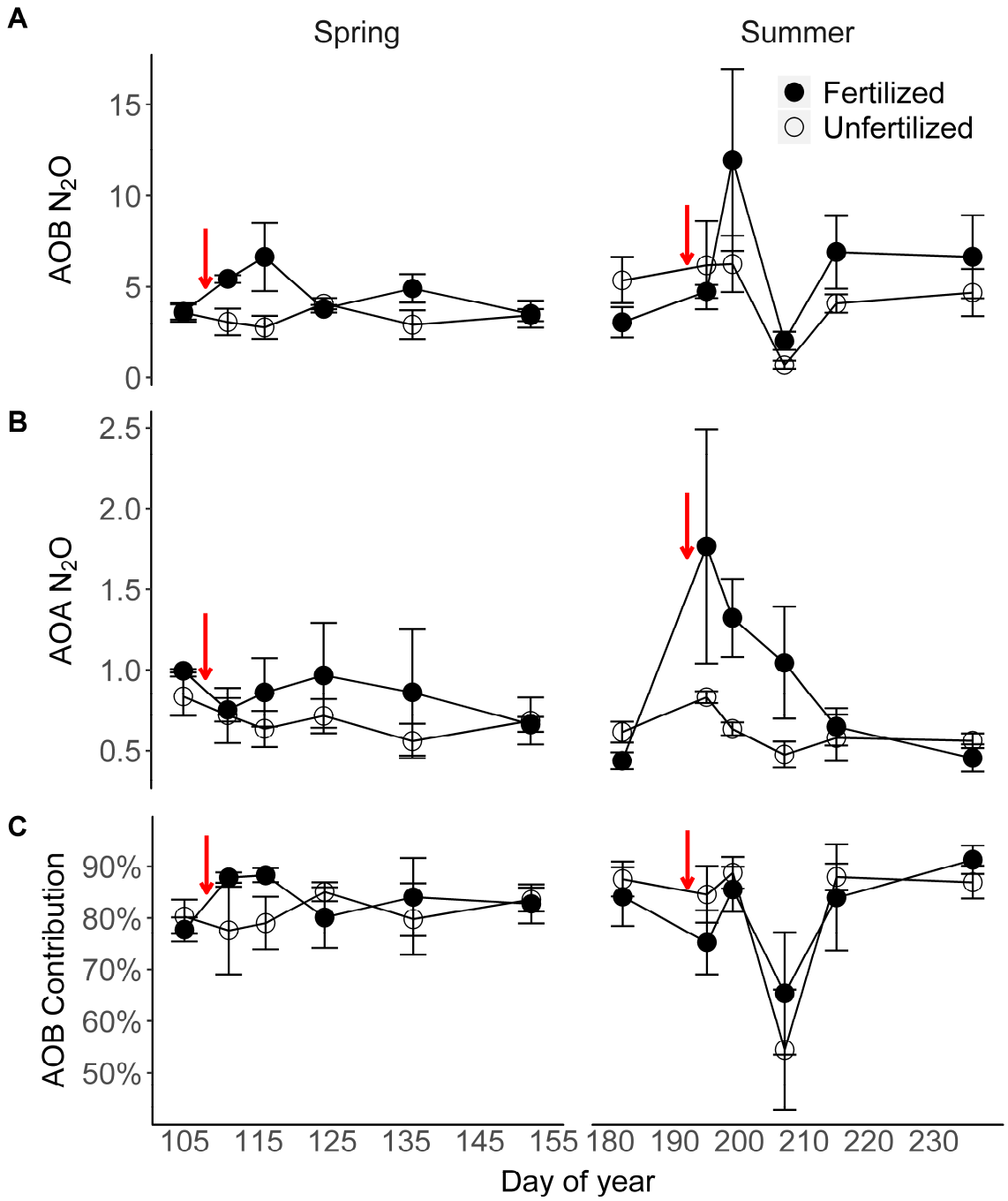

Figure 4.5 Seasonal N_2O emissions (µg N kg^{-1} day^{-1}) of AOB (A) and AOA (B) and the relative contribution of AOB to total nitrification-derived N_2O (C) in unfertilized (open circles) and fertilized (solid circles) treatments in the corn system; red arrows indicate the dates when N fertilizer was applied. Each column represents a different season. Bars represent standard errors based on n=3-4 field replicates.

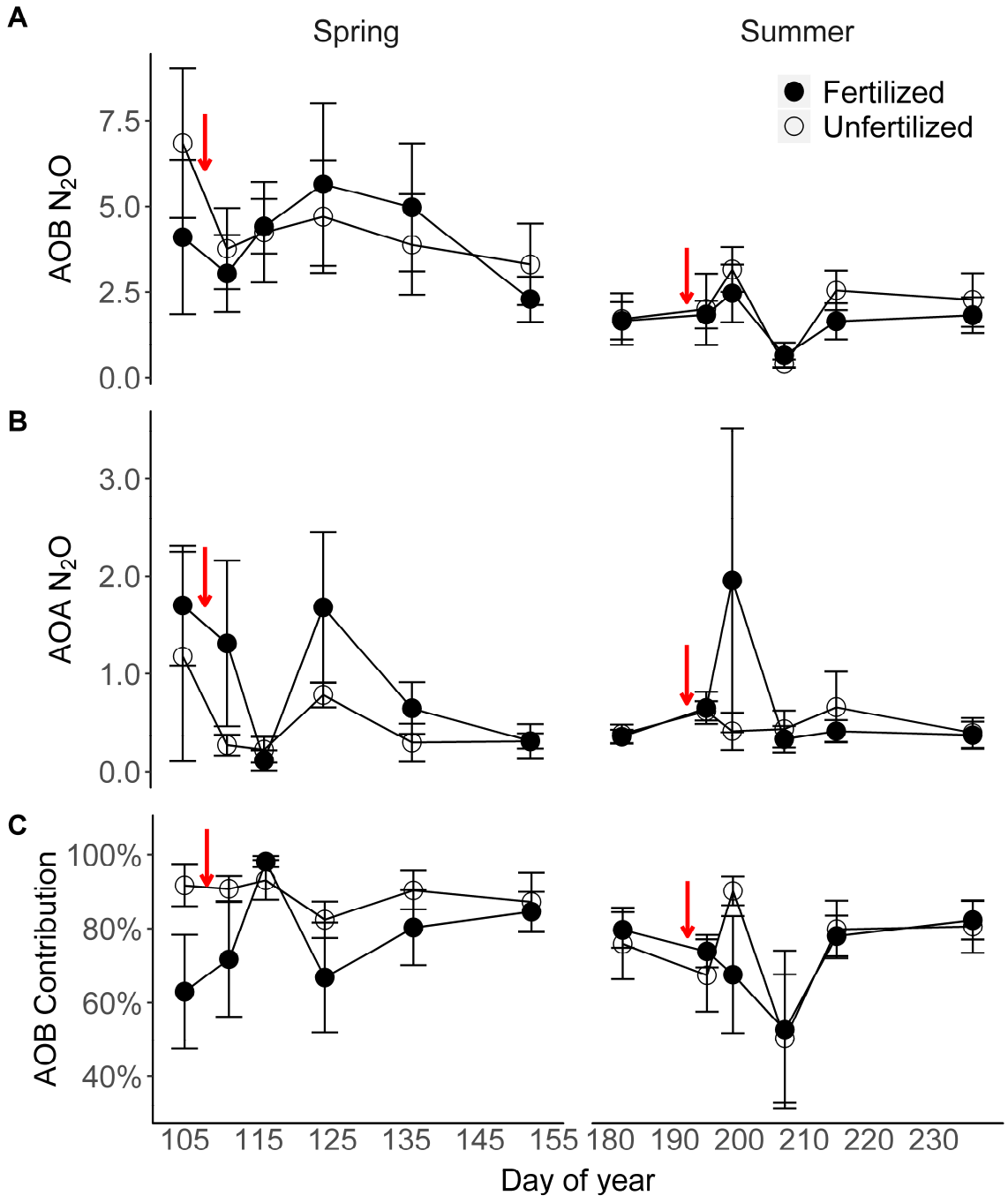

Figure 4.6 Seasonal N_2O emissions (µg N kg^{-1} day^{-1}) of AOB (A) and AOA (B) and the relative contribution of AOB to total nitrification-derived N_2O (C) in unfertilized (open circles) and fertilized (solid circles) treatments in the switchgrass system; red arrows indicate the dates when N fertilizer was applied. Each column represents a different season. Bars represent standard errors based on n=3-4 field replicates.

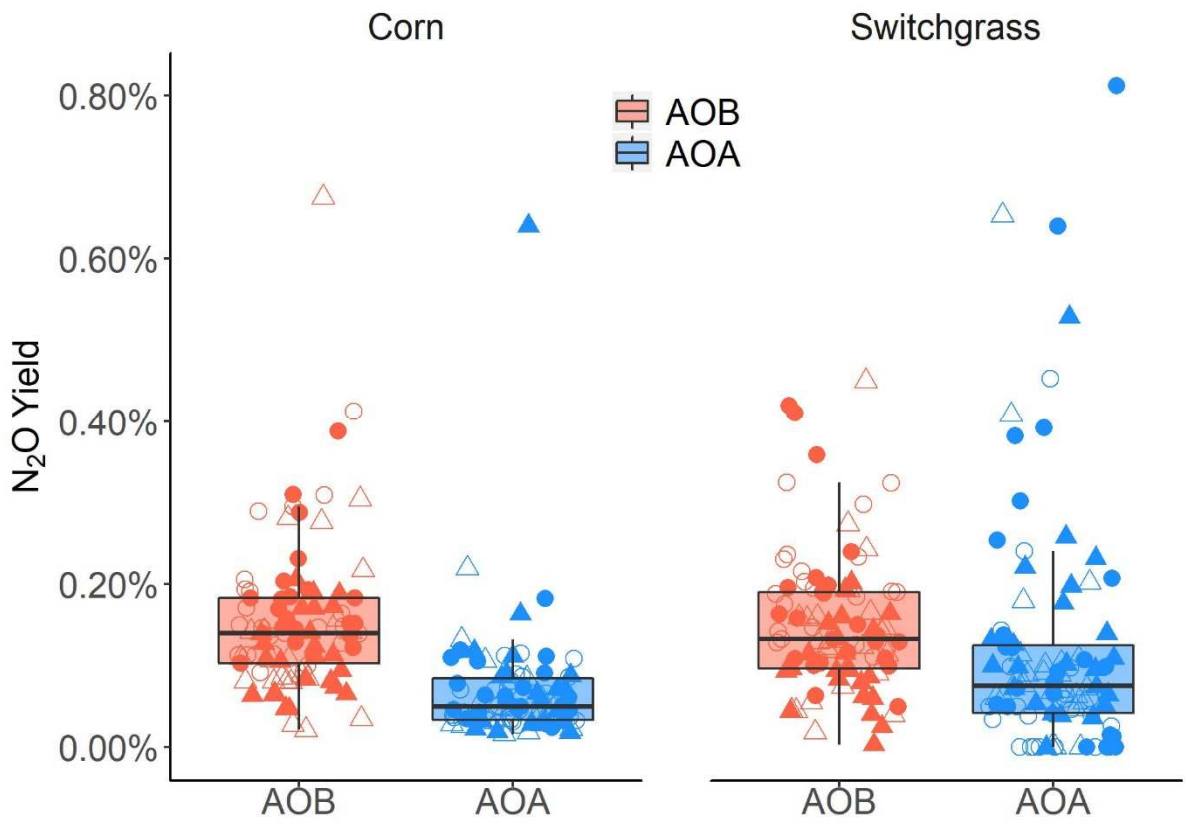

Figure 4.7 N_2O yield (N_2O-N per NO_2^- + NO_3^--N produced) of AOB (orange) and AOA (blue) in corn and switchgrass systems; the upper, mid, and lower lines of each boxplot indicate 25th, median, and 75th percentiles. The upper and lower whiskers indicate 1.5 × inter-quartile range (IQR). Each data point represents individual samples taken at each sampling date. Circles and triangles represent spring and summer, and open and solid symbols represent unfertilized and fertilized treatments.

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Chapter 5: Conclusions

Understanding the microbial sources of N_2O emissions is critical for improving future greenhouse gas mitigation practices. However, current methods for differentiating sources of N_2O between nitrifiers and denitrifiers, such as specific inhibitors, stable isotope enrichment, and isotopomer analysis, suffer from various technical difficulties and assumptions that limit application to field fluxes. As a result, the importance of nitrification versus other microbial processes, and the relative contribution of AOA versus AOB in nitrification-derived N_2O emissions are still largely unknown *in situ*. In this study I sought to constrain the contributions of nitrifiers to N_2O emissions by combining nitrification-derived N_2O kinetics with long-term field NH_4^+ concentrations and N_2O fluxes, and explored potential mechanisms for reducing N_2O emissions from nitrification.

Major results show that soil nitrification is highly unlikely to be the dominant source of N_2O for ecosystems under a range of management intensities, and is likely to be especially low in annual row crops. This suggests that the development of N_2O mitigation strategies should prioritize other N_2O generating processes, in particular denitrification. Results also show that AOB, although dominated numerically in all soils by AOA, almost always dominate nirification rates, and in almost all systems can be inhibited by high NH_4^+ concentrations. This finding implies that the short-term reduction of N_2O emissions could be achieved by creating soil microsites with high NH_4^+ concentrations, as might occur when N fertilizer is band-applied, althought the overall N budget and N use efficiency of such a practice change merits further investigation. Finally, results also show that plants appear to compete with soil nitrifiers for NH_4^+ during the growing

season and thereby dimishes N_2O emissions from nitrification for several weeks following fertilizer application. This indicates the importance of matching plants nutrient demands with timing of fertilizer application for mediating N_2O emissions from nitrification.