AN ABSTRACT OF THE DISSERTATION OF

Andrew T. Giguere for the degree of Doctor of Philosophy in Soil Science presented on March 20, 2017.

Title: An Examination of Factors Controlling the Activity of Ammonia- and Nitrite-oxidizers in Diverse Soils

Abstract approved:

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Peter J. Bottomley David D. Myrold

Nitrification is a critical step in the global nitrogen cycle involving the biological oxidation of ammonia (NH_3) to nitrite (NO_2^-) and then to nitrate (NO_3^-). The first step in nitrification is carried out by NH_3-oxidizing bacteria (AOB) and archaea (AOA), and the second by NO_2^--oxidizing bacteria (NOB). In addition to NO_2^- and NO_3^- being products of nitrification, nitrous oxide (N_2O) can also be a by-product of NH_3 oxidation. Despite the importance of nitrification in agriculture, wastewater treatment, and greenhouse gas accumulation, much remains unknown about the factors controlling nitrification activity, particularly in soils. In the studies presented here, I examined factors controlling the relative contributions of AOA and AOB to nitrification activity. A survey of cropped and non-cropped soils from diverse regions of Oregon showed that AOB activity was more responsive to NH_4^+ additions in cropped soils than was AOA activity, whereas the

opposite situation occurred in non-cropped soils. A larger addition of NH_4^+ was required to stimulate nitrification in cropped soils than in non-cropped soils (67 and 16 mg N kg soil respectively), and summer sampled soils had greater nitrifying activity than winter sampled soils. Upon further examination of the nitrifying response of non-cropped soils to NH_4^+ addition, both AOA and AOB-driven activities gave rise to NO_2^- accumulation and was accompanied by N_2O formation. Nitrite additions to these soils stimulated acetylene-sensitive N_2O production, and a positive, non-linear relationship was revealed between the concentration of accumulated NO_2^- and N_2O production rates. Additions of the NO_2^- oxidizing bacterium, *Nitrobacter vulgaris*, to either prevent NO_2^- accumulation, or to remove accumulated NO_2^-, effectively eliminated N_2O formation in two of three soils. Additional investigation showed that the dynamic nature of NO_2^- accumulation was driven by shifts in the kinetic properties of soil NO_2^- oxidizing activity. Although no significant changes were detected in population size of NOB during the 48 h experiments, an increase in the maximum rate of NO_2^- oxidizing capacity (apparent V_{max}) was detected in the three soils and proven to be protein synthesis dependent in two of the three soil. When protein synthesis and V_{max} increase was prevented by addition of antibiotics, the rate of NO_3^- production also increased in response to the increase in the NO_2^- concentrations; suggesting that both protein synthesis dependent and independent mechanisms can be used to attempt to recouple the rate of NH_3 oxidation to NO_2^- oxidation. Recoupling occurred in all three soils, and was attributed to protein synthesis in two of the three soils, while protein synthesis independent recoupling occurred in one soil. Significant statistical interactions were detected among the soils, indicating that

unknown soil properties and environmental factors, as well as metabolic properties of AOA, AOB, and NOB, are interlinked in these phenomena.

An Examination of Factors Controlling the Activity of Ammonia- and Nitrite-oxidizers in Diverse soils

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I understand that my dissertation will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my dissertation to any reader upon request.

Andrew T. Giguere, Author

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CONTRIBUTION OF AUTHORS

Peter Bottomley, Dave Myrold, and Anne Taylor were responsible for funding this research, and contributed to experimental design, data interpretation, manuscript preparation. Yuichi Suwa contributed to data interpretation and manuscript preparation for Chapter 3.

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Chapter 1

General Introduction

Nitrification is a critical step in the nitrogen (N) cycle, and is the biologically mediated, two-step, oxidation of ammonia (NH_3) to nitrite (NO_2^-) and finally to nitrate (NO_3^-) (Ward et al., 2011). In the late 19th century nitrification was discovered to be carried out by two groups of organisms, the NH_3-oxidizing bacteria (AOB) and the NO_2^--oxidizing bacteria (NOB) (Winogradsky, 1890; Frankland and Frankland 1890). Until the early 21st century the first step was thought to be solely carried out by AOB, until it was demonstrated in 2004 that archaea have the genes encoding for NH_3 oxidizing enzymes and could oxidize NH_3 (AOA) (Venter et al., 2004, Treusch et al., 2005, Lenninger 2006, Konneke et al., 2005). In addition, a complete nitrifier, *Nitrospira inopinata* had been observed to carry out both NH_3 and NO_2^- oxidation however, it remains unknown if, or to what extent comammox contributes to soil nitrification (Daims et al., 2016). Since AOB have been extensively studied for 130+ years, much more is known about these NH_3 oxidizers than is known about the AOA. In most soils AOA and AOB coexist, and AOA frequently outnumber AOB, yet much remains unknown what controls their relative activities (Alves et al., 2013; Leininger et al., 2006; Lu et al., 2015; Nicol et al., 2008; Wessen et al., 2011). Many studies have examined AOA and AOB abundance and genetic diversity, only a few studies have examined their relative activities in soil (Chen et al., 2013; Daebeler et al., 2015; Taylor et al., 2010, 2013; Wessén et al., 2010; Lu et al., 2015). Some studies have suggested that NH_4^+ availability might be a major factor controlling the relative contributions to nitrification, as AOA have been shown to have a

higher affinity for NH_3 than many AOB (Martens-Habbena et al., 2009, Prosser and Nicol, 2012). Furthermore, evidence suggests that pH also separates AOA and AOB contributions, with AOA dominating at low pH. This may be linked to the pH dependent equilibrium (pKa: 9.25) between NH_4^+ and NH_3, which may affect NH_4^+ availability (Gubry-Rangin et al., 2010; Lehtovirta-Morley et al., 2011; Nicol et al., 2008). However, it remains unclear what factors control AOA and AOB contributions to nitrification and how AOA and AOB respond to NH_4^+ additions in soil.

Nitrous oxide production from nitrification

It has been demonstrated in pure cultures studies and in marine environments that both AOA and AOB produce nitrous oxide (N_2O) while oxidizing NH_3 to NO_2^- (Kozlowski et al., 2014; Poth and Focht, 1985; Santoro et al., 2011; Shaw et al., 2006; Stieglmeier et al., 2014; Stein, 2011). There is considerable interest in determining the relative contributions of soil AOA and AOB to N_2O production, and the factors that influence N_2O formation (Jung et al., 2013; Mørkved et al., 2007; Shaw et al., 2006; Stieglmeier et al., 2014). In pure culture studies the production of N_2O by AOB has been demonstrated to be stimulated by the presence of NO_2^- (Shaw et al., 2006), and there is a growing body of evidence that aerobic N_2O production in soil may be associated with NO_2^- accumulation (Maharjan and Venterea, 2013; Venterea, 2007; Venterea et al., 2015). Analysis of AOB genomes reveal that most AOB possess the two enzymes (nitrite reductase and nitric oxide reductase) required to carry out NO_2^--dependent N_2O production (Cantera and Stein, 2007; Kozlowski et al., 2014); however only one of these genes (nitrite reductase) has been identified in the AOA (Spang et al., 2012; Walker et

al., 2010, Hatzenpichler, 2012, Kozlowski et al., 2016). Although it has been suggested that AOA abiologically produce N_2O (Kozlowski et al., 2016), the isotopic signature of N_2O produced from AOA enrichments suggests that biological reduction of NO_2^- is the source of N_2O production (Jung et al., 2013; Stieglmeier et al., 2014). Despite the interest in the contributions of AOA and AOB to N_2O emissions, only one study has examined AOA and AOB contributions to N_2O production in soils (Hink et al., 2016); therefore, it remains unclear what factors control the relative contributions of soil AOA and AOB to aerobic N_2O production.

The NOB and NO_2^- accumulation

Soil NOB are phylogenetically diverse, predominantly belonging to the genera *Nitrobacter* and *Nitrospira* (Freitag et al., 2005, Pester et al., 2015, Poly et al., 2008 Wetrz et al., 2008). Despite their importance in nitrification, very little is known about the factors that influence their NO_2^- oxidizing activity in soil environments, or how the soil NOB activity stays 'coupled' with that of the NH_3 oxidizers. During nitrification in soil, NH_3 oxidation is generally thought to be the rate limiting step (Kowalchuk and Stephens 2001); however, transient NO_2^- accumulation in soil has been demonstrated for decades, suggesting that rates of NH_3 and NO_2^- oxidation can become uncoupled (Burns et al., 1995; Chapman and Liebig, 1952, Müller et al., 2014; Maharjan and Venterea, 2013; Nelson 1982). Studies examining NO_2^- accumulation in soil suggest that it is associated with applications of either anhydrous NH_3 or urea promoting increases in pH to levels which inhibit NOB (Burns et al., 1995; Chapman and Liebig, 1952; Ma et al., 2015; Shen et al., 2003; Venterea, 2007), and/or stimulation of NH_3-oxidizing activity

beyond that of NO_2^- oxidizing activity (Müller et al., 2006). A few studies have examined NOB in soil and focused on their genetic diversity and distribution (Freitag et al., 2005, Pester et al., 2015, Poly et al., 2008 Wetrz et al., 2008); a few other studies specifically examined NOB activity in soil showing that soil NOB activities are affected by tillage (Attard et al., 2010), location within the soil matrix (Ke et al., 2013), and associations with AOA and AOB (Wang et al., 2015). However, these studies did not consider the effects of NO_2^- accumulation on NOB, or how it could potentially influence the recoupling of NH_3 oxidation to NO_2^- oxidation.

Thesis objectives

The objectives of the research presented in this thesis were to characterize some of the factors that control AOA, AOB, and NOB contributions to soil nitrification. To achieve this, three studies were conducted to examine: (1) AOA and AOB contributions to nitrification in response to NH_4^+ additions, cropping status, and season; (2) the impact of AOA and AOB contributions to NO_2^- accumulation and N_2O formation; and (3) the role of NOB in responding to NO_2^- accumulation and recoupling the rate of NO_2^- oxidation with that of NH_3 oxidation.

(i) Soil AOA and AOB response to NH_4^+ additions

In the second chapter of this thesis, the nitrification responses of AOA and AOB to additions of gaseous NH_3 in cropped and non-cropped soils, sampled in summer and winter are presented.

The hypotheses were that: i) AOA respond to lower concentrations of NH_3 than AOB, given that AOA have been shown to have a much higher affinity for NH_4^+ ii) that AOA

activity would dominate in non-cropped soils, as they and are likely more adept at scavenging NH_3, and AOB dominate cropped soils because they receive regular NH_4^+ additions, and respond to large inputs of NH_3 and iii) that there is greater nitrification activity in summer, compared to winter sampled soils for both AOA and AOB.

(ii) AOA and AOB contributions to soil N_2O production

In the third chapter I utilized several non-cropped Oregon soils to examine the contributions of AOA and AOB driven NH_3 oxidation contributions to NO_2^- accumulation, and N_2O formation. The hypotheses were that i) both AOA and AOB nitrification activity have the potential to contribute to NO_2^- accumulation and N_2O production ii) and that NO_2^- is critical in N_2O production from nitrification.

(iii) Role of NOB in the coupling of nitrification

In the fourth chapter, I further examined NO_2^- accumulation and the mechanisms of recoupling the rate of NO_2^- oxidation with that of NH_4^+ oxidation. The hypotheses were that i) protein synthesis by soil NOB is required to recouple the rate of NO_2^- oxidation with that of NH_3 oxidation, and that ii) protein synthesis changes the kinetic properties of NO_2^- consumption due to increases in NO_2^- oxidizing potentials, changes in affinity for NO_2^-, or a combination of both.

The studies presented here provide new insights into the factors controlling AOA, AOB, and NOB contributions to soil nitrification. In the soils used in these studies I found that AOA and AOB responses to NH_4^+ are influenced by cropping and season, that NO_2^- accumulation plays a critical role in NO_2^- formation from nitrification, and that soil NOB quickly adapt in response to NO_2^- accumulation. I also found that within these

trends that individual soils demonstrated different behaviors, suggesting that undefined soil properties and environmental factors as well as metabolic flexibility are interlinked in these phenomena.

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Chapter 2

Nitrification responses of soil ammonia-oxidizing archaea and bacteria to ammonium concentrations

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Abstract

Although ammonia-oxidizing archaea (AOA) and bacteria (AOB) co-exist in most non-acidic agricultural soils, the factors that influence their relative contributions to soil nitrification activity remain unclear. A 2-4 d whole soil microcosm assay was developed, utilizing the aliphatic C8-alkyne, 1-octyne, to inactivate AOB driven nitrification activity without impacting AOA nitrification activity. Responses of AOA and AOB supported net nitrification activities (accumulation of NO_2^- + NO_3^-) to different concentrations of extractable ammonium (NH_4^+) were examined in four diverse, paired cropped and non-cropped Oregon soils sampled in summer and winter. Maximum AOA supported net nitrification rates were significantly higher in non-cropped (3.7 mg N kg^{-1} soil d^{-1}) than in cropped soils (1.0 mg N kg^{-1} soil d^{-1}), and in soils sampled in summer (3.1 mg N kg^{-1} soil d^{-1}) compared to soils sampled in winter (1.6 mg N kg^{-1} soil d^{-1}). The NH_4^+ concentration required to significantly stimulate AOB nitrification activity was significantly higher in cropped soils (67 mg N kg^{-1} soil) than in non-cropped soils (12 mg N kg^{-1} soil). Maximum AOB activity was significantly higher in cropped (8.6 mg N kg^{-1} soil d^{-1}) than in non-cropped soils (2.9 mg N kg^{-1} soil d^{-1}), and in summer (7.8 mg N kg^{-1} soil d^{-1}) compared to winter soils (3.8 mg N kg^{-1} soil d^{-1}). This study has revealed that AOA and AOB supported nitrification rates in cropped and non-cropped soils respond differently to season and NH_4^+ concentration, and raises the possibility that AOA and AOB nitrification activities might be differentially managed to improve N use efficiency.

Abbreviations: AOA, Ammonia oxidizing archaea; AOB ammonia oxidizing bacteria; SC summer cropped; WC, winter cropped; SNC, summer non-cropped; WNC, winter non-cropped.

Introduction

Nitrification is the microbially mediated transformation of ammonium (NH_4^+) to nitrite (NO_2^-) and subsequently to nitrate (NO_3^-). The first and rate limiting step in the nitrification process, is carried out by ammonia-oxidizing archaea (AOA) and bacteria (AOB). Although AOB have been extensively studied for 130+ years, AOA were only discovered recently (Konneke et al., 2005; Treusch et al., 2005). Since the discovery of AOA, it has been revealed that AOA are abundant in soil and frequently outnumber AOB (Alves et al., 2013; Leininger et al., 2006; Nicol et al., 2008; Wessen et al., 2011). Despite AOA abundance, it remains unclear what factors control the contributions of AOA to soil nitrification. There is evidence from marine systems to suggest that AOA and AOB exhibit a niche separation based on their respective affinities for NH_3, and that AOA are dominant under low NH_3 conditions (Martens-Habbena et al., 2009). In soil systems there is evidence that pH separates AOA and AOB contributions, with AOA dominating at low pH, which may be linked to NH_3 availability (Gubry-Rangin et al., 2010; Lehtovirta-Morley et al., 2011; Nicol et al., 2008). In most soils AOA and AOB coexist, yet it remains unknown what controls their relative activities. Recently Taylor et al. (2013) described a procedure that discriminates between AOA and AOB activities and

obtained evidence for seasonal and cropping effects on the contributions of AOA and AOB to nitrification in soil slurries.

The aim of this study was to extend the above work and examine the response of both total and relative contributions of AOA and AOB nitrification activities to incremental increases in NH_4^+ concentrations in cropped and non-cropped soils sampled in summer and winter. Gaseous additions of 1-octyne and NH_3 to the soils allowed these experiments to be performed in unsaturated whole soils. Previous studies have used gaseous NH_3 additions to examine nitrification in soil at unsaturated water contents (Murphy et al., 1999, 1997; Stark and Firestone, 1995; Taylor et al., 2013). I hypothesized: i) that AOA would respond to lower concentrations of NH_4^+ than AOB, given that AOA have been shown to have a much higher affinity for NH_4^+ (Martens-Habbena et al., 2009); ii) that AOA activity would dominate in non-cropped soils, as they do not receive NH_4^+ additions, and AOB would dominate cropped soils, as they regularly receive NH_4^+ fertilization (Taylor et al., 2010, 2013); and iii) that there would be greater nitrification activity in soils sampled in summer, compared to soils sampled in winter for both AOA and AOB (Taylor et al., 2010).

Materials and methods

Soil sampling

Cropped and non-cropped soils were sampled from four locations in Oregon. Samples were collected from: i) Columbia Basin Agricultural Research Center, Pendleton; ii) Central Oregon Agricultural Research Center, Madras; iii) Klamath Basin

Research & Extension Center, Klamath Falls; iv) Hyslop Crop Science Field Research Laboratory, Corvallis. From each location three samples were collected from cropped and adjacent non-cropped surface soils (0-20 cm). Samples were collected in the summer of 2012 and the winter of 2013, and stored at 4ºC until used.

Site Description

The Columbia Basin Agricultural Research Center, is located in northeast Oregon (45°43'9.92"N, 118°37'37.24"W). It receives a mean of 360 mm of precipitation annually and has a mean annual temperature of 11ºC. The soil at this site is classified as a coarse-silty, mixed, superactive, mesic Typic Haploxerolls (Soil Survey Staff, 2014). The cropped soil was in a wheat-fallow cropping rotation and the adjacent non-cropped soil component represents a remnant grassland that has never been cultivated. The Central Oregon Agricultural Research Center is located in central eastern Oregon (44°40'52.38"N, 121° 8'56.14"W). It receives a mean of 250 mm of precipitation annually and has a mean annual temperature of 9ºC. The soil at this site is classified as fine-loamy, mixed, superactive, mesic Aridic Argixerolls (Soil Survey Staff, 2014). The cropped soil is cultivated for root crop seed production and the non-cropped soil occurs under sage brush. Klamath Basin Research & Extension Center is located in south central Oregon. (42° 9'57.09"N, 121°45'27.53"W). It receives a mean of 300 mm of precipitation annually and has a mean annual temperature of 8ºC. The soil on this site is classified as sandy, mixed, mesic Typic Durixerepts (Soil Survey Staff, 2014). Cropped soils are under a wheat rotation and the adjacent non-cropped soil occurs under a pine woodlot, which has never been cultivated. Hyslop Crop Science Field Research Laboratory in

Corvallis is located in western Oregon (44°38'1.64"N, 123°11'38.99"W). It receives a mean of 1140 mm of annual rainfall and has a mean annual temperature of 11ºC. Soil at this site is classified as fine-silty, mixed, superactive, mesic Aquultic Argixerolls (Soil Survey Staff, 2014). Cropped soils are under a wheat-fallow rotation and non-cropped soils were removed from cultivation and seeded over with mixed grass species ~20 years ago. Soil properties are described in Table 2.1.

Determination of NO_3^-, NO_2^- and NH_4^+

Net nitrification activity was determined by quantifying total NO_3^- and NO_2^--N accumulation. Soil (2.5 g) was extracted with 15 ml distilled water for 15 min. Samples were centrifuged, and the supernatants analyzed colorimetrically using the method described by Miranda et al. (2001). Extractable NH_4^+ was determined after extracting 2.5 g soils with 15 ml 2 M KCl for 1 h using the method described in Mulvaney (1996).

Whole soil incubations to determine net nitrification activities

Prior to incubations the gravimetric water content of soil samples was determined. The three field samples of cropped or non-cropped soil from each location were composited and homogenized prior to incubation. Soils (10 g) were added to 125-ml Wheaton bottles and wet to field capacity and allowed to pre-incubate for 24 h at room temperature (23ºC). Pre-incubation minimized the influence of storage at 4ºC and allowed the added water to equilibrate with the soil prior to substrate and inhibitor addition. Bottles were capped and sealed with n-butyl stoppers. Anhydrous NH_3 was added in amounts sufficient to achieve approximately 14, 28, 70, and 140 mg NH_4^+-N kg^{-1} dry soil. KCl-extractable NH_4^+ concentrations were measured in soil samples recovered

from bottles treated with acetylene, to obtain an accurate measurement of the final NH_4^+ concentrations achieved in the soils. Acetylene was prepared by making a 10-fold dilution into 155 ml air, then adding 300 µl aliquots of the dilution to the 125-ml bottles to give a final aqueous concentration of 6 µM (0.02 % v/v). A stock preparation of the AOB inhibitor, 1-octyne, was prepared and added to bottles as described by Taylor et al. (2013). Briefly, several glass beads were added to a 125-ml screw cap media bottle fitted with an n-butyl rubber stopper, 40 µl liquid octyne was added, and the bottle over pressured with 100 ml air. The bottle was shaken vigorously, and 2.7 ml aliquots of octyne gas were added to soil amended bottles with a gas tight syringe, to give a final aqueous concentration of ~4 µM (1.9% v/v). To achieve measureable net nitrification activity, soils sampled in summer were incubated and sampled at 2 d; soils sampled in winter were incubated and sampled at 2 and 4 d. After each sampling the bottles were left open for 1 h to release the acetylene and octyne, whereupon the bottles were resealed and fresh octyne and acetylene added to achieve the initial concentrations. Three analytical replications were used for each treatment. Total net nitrification rates were based on the accumulation of $NO_3^- + NO_2^-$ in the absence of gaseous inhibitors. Net nitrification in the presence of 1-octyne (i.e., octyne resistant) was attributed to AOA activity, with AOB activity was calculated as the difference between the total and AOA nitrification rates (i.e., octyne-sensitive).

Determination of Net N Mineralization rates

Net N mineralization was determined with whole soil incubations of 28 d duration. Gravimetric water content was determined, and 40 g portions of soil were added

to 125-ml bottles. Water content was adjusted to field capacity, and soils incubated at 25ºC in the presence and absence of 6 μM_{aq} acetylene. The accumulation of NO_3^- + NO_2^--N and NH_4^+-N were measured every 7 d. Rates of mineralization were calculated as the accumulation of NH_4^+ in the presence of acetylene from 0-7d. NO_2^- plus NO_3^- did not accumulate during the incubation.

Statistics

Significant differences in the accumulation of NO_3^- + NO_2^- at different NH_4^+ concentrations were determined using an analysis of variance with Tukey-Kramer adjustment for all pairwise comparisons (Fig. 2.1, 2.2). From these data, three parameters related to total, AOA, and AOB nitrification activity were determined using an analysis of variance with Tukey-Kramer adjustment: i) the minimum concentration of NH_4^+ needed to stimulate nitrification activity was chosen as the lowest NH_4^+ that stimulated net nitrification activity above that observed without added NH_4^+; ii) the maximum rate of net nitrification activity was the highest rate of observed net nitrification; and iii) the concentration of NH_4^+ required to saturate nitrification activity was selected as the concentrations above which there was no further significant stimulation of nitrification activity (Fig. 2.1). Differences in rates of nitrification, and NH_4^+ concentrations between cropped/non-cropped and summer/winter and fraction of octyne-resistant activity were determined using a two-way analysis of variance. Analysis was performed using Statgraphics X64 software (Statpoint Technologies, Warrenton, VA, USA).

Results

Figure 1 demonstrates the total, AOA and AOB nitrification responses in one representative pair of cropped and non-cropped soils. These nitrification response curves were generated at all locations, for cropped and non-cropped in both summer and winter. Significant $NO_2^- + NO_3^-$ accumulation did not occur in the acetylene controls, suggesting that all net nitrification activity was due to lithotrophic NH_3 oxidation.

Total net nitrification activity

There were no significant differences in background rates (without the addition of NH_4^+) of nitrification by season or cropping treatment (Table 2.2). Net mineralization rates in winter cropped (referred to as WC) ranged from 0.9-2.9 mg N kg^{-1} soil d^{-1}, and in winter non-cropped (referred to as WNC) rates ranged from 1.3-9.5 mg N kg^{-1} soil d^{-1}. Net mineralization rates in summer cropped (referred to as SC) ranged from 4.2-11.6 mg N kg^{-1} soil d^{-1}, and in summer non-cropped (referred to as SNC) rates ranged from 0.6-3.6 mg N kg^{-1} soil d^{-1} (Table 2.1). The minimum NH_4^+ concentration required to significantly stimulate total nitrification above background in WC varied about four-fold among the soils (15-67 mg N kg^{-1} soil, Fig. S2.1), whereas total nitrification activity was only stimulated in one of four WNC by NH_4^+ additions. In SC, nitrification activity was significantly stimulated by NH_4^+ concentrations that were higher than needed for WC and varied more than six-fold (22-145 mg N kg^{-1} soil, Fig. 2.2). In SNC, total nitrification activity was stimulated by NH_4^+ concentrations that were lower than needed for SC (14-29 mg N kg^{-1} soil, Fig. 2.3).

The concentration of NH_4^+ needed to saturate total nitrification activity was significantly higher in cropped soils (127±96 mg N kg^{-1} soil) compared to non-cropped soils (28±24 mg N kg^{-1} soil; p=0.01) (Fig. 2.2, Fig. S2.1). The mean maximum nitrification activity in summer soils (8.5±5 mg N kg^{-1} soil d^{-1}) were nearly twice that of winter soils (4.9±2.3 mg N kg^{-1} soil d^{-1}; p=0.04). Maximum activity in SC soils was achieved by NH_4^+ concentrations with a mean of 115±23 mg N kg^{-1} soil, and in two cases could not be saturated even at the highest NH_4^+ concentrations (119 and 146 mg N kg^{-1} soil). Maximum nitrification activity in SNC soils were achieved by NH_4^+ concentrations that were substantially lower than SC (28±18 mg N kg^{-1} soil; p=0.01).

Net AOA nitrification activity

Background AOA activity was detected in five of eight non-cropped soils (two of four WNC and three of four SNC) ranging from 0.7-1.9 mg N kg^{-1} soil d^{-1}. Background AOA activity was detected in two of eight cropped soils, (two of four SC) with rates ranging from 0.8-1.4 mg N kg^{-1} soil d^{-1}. There were no significant differences in background AOA nitrification activity between seasons or treatments.

The addition of NH_4^+ stimulated AOA activity in non-cropped soil, while additional NH_4^+ did not stimulate AOA nitrification activity in cropped soils, implying that in cropped soils, AOA activity was saturated by background NH_4^+ concentrations (4.7±3.7 mg N kg^{-1} soil). The minimum NH_4^+ concentration required to stimulate AOA activity in non-cropped soils (16±13 mg N kg^{-1} soil) was significantly higher than the background NH_4^+ concentrations in cropped soils (p=0.015) (Fig. 2.4). The concentration of NH_4^+ required to stimulate AOA activity was also significantly higher in summer soils

(15±12 mg N kg^{-1} soil) than in winter soils (5.3±5 mg N kg^{-1} soil; p=0.02) (Fig. 2.4). Ammonium-stimulated AOA nitrification activity was significantly higher in non-cropped soils (2.9±1.3 mg N kg^{-1} soil d^{-1}) compared to cropped (0.6±0.4 mg N kg^{-1} soil d^{-1}; p=0.0001) soils, and was higher in summer (2.2±1.8 mg N kg^{-1} soil d^{-1}) than in winter (1.2±1 mg N kg^{-1} soil d^{-1}; p=0.03) soils. Ammonium-stimulated rates in non-cropped soils were compared to background rates in cropped soils, as there was no additional stimulation of AOA nitrification activity by NH_4^+ additions in cropped soils. Maximum AOA nitrification activity was significantly higher in non-cropped (3.7±2.3 mg N kg^{-1} soil d^{-1}) than in cropped soils (0.9±0.5 mg N kg^{-1} soil d^{-1}) (p= 0.004) (Fig 2.5). The mean concentration of NH_4^+ required to saturate AOA nitrification activity was significantly higher in non-cropped (21±17 mg N kg^{-1} soil) soils compared to cropped soils (4.5±3.8 mg N kg^{-1} soil; p=0.009) (Fig 2.5).

Fraction of AOA/total nitrification activity

The fraction of AOA activity was significantly greater in SNC (73%±9) than in SC (24%±20) across all NH_4^+ concentrations (p<0.0001). The fraction of AOA activity was also significantly greater in WC (54%±30) than in WNC (16%±8) (p<0.0001). The fraction of octyne resistant nitrification activity in SNC was also significantly greater than in WNC soils (p=0.0002), but did not differ between SC and WC (p=0.23). There was a significant interaction (p=0.04) between cropped/non-cropped and season, so soils were separated for analysis to allow comparison of SNC to SC, WC to WNC, SNC to WNC and SC to WC.

Net AOB nitrification activity

AOB net nitrification rates were calculated as the difference between total and AOA net nitrification rates. Background AOB activity was detected in only three of eight winter soils (0.5 -1.9 mg N kg^{-1} soil d^{-1}), and undetected in summer soils.

The NH_4^+ concentration required to significantly stimulate AOB activity above background was significantly higher in cropped (67±49 mg N kg^{-1} soil) than in non-cropped (12±10 mg N kg^{-1} soil) soils (p=0.004) (Fig.2.4). AOB activity was stimulated by NH_4^+ additions in all cropped soils, while it was only stimulated in two of eight non-cropped soils. When there was no stimulation of AOB nitrification activity, the background KCl extractable NH_4^+ was considered to be the saturating concentration of NH_4^+. There was no effect of season on the concentration of NH_4^+ required to stimulate AOB activity.

The concentration of NH_4^+ required to support the maximum rate of AOB nitrification activity was significantly higher in cropped (116±31 mg N kg^{-1} soil) than in non-cropped (30±47 mg N kg^{-1} soil) soils (p=0.0036) (Fig. 2.5). Mean maximum AOB activity was significantly higher in cropped (8.6±6.0 mg N kg^{-1} soil d^{-1}) than in non-cropped (2.9±1.9 mg N kg^{-1} soil d^{-1}) soils (p=0.009) (Fig. 2.5).

Discussion

In this study I built upon earlier work that showed that the linear C8 alkyne, 1-octyne, selectively and irreversibly inactivates NH_3 oxidation by AOB at very low concentrations (1 μM_{aq}), but does not inhibit AOA activity unless used at 10 to 20-fold higher concentrations (Taylor et al., 2013). Using this method, I examined the influence

of season, cropping, and NH_4^+ additions on short-term (≤4 d) rates of AOA (octyne-resistant) and AOB (octyne-sensitive) nitrification, in adjacent cropped and non-cropped soils from four of the major agricultural production regions of Oregon. As mentioned in the introduction, although several studies have been reported in the literature which show that NH_3/NH_4^+ availability, cropping practice, and season are major factors influencing the relative sizes of AOA and AOB populations in soil, there has been little work to compare the relative nitrifying activities of AOA and AOB in soil in response to these different cropping and seasonal soil conditions (Taylor et al., 2012).

In this study the most important factor influencing the relative magnitudes of AOA and AOB nitrification activities was whether the soils were cropped or non-cropped. The maximum AOA rates of nitrification in cropped soils were generally lower than non-cropped soils. For example, SC soils had a mean AOA rate of 1.3±0.7 versus 4.8±2.4 mg N kg^{-1} soil d^{-1} in SNC soils. In addition, the AOA rates in cropped soils were not significantly stimulated by additions of NH_4^+, whereas AOA activity was stimulated by NH_4^+ additions in all SNC, suggesting that AOA activity was NH_4^+ limited in the latter soils. Because non-cropped soils had no history of either cultivation or N fertilization, NH_4^+ limitation of AOA activity presumably reflects the fact that the indigenous pool of mineralizable N was insufficient to meet the AOA nitrifying potential at the time of sampling. Furthermore, because the maximum AOA rates were two to four-fold higher in SNC than WNC, the data confirm that the potentially active AOA population was larger in summer than winter, or that the *per cell* activity potential was greater in summer than in winter. Research findings have been mixed on whether nitrification activity by soil

AOA depends upon exogenous additions of NH_4^+. For example, several studies have shown that soil AOA will proliferate and/or incorporate $^{13}CO_2$ into thaumarchaeal DNA when N mineralization is the sole source of NH_4^+ (Jia and Conrad, 2009; Zhang et al., 2010). This result might be expected if soil AOA possess a high affinity for NH_4^+ as shown in the marine thaumarcheon, *N. maritimus* (Martens-Habbena et al., 2009). Other soil studies have shown, however, that AOA population growth can be stimulated above background by additions of low concentrations of NH_4^+ in the order of 14-28 mg N kg^{-1} soil; implying that AOA are NH_4^+ limited under some soil conditions (Taylor et al., 2013; Verhamme et al., 2011). Clearly, our data illustrate that the NH_4^+ concentration required to support maximum activity of AOA varies among soils and that season of sampling might also be influential.

In contrast to AOA activity, AOB nitrification rates were stimulated by NH_4^+ additions to higher maximum activities in cropped soils than in non-cropped soils, suggesting that cropped soils contain higher population densities of active AOB than non-cropped soils, or that the *per cell* activity potential was higher in cropped soils. This is not too surprising since the SC soils were sampled from under crops several weeks after spring N fertilization. In SC, the rates of AOB nitrification were significantly stimulated above background by a mean NH_4^+-N concentration of 95.9± 55.4 mg N kg^{-1} soil, whereas in SNC, AOB activities were significantly stimulated above background by lower concentrations of NH_4^+ (22.2 ±13.7 mg N kg^{-1} soil). This observation indicates that the active AOB populations in non-cropped are NH_4^+ limited. Evidence has been obtained from pure culture studies that the K_s for NH_4^+/NH_3 varies among different members of

the soil dominant *Nitrosospira* lineage (Bollmann et al., 2005; Taylor and Bottomley, 2006), and also that sensitivity to high NH_4^+ concentrations differs among subgroups of *Nitrosospira* (Webster et al., 2005). Although I did not compare AOB community composition between cropped and non-cropped soil, AOB population composition has been shown to differ between soils that are N fertilized versus those not fertilized with N, and that AOB abundance increases in N fertilized soils (Di et al., 2009; Prosser and Nicol, 2012; Taylor et al., 2010; Zeglin et al., 2011). In SC soils, the AOA fraction of total nitrification was highest at NH_4^+ concentrations $\leq$70 mg N kg^{-1} soil, and the increase in the fraction of AOB nitrification at higher NH_4^+ concentrations is most readily explained by the presence of AOB populations that develop greater NH_3 oxidizing capacity albeit with lower affinity for NH_4^+/NH_3. I also noted that whereas the AOB activity of WC soils saturated at ~70 mg N kg^{-1} soil, it could not be saturated in two of the SC soils. Again, this result suggests that the AOB populations responsive to NH_4^+ in SC soils possessed different kinetic properties of NH_3 oxidation than those potentially active in WC soils. The difficulty in saturating nitrification in some SC might be due to the fact that most of the added NH_4^+ was bound to soil exchange sites and soil solution NH_4^+ concentrations did not rise >2mM (Data not shown); K_m values of some AOB fall in the range of 1-2 mM NH_4^+ at circumneutral pH (Hyman and Wood, 1985; Suwa et al., 1994; Suzuki et al., 1974).

Lower AOA nitrification activity in cropped soils compared to non-cropped soils may infer that long-term N fertilization negatively impacts AOA populations. Evidence from enrichment and pure culture studies has shown that some AOA are sensitive to

moderate concentrations of NH_4^+ > 2-3 mM (French et al., 2012; Hatzenpichler, 2012; Konneke et al., 2005). In our study, although nitrification by AOA saturated at low NH_4^+, this activity was not reduced by adding NH_4^+ concentrations realistic of fertilizer N applications. This lack of sensitivity to NH_4^+ can be explained by NH_4^+ concentrations in soil solution not exceeding 2 mM even at the highest NH_4^+ concentrations applied (data not shown). 2 mM NH_4^+ is a value often used to culture AOA in the laboratory (Hatzenpichler, 2012; Martens-Habbena et al., 2009; Tourna et al., 2011).

Evidence was obtained in this study that season of sampling significantly influenced AOB maximum nitrification rates, and weakly influenced maximum AOA rates (p=0.07). Other studies have shown that season influences AOA and AOB *amoA* gene abundances, and also that nitrification potential rates fluctuate throughout the year (O'Sullivan et al., 2013; Taylor et al., 2012)**.** In our study, the soil incubations were conducted at 25ºC regardless of season of sampling, yet, some studies indicate that soil AOA may show preference for either higher or lower temperatures than 25°C. For example, *N. viennensis* is a soil AOA isolate that exhibits maximum nitrification activity at >35ºC (Tourna et al., 2011), and another study demonstrated that AOA community composition shifted when soil was incubated at 30ºC with little discernible change occurring at incubations ≤25°C (Tourna et al., 2008). In contrast, Alves et al. (2013) showed that the AOA composition of Arctic soil enrichment cultures shifted in response to incubation at 4ºC versus 20ºC, and nitrification activity did not persist in enrichments made at 28ºC suggesting that differences in temperatures between 4 ºC and 20°C might be sufficient to influence AOA community composition and their nitrification activity.

Previous research has examined the potential of acetylenic compounds to inhibit nitrification in soils. For example, McCarty and Bremner (1986) demonstrated that a wide range of acetylenic compounds inhibit nitrification to varying degrees, and that 1-octyne inhibited 49-77% of nitrification activity in 7-d incubations of three Iowa soils. Our study raises the possibility that selective inhibitors could be employed to reduce the rate of nitrification as a technique in ammoniacal N management. Our data demonstrate that nitrification activity of AOA respond generally to lower NH_4^+ concentrations than AOB, and express lower maximum nitrification rates than AOB in cropped soils. Placing this into a cropping perspective, two of the largest acreage field crops produced in Oregon are grass seed and winter wheat with recommended fertilizer N rates of 106 and 185 kg N ha^{-1}, respectively (Gardner et al., 2000; Petrie et al., 2006). Our study demonstrated that these rates of fertilization were often sufficient to saturate total nitrification activity, and I calculated that under ideal conditions, AOB activity could nitrify all NH_4^+-N applied to grass seed and wheat in 12-22 d, while AOA activity would take 88-154 d to nitrify the same quantity of NH_4^+. The data collected in this study suggest that if a suitable inhibitor for field use could be found, selective inhibition of AOB activity might be a simple N management strategy to reduce N loss from some cropping systems.

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Table 2.1: Soil Physical and chemical properties of soils used in this study

Location	Pendleton		Madras		Klamath		Corvallis	
Land use	Non-cropped	Cropped	Non-cropped	Cropped	Non-cropped	Cropped	Non-cropped	Cropped
% sand/silt/clay	14.2/71.8/14		38.5/35.7/25.8		83/4/13		19.9/57.5/22.6	
pH	7.26	6.15	7.68	6.87	7.36	6.42	6.18	6.38
WHC -33 kPa†	0.45	0.35	0.38	0.39	0.32	0.22	0.26	0.32
Total C (g kg^{-1})#	20.7	10.6	8.7	8.7	13.4	6.6	25.7	12.9
Total N (g kg^{-1})#	1.8	0.9	0.9	0.8	1.1	0.6	1.7	0.6
NH_4^+ summer‡‡	3.61	6.8	8.48	11.6	0.56	8.26	2.09	4.18
NH_4^+ winter‡‡	3.18	3.1	1.29	2.92	9.54	0.92	1.93	1.44
CEC ($cmol_c$ kg^{-1})‡	21.9	15.1	20.5	22.0	13.6	10.7	16.9	14.2
AOA amoA§	352±197	123±73	474±47	283±244	419±228	307±48	3.9±2.7††	0.9± 0.7††
AOB amoA§	5.9±2.6	5.6±0.9	0.5±0.2	15.6±15	9.4±8.7	9.8±2.1	1.0±0.5††	0.8±0.2††
N-mineralization¶	1.5±2.4	0.7±0.1	1.3±0.4	0.8±0.2	1.0±0.3	1.5±0.3	1.2±0.56	0.5±0.09

†: Water holding capacity
‡: Cation exchange capacity
§: Gene copies 10^6 from Taylor et al. (2013)
¶: NH_4^+ accumulation rates in the presence of acetylene (mg N kg^{-1} DW soil d^{-1})
#: Determined by the Central Analytical lab, Oregon State University.
†† Gene copies 10^6 g^{-1} soil from Taylor et al. (2010)
‡‡: Background KCl extractable NH_4^+ mg N kg^{-1} soil

Table 2.2: Background total net nitrification rates

Season	Site	Background Nitrification† Cropped	Non-cropped
Winter			
	Pendleton	0.37±0.2	0.17±0.3
	Madras	0.76±0.3	0.08±0.8
	Klamath	0.61±0.04	2.8±1.0
	Corvallis	0.60±0.2	1.4±0.13
Summer			
	Pendleton	0.31±0.3	0.37±0.4
	Madras	0.78±1.4	0.92±0.3
	Klamath	1.7±0.2	0.81±0.04
	Corvallis	0.14±0.2	0.59±0.06

Means given ± standard deviation

† Background net nitrification mg NO_3^- + NO_2^--N kg^{-1} soil d^{-1} measured without the addition of NH_4^+

Figure Legends

Figure 2.1: Total, AOA and AOB nitrification rates in soil. Closed circles represent total nitrification activity, open circles represent AOA nitrification activity, and closed triangles represent mean AOB activity, calculated as the difference between total and AOA activity. † represents the minimum concentration of NH_4^+ required to significantly stimulate nitrification activity, determined using an ANOVA with Tukeys HSD for all pairwise comparisons. ‡ represents the maximum observed mean nitrification activity. § represents the minimum level of NH_4^+ required to saturate nitrification activity, determined using an ANOVA with Tukeys HSD for all pairwise comparisons. Error bars represent the standard deviation (n=3).

Figure 2.2: Rates of total nitrification activity of soils sampled in summer 2012. Values with different letters are significantly different as determined with an ANOVA and Tukeys HSD test (p-value ≤ 0.05). Closed circles represent cropped soils, open circles represent non-cropped soils and error bars represent standard deviation (n=3).

Figure 2.3: Octyne resistant nitrification activity of soils sampled in summer 2012. Values with different letters are significantly different as determined with an ANOVA and Tukeys HSD test (p-value ≤ 0.05). Closed circles represent cropped soils, open circles represent non-cropped soils and error bars represent standard deviation (n=3).

Figure 2.4: Minimum concentration of NH_4^+ required to stimulate nitrification activity. Black bars represent the concentration of NH_4^+ required to stimulate AOA activity, and grey bars represent the concentration of NH_4^+ required to stimulate AOB activity. Error bars represent the standard deviation (n=4).

Figure 2.5: Maximum nitrification activity. Black bars represent maximum AOA nitrification activity, and grey bars represent AOB nitrification activity. Error bars represent the standard deviation (n=4).

Figure S2.1: Rates of total nitrification activity of soils sampled in winter 2013. Values with different letters are significantly different as determined with an ANOVA and Tukeys HSD test (p-value ≤ 0.05). Closed circles represent cropped soils, open circles represent non-cropped soils and error bars represent standard deviation (n=3).

Figure S2.2: Octyne resistant nitrification activity of soils sampled in winter 2013. Values with different letters are significantly different as determined with an ANOVA and Tukeys HSD test (p-value ≤ 0.05). Closed circles represent cropped soils, open circles represent non-cropped soils and error bars represent standard deviation (n=3).

Figure 2.1

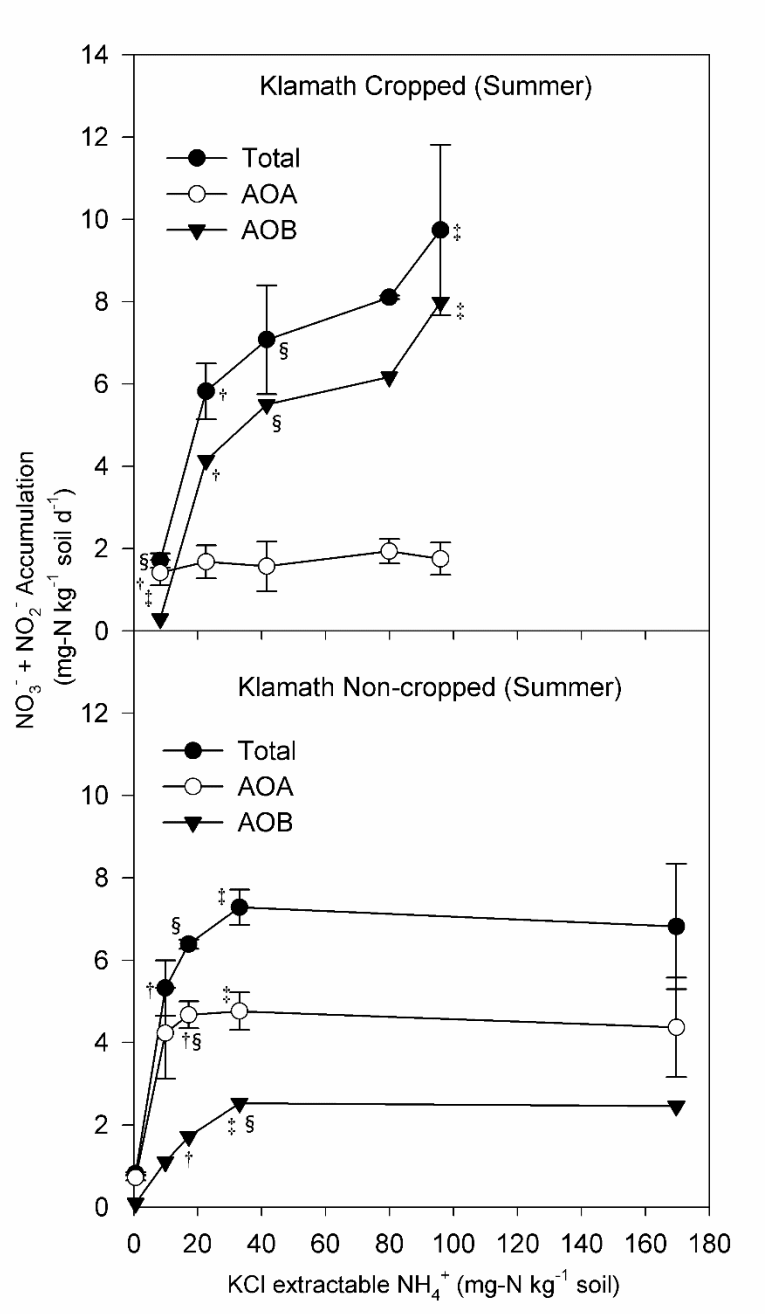

Klamath Cropped (Summer)
Total
AOA
AOB
Klamath Non-cropped (Summer)
Total
AOA
AOB
$NO_3^- + NO_2^-$ Accumulation (mg-N kg^{-1} soil d^{-1})
KCl extractable NH_4^+ (mg-N kg^{-1} soil)
0
2
4
6
8
10
12
14
0
20
40
60
80
100
120
140
160
180

Figure 2.2

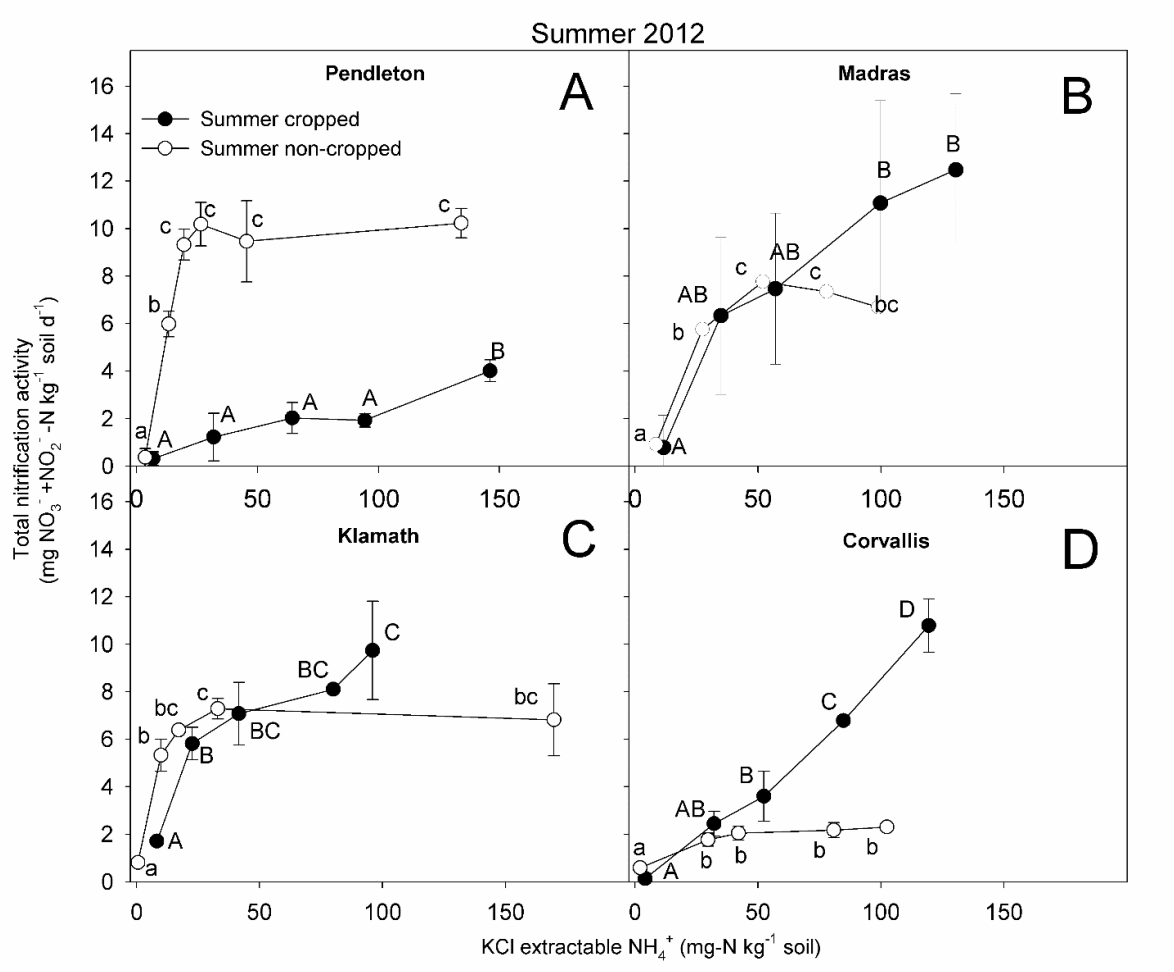

Summer 2012
Pendleton
A
Summer cropped
Summer non-cropped
Madras
B
Klamath
C
Corvallis
D
Total nitrification activity
(mg NO3- +NO2- -N kg-1 soil d-1)
KCl extractable NH4+ (mg-N kg-1 soil)

Figure 2.3

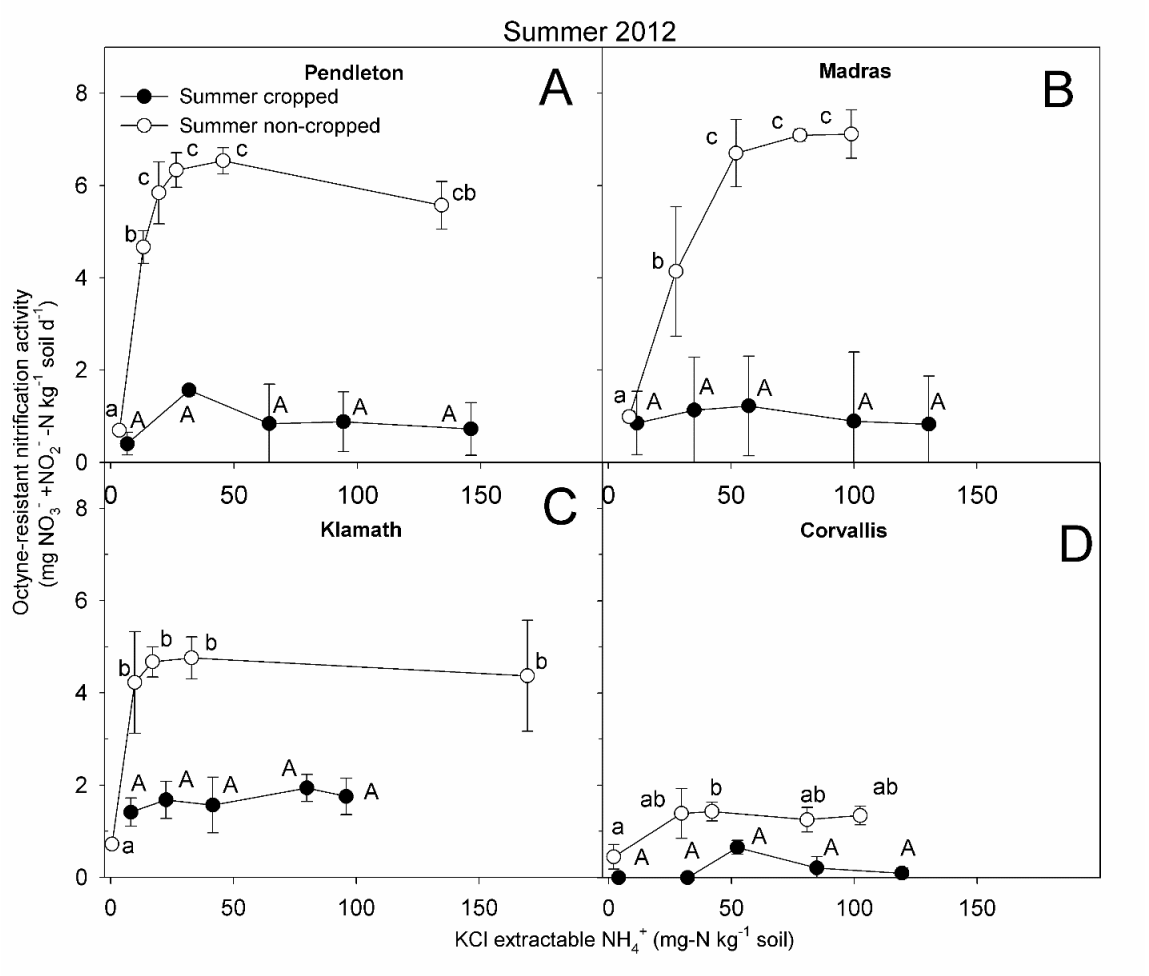

Summer 2012
Pendleton
A
Summer cropped
Summer non-cropped
Madras
B
Klamath
C
Corvallis
D
Octyne-resistant nitrification activity
(mg NO3- +NO2- -N kg-1 soil d-1)
KCl extractable NH4+ (mg-N kg-1 soil)

Figure 2.4

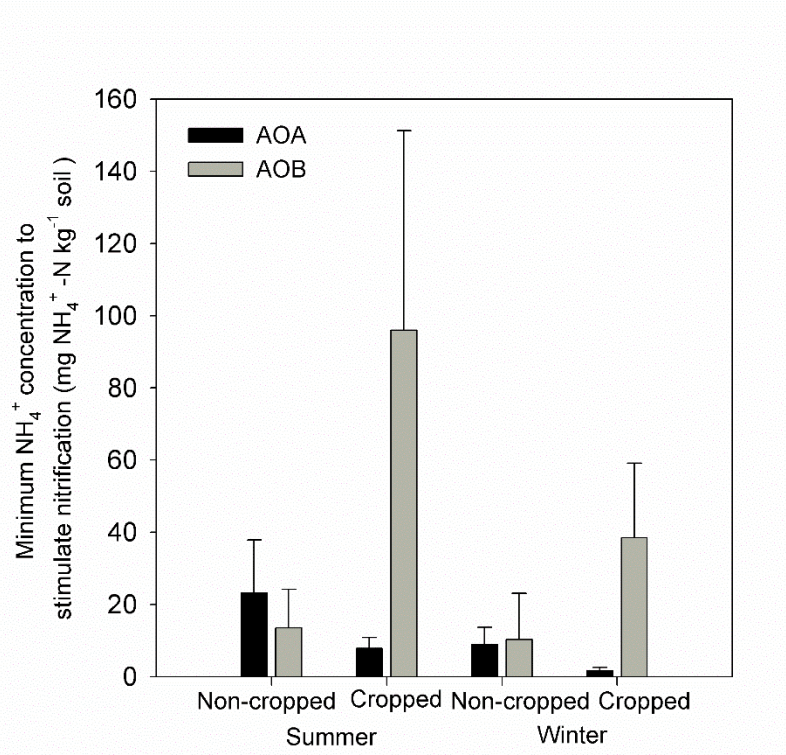

AOA
AOB
Minimum NH_4^+ concentration to stimulate nitrification (mg NH_4^+ -N kg^{-1} soil)
160
140
120
100
80
60
40
20
0
Non-cropped
Cropped
Non-cropped
Cropped
Summer
Winter

Figure 2.5

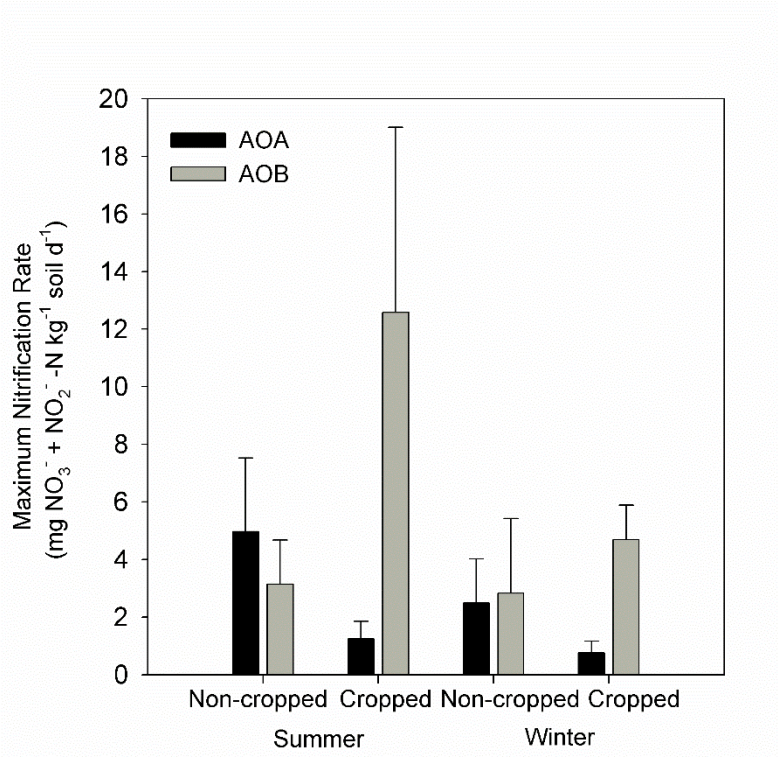

AOA
AOB
Maximum Nitrification Rate
(mg NO_3^- + NO_2^- -N kg^{-1} soil d^{-1})
0
2
4
6
8
10
12
14
16
18
20
Non-cropped
Cropped
Non-cropped
Cropped
Summer
Winter

Figure S2.1

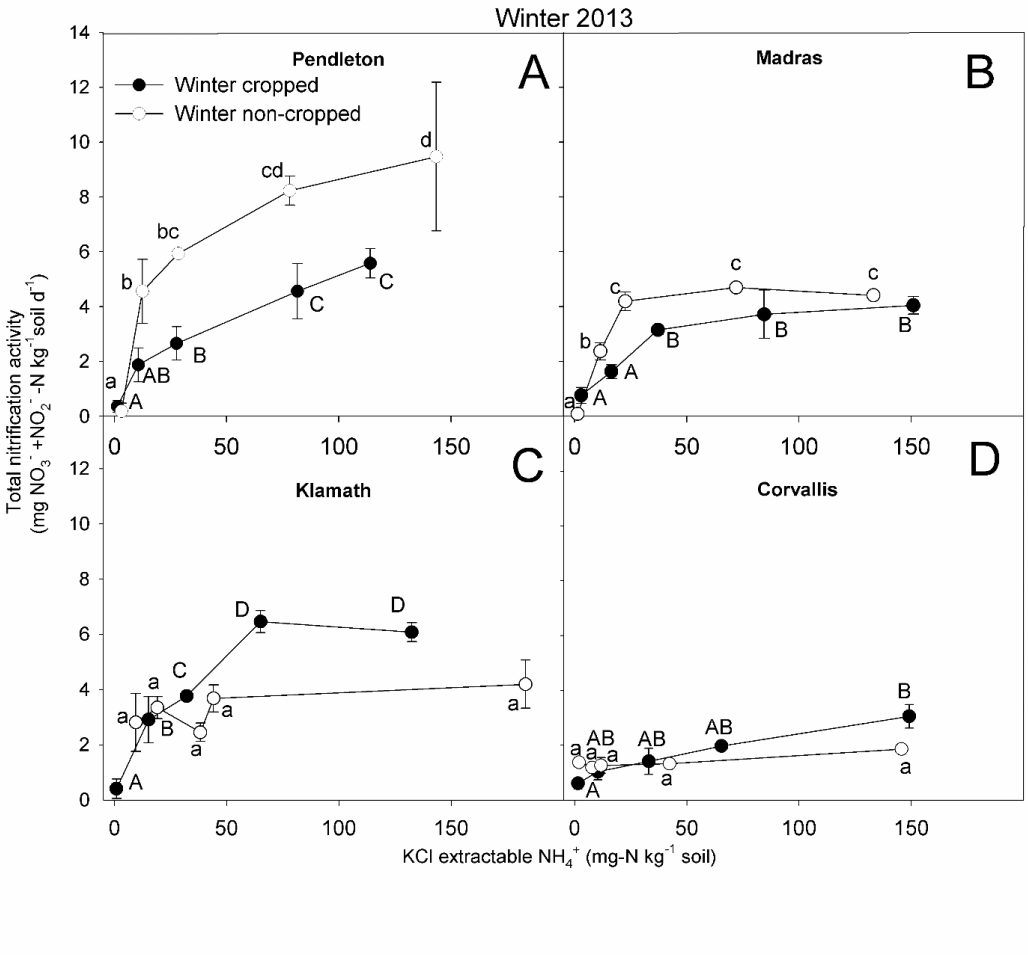
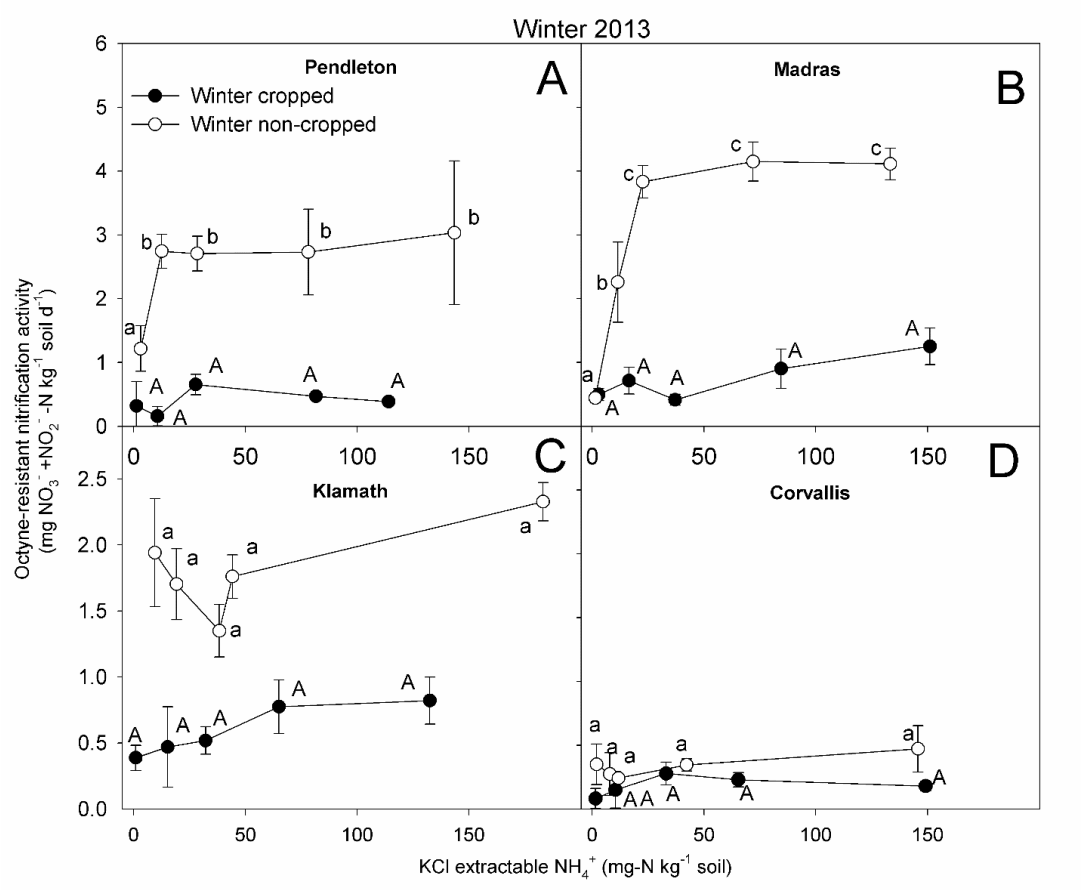

Winter 2013
Pendleton
A
Winter cropped
Winter non-cropped
Madras
B
Klamath
C
Corvallis
D
Total nitrification activity
(mg NO3- +NO2- -N kg-1 soil d-1)
KCl extractable NH4+ (mg-N kg-1 soil)

Figure S2.2


Winter 2013
Pendleton
Winter cropped
Winter non-cropped
A
Madras
B
Klamath
C
Corvallis
D
Octyne-resistant nitrification activity
(mg NO3- +NO2- -N kg-1 soil d-1)
KCl extractable NH4+ (mg-N kg-1 soil)

Chapter 3

Uncoupling of ammonia oxidation from nitrite oxidation: impact upon nitrous oxide production in non-cropped Oregon soils

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Abstract

The factors controlling the relative contributions of ammonia- (NH_3) oxidizing archaea (AOA) and bacteria (AOB) to nitrification and nitrous oxide (N_2O) production in soil remain unclear. A study was conducted to examine the contributions of AOA and AOB to nitrification, nitrite (NO_2^-) accumulation, and NO_2^--affected N_2O production in three non-cropped Oregon soils. Nitrification potential rates in the three soils ranged seven-fold from 0.15-1.08 μmol N g^{-1} d^{-1}, with AOA contributing 64-71% of the total activity. AOA- and AOB-driven NO_2^- accumulation represented 8-100% of total NO_2^- + NO_3^- accumulation, persisted over 48 h, and was accompanied by acetylene-sensitive, ammonium- (NH_4^+) stimulated N_2O production. Ammonium- and NO_2^--dependent N_2O production occurred when both AOA and AOB, or AOA alone were active. By adding the NO_2^--oxidizing bacteria, *Nitrobacter vulgaris*, to soil slurries to increase NO_2^--oxidizing capacity, both NO_2^- accumulation and N_2O production were prevented, while the overall rate of nitrification was unaffected. Yields of N_2O-N amounted to 0.05±0.01% of total NO_2^- + NO_3^--N accumulation in the presence of supplemental NH_4^+, and 0.28±0.11% in the presence of both supplemental NH_4^+ + NO_2^-. Regression analysis of the N_2O production against NO_2^- accumulation over 24 h revealed a positive, non-linear relationship for N_2O production by both AOA plus AOB and by AOA alone. Values of V_{max} ranged 12-fold from 0.05-0.62 nmol N_2O g^{-1} d^{-1}, and predicted K_m values for NO_2^- ranged 15-fold from 0.02-0.30 μmol NO_2^- g^{-1} soil. These findings provide new insights into the impact of NO_2^- accumulation in soils on N_2O production by both AOA and AOB,

and show that NO_2^- accumulation primarily drives N_2O formation in these soils, and increases N_2O yield by both AOA and AOB.

Introduction

Nitrification is the process whereby ammonia (NH_3) is oxidized sequentially to nitrite (NO_2^-) and nitrate (NO_3^-). The first step of nitrification is carried out by NH_3-oxidizing bacteria (AOB) and thaumarchaea (AOA) (Arp and Stein, 2003; Leininger et al., 2006; Vajrala et al., 2013). Several studies have shown that the process of NH_3 oxidation can be a major source of aerobically produced N_2O, and can contribute 36-57% of total N_2O production from soils (Kool et al., 2011; Wrage et al., 2001; Zhu et al. 2013). Whereas AOA and AOB are generally abundant and widely distributed in soils (Leininger et al., 2006; Prosser and Nicol, 2012; Taylor et al., 2012, 2013), few studies have examined the relative contributions of AOA and AOB to soil nitrification (Chen et al., 2013; Daebeler et al., 2015; Giguere et al., 2015; Taylor et al., 2010, 2013; Wessén et al., 2010; Lu et al., 2015). Furthermore, despite the activities of AOA and AOB having the potential to produce N_2O (Kozlowski et al., 2014; Poth and Focht, 1985; Santoro et al., 2011; Shaw et al., 2006; Stieglmeier et al., 2014; Stein, 2011), to our knowledge there is only one study in the literature that has examined the relative contributions of AOA and AOB to nitrifier-dependent N_2O production in soil (Hink et al., 2016). There is considerable interest in determining the factors that influence the proportion of NH_3 oxidized that is transformed to N_2O, and if the relative contributions of AOA and AOB

might influence the latter value (Jung et al., 2013; Mørkved et al., 2007; Shaw et al., 2006; Stieglmeier et al., 2014).

There is a growing body of evidence that aerobic N_2O production in soil may be associated with NO_2^- accumulation (Maharjan and Venterea, 2013; Venterea, 2007; Venterea et al., 2015). Several studies have demonstrated that NO_2^- accumulates in soil under conditions where NH_3-oxidizing activity is stimulated (Müller et al., 2006), and/or NO_2^--oxidizing activity is negatively affected by additions of urea (Burns et al., 1995; Chapman and Liebig, 1952; Ma et al., 2015; Shen et al., 2003; Venterea, 2007) or anhydrous NH_3 (Maharjan and Venterea, 2013; Venterea et al., 2015). Production of N_2O by AOB has been demonstrated to be stimulated by NO_2^- (Shaw et al., 2006) and most AOB possess both nitrite reductase (NirK) and nitric oxide reductase (NorB) which enable them to carry out NO_2^--dependent N_2O production (Cantera and Stein, 2007; Kozlowski et al., 2014). In the case of AOA, although they possess the putative gene encoding for NirK (Spang et al., 2012; Walker et al., 2010), a gene encoding for nitric oxide reductase has not been identified (Hatzenpichler, 2012, Kozlowski et al., 2016). Although it has been suggested that AOA can abiologically produce N_2O, the isotopic signature of N_2O produced from AOA enrichments suggests that NO_2^- is involved in N_2O production (Jung et al., 2013; Stieglmeier et al., 2014), and a positive relationship was observed between NO_2^- concentration and N_2O production by marine AOA enrichment cultures (Santoro et al., 2011).

Nonetheless, only one study has examined the relative importance of AOA and AOB driven NH_3 oxidation to N_2O production (Hink et al., 2016), and no study has

examined the importance of NO_2^- accumulation on AOA- and AOB-dependent N_2O production. Indeed, Hink et al. (2016) measured both AOA- and AOB-dependent N_2O production over a 28-d incubation of a cropped UK sandy loam soil and found KCl-extractable NO_2^- levels to be undetectable. I have identified Oregon soils with significant nitrification contributions from both AOA and AOB (Taylor et al., 2013, Giguere et al. 2015), and that also accumulate NO_2^- when nitrification is stimulated by NH_4^+ additions. In addition, with our recent discovery of the selective AOB inactivator, 1-octyne (Taylor et al., 2013), I have formulated the following objectives. These are: to determine to what extent AOA and AOB-driven NH_3 oxidizing activities contribute to N_2O production, and to determine the influence of NO_2^- accumulation on AOA and AOB-driven N_2O production.

Materials and Methods

Soil Sampling and Location

Three locations in Oregon (Pendleton, Madras, and Klamath Falls) were selected for this study and are described in detail elsewhere (Giguere et al., 2015). At each location, four replicate samples of cropped and non-cropped soils were collected from adjacent sites on the same soil series Pendleton (Walla Walla silt loam), Madras (Madras loam), and Klamath (Fordney loamy fine sand). A preliminary survey showed that non-cropped soils accumulated NO_2^- after nitrification was stimulated with 1 mM NH_4^+ additions as described elsewhere (Giguere et al., 2015; Taylor et al., 2012).

Soil slurry design

Soils were removed from 4ºC storage and composite 5-g portions of soil were added to 125-ml Wheaton bottles, wet to approximately field capacity, capped loosely with butyl stoppers, and pre-incubated at room temperature (21ºC) for 24 h. Each bottle received 15 ml of water, was amended depending on the experiment, and was capped tightly. Soil slurries were shaken continuously at 200 rpm at 25ºC. Gas samples for N_2O analysis were collected through the butyl stoppers at 24 and 48 h for all experimental incubations. Acetylene (6 μM_{aq}) was used to inhibit ammonia-oxidizing activity. Previous studies of these soils found no evidence of acetylene-insensitive nitrification, implying that all ammonia oxidation was chemolithotrophic (Giguere et al., 2015; Taylor et al., 2013). Octyne (4 μM_{aq}) was used to inactivate AOB activity, leaving AOA activity unaffected (Giguere et al., 2015; Hink et al., 2016; Lu et al., 2015; Taylor et al., 2013, 2015). Octyne was prepared by adding 40 µl liquid octyne to a Wheaton bottle with a 155 ml headspace, with several glass beads and over-pressured with 100 ml air, and a 2.8 ml aliquot was added to each sample bottle.

Analysis of NO_2^-, NO_3^-, NH_4^+, pH and N_2O

Initial pH measurements were made in a 2:1 soil water slurry and ranged from 7.2-7.6. Concentrations of NO_2^- and NO_3^- were determined as described elsewhere (Miranda et al., 2001; Taylor et al., 2013). Briefly, aliquots of soil slurries were sampled from sealed Wheaton bottles, centrifuged, and were immediately analyzed. Nitrite was measured colorimetrically using Griess reagents, and NO_3^- was measured using a vanadium reduction assay in which NO_3^- is reduced to NO_2^- and the total NO_2^-+ NO_3^- measured (Miranda et al., 2001). The NO_3^- concentration was calculated as the difference

between NO_2^- + NO_3^- and NO_2^- accumulations. Nitrification rates were calculated as the net accumulation of NO_2^- + NO_3^- above the acetylene controls. Detection limits for NO_2^- were 0.02 µmol NO_2^- g^{-1} soil, and 0.05 µmol NO_3^- g^{-1} soil for NO_3^-.

NH_4^+ extractions were conducted independently from NO_2^- or NO_3^- by extracting 5 g portions of soil in 15 ml 2 M KCl for 1 h. Extracts for NH_4^+ analysis were frozen until analysis and measured colorimetrically as described by Mulvaney et al (1996).
N_2O concentration in the gas phase was determined using a Varian Model 3700 gas chromatograph equipped with an electron capture detector as described previously (Mellbye et al., 2016). Total N_2O production from the soil was calculated as described by Tiedje (1994) using the equation

$$M = C_s(V_g + V_l * \alpha) \quad [1]$$

where, M is total N_2O, C_s is N_2O concentration in the gas phase, V_g is total gas volume, V_l is volume of the liquid and α is the Bunsen absorption coefficient for N_2O at 25ºC (0.544). The detection limits for N_2O production were 0.015 nmol g^{-1} soil. Rates of N_2O formation were calculated as the difference between the acetylene control N_2O levels and N_2O accumulation at 24 h and 48 h. N_2O yields were calculated using the equation

$$\frac{N_2O-N}{(NO_2^- -N + NO_3^- -N)} \quad [2]$$

Incubations to establish the impact of NH_4^+, and NO_2^- on N_2O production by AOB+AOA and AOA alone.

An experiment was conducted to examine the effect of supplemental NH_4^+ and NO_2^- on nitrification activity and N_2O production by the combination of AOA + AOB (-

octyne) and by AOA alone (+octyne). Soil slurry incubations for each of the three soils were conducted in the presence or absence of supplemental 1mM NH_4^+ and in the presence or absence of supplemental 1mM NO_2^-. NO_2^- and NO_3^-, concentrations were measured at 0, 6, 24, and 48 h. Subtraction of the octyne resistant rate from the rate measured in the minus octyne treatment provides the rate attributed to AOB.

Using *Nitrobacter vulgaris* to prove NO_2^- accumulation is required for N_2O production.

Experiments were conducted using *Nitrobacter vulgaris* to either prevent NO_2^- accumulation, or reduce pre-formed NO_2^- levels and assess the impact on N_2O formation. *N. vulgaris* was grown in mineral salts media as described elsewhere (Spieck and Lipski, 2011). Cells were harvested after consuming 30 mM NO_2^- and reaching stationary phase (OD_{600} = 0.07) by centrifuging 500-ml portions (10,000 g, 20 min). Cells were re-suspended in 50 ml of 2.5 mM sodium phosphate buffer, pH 7.5, and centrifuged and rinsed three times. Cells were concentrated 10-fold (500 ml to 50 ml) and 1-ml portions were added to each soil slurry, either at the beginning of the incubation or after 24 h, to achieve a final density equivalent to the initial density of the stationary phase culture (OD_{600} = 0.07). Samples for NO_2^- and NO_3^- analysis were taken at 0, 24, and 48 h. When NOB were added at 24 h, a sample was also taken 1 h later. Portions of heat-killed *N. vulgaris* were used as controls to determine if there were any abiotic effects of adding NOB to the levels of NO_2^- and N_2O.

Determination of abiotic N_2O production potential

An independent experiment was conducted to look for evidence for abiotic N_2O production in sterile soil samples using a range of NO_2^- concentrations as previously described (Harper Jr. et al., 2015; Heil et al., 2015; Ni et al., 2011; Zhu-Barker et al., 2015). Soil (5 g) was added to 125-ml Wheaton bottles, loosely capped, autoclaved at 120ºC for 20 min, and subsequently incubated at room temperature for 24 h. This was followed by a second autoclaving treatment. After cooling, portions of soil were amended with either 15-ml aliquots of deionized water or of 1 mM NO_2^- with 1 mM NH_4^+. NO_2^- was measured at 0, 24, and 48 h. There was no measureable production or consumption of NO_2^-, or production of N_2O.

Statistics

Analysis of N_2O formation in response to NH_4^+, and NO_2^-, were analyzed using a multi-way ANOVA analysis. Interactions were detected, and treatment effects within each soil were analyzed independently. Differences in NO_2^-, NO_3^-, and N_2O accumulations between treatments at a specific sampling time were determined using multi-way ANOVA. Significant differences in NO_2^- and NO_3^- accumulation measured over time were determined using repeated measures ANOVA. Statistical analysis was performed using Statgraphics 17.1.06. Data in text are given as mean ± standard deviation. Predicted values from regression analysis are given ± standard error. Non-linear regression analysis was performed with Michaelis-Menten kinetics using the equation

$$V = \frac{V_{max}[S]}{K_m + [S]} \quad [3]$$

Where V is the rate of the reaction (N_2O production), V_{max} is the maximum potential rate (maximum rate of N_2O production), [S] is NO_2^-concentration (NO_2^-), and K_m is the concentration of substrate that supports one half the V_{max} rate of N_2O production. Data for the regression analysis was compiled from several different experiments.

Results

Rates of nitrification and NO_2^- accumulation

Background KCl-extractable NH_4^+ concentrations ranged from 0.17- 0.23 µmol NH_4^+ g^{-1} soil among the three soils. Rates of nitrification in the three soils were determined in the absence (-NH_4^+) and presence (+NH_4^+) of added NH_4^+. Total rates of nitrification (NO_2^- + NO_3^- accumulation) in -NH_4^+ treatments ranged from 0.08-0.44 µmol NO_2^- +NO_3^- g^{-1} soil d^{-1}, and the contributions of AOA (+octyne) ranged from 13-100% of the total nitrification activity across the three soils (Table 3.1). Total rates of nitrification (-octyne) were stimulated 1.3- to 3.5-fold by +NH_4^+ treatments, and AOA-dependent nitrification rates were stimulated 1.3- to 1.6-fold by the +NH_4^+ treatment across the three soils. The rates of nitrification in the +NH_4^+ treatment varied from 0.15-1.08 µmol NO_2^- + NO_3^- g^{-1} soil d^{-1} across the three soils, with the AOA contributions ranging from 64-71% of the total activity (Table 3.1).

Dynamics of NO_2^- and NO_3^- accumulation

Nitrite accumulated during incubation of all three soils in both the presence and absence of NH_4^+ and of octyne, and the fraction of NO_2^-+NO_3^- that accumulated as NO_2^- varied across the soils (Table 3.1). In +NH_4^+ treatments, the fraction that remained as

NO_2^- ranged from 8-100% after 24 h, whereas the proportion of NO_2^- that accumulated in the -NH_4^+ treatment ranged between 1-5% in Pendleton and Klamath soils; in Madras soil the proportion ±NH_4^+ was 100% (Table 3.1). Accumulations of NO_2^- were lower in the -NH_4^+ treatment compared to the +NH_4^+ treatment, being two-fold lower at 24 h compared to +NH_4^+ treatments among the three soils (Data not shown, p=0.02).

Because the proportions of NO_2^- accumulation varied among the soils, a more detailed temporal study of the nitrification response to NH_4^+ was conducted (Fig. 3.1). In +NH_4^+, -octyne treatments, NO_2^- significantly accumulated in all three soils after 6 h of incubation to a mean of 0.08±0.03 µmol NO_2^- g^{-1} soil (p=0.001, Fig 3.1 A), and to a lesser extent in the +octyne treatment to 0.05±0.02 µmol g^{-1} soil (p=0.03, Fig 3.1 B).

The dynamics of NO_2^- accumulation in the -octyne treatment varied among the three soils. Nitrite accumulated to its highest concentration at 6 h in Pendleton (0.13±0.003 µmol g^{-1} soil) and Klamath (0.06±0.01 µmol g^{-1} soil), and subsequently declined over 48 h. In Madras soil, NO_2^- concentrations continued to increase between 6 and 24 h, and persisted over the 48-h incubation (Fig. 3.1A). Dynamics of NO_2^- accumulation were similar in the ±octyne treatments in all soils.

Nitrate accumulated in all three soils, illustrating that NO_2^- oxidation was occurring, and that the NO_2^- pool was in flux; however, there were differences among the soils in the appearance of NO_3^- accumulation. In the -octyne treatment, NO_3^- accumulation was observed at 24 h in Pendleton and Klamath soils, whereas 48 h was required for NO_3^- to accumulate in Madras soil (Fig. 3.1, Table 3.1). In the +octyne treatment, the timing of NO_3^- accumulation was similar to -octyne (Fig. 2.1). The data

show that the overall rates of NO_2^-+NO_3^- accumulation were generally linear over 48 h, whereas NO_3^- accumulation generally increased over the 48 h incubation.

Effect NH_4^+ and NO_2^- on N_2O production

I characterized to what extent additions of NH_4^+, and NO_2^- influenced N_2O production (Fig. 3.2). Multi-way ANOVA revealed significant stimulation of N_2O production by NH_4^+ and NO_2^-; however, soil x NH_4^+ ($p=0.027$) and soil x NO_2^- ($p\leq0.001$) interactions were detected. Thus, N_2O production was analyzed independently for each soil with and without octyne. Acetylene-sensitive N_2O production in -octyne treatments was stimulated by additions of 1 mM NH_4^+ alone: 7-fold in Madras ($p<0.001$) and 3.8-fold in Pendleton ($p=0.068$) soils, but not in Klamath soil ($p=0.329$). The addition of supplemental 1 mM NO_2^- alone also stimulated N_2O production within each soil (-octyne) about 10-fold, from 0.04±0.01 to 0.41±0.34 nmol g^{-1} soil d^{-1} ($p<0.001$). The combination of 1 mM NO_2^- and 1 mM NH_4^+ further stimulated N_2O production in all three soils (-octyne) ($p<0.001$) to an average of 0.89±0.56 nmol g^{-1} soil (Fig. 3.2). AOA-dependent N_2O production was detected in +octyne treatments, being significantly lower compared to -octyne treatments across the three soils ($p=0.01$). In the +octyne treatment, N_2O was not significantly stimulated by the addition of NH_4^+ alone (Fig. 3.2, $p>0.167$), whereas the addition of NO_2^- alone significantly stimulated N_2O production 6.5-fold in two of three soils ($p<0.001$), but not in the soil from Madras ($p=0.216$). Production of N_2O was further stimulated in the presence of octyne within all soils by the addition of a combination of NH_4^+ and NO_2^- to an average of 2.7-fold above NO_2^- alone to 0.66±0.5 nmol g^{-1} soil (Fig. 3.2, $p< 0.020$). When NO_3^- was added in place of NO_2^- there was no

significant stimulation of N_2O production after 1 mM NO_3^- additions to any soil (data not shown, p=0.404). Sterile soils incubated in the presence of NH_4^+ and NO_2^- did not produce N_2O (data not shown, p=0.395).

N_2O Yield

Yields of N_2O based on the data shown in Figure 2 were calculated as N_2O-N accumulation divided by the accumulation of NO_2^- +NO_3^--N in the presence of 1 mM NH_4^+ with and without 1 mM NO_2^- (Table 3.2). The yields (expressed as percentages) were significantly higher in +NO_2^- than in -NO_2^- treatments across all three soils (p=0.011). In the -octyne, +NH_4^+ treatment, where both AOA and AOB contribute to nitrification and N_2O production, N_2O yields were 0.05±0.01% in -NO_2^- and 0.28±0.11% in +NO_2^- treatments. In the +octyne, +NH_4^+ treatment, the yields were 0.06±0.03% in -NO_2^- and 0.22±0.15% in +NO_2^- treatments.

The N_2O yield for the AOB contribution to N_2O production was calculated as the difference between the total N_2O production and the octyne-resistant fraction of N_2O production. The N_2O yield for AOB was 0.06±0.01% in the -NO_2^- treatments and 0.25±0.07% in the +NO_2^- treatments across the three soils (Table 3.2). There were no significant differences in the N_2O yields between AOA and AOB activities with or without supplemental NO_2^- for the average across the three soils (p=0.941, Table 3.2). Statistical analysis was unable to detect differences in N_2O yields *among* the three soils, however, there were differences within individual soils between the N_2O yields of AOA and AOB. In the presence of NH_4^+ and NO_2^-, AOA yield (0.36±0.06%) was significantly higher than AOB (0.17±0.07%) (p=0.023) in Pendleton soil, whereas in Madras soil,

AOA yield (0.09±0.03%) was lower than AOB (0.28±0.05%) ($p=0.001$). When only NH_4^+ was added, the N_2O yield was higher for AOB (0.06±0.02%) than AOA (0.03±0.01%) in Pendleton soil ($p=0.014$).

Influence of preventing NO_2^- accumulation or removing pre-accumulated NO_2^- on N_2O production by increasing the NO_2^--oxidizing potential (NOP) of soil slurries with *Nitrobacter vulgaris*

Accumulation of NO_2^- was successfully prevented by the addition of *N. vulgaris* (+NOB). In the -NOB treatments, NO_2^- accumulated to 0.14±0.02 μmol g^{-1} soil in Pendleton, 0.22±0.02 μmol g^{-1} soil in Madras, and 0.04±0.003 μmol g^{-1} soil in Klamath soils (Fig. 3.3). The reduction of NO_2^- concentrations to below the detection limit (0.02 μmol g^{-1} soil) was significant within each of the three soils ($p\leq0.001$). The +NOB treatment significantly reduced N_2O production from a mean of 0.08±0.02 nmol g^{-1} soil ($p<0.014$) to a concentration not significantly different from acetylene control N_2O concentrations. There were indications in the Klamath soil of NO_2^--independent N_2O production accumulating to ~25% of the -NOB treatment (Fig. 3.3C). Corresponding with enhanced NO_2^--oxidizing capacity, NO_3^- significantly increased within each of the three soils ($p\leq0.01$) demonstrating that the majority of NO_2^- was oxidized to NO_3^- by supplementing the NO_2^--oxidizing capacity with *N. vulgaris* (Fig. 3.3). There were no significant differences in NO_2^- or N_2O production between -NOB treatments and those amended with heat-killed *N. vulgaris* (data not shown, $p>0.05$).

I also considered the possibility that the effect of NO_2^- accumulation on N_2O production might require only a *transient* accumulation of NO_2^-. Experiments were

conducted with soils that were incubated for 24 h without NOB addition to allow NO_2^- accumulation and N_2O production. Then, NOB were added to consume the NO_2^- that had accumulated. Introduction of NOB to soil slurries at 24 h reduced the NO_2^- pool to below the detection limit within 1 h ($p \leq 0.001$), and effectively stopped further accumulation of N_2O between 24 and 48 h within each soil. ($p > 0.05$, Fig. 3.4). By allowing NO_2^- to accumulate before removing the NO_2^- pool, I demonstrated there was no NO_2^--dependent induction of a NO_2^--independent mechanism of N_2O formation. Although Klamath soil showed some N_2O production when NOB were added at the beginning of the experiment, N_2O production was completely prevented when NOB were added at 24 h.

Regression Analysis

Data from several different experiments were compiled to reveal a positive relationship between NO_2^- accumulation and N_2O production during 24-h incubations (Fig. 3.5). These data were fit to the Michaelis-Menten equation using non-linear regression to determine K_m and V_{max} for NO_2^--stimulated N_2O production (Table 3.3). For the Pendleton soil, this resulted in a predicted K_m value (half-saturation concentration) of 0.30±0.07 μmol NO_2^- g^{-1} soil and a V_{max} (predicted maximum rate of N_2O production) of 0.62±0.07 nmol N_2O g^{-1} soil d^{-1} ($R^2=0.86$, $p \leq 0.001$). For Madras soil, a three-fold lower K_m of 0.08±0.04 μmol NO_2^- g^{-1} soil was determined, and a V_{max} value of 0.08±0.02 nmol N_2O g^{-1} soil d^{-1} ($R^2=0.51$, $p \leq 0.001$). In Klamath soil, analysis revealed K_m and V_{max} values more similar to Madras than Pendleton soils, with a K_m value of 0.04±0.02 μmol NO_2^- g^{-1} soil and a V_{max} value of 0.07±0.02 nmol N_2O g^{-1} soil d^{-1} ($R^2=0.37$, $p \leq 0.001$).

Non-linear regression of the +octyne treatment of Pendleton soil (R^2=0.54) predicted a V_{max} of 0.15±0.03 nmol N_2O g^{-1} soil d^{-1} ($p \leq 0.001$) and a K_m value of 0.02±0.02 µmol NO_2^- g^{-1} soil. Analysis of +octyne treatment of Madras soil (R^2=0.57) predicted a non-significant K_m value of 0.02±0.02 µmol NO_2^- g^{-1} soil and a V_{max} of 0.05±0.01 nmol N_2O g^{-1} soil d^{-1}. Plus octyne data from Klamath soil was excluded from the regression analysis as NO_2^- concentrations did not accumulate above ~0.05 µmol g^{-1} soil.

Discussion

NO_2^- accumulation

Although NO_2^- accumulation in soil has been observed for decades (Chapman and Liebig, 1952, Müller et al., 2006; Nelson 1982), it usually accumulates under specific conditions that cause NO_2^- oxidation to be suppressed relative to NH_4^+ oxidation. For example, additions of either high levels of urea or anhydrous NH_3 stimulate NH_3 oxidation and also induce transient pH increases that inhibit NO_2^- oxidation (Burns et al., 1995; Maharjan and Venterea, 2013). In our non-cropped Oregon soils, however, I observed that NO_2^--oxidizing activity was "under capacity" even when NH_3-oxidizing capacity was limited by NH_4^+ availability, and when the contribution of AOB to total nitrification activity was specifically inactivated with octyne. Although I did not study specifically why NO_2^- oxidation was limiting relative to NH_3-oxidizing potential, it is well known that AOB are quite resistant to NH_4^+ starvation and retain their capacity to oxidize NH_4^+ after long periods of NH_4^+ deprivation (Bollmann et al., 2005; Elawwad et al., 2013; Johnstone and Jones, 1988). Data on NOB starvation are limited, but

Nitrobacter winogradskyi has been shown to lose 80% of its NO_2^--oxidizing capacity after deprivation of NO_2^- for 6 d (Tappe et al., 1999). Also of interest, the three non-cropped Oregon soils used in our study displayed a range of NH_3-oxidizing capacities and accumulated NO_2^- to different degrees, further emphasizing the need for a better understanding of the reasons behind why NO_2^- oxidizing activity is limited in these soils and to expand our knowledge about the physiological ecology of soil-borne NOB in general. In non-cropped soils, NH_4^+ stimulated NO_2^- accumulation might occur if NO_2^- oxidizing capacity is compromised more by a period of NH_4^+ deprivation and/or soil stresses than is NH_3 oxidizing capacity.

In recent years, considerable amounts of new information have emerged about the genomics and physiologies of novel NOB isolates obtained from hot springs, tundra, and marine waters (Alawi et al., 2007, 2009; Koch et al., 2015; Lebedeva et al., 2011), and about NOB community composition/dynamics in wastewater treatment plants (Lücker et al., 2010; Pester et al., 2014; Sorokin et al., 2012). Furthermore, the recent discovery of a complete nitrifier, *Nitrospira inopinata,* and observations that comammox activity can lead to NO_2^- accumulation during NH_3 oxidation suggests comammox could contribute to NO_2^- accumulation (Daims et al., 2015). However, fewer studies have been devoted to soil NOB (Attard et al., 2010; Ke et al., 2013, Wang et al, 2015) and it remains unknown if, or to what extent, comammox contributes to soil nitrification.

In our study, NO_2^- accumulation ranged from 2 µM NO_2^- (the detection limit) to a maximum of ~200 µM. Nowka et al. (2015) characterized the NO_2^- oxidation kinetics of a diverse group of NOB isolates from the *Nitrospira* and *Nitrobacter* genera and found a

wide range of K_m values for NO_2^- oxidation to NO_3^- ranging from 9-544 µM NO_2^-. Clearly, at the lower range of NO_2^- accumulation detected in our study, the rates of NO_2^- oxidation could be substrate limiting if the soil NOB have similar K_m values to the laboratory cultures. On a cautionary note, however, the soil slurry experimental system employed in this study (1:5 soil: water ratio) may have contributed to NO_2^- accumulation by diluting the NO_2^- to a concentration that was rate limiting for the native soil NOB. Nonetheless, because I was successful at augmenting the NO_2^--oxidizing capacity of soil slurries by adding an NOB of moderately high K_m for NO_2^- (*N. vulgaris*, K_m = 49 µM), I do not believe that soil slurry dilution of NO_2^- would have been an insurmountable problem if the soil NO_2^--oxidizing capacities had been adequate in the first place.

NO_2^- accumulation and N_2O production

Several studies have suggested that nitrifier denitrification is a significant contributor to N_2O production in soil (Kool et al., 2011; Wrage et al., 2001; Zhu et al., 2013), and there is evidence for both NO_2^--dependent (nitrifier denitrification) and NO_2^--independent mechanisms of N_2O production by NH_3 oxidizers (Cantera and Stein, 2007; Jung et al., 2013; Kozlowski et al., 2014; Stieglmeier et al., 2014). Our novel approach of enhancing the NO_2^--oxidation capacity of soil slurries with *N. vulgaris* to prevent NO_2^- from accumulating above the limit of detection has provided conclusive evidence that, in two of three soils, N_2O production was completely dependent on NO_2^- accumulation. Because a minor fraction of NO_2^--independent N_2O production persisted in Klamath soil, the data also support the existence of a NO_2^--independent mechanism in this soil. Although Kozlowski et al. (2016) have proposed a new abiotic mechanism of AOA

driven N_2O production, I observed no NO_2^- independent N_2O production in two of three soils during AOA driven nitrification activity. However, because the NO_2^--independent rate of one soil was greatly surpassed (4-fold) when NO_2^- was allowed to accumulate, I conclude that the capacity for AOA-driven NO_2^--dependent N_2O production was greater in the three Oregon soils, at least under our study conditions.

Possible relationship between NO_2^- accumulation and the magnitude of the N_2O yield

There is considerable interest in determining the contributions of nitrification to N_2O production. N_2O yields reported in the literature generally ranged between 0.02-0.1% of $NO_2^- + NO_3^-$ produced (Hink et al., 2016; Jung et al., 2013; Mørkved et al., 2007; Santoro et al., 2011; Shaw et al., 2006; Stieglmeier et al., 2014; Zhu et al., 2013), with a few higher values ranging from 0.45-7.6% (Jung et al., 2013; Mørkved et al., 2007; Shaw et al., 2006). In our study, N_2O yields ranged from 0.04-0.08% across the three soils, with no significant differences between AOA and AOB yield values. However, when supplemental NO_2^- was added to soil slurries, the N_2O yields significantly increased for both AOA and AOB treatments to 0.16-0.30%, and statistically significant differences emerged between AOA and AOB yields in two of three soils. These results raise the question to what extent the N_2O yield values reported in previous studies might have been influenced by NO_2^- accumulation. For example, our results can be compared with Hink et al. (2016) who performed a four-week, NH_4^+-supplemented incubation of one UK soil and found a statistically significant difference between N_2O yields derived from AOA- (0.05%) and AOB-driven (0.09%) nitrification.

This yield range spanned that of our study when supplemental NO_2^- was not added, and where only one of three soils produced a significant difference between AOA and AOB N_2O yields.

Although the extent of NO_2^- accumulation could be one factor that influences N_2O yield, other factors that might influence the response of N_2O production to NO_2^- accumulation in a soil are the K_m and V_{max} values of NO_2^- for N_2O production. To our knowledge only two studies have measured and modeled the kinetic relationship between NO_2^- concentration and N_2O production rates (Venterea, 2007; Venterera et al., 2015). The K_m and V_{max} values for the response of N_2O production to added NO_2^- concentration in the five soils used in those two studies ranged 10-fold, as did the K_m and V_{max} values of our three soils. The wide range of K_m values for NO_2^--stimulated N_2O production might serve to highlight the variability of NH_4^+ oxidizer affinities for NO_2^- during nitrifier denitrification, and also raises the possibility that nitrifier denitrification might be stimulated by low accumulations of NO_2^-, particularly in cases where AOA-driven activity is a major contributor to overall nitrification activity. Despite our study being unable to precisely measure AOA K_m values for NO_2^--dependent N_2O production, the regression analysis suggests that very low concentrations of NO_2^- are needed to stimulate N_2O production by AOA. Finally, it is also possible that the contribution of NO_2^- accumulation to nitrifier-dependent N_2O production in soils may get overlooked because NO_2^- is unstable in unbuffered KCl or frozen soil extracts and can be underestimated, or even undetected, if analysis of extracts is delayed (Stevens and Laughlin, 1995; Takenaka et al., 1992).

Results from this study highlight the need for a much better understanding of soil NOB, and the conditions that impact their activity relative to the activity of NH_3 oxidizers. In addition, the role of NO_2^- accumulation in nitrifier denitrification by AOA and AOB needs to be further examined to determine if and when the accumulation of NO_2^- is a requirement for aerobic N_2O production in soils, and to determine how the relative contributions of AOA and AOB to soil nitrification activity, and their associated kinetic properties influence nitrifier denitrification.

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Table 3.1. Characteristics of the impact of NH_4^+ on the contributions of AOA and AOB to nitrification potential activities and NO_2^- accumulation in three Oregon soils over 24 h.

	No added NH_4^+				1 mM NH_4^+			
	Total[a] (-octyne)	AOA[a] (+octyne)	AOB[a] (octyne sensitive)	(NO_2^-/NO_2^- +NO_3^-)%[b]	Total[a] (-octyne)	AOA[a] (+octyne)	AOB[a] (octyne sensitive)	(NO_2^-/NO_2^- +NO_3^-)%[b]
Pendleton	0.44(0.02)	0.44(0.12)	0.01(0.2)	1(0.8)	1.08(0.1)	0.69(0.09)	0.39(0.07)	8(0.01)
Madras	0.08(0.04)	0.01(0.02)	0.07(0.02)	100(0.01)	0.15(0.01)	0.06(0.03)	0.08(0.01)	100(0.01)
Klamath	0.12(0.08)	0.11(0.07)	0.01(0.02)	5(7.8)	0.26(0.01)	0.16(0.07)	0.11(0.08)	13(0.02)

Rates (mean with standard deviation in parentheses, n=4) given as µmol NO_2^-+NO_3^- accumulated g^{-1} soil d^{-1}

[a] Nitrification potential activities for Total (AOA+AOB activity, -octyne), AOA activity (+octyne) and AOB activity (octyne sensitive).

[b] Percentage of total NO_2^-+NO_3^- accumulated

Table 3.2. The impact of supplemental NO_2^- upon N_2O-N yield from AOA and AOB-driven nitrification activity expressed as a percentage of total nitrification activity (N_2O-N/(NO_2^- +NO_3^--N))*.

	Total nitrification activity (-octyne)‡		AOA dependent activity (+octyne) ‡§		AOB dependent activity (octyne sensitive) ‡§	
	No NO_2^- added	1 mM NO_2^-	No NO_2^- added	1 mM NO_2^-	No NO_2^- added	1 mM NO_2^-
Pendleton	0.04(0.01)^{a}	0.28(0.05)^{b}	0.03(0.01)^{aA}	0.36(0.06)^{bA}	0.06(0.02) ^{aB}	0.17(0.07)^{bB}
Madras	0.06(0.02)^{a}	0.16(0.02)^{b}	0.06(0.03)^{aA}	0.09(0.03)^{aA}	0.06(0.05)^{aA}	0.28(0.05)^{bB}
Klamath	0.06(0.02)^{a}	0.39(0.05)^{b}	0.08(0.05)^{aA}	0.22(0.05)^{bA}	0.05(0.03)^{aA}	0.30(0.12)^{bA}
Mean	**0.05(0.01)**	**0.28(0.11)**	**0.06(0.03)**	**0.22(0.15)**	**0.06(0.01)**	**0.25(0.07)**

*The percentage of the total NO_2^-+NO_3^--N accumulation converted to N_2O-N in the presence of 1 mM NH_4^+ over 24 h. Given as mean (standard deviation, n=4).
‡Different lower case letters represent significant differences between with and without NO_2^- at each specific location.
§Different upper case letters represent significant differences between AOA and AOB dependent activity yields within no NO_2^- added or 1mM NO_2^- treatments ($p<0.05$).

Table 3.3. Kinetic parameters of N_2O production derived from the regression analysis of the relationship between NO_2^- concentrations and N_2O production rates from total AOA + AOB (- octyne) and AOA driven (+ octyne) nitrification activities.

		V_{max}[ad]	K_m[bd]	R^2	p-value[c]
Total (-octyne)	Pendleton	0.62(0.07)***	0.30(0.07)***	0.82	<0.0001
	Madras	0.09(0.02)***	0.10(0.06)ns	0.51	<0.0001
	Klamath	0.07(0.02)***	0.04(0.02)*	0.37	<0.0001
AOA (+octyne)	Pendleton	0.15(0.03)**	0.02(0.02)ns	0.54	0.0040
	Madras	0.05(0.01)*	0.02(0.03)ns	0.57	0.0004

[a] V_{max} values given as nmol N_2O g^{-1} soil d^{-1}.
[b] K_m values given as µmol NO_2^- g^{-1} soil.
[c] P-values given in the table represent significance of the model.
[d] Asterisks represent significance of predictions for V_{max} and K_m values. *p<0.05, **p<0.001, ***p<0.0001, ns nonsignificant p>0.05. Values are given as predicted with standard error in parentheses.
Regression analysis for Klamath AOA (+ocytne) activity was excluded.

Figure Legends

Figure 3.1. Accumulation of NO_2^- (left axis) or NO_3^- (right axis) in soil slurry incubations with 1 mM NH_4^+. Upper and lowercase letters represent significant differences in NO_2^- and NO_3^-, respectively, over time within each location ($p<0.05$). Panel A (-octyne) represents total AOA+AOB nitrification activity, panel B (+octyne) represents AOA activity. Error bars represent the standard deviation of the mean (n=4).

Figure 3.2: N_2O accumulation in the presence (black bars) or absence (grey bars) of 1 mM NH_4^+, the presence (left panels) or absence (right panels) of octyne, and presence (left pair) or absence (right pair) of 1 mM NO_2^-. Panel A, Pendleton over 24 h; Panel B, Madras over 48 h; Panel C, Klamath over 24 h. Different lowercase letters represent significant differences between $+NH_4^+$ and $-NH_4^+$ treatments. Different upper case letters represent differences between $+NO_2^-$ and $-NO_2^-$ treatments within each NH_4^+ treatment. Error bars represent the standard deviation of the mean (n=4)

Figure 3.3: Accumulation of NO_3^-, NO_2^-, and N_2O, in the presence of 1 mM NH_4^+, either in the presence or absence of *N. vulgaris* (NOB). The left y-axis represents NO_2^- or NO_3^- accumulation, and the right y-axis represents N_2O production. Panel A, Pendleton over 24 h; Panel B, Madras over 48 h; Panel C, Klamath over 24 h. Different lower case letters represent significant differences between +NOB and -NOB treatment. Error bars represent the standard deviation of the mean (n=4).

Figure 3.4: Accumulation of NO_2^- before and after *N. vulgaris* additions to soil slurry incubations, and the production of N_2O during 24 h following *N. vulgaris* additions. Panel A Pendleton; Panel B, Madras; Panel C, Klamath. Different upper case letters represent differences between -NOB and +NOB treatments. Bars represent the mean, error bars represent the standard deviation of the mean (n=4).

Figure 3.5: Relationship between accumulated NO_2^- concentration and N_2O production rate. Dark circles represent total AOB + AOA activity (-octyne), and open circles represent AOA (+octyne) activity. Panel A, Pendleton; Panel B, Madras; Panel C, Klamath. Dashed and solid lines represent non-linear regression fit for total (-octyne) AOA-dependent (+octyne) N_2O production, respectively. Asterisks represent significance of the regression **$p<0.001$, ***$p<0.0001$. Regression analysis for +octyne (AOA) data from Klamath was non-significant.

Figure 3.1

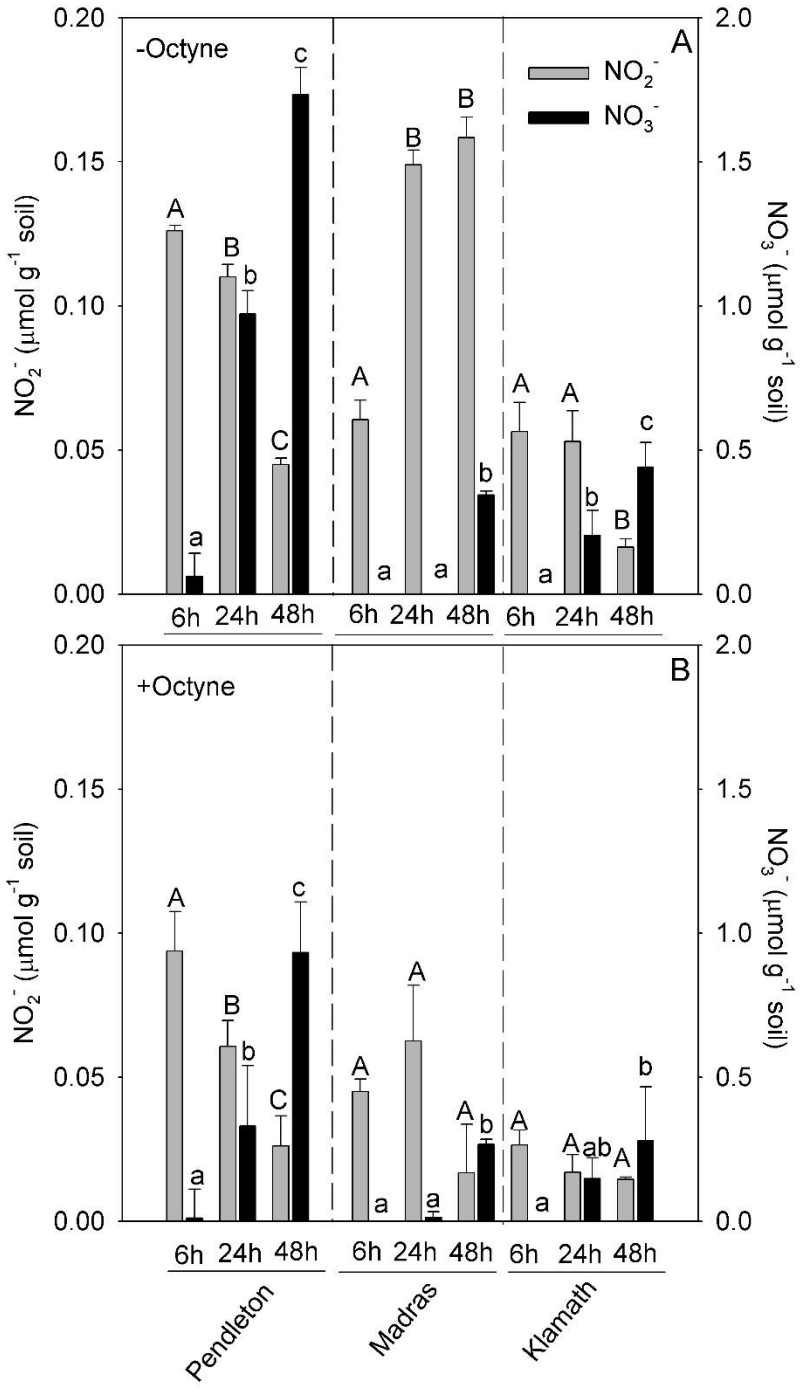

-Octyne
+Octyne
A
B
NO_2^-
NO_3^-
NO_2^- (µmol g^{-1} soil)
NO_3^- (µmol g^{-1} soil)
0.00
0.05
0.10
0.15
0.20
0.0
0.5
1.0
1.5
2.0
6h 24h 48h
Pendleton
Madras
Klamath

Figure 3.2

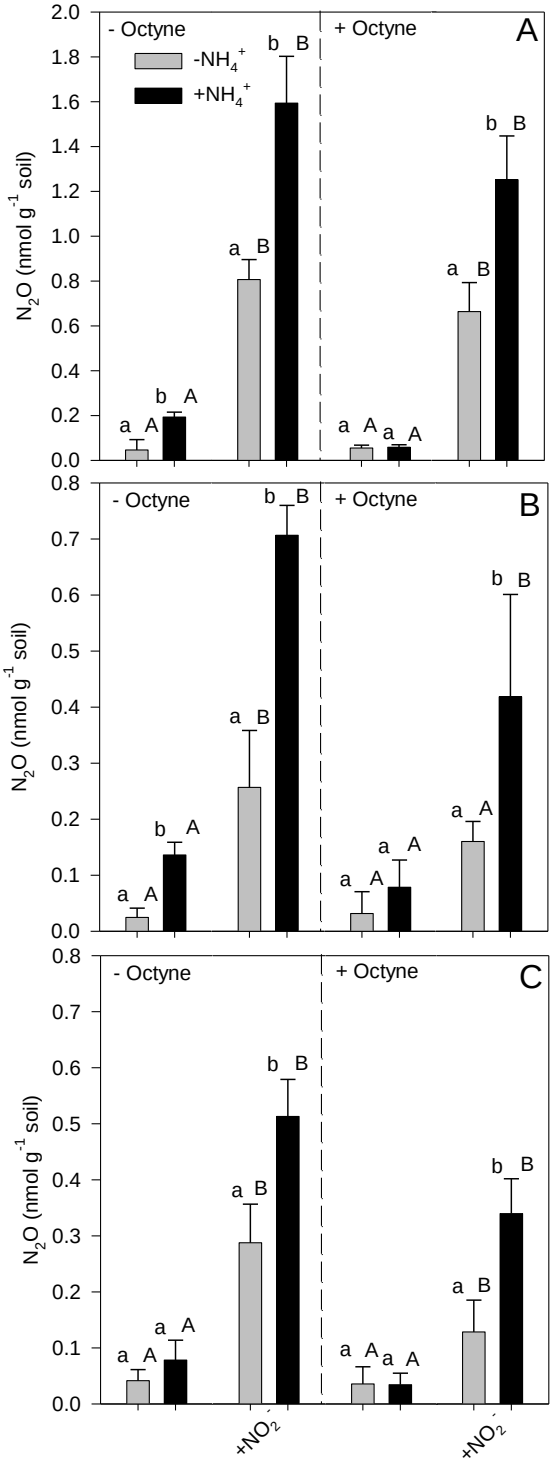


Figure 3.3

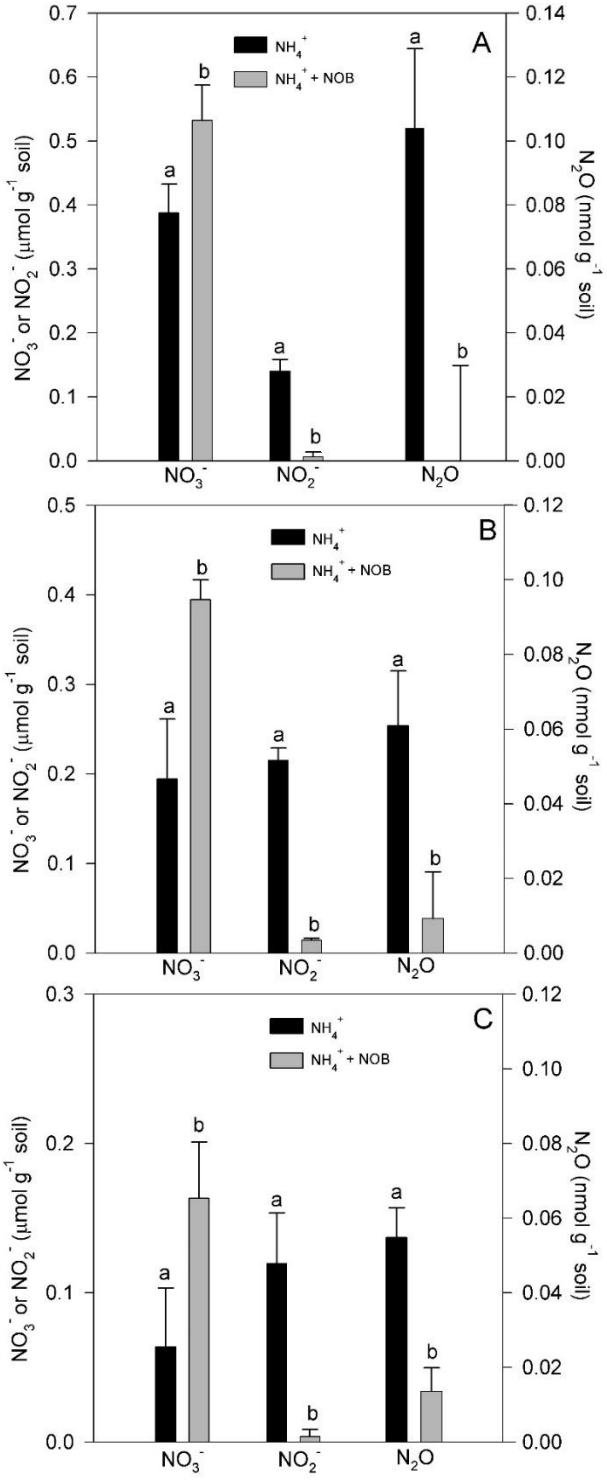

A
B
C
NH4+
NH4+ + NOB
NO3- or NO2- (µmol g-1 soil)
N2O (nmol g-1 soil)
NO3-
NO2-
N2O
a
b

Figure 3.4

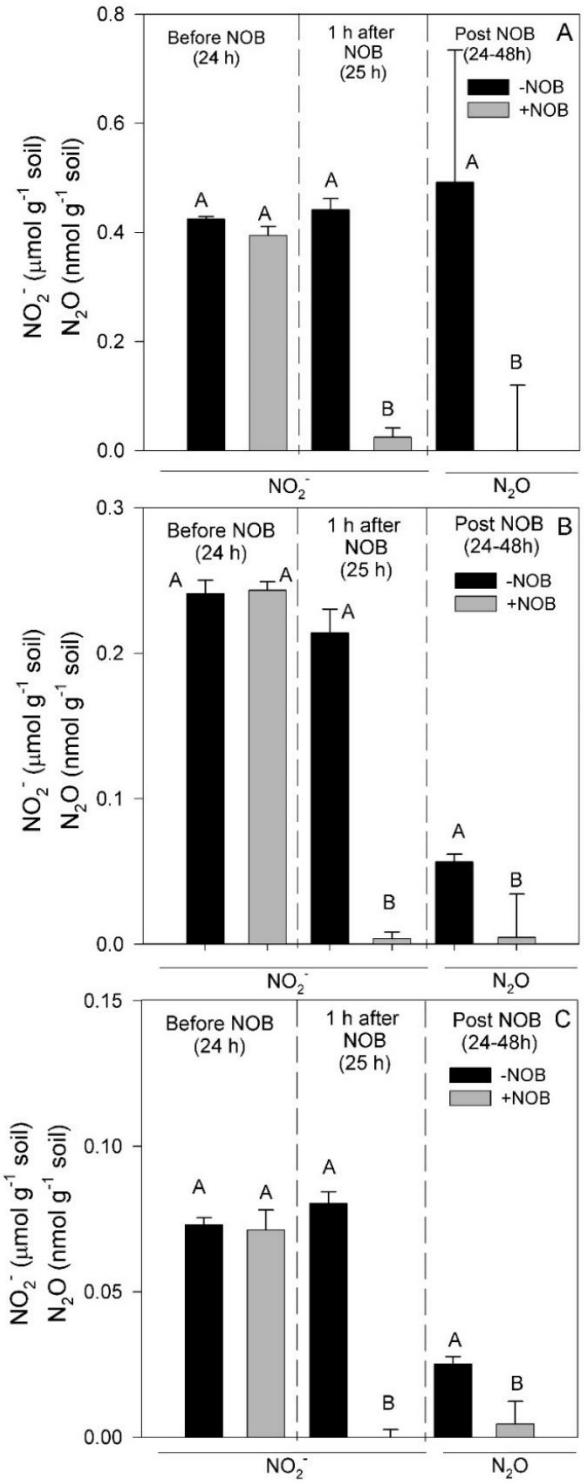
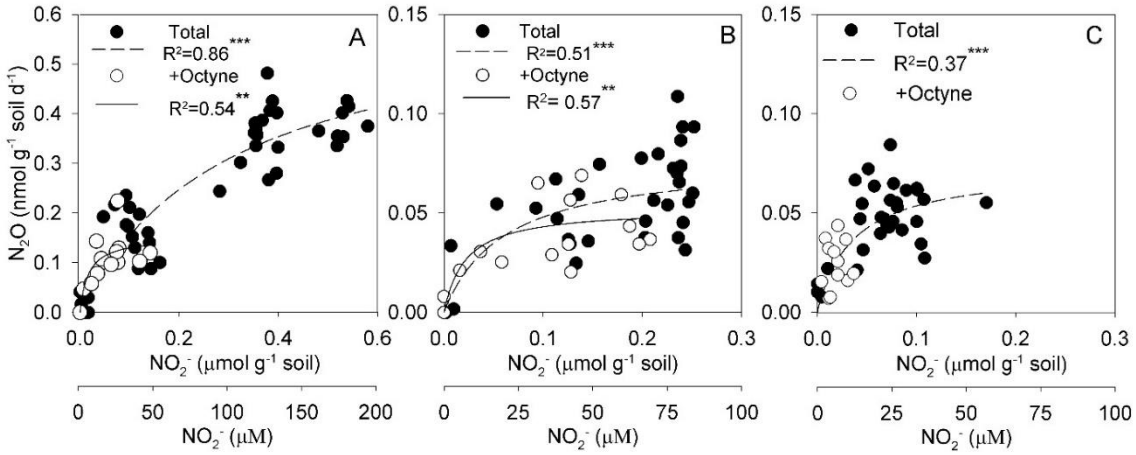

A
Before NOB (24 h)
1 h after NOB (25 h)
Post NOB (24-48h)
-NOB
+NOB
NO_2^- (µmol g^{-1} soil)
N_2O (nmol g^{-1} soil)
0.8
0.6
0.4
0.2
0.0
A
A
A
B
A
B
NO_2^-
N_2O
B
Before NOB (24 h)
1 h after NOB (25 h)
Post NOB (24-48h)
-NOB
+NOB
NO_2^- (µmol g^{-1} soil)
N_2O (nmol g^{-1} soil)
0.3
0.2
0.1
0.0
A
A
A
B
A
B
NO_2^-
N_2O
C
Before NOB (24 h)
1 h after NOB (25 h)
Post NOB (24-48h)
-NOB
+NOB
NO_2^- (µmol g^{-1} soil)
N_2O (nmol g^{-1} soil)
0.15
0.10
0.05
0.00
A
A
A
B
A
B
NO_2^-
N_2O

Figure 3.5


Total
R²=0.86***
+Octyne
R²=0.54**
A
Total
R²=0.51***
+Octyne
R²= 0.57**
B
Total
R²=0.37***
+Octyne
C
N2O (nmol g-1 soil d-1)
NO2- (μmol g-1 soil)
NO2- (μM)

Chapter 4

Short-term protein synthesis dependent and independent adaptation of soil nitrite oxidizing bacteria in response to NO_2^- accumulation

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Abstract

The factors controlling nitrite-(NO_2^-) oxidizing activity in response to and the accumulation of NO_2^- in soil remain unclear. A study was conducted to determine the driving factors behind NO_2^- accumulation, and recoupling of ammonia (NH_3) oxidation to NO_2^- oxidation. Acetylene sensitive, NO_2^- accumulation was observed in microcosm incubations of the all three soils in the absence of supplemental NH_4^+, was stimulated by the addition of 1 mM NH_4^+ ($p<0.001$) in two of three soils, but was not further stimulated by the addition of 2 mM NH_4^+ ($p>0.060$). The subsequent decline of the NO_2^- pool during the 48 h incubation indicated that NO_2^- oxidation kinetics may change in response to NO_2^- accumulation. The presence of bacterial protein synthesis inhibitors resulted in a significantly larger accumulation of NO_2^- in all three soils ($p<0.005$). The timing of the antibiotic effect varied from 9 to 48 h among the soils. Although no significant increases in NO_2^--oxidizing bacteria *nxr*A and *nxr*B gene abundances were detected ($p>0.110$), maximum NO_2^- consumption rates increased 1.8- to 1.9-fold in the treatment without antibiotics compared to no change with antibiotics ($p<0.050$); no significant changes were observed in the apparent half-saturation constant (K_m) values. In the presence of antibiotics in response to AB treatments the greater accumulation of NO_2^- also resulted in an increase in the rate of NO_3^- formation. This study demonstrates that the kinetics of NO_2^- oxidation in soil change, and that NOB can quickly undergo protein synthesis dependent adaptation in response to the accumulation of NO_2^-. Furthermore, and that inflation of NO_2^- accumulation with antibiotics has the potential to drive faster NO_3^- production. Demonstrating that both protein synthesis dependent and independent

mechanism may be used to increase NO_2^- consumption rates to match NH_3 oxidation rates and recouple nitrification.

Introduction

Nitrification consists of the biological oxidation of ammonia (NH_3) to nitrite (NO_2^-) that is carried out by (NH_3)-oxidizing archaea (AOA) and bacteria (AOB), combined with the oxidation of NO_2^- to nitrate (NO_3^-) carried out by phylogenetically diverse NO_2^--oxidizing bacteria (NOB). Much of recent research into soil nitrification has focused on the factors that control AOA and AOB contributions to nitrification (Giguere et al., 2015, 2017; Gurby-Rangin et al., 2010, 2017; Lu et al., 2015; Taylor et al., 2012, 2013, 2016). Few studies have examined the factors controlling NOB contributions to soil nitrification. Furthermore, much of the limited literature on soil NOB has focused on the distribution and diversity of soil NOB populations (Freitag et al., 2005, Pester et al., 2015, Poly et al., 2008 Wertz et al., 2008); few studies have directly measured soil NO_2^- oxidation rates or examined the response of NOB activity to situations where NH_3 oxidation is stimulated (Atttard et al., 2010, Ke et al., 2013, Wang et al., 2015).

Although NH_3 oxidation is thought of as the rate limiting step in soil (Kowalchuk and Stephen, 2001), there are instances of NO_2^- accumulation in soil that have been observed under specific conditions where NH_3-oxidizing activity was stimulated (Muller et al., 2006, Giguere et al., 2017) and/or when NOB activity was negatively affected by urea- or anhydrous NH_3-induced increase in soil pH (Burns et al., 1995; Chapman and Liebig, 1952; Ma et al., 2015; Shen et al., 2003; Venterea, 2007; Maharjan and Venterea,

2013; Venterea et al., 2015). To our knowledge however, nothing is known about the influence of NO_2^- accumulation on NO_2^--oxidation rates or on NOB physiological regulation.

The importance of this phenomenon lies in the observations from field and laboratory-based studies that NO_2^- accumulation in soils, associated with N fertilization, increases nitrifier-dependent N_2O production (Ma et al., 2015; Giguere et al., 2017; Maharjan and Venterea, 2013; Venterea, 2007; Venterea et al., 2015). Furthermore, our own work has shown that, when the NO_2^--oxidizing capacity of some Oregon soils was increased by adding *Nitrobacter vulgaris*, both NO_2^- accumulation and N_2O production were prevented (Giguere et al., 2017). In that study I reported evidence of NO_2^-, accumulating during NH_3 oxidation, reaching a maximum pool size after 9-24 h depending on the soil, and subsequently declining (Giguere et al., 2017). I hypothesized that i) stimulation of NH_3 oxidation rates contributes to uncoupling of NH_3-oxidation rates from NO_2^--oxidation rates ii) Protein synthesis by soil NOB is required to recouple the rate of NO_2^- oxidation with that of NH_3 oxidation and iii) protein synthesis changes the kinetic properties of NO_2^- consumption.

Methods and Materials

Study Soils

Three locations in Oregon (Pendleton, Madras, and Klamath Falls) were selected for this study and are described in detail elsewhere (Giguere et al., 2015). At each location, four replicates of cropped and non-cropped soils were sampled from adjacent

sites on the same soil series at Pendleton (Walla Walla silt loam), Madras (Madras loam), and Klamath (Fordney loamy fine sand). A preliminary survey showed that non-cropped soils accumulated NO_2^- after nitrification was stimulated with 1 mM NH_4^+ additions as described elsewhere (Giguere et al., 2015; Taylor et al., 2012).

Soil slurry assays and incubations to determine the effect of NH_4^+ concentration on NO_2^- and NO_3^- accumulation.

A soil slurry design was employed using four technical replicates of composited field replicates and described in further detail elsewhere (Giguere et al. 2017). Soil slurries were incubated in the absence or presence of 1 and 2 mM NH_4Cl. Aliquots were taken at 9, 24, and 48 h, and NO_2^- and NO_3^- were measured colorimetrically as described by Giguere et al. (2017). Subsequently, only 1mM NH_4^+ was used in the following experiments.

Effect of protein synthesis inhibiting antibiotics on the adaptive behavior of NO_2^- oxidation.

Soil slurries were incubated in the presence of 1 mM NH_4^+ and a combination of kanamycin and spectinomycin (hereafter, AB) at either 200/150, 400/300, 800/600 µg kanamycin/spectinomycin ml^{-1} soil slurry. Aliquots of slurry were taken at 3, 6, 9, 12, 24, 32, and 48 h, and analyzed for NO_2^- and NO_3^-. A concentration of 800/600 µg kanamycin/spectinomycin ml^{-1} soil slurry solution was required to allow NO_2^- accumulation to proceed at its initial rate beyond the time when the NO_2^- pool ceased to increase in the -AB treatments.

Kinetics of NO_2^- oxidation pre-and post-protein synthesis

A series of experiments were conducted to assess the effect of protein synthesis on the kinetic properties of NO_2^- oxidation. First, NO_2^- consumption was performed on pre-incubated soils, to establish initial values of the apparent V_{max} and K_m. Second, soil slurries were incubated in the presence and absence of AB for sufficient time to observe divergence of the NO_2^- accumulation in +AB versus -AB treatments, and third NO_2^- consumption properties were re-examined after the NO_2^- accumulation diverged between +AB and -AB treatment to determine if the AB treatments had affected the NO_2^--oxidizing properties of the slurries.

Nitrite consumption rates were determined to evaluate apparent V_{max} and K_m in soil slurries incubated with a range of NO_2^- concentrations (0 to 500 µM NO_2^-) in the presence of acetylene (0.02%) to eliminate all NO_2^- production from NH_3 oxidation. Aliquots were sampled every hour for up to 6 h. Linear regression analysis of NO_2^- consumption versus time was used to calculate the rates of NO_2^- oxidation. To compare NO_2^--consumption rates pre-and post-protein synthesis, soil slurries were incubated in the presence of 1 mM NH_4^+ for either 24 h (Pendleton and Klamath soils) or 48 h (Madras soil). Acetylene was injected into the slurries to inactivate NH_3 oxidation and, after all NO_2^- had been consumed, NO_2^- consumption rates were determined as described above.

Quantification of AOA *amoA*, AOB *amoA*, *Nitrobacter*-like *nxrA*, *Nitrospira*-like *nxrB* and per cell activity calculations

DNA was extracted from aliquots of soil slurries incubated in the presence of 1 mM NH_4^+ for 0, 24, and 48 h, using a standard method described previously (Griffiths et al., 2000). DNA standards were prepared from genomic DNA extracted from

Nitrososphaera viennensis (AOA *amoA*), *Nitrosomonas europaea* (AOB *amoA*), *Nitrobacter winogradskyi* (*nxrA*), and *Nitrospira defluvii* (*nxrB*). Primers and PCR conditions are listed in Table S1. PCR efficiencies were checked were performed as described by Mellbye et al., (2016). Theoretical rates of NO_2^- oxidation for *Nitrobacter* and *Nitrospira* were calculated from gene abundances, using the highest and lowest reported per-cell activities for each respective group obtained from the literature (Table S4.2) (Nowka et al., 2015). It was assumed that both *Nitrobacter* and *Nitrospira* contain two copies of the functional gene per genome.

Statistics

Statistical analysis was performed using Statgraphics 17.1.12 (Warrenton, VA). Determinations of significant differences in NO_2^- , NO_3^- concentrations and gene abundances were performed using repeated measured analysis of variance (ANOVA). When soil interactions were detected, soils were analyzed independently. Nonlinear regression analysis was performed using the Michaelis-Menten equation:

$$v = \frac{V_{max}[s]}{K_m + [s]}$$

where v = the rate of reaction, V_{max} = maximum rate of the reaction, K_m = concentration of substrate that gives a rate that is one half of V_{max}, and [s] is the substrate (NO_2^-) concentration. In the case of soils where non-constant variance was detected, inverse y-weighted regression analysis was used. Data given in text are mean ± standard deviation of the mean, and model parameters are given as mean ± standard error. It should be noted

that as this study was not conducted with a pure protein or single microorganism, we only were able to determine the apparent V_{max} and K_m values of the overall process.

Results

NH_4^+ effects on rates of nitrification and NO_2^- and NO_3^- accumulations

Rates of total nitrification were significantly stimulated by the addition of 1 mM NH_4^+ in Pendleton and Madras soil ($p<0.013$), but not in Klamath soil (Fig 4.1). Soil x time interactions were detected for both NO_2^- + NO_3^- ($p=0.001$) and NO_3^- only accumulations ($p=0.0002$), so NH_4^+ effects were analyzed independently for each soil. Ammonium stimulated the rates of total nitrification (NO_2^- + NO_3^- accumulation) 6-fold over the 0-24 h interval in Pendleton ($p=0.0001$) and 1.5 fold in Madras ($p=0.001$) soils. There was no stimulation of total nitrification in Klamath soil from the addition of 1 mM NH_4^+ ($p=0.221$). Supplemental 1 mM NH_4^+ stimulated NO_2^- accumulation by 4.6-fold in Pendleton soil over the 0-24 h interval ($p<0.001$), which was followed by a 5.6-fold decrease in the NO_2^- concentration between 24-48 h. In Madras soil, 1 mM NH_4^+ stimulated NO_2^- accumulation 4-fold over the 0-24 h interval ($p<0.02$), which was followed by a 1.6-fold decrease in the rate of NO_2^- accumulation during the 24-48 h interval. In Klamath soil there was no stimulation of NO_2^- accumulation, however the NO_2^- pool increased 1.3 fold ($p=0.01$) between 0 and 24 h, which was followed by a 3-fold decrease between 24 and 48 h (Fig 4.1). All NO_2^- accumulation was completely inhibited by acetylene (data not shown). Nitrate accumulated in both the presence and absence of supplemental NH_4^+ in all soils suggesting that NO_2^- was being oxidized to

NO_3^-. There were no significant differences in accumulation of NO_2^- ($p>0.06$), NO_3^- ($p>0.140$) or $NO_2^- + NO_3^-$ ($p>0.503$) between 1 and 2 mM NH_4^+ in any soil (Fig. 4.1).

Effects of bacterial protein synthesis inhibitors on NO_2^- and NO_3^- accumulation

After the addition of NH_4^+ a decrease in NO_2^- accumulation, accompanied by an increase in NO_3^- production over the time course of the incubation, suggested that NO_2^--oxidizing activity increased. I compared the responses of NO_2^- and NO_3^- accumulation in the presence (+AB) and absence (-AB) of bacterial protein synthesis inhibitors to query this phenomenon (Fig. 4.2). Total rates of nitrification ($NO_2^- + NO_3^-$) were not significantly different in +AB and -AB treatments over 48 h in the three soils ($p>0.07$). Short-term NO_2^- consumption rates (measured <6 h after initiation of the experiment) were not significantly different in the presence or absence of AB in any soil ($p=0.440$; Fig. S4.1). However, timing of the antibiotic effect on NO_2^- accumulation varied among the soils (Fig. 4.2). Furthermore, statistical analysis of the data revealed a soil x AB treatment interaction on NO_2^- accumulation ($p=0.004$), therefore the soils were analyzed independently. Nitrite had accumulated to a significantly higher concentration in +AB than in -AB treatment in Pendleton soil ($p=0.005$) after 9 h of incubation, after 24 h of incubation in Klamath soil ($p<0.0001$), and after 48 h in Madras soil ($p<0.0001$; Fig. 4.2).

Nitrate production was observed in the three soils in both the presence and absence of AB; again, soil x time interactions were detected ($p=0.0003$). Significant NO_3^- accumulation required at least 24 h of incubation. In Pendleton soil, NO_3^- concentrations were significantly higher ($p<0.002$) in -AB than in +AB treatments by 24 h, while 48 h of incubation was required in Madras ($p<0.0001$) and Klamath ($p<0.008$) soils.

Adaptation of NOB

Data on NO_2^- and NO_3^- pool dynamics presented in Fig. 4.2 suggested that the characteristics of NO_2^--oxidizing activity changed during the incubation, both in the presence and absence of AB. These changes in activity could be caused by: (a) an increase in soil NOB population density and/or (b) shifts in the kinetic properties of NO_2^- oxidation. qPCR analysis showed that AOB *amoA*, AOA *amoA*, *Nitrobacter nxrA,* and *Nitrospira nxrB* were present in all soils, and a repeated measures ANOVA showed there were no significant changes in gene abundances over the 48 h incubation ($p>0.110$; Fig. S4.2).

Assessment of initial NO_2^--oxidizing kinetics

Nitrite consumption curves were generated to assess if shifts had occurred in kinetic properties. Non-linear regression analysis of NO_2^--consumption curves generated from pre-incubated soil showed that V_{max} rates ranged 3-fold among the soils (Pendleton =1.13±0.08 µmol g^{-1} d^{-1}, Klamath =1.14±0.13 µmol NO_2^- g^{-1} d^{-1}; Madras = 0.36±0.03 µmol NO_2^- g^{-1} soil d^{-1}; Fig. 4.3). Apparent K_m values ranged 4.4-fold, with Pendleton and Madras soils possessing similar K_m values (34±13 and 24±6 µM NO_2^-, respectively; Fig. 4.3 A,B), whereas the K_m of Klamath soil was higher (151±37 µM NO_2^-; Fig 4.3 C). The maximum NO_2^- oxidation rates of the soils were 2.7-fold higher than maximum NH_3-oxidation rates in Pendleton soil, 2.4-fold higher in Madras soil, and 4.9-fold higher in Klamath soil ($p<0.0001$).

Assessment of NO_2^--oxidizing kinetics after incubation with and without AB

To assess if prevention or allowance of protein synthesis had any influence on V_{max} and K_m values, NO_2^--consumption rates were determined in soils that had been incubated for 24 h (Pendleton and Klamath) or 48 h (Madras), in the presence or absence of AB. To accomplish this, acetylene was added at 24 or 48 h to inactivate NH_3 oxidation, and NO_2^- consumption was monitored. In the case of Madras soil, the rate of NO_2^- consumption in the +AB treatment was less than the initial rate of pre-incubated soil implying that +AB had negatively affected the preexisting NO_2^--oxidizing properties of the soil during the 48-h incubation. As a consequence, I could not confidently make the ±AB comparison in Madras soil. As soon as NO_2^- was consumed below the detection limit (<2 uM) in Pendleton and Klamath soils, a range of NO_2^- concentrations were added to assess V_{max} and K_m values in both plus and minus AB treatments. V_{max} values increased in the -AB treatment of Pendleton (1.9-fold) and Klamath (1.8-fold) soils compared to the +AB treatment ($p<0.05$) where V_{max} values remained the same as the initial values (Fig. 4.4; $p>0.05$). The antibiotic treatment did not significantly affect K_m values in either Pendleton or Klamath soils ($p>0.05$).

Protein synthesis dependent and independent adaptation of NO_2^--oxidizing activity

Adaptive behavior of NO_2^- consumption was observed in all three soils; however, the manner of adaptation differed among the soils. In the case of Pendleton soil, NO_3^- production rates increased in both +AB and -AB treatments between the 9-24 h and 24-48 h intervals. Over the 9-24 h interval the rate of NO_3^- production in -AB treatment was 2.4-fold greater ($p=0.0002$) than in the +AB treatment. The rates of NO_3^- formation increased further during the 24-48 h interval by 4.3-fold in +AB and 5.8-fold in -AB

treatments ($p<0.0001$). By rearranging the Michaelis-Menten equation it was calculated that similar concentrations of NO_2^- (17 and 6 µM NO_2^-) would be required to support the 9-24 h NO_3^- production rates in the -AB and +AB treatments, respectively. The actual NO_2^- concentrations measured at 9 h were more than adequate to support the -AB and +AB rate (27±2.5 µM and 35±2.7 µM). In contrast, the concentration of NO_2^- required to support the NO_3^- production rates measured during the 24-48 h interval differed (38 µM -AB and 112 µM +AB treatments). The actual NO_2^- concentration in the -AB treatment had reached 44±3 µM at 24 h and 76 ±6.1 µM in +AB treatment. As predicted from the observed increase in V_{max}, by 48 h the NO_2^- concentration had declined to 14±3.4 µM in the -AB treatment. In contrast, the NO_2^- concentration continued to increase to 95±10 µM in +AB treatment, supporting the idea that protein synthesis independent adaptation of the secondary rate of NO_3^- formation can occur, provided that sufficient NO_2^- accumulates to meet the kinetic needs of the preexisting NO_2^--oxidizing capacity.

In contrast, in Klamath soil the rates of NO_3^- formation were linear over the 9-48 h interval, and were significantly different in -AB and +AB treatments ($p<0.06$). However, the NO_2^- concentration was 1.8-fold lower in -AB than in the +AB treatment at 24 h (16±2.1 versus 29±2.8 µM; $p=0.0005$), and 4-fold lower (11±1.0 versus 41±3.2 µM; $p<0.0001$) at 48 h. Again, this result demonstrates that if V_{max} increases, it reduces the NO_2^- concentration required to drive similar rates of NO_3^- production and causes the NO_2^- pool to decrease.

In Madras soil, although the ±AB treatment comparison could not be made, in the -AB treatment, NO_3^- accumulation increased 4.6-fold ($p=0.02$) between the 9-24 and 24-

48 h intervals. The concentrations of NO_2^- required to drive the observed rate of NO_3^- formation over the 9-24 h and 24-48 h intervals were 24 µM and 149 µM respectively. However, NO_2^- concentrations only reached 57-63 µM suggesting that V_{max} would need to increase to support the higher rates of NO_3^- formation. By using the initial K_m value and NO_2^- concentration at 24 h, and NO_3^- formation rates between 24-48 h, a V_{max} was calculated to be 1.5-fold higher than the initial V_{max} rate, suggesting that adaptation had occurred. Without a valid +AB control, however, the higher V_{max} cannot be unequivocally attributed to protein synthesis.

The proportion of NO_2^- plus NO_3^- that remained in the NO_2^- pool (NO_2^- / NO_2^- + NO_3^-) was significantly higher in +AB than in -AB treatments ($p<0.0001$). In further support of differences among the soils, soil x AB treatment ($p=0.01$) and time x AB treatment ($p=0.0005$) interactions were measured and soils were analyzed separately. In the -AB treatment, the ratio of NO_2^- / NO_2^- + NO_3^- significantly decreased between 9 h and 48 h for each of the three soils ($p<0.0002$), while in the +AB treatment, NO_2^- / NO_2^- + NO_3^- significantly decreased in Pendleton and Klamath soils ($p<0.008$).

Relationship between nitrifier functional gene abundances and uncoupling

Regression analysis revealed that the ratio of NOB functional gene abundances (*nxr*A + *nxr*B) relative to AOA+AOB *amoA* abundances was not related to the magnitude of the initial uncoupled state, but indicated that it might play a role in the recovery of NO_2^- oxidation capacity. A negative relationship was found between NOB:AOA+AOB functional gene ratios and NO_2^- /NO_2^- + NO_3^- ratio at 24 h ($R^2=0.42$); the relationship was not evident using the data at 9 h ($R^2= 0.01$) or 48 h ($R^2=0.23$; Fig. 4.5). There was a

strong positive linear (R^2=0.86) relationship between the abundances of *Nitrospira nxrB* and *Nitrobacter nxrA.* There were also positive relationships between AOB *amoA* and *Nitrobacter nxrA* (R^2=0.41) and between AOB *amoA* and *Nitrospira nxrB* (R^2= 0.31; Fig. 4.5). No other significant relationships were found.

Discussion

In the following sections, the data presented in this study will be placed into context with a range of literature directed at NOB physiology and at the accumulation of NO_2^- in soils. Few studies have focused on soil NOB and our data provides new insights into the factors controlling activity and physiological regulation of soil NOB.

NO_2^- concentration and soil NOB affinity for NO_2^-

To our knowledge, this study is the first to determine the response of soil NOB activity to NO_2^- additions, determining both apparent V_{max} and K_m of NO_2^- consumption. Apparent K_m values for NO_2^- consumption observed in this study ranged from 25-151 µM among the three soils, and aligns with values obtained from studies of NOB pure cultures and enrichments which possess K_m values for NO_2^- ranging from 49-544 µM NO_2^- for *Nitrobacter* and 9-27 µM NO_2^- for *Nitrospira* (Nowaka et al., 2015, Maxiner et al., 2006). Despite the high affinity K_m values reported for NO_2^- by *Nitrospira* isolates, other evidence suggests that some natural populations of *Nitrospira* are limited for NO_2^- even at concentrations higher than found in our study. For example, Gruber-Dorninger et al. (2015) showed that *Nitrospira* Cluster Ig grew faster when incubated with 1 mM NO_2^- than with 0.1 mM NO_2^-, raising the possibility that, the concentrations of NO_2^- that

accumulated in the soils (16-48 μM NO_2^-) overlap the apparent K_m and may have limited NOB activity. This would also explain why little NO_3^- formation occurs until NO_2^- accumulates to a concentration high enough to drive significant NO_2^--oxidizing activity. Although our experiments were conducted in soil slurries, which could have diluted soil NO_2^- relative to an intact whole soil system, when soils from this study were incubated at field capacity and nitrification activity stimulated by supplementing with 10 μmol NH_4^+ g^{-1} soil (Fig. S4.4), NO_2^- accumulated to values ranging from 0.025-0.1 μmol g^{-1} soil (50-245 μM NO_2^-) suggesting that NO_2^- accumulation is not simply an artifact of the soil slurry method.

In this study, evidence was obtained for NOB to quickly synthesize more NO_2^--oxidizing capacity when NO_2^- accumulated to low concentrations. Surprisingly, the role of NO_2^- in regulation of the physiology of NOB remains unexplored. In our study there was evidence to suggest that NO_2^- at relatively low (27 μM in Pendleton and 16 μM in Klamath), concentrations can induce protein synthesis suggesting that the induction of NXR synthesis might be promoted by concentrations lower than those required to support optimal NO_2^- oxidizing activity. Other evidence suggests that NOB retain a fraction of their NO_2^--oxidizing activity when grown on other substrates in the absence of NO_2^- (Starkenburg et al., 2008) and, *N. defluvii* retained NXR after 110 d of NO_2^- starvation and synthesized new protein within 8 d of NO_2^- (300 μM) addition (Lucker et al., 2010). Evidence from soil studies suggests that *Nitrobacter nxrA* transcript abundance increases within 0.5 to 3 h of rewetting a dry soil which was also associated with an increase in NH_3-oxidizing activity (Placella and Firestone, 2013).

Retention of NO_2^--oxidizing activity, and regulation of protein synthesis, and initial NO_2^--oxidizing activity

In this study I observed that the initial NO_2^--oxidizing capacity was 2.7- to 4.5-fold higher than NH_3-oxidizing activity which agrees with another study, where NO_2^--oxidizing potentials were up to an order of magnitude higher than NH_3-oxidizing potentials (Ke et al., 2013). One potential explanation for NO_2^- oxidation capacity being greater than NH_3-oxidizing capacity, could be that soil NOB have an insufficient affinity to oxidize NO_2^- at soil NO_2^- concentrations. As a consequence, a high V_{max} is required to compensate for NO_2^- oxidation at lower concentrations. This is demonstrated by the 3.2-fold decrease in the critical NO_2^- concentration required to drive NO_2^- oxidation at the same rate as NH_3 oxidation (Fig 4.6). Another possible explanation for a higher NO_2^--oxidizing potential than NH_3-oxidizing potential is the potential for mixotrophic growth inflating the population density of NOB. NOB demonstrate metabolic versatility and studies have shown that strains of both *Nitrobacter* and *Nitrospira* can use a range of substrates including lactate, pyruvate, formate, acetate, and hydrogen (Bock et al, 1986, Starkenburg et al., 2008, Daims et al., 2001, Koch et al., 2014, 2015; Gruber-Dorninger et al., 2015). Starkenburg et al. (2008) demonstrated that *N. hamburgensis* grown heterotrophically on lactate retained 50% of the NO_2^--oxidizing capacity of cells grown on NO_2^- as a sole energy source. Recently, it was shown that *Nitrospira moscoviensis* has the capacity to simultaneously oxidize both formate and NO_2^- (Koch et al., 2014). A wide metabolic versatility and constitutive expression of NXR could explain why NO_2^--oxidizing potentials are higher than NH_3-oxidizing potentials.

Spatial arrangement and NO_2^- concentration in soil

The community structure and spatial orientation of NH_3-oxidizers and NOB could influence the "critical" concentration of NO_2^- in soil. In soil environments, NO_2^- produced from NH_3 oxidation could be present within aggregations of NH_3-oxidizers and NO_2^- oxidizers on mineral surfaces, in biofilms, or diffused into soil water films. NOB within these structures could be reactive to shifts in the concentrations of NH_4^+, NO_2^-, or cell-cell signaling molecules. *Nitrobacter winogradsky* adjusted expression of 12% of its genome in response to co-culturing with *N. europaea* (Perez et al., 2015) and 24% of its genes in response to being exposed to NH_4^+ (Sayavedra-Soto et al., 2015). Another interesting possibility for fine tuning NO_2^- oxidizing activity with NH_3 oxidizing activity, is the role of quorum sensing in NOB physiological regulation. Studies have shown that the quorum sensing molecule acyl-homoserine lactone is used for cell-cell signaling, is produced by *N. winogradskyi*, and that it regulates genes associated with NO_2^- reduction, and motility and chemotaxis (Mellbye et al., 2016). These data demonstrate that cell-to-cell signaling may be important within NOB populations, and that communication between NOB, or NH_3-oxidizers and NOB might be important. Studies from soil have shown correlations between AOA *amoA* and *Nitrobacter* gene abundances in the rhizosphere of rice, whereas there was a relationship between AOB *amoA* and *Nitrospira* gene abundances in bulk soils, suggesting that nitrifiers in soil many also exhibit non-random spatial arrangements (Ke et al., 2015). Maxiner et al. (2006) suggested that spatial arrangement and proximity of NOB to NH_3 oxidizers influence access of NOB to NO_2^-, affecting rates of NO_2^- oxidation, and contributing to uncoupling of nitrification,

and NO_2^- accumulation. By utilizing FISH probes it has been demonstrated that spatial configuration of NOB in wastewater treatment plants plays a role in the persistence of phylogenetically distinct NOB (Maxiner et al., 2006; Gurber-Dorninger et al., 2015). In soil studies, microdissection and modeling studies of soil aggregates have shown that there can be spatial associations between *Nitrobacter* and NH_3-oxidizers (Grundmann et al., 2001; Grundmann and Debouzie, 2000). Close physical associations between NH_3-oxidizers and NOB provides a potential explanation of how NO_2^- oxidation occurs rapidly in soils and without NO_2^- accumulation. Disassociation of the two might be a simple reason to explain uncoupling of NH_3 and NO_2^- oxidations, and highlights the need of greater effort to understand the factors influencing the assembly and disassembly of these associations. Also, raises the possibility that NOB not physically associated with NH_3 oxidizers might be inactive and require protein synthesis to contribute to NO_2^- oxidation.

In agreement with previous soil studies I observed that accumulations of NO_2^- were transient, yet the reasons for NO_2^- accumulation and subsequent decline in soil remain unclear (Ma et al., 2015; Shen et al., 2003 Maharjan and Venterea, 2013; Venterea et al., 2015). In some cases, it appears that NO_2^- does not persist due to a decline in the rate of NH_3 oxidation to support the NO_2^- pool (Cai et al., 2016; Maharjan and Venterea, 2013). In other cases however, the NO_2^- pool was shown to decrease even when NH_3 oxidation continued at a constant rate, demonstrating that there is adaptive behavior by soil NOB (Giguere et al., 2017; Shen et al., 2003; Venterea et al., 2015). Although NO_2^- accumulation is generally transient, it can persist for days (Venterea et al., 2015) or weeks (Maharjan and Venterea, 2013), and understanding the factors that contribute to

NO_2^- is reactive and is persistence is important because it becomes vulnerable to loss via bacterial or chemo- denitrification to NO_x, N_2O, or HONO (Giguere et al., 2017; Kozlowski et al., 2014; Poth and Focht, 1985; Santoro et al., 2011; Shaw et al., 2006; Stieglmeier et al., 2014; Spott et al., 2011; Maharjan and Venterea, 2013; Oswald et al., 2013; Zhu et al., 2013; Heil et al., 2016). More remains to be done to determine the factors that drive NO_2^- accumulation, and what controls recoupling, and reduction of NO_2^- pools in soil environments.

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Figure Legends

Figure 4.1: Accumulation of NO_2^- (grey bars, left y-axis) and NO_3^- (black bars, right y-axis) in Pendleton (panel A), Madras (panel B) or Klamath soil (panel C) the either the absence of supplemental NH_4^+, or the presence of 1 mM NH_4^+ , or 2 mM NH_4^+. Within each soil, different lower case letters represent differences in NO_2^- accumulation, and different uppercase letters represent differences in NO_3^- accumulation. Bars represent the mean and error bars represent the standard deviation of the mean (n=4).

Figure. 4.2: Accumulation of NO_2^- and NO_3^- over 48-h incubations in the presence (+AB) and absence (-AB) of bacterial protein synthesis inhibitors. Panels A, B, and C represent Pendleton, Madras, and Klamath soils respectively. Light grey bars represent the accumulation of NO_2^- in -AB treatments, light grey striped bars represent NO_2^- accumulation in +AB treatments, white bars represent NO_3^- accumulation in the -AB treatments, and white stripped bars represent NO_3^- accumulation in the +AB treatments. Different lower case letters represent differences in NO_2^- accumulation over time, and different upper case letters represent differences in NO_3^- accumulation over time. * represents differences in NO_2^- accumulation between -AB and +AB treatments, and † represents differences between NO_3^- accumulation between -AB and +AB treatments. Bars represent the mean and error bars represent the standard deviation of the mean (n=4).

Figure 4.3: Nitrite consumption rates in Pendleton (panel A), Madras (panel B,) and Klamath (panel C). Solid lines represent modeled Michaelis-Menten kinetics for rate of NO_2^- consumption against NO_2^- concentration.

Figure 4.4: Nitrite consumption rates in the presence and absence of the bacterial protein synthesis inhibitors kanamycin and spectinomycin after 24 h of incubation with NH_4^+, and after inactivation with acetylene and subsequent NO_2^- consumption. Panel A represents Pendleton and Panel B represents Klamath.

Figure 4.5: Regression analysis for AOB *amoA* and *Nitrobacter*-like *nxrA* (panel A), AOB *amoA* and *Nitrospira*-like *nxrB* (panel B), *Nitrobacter*-like *nxrA* and *Nitrospira*-like *nxrB* (panel C), and the ratio of total NOB/total AOA+AOB and the extent of uncoupling (NO_2^- / NO_2^- + NO_3^-) after 24 h of incubation (black symbols) and 48 h (white symbols), (Panel D). Circles represent Klamath, triangles represent Madras, and squares represent Pendleton.

Figure 4.6: The dashed lined represent modeled initial NO_2^- consumption curve from Pendleton soil and solid line represent modeled consumption curve after adaptation in the absence of AB. Both curves have the initial K_m for NO_2^- as observed in pre-incubated Pendleton soil (34 µM). The horizontal dotted line represents the NH_3 oxidation potential rate, and the vertical solid lines show the concentration of NO_2^- required to drive NO_2^- at

the same rate as the NH_3 oxidizing potential: A lower NO_2^- concentration is needed when V_{max} is higher

Figure S4.1: Short-term consumption NO_2^- consumption in the presence of 250 µM NO_2^-, 1 mM NH_4^+, and 0.02% acetylene with (+AB, grey bars) and without (-AB, black bars) the bacterial protein synthesis inhibitors kanamycin and spectinomycin measured over 6 h. Within each soil, different upper case letters represent differences in the rates of NO_2^- consumption with and without antibiotics. Bars represent the mean and error bars represent the standard deviation of the mean (n=4).

Figure S4.2: Quantification of AOA *amoA* (panel A), AOB *amoA* (panel B), *Nitrobacter nxrA* (panel C), and *Nitrospira nxrB* (panel D) genes in soil slurry incubations over 48 h. Within each soil, different letters represent differences between gene abundances over time, within each soil. Black, light grey, and dark grey represent samples taken at 0, 24, and 48 h respectively. Bars represent the mean and error bars represent the standard deviation of the mean (n=4).

Figure S4.3: Panel A: accumulation of NO_3^- and NO_2^- in whole soil incubations conducted in the presence of 10 µmol NH_4^+ g^{-1} soil, wet to field capacity. Panel B: represents soil solution NO_2^- concentrations. Bars represent the mean and error bars represent the standard deviation of the mean (n=4).

Figure 4.1

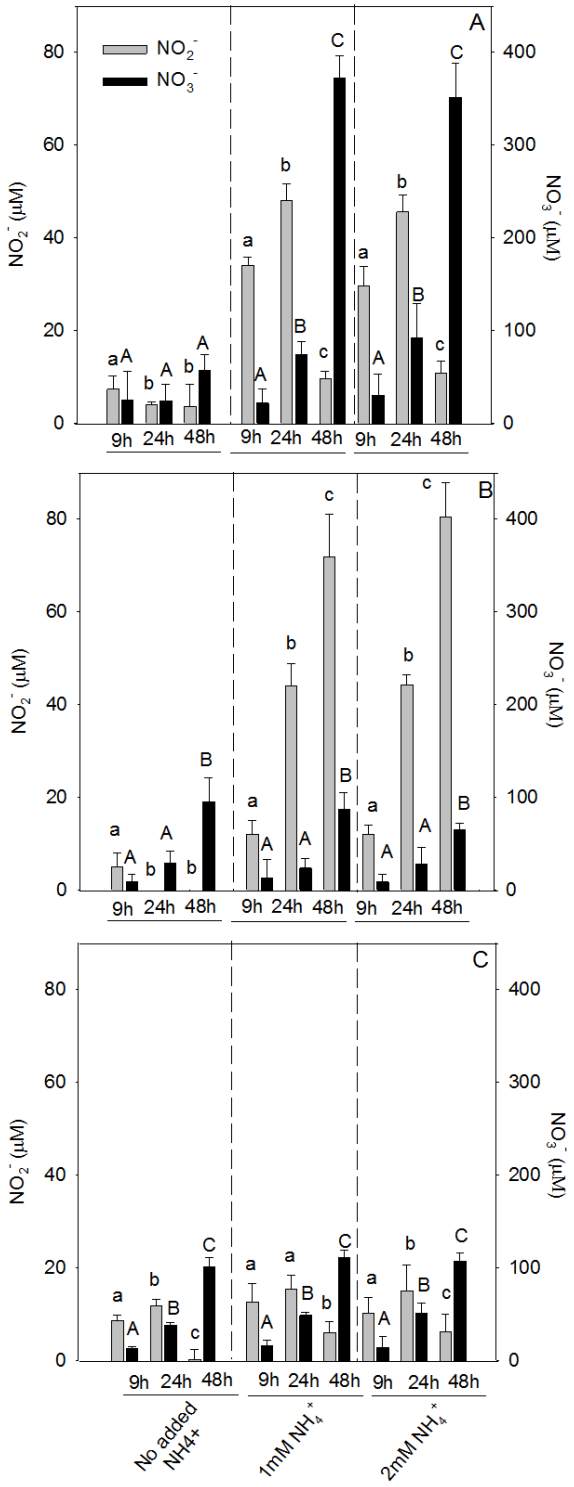


Figure 4.2

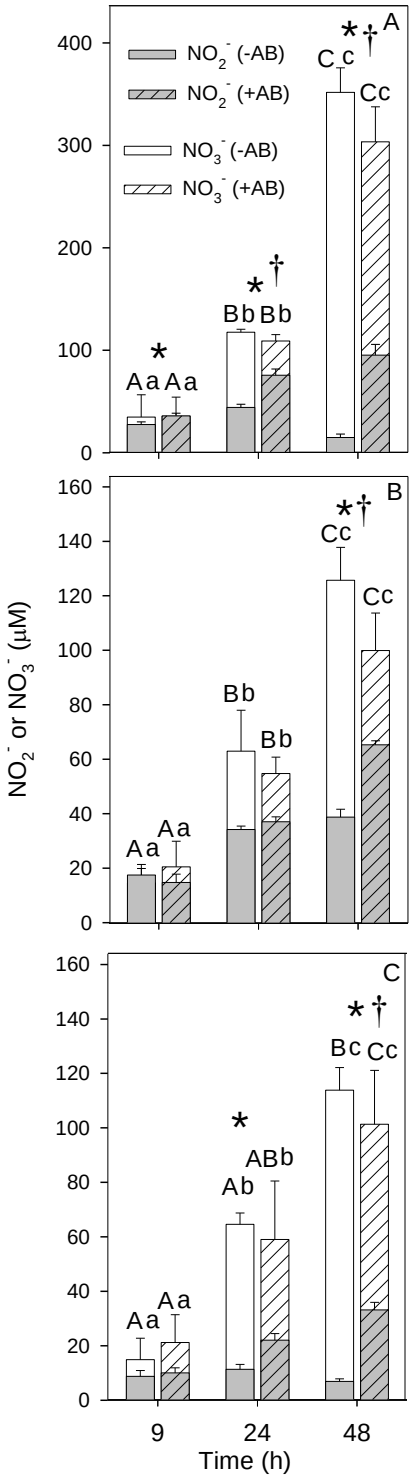

A
B
C
NO_2^- (-AB)
NO_2^- (+AB)
NO_3^- (-AB)
NO_3^- (+AB)
NO_2^- or NO_3^- (μM)
Time (h)
9
24
48
Aa Aa
Bb Bb
C c
Cc
Aa Aa
Bb
Bb
Cc
Cc
Aa Aa
Ab
ABb
Bc Cc

Figure 4.3

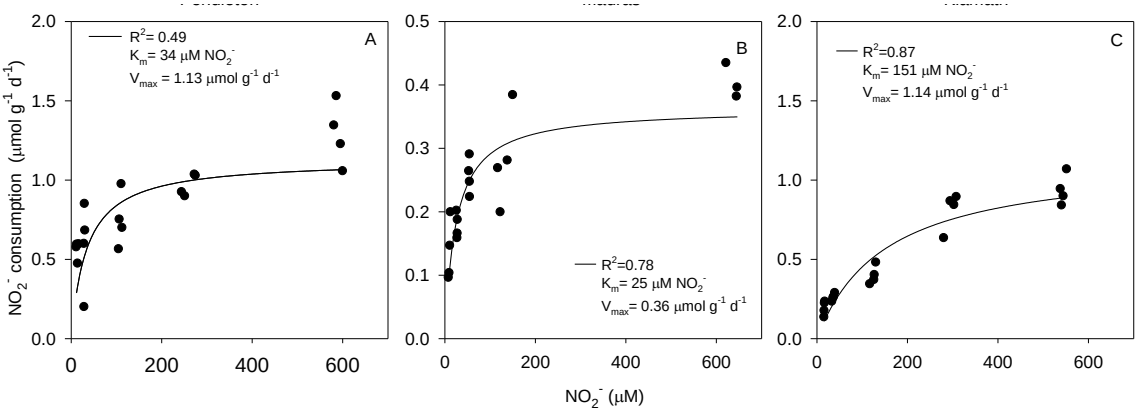

A
R^2= 0.49
K_m= 34 µM NO_2^-
V_{max} = 1.13 µmol g^{-1} d^{-1}
B
R^2=0.78
K_m= 25 µM NO_2^-
V_{max}= 0.36 µmol g^{-1} d^{-1}
C
R^2=0.87
K_m= 151 µM NO_2^-
V_{max}= 1.14 µmol g^{-1} d^{-1}
NO_2^- consumption (µmol g^{-1} d^{-1})
NO_2^- (µM)

Figure 4.4

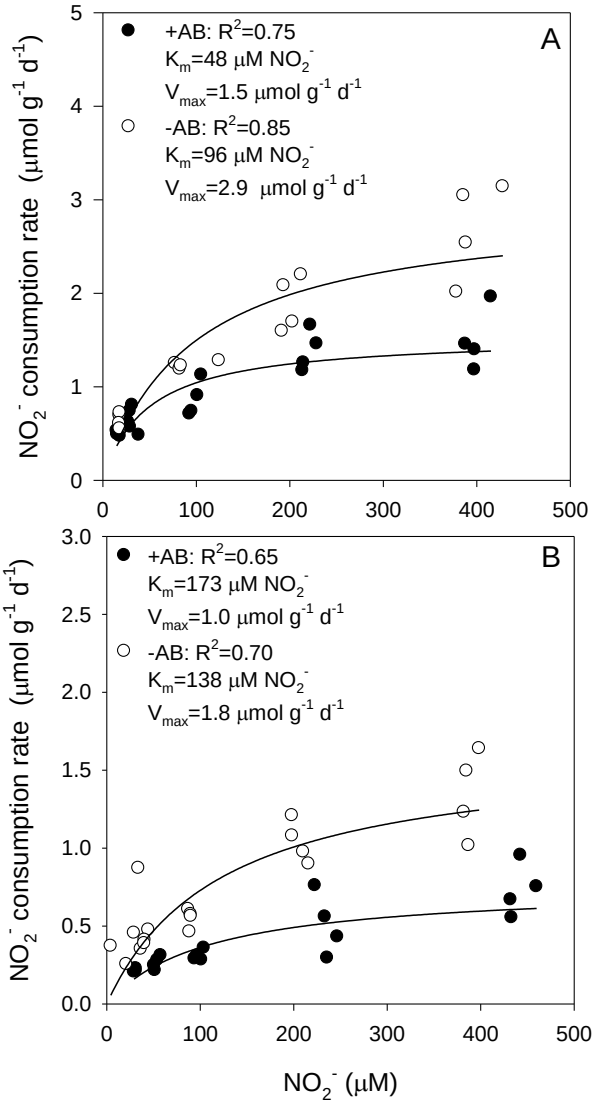

A
+AB: R2=0.75
Km=48 μM NO2-
Vmax=1.5 μmol g-1 d-1
-AB: R2=0.85
Km=96 μM NO2-
Vmax=2.9 μmol g-1 d-1
NO2- consumption rate (μmol g-1 d-1)
B
+AB: R2=0.65
Km=173 μM NO2-
Vmax=1.0 μmol g-1 d-1
-AB: R2=0.70
Km=138 μM NO2-
Vmax=1.8 μmol g-1 d-1
NO2- consumption rate (μmol g-1 d-1)
NO2- (μM)

Figure 4.5

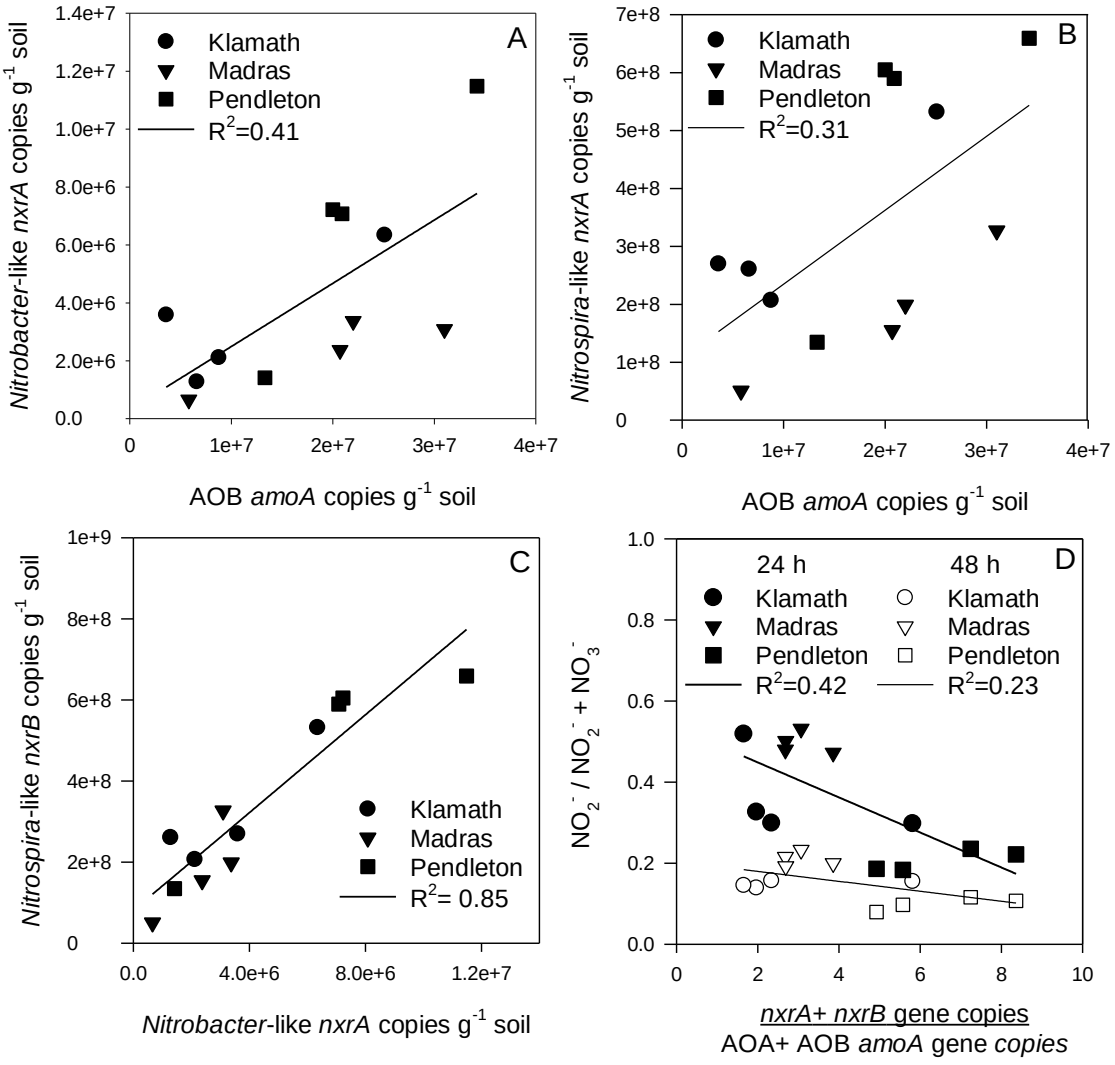

A
Klamath
Madras
Pendleton
R2=0.41
Nitrobacter-like nxrA copies g-1 soil
AOB amoA copies g-1 soil
B
R2=0.31
Nitrospira-like nxrA copies g-1 soil
C
R2= 0.85
Nitrospira-like nxrB copies g-1 soil
Nitrobacter-like nxrA copies g-1 soil
D
24 h
48 h
R2=0.42
R2=0.23
NO2- / NO2- + NO3-
nxrA+ nxrB gene copies
AOA+ AOB amoA gene copies

Figure 4.6

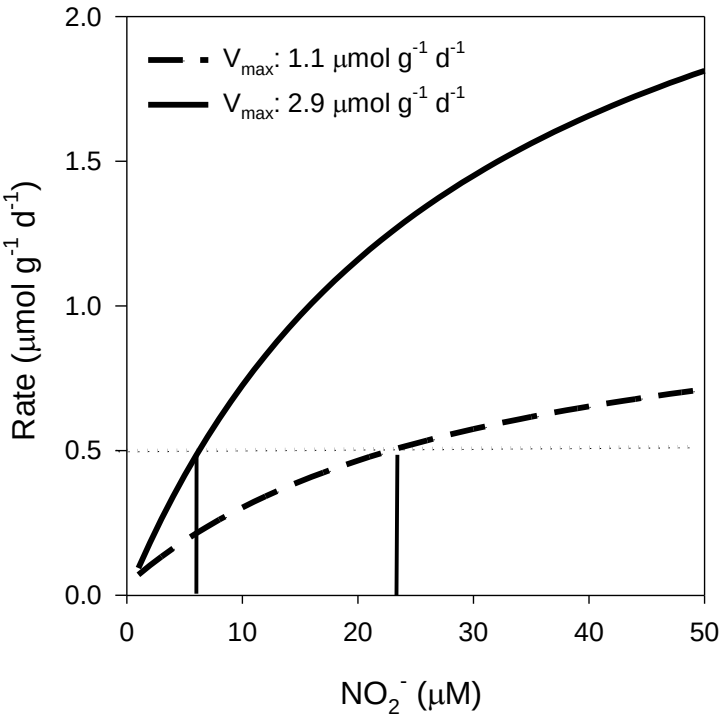

2.0
1.5
1.0
0.5
0.0
0
10
20
30
40
50
Rate (µmol g-1 d-1)
NO2- (µM)
Vmax: 1.1 µmol g-1 d-1
Vmax: 2.9 µmol g-1 d-1

Table S4.1: qPCR reagents, primers, and conditions.

	AOA *amoA*	AOB *amoA*	*Nitrobacter nxrA*	*Nitrospira nxrB*
Thermoc-ycler protocol	95º C, 5 min, 1x 95º C, 30 sec 40x 55º C, 30 min 40x 72º C, 1 min 40x Melt curve starting at 55ºC	95º C, 5 min, 1x 95º C, 30 sec 40x 60º C, 1 min 40x 72º C, 1 min 40x Melt curve starting at 60ºC	95ºC, 5 min, 1x 94, 30 sec, 40x 55 ºC, 45 sec, 40x 72ºC 45 sec, 40x Melt curve starting at 65ºC	95ºC, 5 min, 1x 95ºC, 40 sec, 40x 56.2 ºC, 40 sec, 40x 72ºC 90 sec, 40x Melt curve starting at 65ºC
Reaction mix recipe	10 µl Bio-rad iQ SYBR master mix 0.5 µM forward primer 0.5 µM reverse primer 0.5 mg BSA ml^{-1} 1 ng (5 µl of 0.2 ng ul^{-1}) template DNA Nuclease free water to 20 µl			
Forward primers	Arch-amoA-104F: GCAGGAGACT AYATHTTCTA (Alves et al., 2013)	*amoA*-1F; GGGGTTTCTA CTGGTGGT (Rotthauwe et al., 1997)	F1norA CAG ACC GAC GTG TGC GAA AG (Poly et al., 2008)	nxrB169f TAC ATG TGG TGG AAC A (Pester et al., 2014)
Reverse primers	Arch-amoA-616R: GCCATCCATCT RTADGTCCA (Alves et al., 2013)	*amoA*-2R; CCCCTCKGSA AAGCCTTCTT C [K 5 G or T; S 5 G or C] (Rotthauwe et al., 1997)	F2843 R2 nxrA TCC ACA AGG AAC GGA AGG TC). (Wertz et al., 2008)	nxrB638r CGG TTC TGG TCR ATC A (Pester et al., 2014)

Table S4.2. Predicted and observed NO_2^- oxidizing potential activities.

		Predicted			
	NOP[†]	*Nitrobacter* high*	*Nitrobacter* low*	*Nitrospira* high*	*Nitrospira* low*
Pendleton	1.15(0.08)	1.1(0.64)	0.17(0.09)	13.3(6.7)	2.8 (1.5)
Madras	0.36(0.03)	0.37(0.19)	0.05(0.03)	6.1(3.8)	1.3(0.8)
Klamath	1.13(0.13)	0.52(0.18)	0.08(0.03)	10.7(4.8)	2.3(1.0)

Rates given as µmol NO_2^- g^{-1} soil, mean (stdev)

[†]Nitrite oxidizing potentials determined as V_{max} calculated from NO_2^- consumption curves.

* Per cell activities from Nowka et al. (2015). *N. vulgaris* and *N. winogradskyi* used as high and low activity *Nitrobacter*, respectively; *N. defluvii* and *N. moscoviensis* used as high and low *Nitrospira*, respectively

Figure S4.1

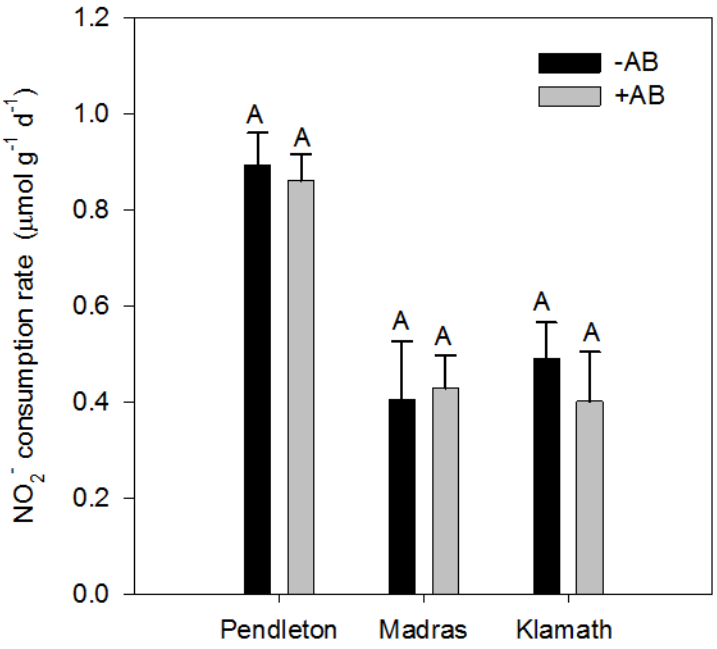

1.2
1.0
0.8
0.6
0.4
0.2
0.0
NO_2^- consumption rate (µmol g^{-1} d^{-1})
-AB
+AB
A
A
A
A
A
A
Pendleton
Madras
Klamath

Figure S4.2

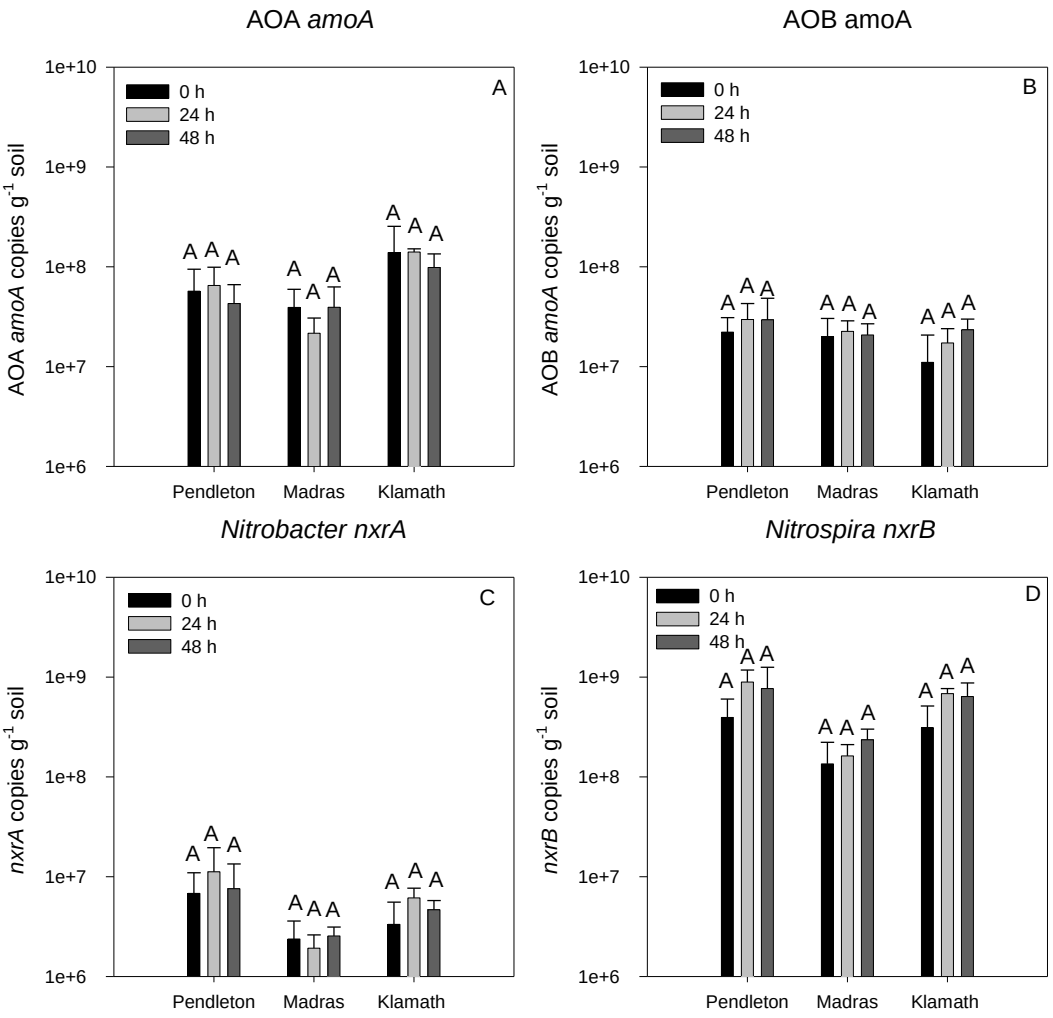
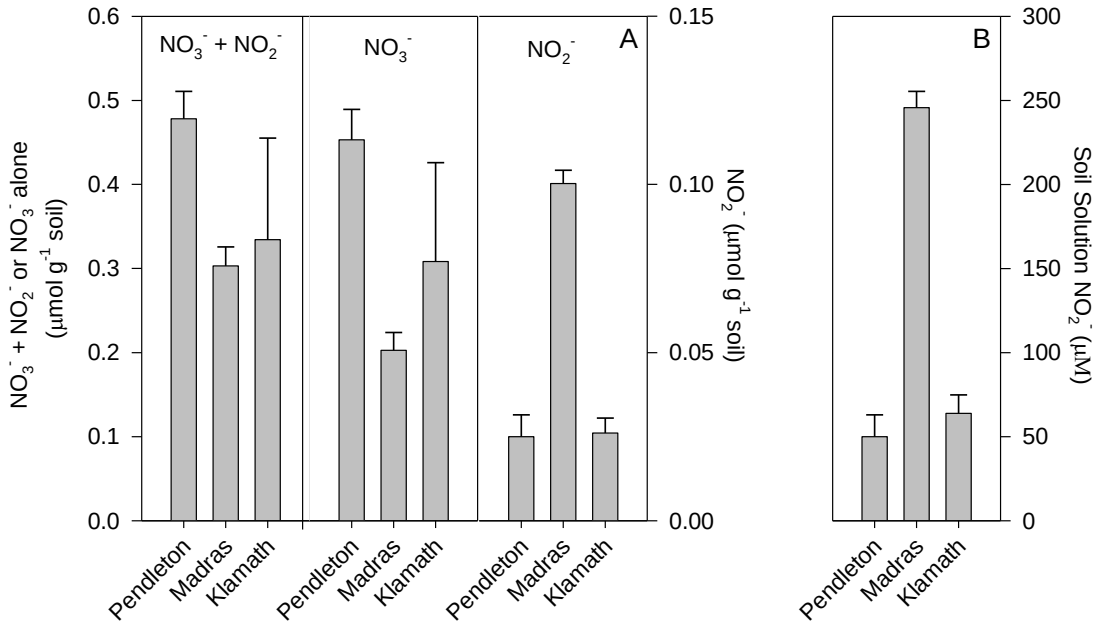

AOA amoA
AOB amoA
Nitrobacter nxrA
Nitrospira nxrB
A
B
C
D
0 h
24 h
48 h
AOA amoA copies g-1 soil
AOB amoA copies g-1 soil
nxrA copies g-1 soil
nxrB copies g-1 soil
1e+10
1e+9
1e+8
1e+7
1e+6
Pendleton
Madras
Klamath

Figure S4.3


$NO_3^- + NO_2^-$
NO_3^-
NO_2^-
A
B
$NO_3^- + NO_2^-$ or NO_3^- alone (µmol g^{-1} soil)
NO_2^- (µmol g^{-1} soil)
Soil Solution NO_2^- (µM)
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.00
0.05
0.10
0.15
0
50
100
150
200
250
300
Pendleton
Madras
Klamath

Chapter 5

General Conclusions

The factors controlling nitrification activity in soil are many. In this thesis I examined some of the factors that regulate the contributions of AOA, AOB, and NOB to nitrification in soil, and assess some of the potential impacts of their nitrification activities.

I demonstrated that in soils from Pendleton, Madras, and Klamath NH_4^+ additions, cropping history, and season of sampling affect the relative contributions of AOA and AOB to nitrification. Nitrification responses to supplemental NH_4^+ additions showed that in cropped soils, AOB activity was more responsive to NH_4^+ than AOA activity, whereas in non-cropped soil, AOA activity contributed to a greater proportion of the response to NH_4^+. Furthermore, AOA and AOB generally expressed a greater response to NH_4^+ additions in soils sampled in the summer than those sampled in the winter. I also determined that the concentration of NH_4^+ required to stimulate AOB activity was higher than the concentration of NH_4^+ required to stimulate AOA activity.

In Chapter 3, I examined how AOA and AOB contributions to overall nitrification activity might influence NO_2^- accumulation and nitrifier-dependent N_2O formation. I found that both AOA and AOB activities contributed to the accumulation of NO_2^- in several Oregon non-cropped soils. Additionally, I demonstrated that the addition of NH_4^+ stimulated the accumulation of NO_2^- and this accumulation was acetylene-sensitive. Furthermore, I showed that the addition of NO_2^- stimulated both AOA-and AOB-

dependent NO_2^- accumulation and N_2O production, and determined there was a positive non-linear relationship between the concentration of accumulated NO_2^- and the N_2O formation rate.

The dynamics of NO_2^- accumulation and the mechanisms of recoupling of NH_3 oxidation to NO_2^- oxidation were examined in Chapter 4. I showed there was protein synthesis by soil NOB in response to an increase in the rate of NH_3 oxidation and concomitant NO_2^- accumulation. Protein synthesis by soil NOB changed the kinetics of NO_2^- oxidation by increasing the maximum NO_2^- oxidation capacity (V_{max}), without modifying the affinity for NO_2^-. The increase in V_{max} effectively reduced the concentration of NO_2^- required to drive NO_2^- oxidation and resulted in a decline in the pool of accumulated NO_2^-. Furthermore, I obtained evidence that a protein synthesis independent adaptive NO_2^- oxidation rate occurred in one soil without an accompanying increase in the maximum oxidation potential by increasing NO_2^- concentrations and increasing the rate of NO_3^- formation. In this case the NO_2^- oxidation rate increased in response to the antibiotic-induced increase in the NO_2^- pool.

Potential implications

The studies presented in this thesis show that a range of factors control AOA and AOB contributions to nitrification. Better understanding of these factors could help lead to the improvement N management strategies. Nitrification in soil is critical for supplying NO_3^--N for plant growth needs, but modifying the rate at which NH_3 is oxidized could maximize crop productivity while reducing excess NO_3^- accumulation which is

susceptible to leaching and reduce NO_2^- accumulation and the production of the greenhouse gas N_2O. AOB dominate the response to NH_4^+ observed in cropped soils, and if a selective inhibitor suitable for field use were applied, AOA and AOB nitrification activities might be differentially managed to improve N use efficiency. This could potentially also reduce NO_3^- loss via leaching or heterotrophic denitrification.

Nitrifier-dependent denitrifying activity in fertilized fields is a major source of atmospheric N_2O with significant environmental implications. Nitrite accumulation in soil has been observed for decades, but the importance of NO_2^- in N_2O production from soil nitrification but been largely overlooked. The studies presented here clearly demonstrate that NO_2^- accumulation is mainly responsible for driving N_2O production from nitrification. If N management practices could be altered to prevent NH_3 oxidation from proceeding at a faster rate than NO_2^- oxidation, preventing NO_2^- accumulation, nitrifier-dependent N_2O emissions could potentially be better managed.

Future Research

There are many potential extensions of the research presented in this thesis that further explore the ecological roles, controls of physiological activity, as well as the phylogenetic and metabolic diversity of AOA, AOB, and NOB in soil environments. For example, little is known about gross rates of NH_3 oxidation by AOA and AOB. Utilizing ^{15}N isotope pool dilution methods in the presence of the selective AOB inhibitor, octyne, would allow assessment of both gross rates of nitrification, and help to determine how competitive AOA and AOB are for NH_3 when compared with heterotrophic bacteria and

fungi. Furthermore, employing these methods in mesocosms or in situ field experiments would provide a better understanding of how NH_3 oxidation occurs under field conditions.

Soil NOB remain understudied, and much remains unknown about their landscape distribution, metabolic and phylogenetic diversity, and what environmental factors regulate their activity. Studies examining the effects of varying environmental conditions such as temperature, soil water content, and NH_4^+ and NO_2^- availability on NO_2^- oxidizing activity would provide useful data that could then be integrated into studies focused on expanding our knowledge of NOB phylogenetic and metabolic diversity.

The spatial arrangements of AOA, AOB, and NOB in soil are also largely unknown. Applying methods used in wastewater treatment plants including florescent in-situ hybridization coupled with probes designed to capture AOA, AOB, and NOB might allow visualization of physical community structures within the soil fabric. Community structure might influence the concentration of NO_2^- that AOA and AOB are exposed to, influencing N_2O formation. Community structure could also influence the availability and concentration of NO_2^- soil NOB are exposed to, which would influence its rate of consumption.

Another largely unexplored area of research is that of niche specialization. Evidence from the literature supports the concept of niche specialization/separation of AOA and AOB; much less is known about niche specialization among soil NOB. Quantification and sequencing of AOA, AOB, and NOB functional genes has proven to

be useful in many studies establishing diversity in and among soil populations; but determining which groups are active under differing conditions remains unknown. One potential approach would be sequencing transcripts of functional genes under varying conditions to determine how different environmental conditions and NH_4^+ availability might influence subpopulation activity. This type of data, coupled with activity data from ^{15}N pool dilution experiments could be a powerful tool for providing insights into nitrifying communities and their activities, and the impact of nitrification rates on the overall coupling of the N cycle.

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