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

Andrew T. Giguere ^{a,*}, Anne E. Taylor ^a, Yuichi Suwa ^b, David D. Myrold ^a, Peter J. Bottomley

^{a,c}

^a Department of Crop and Soil Science, Oregon State University, Corvallis OR, USA. ^b

Department of Biological Sciences, Chuo University, Tokyo, Japan. ^c Department of

Microbiology, Oregon State University, Corvallis OR, USA.

* Corresponding author:

3017 Agricultural Life Sciences Building,

Corvallis, OR, 97331

andrew.giguere@oregonstate.edu

Key words: Nitrification, Ammonia-oxidizing archaea (AOA) and bacteria (AOB), Nitrite, Nitrous oxide, Nitrite-oxidizing bacteria

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 $0.15\text{--}1.08\ \mu\text{mol N g}^{-1}\text{ d}^{-1}$, with AOA contributing 64–71% of the total activity. AOA- and AOB-driven NO_2^- accumulation represented 8–100% of total $\text{NO}_2^- + \text{NO}_3^-$ 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 $0.05\pm0.01\%$ of total $\text{NO}_2^- + \text{NO}_3^-$ -N accumulation in the presence of supplemental NH_4^+ , and $0.28\pm0.11\%$ in the presence of both supplemental $\text{NH}_4^+ + \text{NO}_2^-$. 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 $0.05\text{--}0.62\ \text{nmol N}_2\text{O g}^{-1}\text{ d}^{-1}$, and predicted K_m values for NO_2^- ranged 15-fold from $0.02\text{--}0.30\ \mu\text{mol NO}_2^- \text{ g}^{-1}\text{ soil}$. 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.

1. 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; Vajjala 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 (Kozłowski 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 NO_2^- nitrite reductase (NirK) and nitric oxide reductase (NorB) which enable them to carry out NO_2^- -dependent N_2O production (Cantera and Stein, 2007; Kozłowski et al., 2014). In the case of AOA, although they possess the gene encoding for NirK (Spang et al., 2012; Walker et al., 2010), the gene encoding for nitric oxide reductase has not been detected (Hatzenpichler, 2012, Kozłowski et al., 2016). Although it has been suggested that AOA can abiotically 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. We 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), we 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.

2. Materials and Methods

2.1. 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, 4 replicates of cropped and non-cropped soils were sampled 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).

2.2. Soil slurry design

Soils were removed from 4°C 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 (21°C) 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 25°C. Gas samples for N_2O analysis were collected through the butyl stoppers at 24 and 48 h for all experimental incubations. Acetylene ($6 \mu\text{M}_{\text{aq}}$) 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 ($4 \mu\text{M}_{\text{aq}}$) 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 100ml air, and a 2.8 ml aliquot was added to each sample bottle.

2.3. Analysis of NO₂⁻, NO₃⁻, NH₄⁺, pH and N₂O

Initial pH measurements were made in a 2:1 soil water slurry and ranged from 7.2-7.6.

Concentrations of NO₂⁻ and NO₃⁻ 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 NO₃⁻ was measured using a vanadium reduction assay in which NO₃⁻ is reduced to NO₂⁻ and the total NO₂⁻ + NO₃⁻ measured (Miranda et al., 2001). The NO₃⁻ concentration was calculated as the difference between NO₂⁻ + NO₃⁻ and NO₂⁻ accumulations. Nitrification rates were calculated as the net accumulation of NO₂⁻ + NO₃⁻ above the acetylene controls. Detection limits for NO₂⁻ were 0.02 µmol NO₂⁻ g⁻¹ soil, and 0.05 µmol NO₃⁻ g⁻¹ soil for NO₃⁻.

NH₄⁺ extractions were conducted independently from NO₂⁻ or NO₃⁻ by extracting 5 g portions of soil in 15 ml 2 M KCl for 1 h. Extracts for NH₄⁺ analysis were frozen until analysis and measured colorimetrically as described by Mulvaney et al (1996).

N₂O 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 N₂O 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 N₂O, C_s is N₂O 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 N₂O at 25°C (0.544). The detection limits for N₂O production were 0.015 nmol g⁻¹ soil. Rates of N₂O formation were

calculated as the difference between the acetylene control N₂O levels and N₂O accumulation at 24 h and 48 h. N₂O yields were calculated using the equation

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

2.4. Incubations to establish the impact of NH₄⁺, and NO₂⁻ on N₂O production by AOB+AOA and AOA alone.

An experiment was conducted to examine the effect of supplemental NH₄⁺ and NO₂⁻ on nitrification activity and N₂O production by the combination of AOA + AOB (-oxygen) and by AOA alone (+oxygen). Soil slurry incubations for each of the 3 soils were conducted in the presence or absence of supplemental 1mM NH₄⁺ and in the presence or absence of supplemental 1mM NO₂⁻. NO₂⁻ and NO₃⁻, concentrations were measured at 0, 6, 24, and 48 h. Subtraction of the oxygen resistant rate from the rate measured in the minus oxygen treatment provides the rate attributed to AOB.

2.5. Using *Nitrobacter vulgaris* to prove NO₂⁻ accumulation is required for N₂O production.

Experiments were conducted using *Nitrobacter vulgaris* to either prevent NO₂⁻ accumulation, or reduce pre-formed NO₂⁻ levels and assess the impact on N₂O formation. *N. vulgaris* was grown in mineral salts media as described elsewhere (Spieck and Lipski, 2011). Cells were harvested after consuming 30 mM NO₂⁻ and reaching stationary phase (OD₆₀₀ = 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 (OD₆₀₀ = 0.07). Samples for NO₂⁻ and NO₃⁻ 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 .

2.6. 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 120°C 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 .

2.7. 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 $\pm$ standard deviation. Predicted values from regression analysis are given $\pm$ 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.

3. Results

3.1. Rates of nitrification and NO_2^- accumulation

Background KCl-extractable NH_4^+ concentrations ranged from 0.17- 0.23 $\mu\text{mol NH}_4^+ \text{ g}^{-1}$ soil among the 3 soils. Rates of nitrification in the three soils were determined in the absence ($-\text{NH}_4^+$) and presence ($+\text{NH}_4^+$) of added NH_4^+ . Total rates of nitrification ($\text{NO}_2^- + \text{NO}_3^-$ accumulation) in $-\text{NH}_4^+$ treatments ranged from 0.08-0.44 $\mu\text{mol NO}_2^- + \text{NO}_3^- \text{ g}^{-1} \text{ soil d}^{-1}$, and the contributions of AOA (+oxygen) ranged from 13-100% of the total nitrification activity across the three soils (Table 1). Total rates of nitrification (-oxygen) were stimulated 1.3- to 3.5-fold by $+\text{NH}_4^+$ treatments, and AOA-dependent nitrification rates were stimulated 1.3- to 1.6-fold by the $+\text{NH}_4^+$ treatment across the three soils. The rates of nitrification in the $+\text{NH}_4^+$ treatment varied from 0.15-1.08 $\mu\text{mol NO}_2^- + \text{NO}_3^- \text{ g}^{-1} \text{ soil d}^{-1}$ across the three soils, with the AOA contributions ranging from 64-71% of the total activity (Table 1).

3.2. 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 oxygen, and the fraction of $\text{NO}_2^- + \text{NO}_3^-$ that accumulated as NO_2^- varied across the soils (Table 1). In $+\text{NH}_4^+$ 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 $-\text{NH}_4^+$ treatment ranged between 1-5% in Pendleton and Klamath soils; in Madras soil the proportion $\pm\text{NH}_4^+$ was 100% (Table 1). Accumulations of NO_2^- were lower in the $-\text{NH}_4^+$ treatment compared to the $+\text{NH}_4^+$ treatment, being two-fold lower at 24 h compared to $+\text{NH}_4^+$ 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. 1). In $+\text{NH}_4^+$, -oxygen treatments,

NO_2^- significantly accumulated in all three soils after 6 h of incubation to a mean of 0.08 ± 0.03 $\mu\text{mol NO}_2^- \text{ g}^{-1}$ soil ($p=0.001$, Fig 1 A), and to a lesser extent in the +oxygen treatment to 0.05 ± 0.02 $\mu\text{mol g}^{-1}$ soil ($p=0.03$, Fig 1 B).

The dynamics of NO_2^- accumulation in the -oxygen treatment varied among the three soils. Nitrite accumulated to its highest concentration at 6 h in Pendleton (0.13 ± 0.003 $\mu\text{mol g}^{-1}$ soil) and Klamath (0.06 ± 0.01 $\mu\text{mol g}^{-1}$ 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. 1A). Dynamics of NO_2^- accumulation were similar in the $\pm$ oxygen 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 -oxygen 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. 1, Table 1). In the +oxygen treatment, the timing of NO_3^- accumulation was similar to -oxygen (Fig. 1). The data show that the overall rates of $\text{NO}_2^- + \text{NO}_3^-$ accumulation were generally linear over 48 h, whereas NO_3^- accumulation generally increased over the 48 h incubation.

3.3. Effect NH_4^+ and NO_2^- on N_2O production

We characterized to what extent additions of NH_4^+ , and NO_2^- influenced N_2O production (Fig. 2). Multi-way ANOVA revealed significant stimulation of N_2O production by NH_4^+ and NO_2^- , however, soil $\times$ NH_4^+ ($p=0.027$) and soil $\times$ NO_2^- ($p \leq 0.001$) interactions were detected. Thus, N_2O production was analyzed independently for each soil with and without oxygen. Acetylene-sensitive N_2O production in -oxygen 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 (-oxygen) 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 NO₂⁻ and 1 mM NH₄⁺ further stimulated N₂O production to in all three soils (-oxygen) ($p < 0.001$) to an average of 0.89 ± 0.56 nmol g⁻¹ soil (Fig. 2). AOA-dependent N₂O production was detected in +oxygen treatments, being significantly lower compared to -oxygen treatments across the three soils ($p = 0.01$). In the +oxygen treatment, N₂O was not significantly stimulated by the addition of NH₄⁺ alone (Fig. 2, $p > 0.167$), whereas the addition of NO₂⁻ alone significantly stimulated N₂O production 6.5-fold in two of three soils ($p < 0.001$), but not in the soil from Madras ($p = 0.216$). Production of N₂O was further stimulated in the presence of oxygen within all soils by the addition of a combination of NH₄⁺ and NO₂⁻ to an average of 2.7-fold above NO₂⁻ alone to 0.66 ± 0.5 nmol g⁻¹ soil (Fig. 2, $p < 0.020$). When NO₃⁻ was added in place of NO₂⁻ there were no significant stimulation of N₂O production by 1 mM NO₃⁻ additions to any soil (data not shown, $p = 0.404$). Sterile soils incubated in the presence of NH₄⁺ and NO₂⁻ did not produce N₂O (data not shown, $p = 0.395$).

3.4. N₂O Yield

Yields of N₂O based on the data shown in Figure 2 were calculated as N₂O-N accumulation divided by the accumulation of NO₂⁻ + NO₃⁻-N in the presence of 1 mM NH₄⁺ with and without 1 mM NO₂⁻ (Table 2). The yields (expressed as percentages) were significantly higher in +NO₂⁻ than in -NO₂⁻ treatments across all three soils ($p = 0.011$). In the -oxygen, +NH₄⁺ treatment, where both AOA and AOB contribute to nitrification and N₂O production, N₂O yields were $0.05 \pm 0.01\%$ in -NO₂⁻ and $0.28 \pm 0.11\%$ in +NO₂⁻ treatments. In the +oxygen, +NH₄⁺ treatment, the yields were $0.06 \pm 0.03\%$ in -NO₂⁻ and $0.22 \pm 0.15\%$ in +NO₂⁻ treatments.

The N₂O yield for the AOB contribution to N₂O production was calculated as the difference between the total N₂O production and the oxygen-resistant fraction of N₂O production. The N₂O

yield for AOB was $0.06 \pm 0.01\%$ in the $-\text{NO}_2^-$ treatments and $0.25 \pm 0.07\%$ in the $+\text{NO}_2^-$ treatments across the three soils (Table 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 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 ($0.36 \pm 0.06\%$) was significantly higher than AOB ($0.17 \pm 0.07\%$) ($p=0.023$) in Pendleton soil, whereas in Madras soil, AOA yield ($0.09 \pm 0.03\%$) was lower than AOB ($0.28 \pm 0.05\%$) ($p=0.001$). When only NH_4^+ was added, the N_2O yield was higher for AOB ($0.06 \pm 0.02\%$) than AOA ($0.03 \pm 0.01\%$) in Pendleton soil ($p=0.014$).

3.5. 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 $0.14 \pm 0.02 \mu\text{mol g}^{-1}$ soil in Pendleton, $0.22 \pm 0.02 \mu\text{mol g}^{-1}$ soil in Madras, and $0.04 \pm 0.003 \mu\text{mol g}^{-1}$ soil in Klamath soils (Fig. 3). The reduction of NO_2^- concentrations to below the detection limit ($0.02 \mu\text{mol g}^{-1}$ soil) was significant within each of the three soils ($p \leq 0.001$). The +NOB treatment significantly reduced N_2O production from a mean of $0.08 \pm 0.02 \text{ nmol g}^{-1}$ soil ($p < 0.014$) to a concentration not significantly different from acetylene control N_2O concentrations. There were indications in Klamath soil of NO_2^- -independent N_2O production accumulating to ~25% of the -NOB treatment (Fig. 3C). Corresponding with enhanced NO_2^- -oxidizing capacity, NO_3^- significantly increased within each of the three soils ($p \leq 0.01$) demonstrating that the majority of NO_2^- was oxidized to NO_3^- by supplementing the

NO₂⁻-oxidizing capacity with *N. vulgaris* (Fig. 3). There were no significant differences in NO₂⁻ or N₂O production between -NOB treatments and those amended with heat-killed *N. vulgaris* (data not shown, $p>0.05$).

We also considered the possibility that the effect of NO₂⁻ accumulation on N₂O production might require only a *transient* accumulation of NO₂⁻. Experiments were conducted with soils that were incubated for 24 h without NOB addition to allow NO₂⁻ accumulation and N₂O production. Then, NOB were added to consume the NO₂⁻ that had accumulated. Introduction of NOB to soil slurries at 24 h reduced the NO₂⁻ pool to below the detection limit within 1 h ($p\leq 0.001$), and effectively stopped further accumulation of N₂O between 24 and 48 h within each soil. ($p>0.05$, Fig. 4). By allowing NO₂⁻ to accumulate before removing the NO₂⁻ pool, we demonstrated there was no NO₂⁻-dependent induction of a NO₂⁻-independent mechanism of N₂O formation. Although Klamath soil showed some N₂O production when NOB were added at the beginning of the experiment, N₂O production was completely prevented when NOB were added at 24 h.

3.6. Regression Analysis

Data from several different experiments were compiled to reveal a positive relationship between NO₂⁻ accumulation and N₂O production during 24-h incubations (Fig. 5). These data were fit to the Michaelis-Menten equation using non-linear regression to determine K_m and V_{max} for NO₂⁻-stimulated N₂O production (Table 3). For the Pendleton soil, this resulted in a predicted K_m value (half-saturation concentration) of 0.30 ± 0.07 $\mu\text{mol NO}_2^- \text{ g}^{-1} \text{ soil}$ and a V_{max} (predicted maximum rate of N₂O production) of 0.62 ± 0.07 $\text{nmol N}_2\text{O g}^{-1} \text{ soil d}^{-1}$ ($R^2=0.86$, $p\leq 0.001$). For Madras soil, a three-fold lower K_m of 0.08 ± 0.04 $\mu\text{mol NO}_2^- \text{ g}^{-1} \text{ soil}$ was determined, and a V_{max} value of 0.08 ± 0.02 $\text{nmol N}_2\text{O g}^{-1} \text{ soil d}^{-1}$ ($R^2=0.51$, $p\leq 0.001$). 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

$\text{NO}_2^- \text{ g}^{-1} \text{ soil}$ and a V_{max} value of $0.07 \pm 0.02 \text{ nmol N}_2\text{O g}^{-1} \text{ soil d}^{-1}$ ($R^2=0.37$, $p \leq 0.001$). Non-linear regression of the +octyne treatment of Pendleton soil ($R^2=0.54$) predicted a V_{max} of $0.15 \pm 0.03 \text{ nmol N}_2\text{O g}^{-1} \text{ soil d}^{-1}$ ($p \leq 0.001$) and a K_m value of $0.02 \pm 0.02 \text{ } \mu\text{mol NO}_2^- \text{ g}^{-1} \text{ soil}$. Analysis of +octyne treatment of Madras soil ($R^2=0.57$) predicted a non-significant K_m value of $0.02 \pm 0.02 \text{ } \mu\text{mol NO}_2^- \text{ g}^{-1} \text{ soil}$ and a V_{max} of $0.05 \pm 0.01 \text{ nmol N}_2\text{O g}^{-1} \text{ soil d}^{-1}$. Plus octyne data from Klamath soil was excluded from the regression analysis as NO_2^- concentrations did not accumulate above $\sim 0.05 \text{ } \mu\text{mol g}^{-1} \text{ soil}$.

4. Discussion

4.1. 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, we 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 we 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 we were 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*, $K_m = 49 \mu\text{M}$), we 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.

4.2. 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; Kozłowski 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, however, the data also support the existence of a NO_2^- -independent mechanism in this soil. Although Kozłowski et al. (2016) have proposed a new abiotic mechanism of AOA driven N_2O production we 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, we 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.

4.3. 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 $\text{NO}_2^- + \text{NO}_3^-$ 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.

Acknowledgements: This research was supported by United States Department of Agriculture, National Institute of Food and Agriculture award no. 2012-67019-3028, the National Science Foundation, East Asia and Pacific Summer Institutes for U.S. Graduate Students, the Japan Society for the Promotion of Science award no. 1414921, and the Oregon Agricultural Research Foundation. We thank Eva Spieck and Boris Nowka for providing *N. vulgaris* and Luis Sayavedra-Soto for technical assistance. We are grateful to members of the Columbia Basin Agricultural Research Center, the Klamath Basin Research and Extension Center, and the Central Oregon Agricultural Research Center for maintaining field sites, and sampling soil for our study. Funding sources had no role in study design, data collection or interpretation.

References

- Alawi, M., Lipski, A., Sanders, T., Eva-Maria-Pfeiffer, Spieck, E., 2007. Cultivation of a novel cold-adapted nitrite oxidizing betaproteobacterium from the Siberian Arctic. *ISME J* 1, 256–264.
- Alawi, M., Off, S., Kaya, M., Spieck, E., 2009. Temperature influences the population structure of nitrite-oxidizing bacteria in activated sludge. *Environmental Microbiology Reports* 1, 184–190.
- Arp, D.J., Stein, L.Y., 2003. Metabolism of inorganic N compounds by ammonia-oxidizing bacteria. *Critical Reviews Biochemistry and Molecular Biology* 38, 471–495.
- Attard, E., Poly, F., Commeaux, C., Laurent, F., Terada, A., Smets, B.F., Recous, S., Roux, X.L., 2010. Shifts between *Nitrospira*- and *Nitrobacter*-like nitrite oxidizers underlie the response of soil potential nitrite oxidation to changes in tillage practices. *Environmental Microbiology* 12, 315–326.
- Bollmann, A., Schmidt, I., Saunders, A.M., Nicolaisen, M.H., 2005. Influence of starvation on potential ammonia-oxidizing activity and *amoA* mRNA levels of *Nitrosospira briensis*. *Applied Environmental Microbiology* 71, 1276–1282.
- Burns, L.C., Stevens, R.J., Smith, R.V., Cooper, J.E., 1995. The occurrence and possible sources of nitrite in a grazed, fertilized, grassland soil. *Soil Biology and Biochemistry* 27, 47–59.
- Cantera, J.J., Stein, L., 2007. Role of nitrite reductase in the ammonia-oxidizing pathway of *Nitrosomonas europaea*. *Archives of Microbiology* 188, 349–354.
- Chapman, H.D., Liebig, G.F., 1952. Field and laboratory studies of nitrite accumulation in soils. *Soil Science Society of America Journal* 16, 276–282.

- Chen, Y., Xu, Z., Hu, H., Hu, Y., Hao, Z., Jiang, Y., Chen, B., 2013. Responses of ammonia-oxidizing bacteria and archaea to nitrogen fertilization and precipitation increment in a typical temperate steppe in Inner Mongolia. *Applied Soil Ecology* 68, 36–45.
- Daebeler, A., Bodelier, P.L.E., Hefting, M.M., Laanbroek, H.J., 2015. Ammonia-limited conditions cause of Thaumarchaeal dominance in volcanic grassland soil. *FEMS Microbiology and Ecology* 91, doi: 10.3389/fmicb.2012.00352
- Daims, H., Lebedeva, E.V., Pjevac, P., Han, P., Herbold, C., Albertsen, M., Jehmlich, N., Palatinszky, M., Vierheilig, J., Bulaev, A., Kirkegaard, R.H., Bergen, M. von, Rattei, T., Bendinger, B., Nielsen, P.H., Wagner, M., 2015. Complete nitrification by *Nitrospira* bacteria. *Nature* 258, 504–509.
- Elawwad, A., Sandner, H., Kappelmeyer, U., Koeser, H., 2013. Long-term starvation and subsequent recovery of nitrifiers in aerated submerged fixed-bed biofilm reactors. *Environmental Technology* 34, 945–959.
- Giguere, A.T., Taylor, A.E., Myrold, D.D., Bottomley, P.J., 2015a. Nitrification responses of soil ammonia-oxidizing archaea and bacteria to ammonium concentrations. *Soil science society of America journal* 79, 1366–1374.
- Harper Jr., W.F., Takeuchi, Y., Riya, S., Hosomi, M., Terada, A., 2015. Novel abiotic reactions increase nitrous oxide production during partial nitrification: modeling and experiments. *Chemical Engineering Journal* 281, 1017–1023.
- Hatzenpichler, R., 2012. Diversity, physiology, and niche differentiation of ammonia-oxidizing archaea. *Applied and Environmental Microbiology* 78, 7501–7510.

- Heil, J., Liu, S., Vereecken, H., Brüggemann, N., 2015. Abiotic nitrous oxide production from hydroxylamine in soils and their dependence on soil properties. *Soil Biology and Biochemistry* 84, 107–115.
- Hink, L., Nicol, G.W., Prosser, J.I., 2016. Archaea produce lower yields of N₂O than bacteria during aerobic ammonia oxidation in soil. *Environmental Microbiology* doi:10.1111/1462-2920.13282
- Johnstone, B.H., Jones, R.D., 1988. Recovery of a marine chemolithotrophic ammonium-oxidizing bacterium from long-term energy-source deprivation. *Canadian Journal of Microbiology* 34, 1347–1350.
- Jung, M.-Y., Well, R., Min, D., Giesemann, A., Park, S.-J., Kim, J.-G., Kim, S.-J., Rhee, S.-K., 2013. Isotopic signatures of N₂O produced by ammonia-oxidizing archaea from soils. *ISME Journal* 8, 1115–1125.
- Ke, X., Angel, R., Lu, Y., Conrad, R., 2013. Niche differentiation of ammonia oxidizers and nitrite oxidizers in rice paddy soil. *Environmental Microbiology* 15, 2275–2292.
- Koch, H., Lückner, S., Albertsen, M., Kitzinger, K., Herbold, C., Spieck, E., Nielsen, P.H., Wagner, M., Daims, H., 2015. Expanded metabolic versatility of ubiquitous nitrite-oxidizing bacteria from the genus *Nitrospira*. *Proceedings of the National Academy of Sciences* 112, 11371–11376.
- Kool, D.M., Dolfing, J., Wrage, N., Groenigen, J.W.V., 2011. Nitrifier denitrification as a distinct and significant source of nitrous oxide from soil. *Soil Biology and Biochemistry* 43, 174–178.

- Kozlowski, J.A., Price, J., Stein, L.Y., 2014. Revision of N₂O-producing pathways in the ammonia-oxidizing bacterium *Nitrosomonas europaea* ATCC 19718. *Applied Environmental Microbiology* 80, 4930–4935.
- Kozlowski, J.A., Stieglmeier, M., Schleper, C., Klotz, M.G., Stein, L.Y., 2016. Pathways and key intermediates required for obligate aerobic ammonia-dependent chemolithotrophy in bacteria and Thaumarchaeota. *ISME Journal* doi: 10.1038/ismej.2016.2.
- Lebedeva, E.V., Off, S., Zumbärgel, S., Kruse, M., Shagzhina, A., Lückner, S., Maixner, F., Lipski, A., Daims, H., Spieck, E., 2011. Isolation and characterization of a moderately thermophilic nitrite-oxidizing bacterium from a geothermal spring. *FEMS Microbiology Ecology* 75, 195–204.
- Leininger, S., Urich, T., Schlöter, M., Schwark, L., Qi, J., Nicol, G.W., Prosser, J.I., Schuster, S.C., Schleper, C., 2006. Archaea predominate among ammonia-oxidizing prokaryotes in soils. *Nature* 442, 806–809.
- Lückner, S., Wagner, M., Maixner, F., Pelletier, E., Koch, H., Vacherie, B., Rattei, T., Damsté, J.S.S., Spieck, E., Le Paslier, D., Daims, H., 2010. A *Nitrospira* metagenome illuminates the physiology and evolution of globally important nitrite-oxidizing bacteria. *Proceedings of the National Academy of Sciences* 107, 13479–13484.
- Lu, X., Bottomley, P.J., Myrold, D.D., 2015. Contributions of ammonia-oxidizing archaea and bacteria to nitrification in Oregon forest soils. *Soil Biology and Biochemistry* 85, 54–62.
- Ma, L., Shan, J., Yan, X., 2015. Nitrite behavior accounts for the nitrous oxide peaks following fertilization in a fluvo-aquic soil. *Biology and Fertility of Soils* 51, 563–572.
- Maharjan, B., Venterea, R.T., 2013. Nitrite intensity explains N management effects on N₂O emissions in maize. *Soil Biology and Biochemistry* 66, 229–238.

- Mellbye, B.L., Giguere, A., Chaplen, F., Bottomley, P.J., Sayavedra-Soto, L.A., 2016. Steady state growth under inorganic carbon limitation increases energy consumption for maintenance and enhances nitrous oxide production in *Nitrosomonas europaea*. *Applied and Environmental Microbiology* 82, 3310–3318.
- Miranda, K.M., Espey, M.G., Wink, D.A., 2001. A rapid, simple spectrophotometric method for simultaneous detection of nitrate and nitrite. *Nitric Oxide* 5, 62–71.
- Mørkved, P.T., Dörsch, P., Bakken, L.R., 2007. The N₂O product ratio of nitrification and its dependence on long-term changes in soil pH. *Soil Biology and Biochemistry* 39, 2048–2057.
- Müller, C., Stevens, R.J., Laughlin, R.J., 2006. Sources of nitrite in a permanent grassland soil. *European Journal of Soil Science* 57, 337–343.
- Mulvaney, R.L., 1996. Nitrogen-Inorganic Forms, in: Weaver et al., *Methods of Soil Analysis Part 3: Chemical Methods*, SSSA Book Series 5. Soil Science Society of America, pp. 1123–1184.
- Nelson D. W., 1982. Gaseous loss of nitrogen other than through denitrification. in: Stevenson, *Nitrogen in agricultural soils, agronomy monograph* 22, 327-363.
- Ni, B.-J., Rusalleda, M., Pellicer-Nàcher, C., Smets, B.F., 2011. Modeling nitrous oxide production during biological nitrogen removal via nitrification and denitrification: extensions to the general ASM models. *Environmental Science and Technology* 45, 7768–7776.
- Nowka, B., Daims, H., Spieck, E., 2015. Comparison of oxidation kinetics of nitrite-oxidizing bacteria: nitrite availability as a key factor in niche differentiation. *Applied and Environmental Microbiology* 81, 745–753.

- Pester, M., Maixner, F., Berry, D., Rattei, T., Koch, H., Lückner, S., Nowka, B., Richter, A., Spieck, E., Lebedeva, E., Loy, A., Wagner, M., Daims, H., 2014. NxrB encoding the beta subunit of nitrite oxidoreductase as functional and phylogenetic marker for nitrite-oxidizing *Nitrospira*. *Environmental Microbiology* 16, 3055–3071.
- Placella, S.A., Firestone, M.K., 2013. Transcriptional response of nitrifying communities to wetting of dry soil. *Applied and Environmental Microbiology* 79, 3294–3302.
- Poth, M., Focht, D.D., 1985. ^{15}N kinetic analysis of N_2O production by *Nitrosomonas europaea*: an examination of nitrifier denitrification. *Applied and Environmental Microbiology* 49, 1134–1141.
- Prosser, J.I., Nicol, G.W., 2012. Archaeal and bacterial ammonia-oxidizers in soil: the quest for niche specialization and differentiation. *Trends in Microbiology* 20, 523–531.
- Santoro, A.E., Buchwald, C., McIlvin, M.R., Casciotti, K.L., 2011. Isotopic signature of N_2O produced by marine ammonia-oxidizing archaea. *Science* 333, 1282–1285.
- Shaw, L.J., Nicol, G.W., Smith, Z., Fear, J., Prosser, J.I., Baggs, E.M., 2006. *Nitrosospira* spp. can produce nitrous oxide via a nitrifier denitrification pathway. *Environmental Microbiology* 8, 214–222.
- Shen, Q., Ran, W., Cao, Z., 2003. Mechanisms of nitrite accumulation occurring in soil nitrification. *Chemosphere* 50, 747–753.
- Sorokin, D.Y., Lucker, S., Vejmekova, D., Kostrikina, N.A., Kleerebezem, R., Rijpstra, W.I.C., Damste, J.S.S., Le Paslier, D., Muyzer, G., Wagner, M., van Loosdrecht, M.C.M., Daims, H., 2012. Nitrification expanded: discovery, physiology and genomics of a nitrite-oxidizing bacterium from the phylum *Chloroflexi*. *ISME Journal* 6, 2245–2256.

- Spang, A., Poehlein, A., Offre, P., Zumbärgel, S., Haider, S., Rychlik, N., Nowka, B., Schmeisser, C., Lebedeva, E.V., Rattei, T., Böhm, C., Schmid, M., Galushko, A., Hatzenpichler, R., Weinmaier, T., Daniel, R., Schleper, C., Spieck, E., Streit, W., Wagner, M., 2012. The genome of the ammonia-oxidizing *Candidatus Nitrososphaera gargensis*: insights into metabolic versatility and environmental adaptations. *Environmental Microbiology* 14, 3122–3145.
- Spieck, E., Lipski, A., 2011. Cultivation, growth physiology, and chemotaxonomy of nitrite-oxidizing bacteria, in: *Methods of Enzymology* 486, 109–130.
- Stieglmeier, M., Mooshammer, M., Kitzler, B., Wanek, W., Zechmeister-Boltenstern, S., Richter, A., Schleper, C., 2014. Aerobic nitrous oxide production through N-nitrosating hybrid formation in ammonia-oxidizing archaea. *ISME J* 8, 1135–1146.
- Stein, L.Y., 2011. Heterotrophic nitrification and nitrifier denitrification. In: Ward et al., 2011 *Nitrification*. American Society for Microbiology, 95-114.
- Stevens, R.J., Laughlin, R.J., 1995. Nitrite transformations during soil extraction with potassium chloride. *Soil Science Society of America Journal* 59, 933–938.
- Takenaka, N., Ueda, A., Maeda, Y., 1992. Acceleration of the rate of nitrite oxidation by freezing in aqueous solution. *Nature* 358, 736–738.
- Tappe, W., Laverman, A., Bohland, M., Braster, M., Rittershaus, S., Groeneweg, J., van Verseveld, H.W., 1999. Maintenance energy demand and starvation recovery dynamics of *Nitrosomonas europaea* and *Nitrobacter winogradskyi* cultivated in a retentostat with complete biomass retention. *Applied and Environmental Microbiology*. 65, 2471–2477.
- Taylor, A.E., Taylor, K., Tennigkeit, B., Palatinszky, M., Stieglmeier, M., Myrold, D.D., Schleper, C., Wagner, M., Bottomley, P.J., 2015. Inhibitory effects of C2 to C10 1-

- alkynes on ammonia oxidation in two *Nitrososphaera* species. *Applied and Environmental Microbiology*. 81, 1942–1948.
- Taylor, A.E., Vajrala, N., Giguere, A.T., Gitelman, A.I., Arp, D.J., Myrold, D.D., Sayavedra-Soto, L., Bottomley, P.J., 2013. Use of aliphatic n-alkynes to discriminate soil nitrification activities of ammonia-oxidizing thaumarchaea and bacteria. *Applied and Environmental Microbiology* 79, 6544–6551.
- Taylor, A.E., Zeglin, L.H., Dooley, S., Myrold, D.D., Bottomley, P.J., 2010. Evidence for different contributions of archaea and bacteria to the ammonia-oxidizing potential of diverse Oregon soils. *Applied and Environmental Microbiology*. 76, 7691–7698.
- Taylor, A.E., Zeglin, L.H., Wanzek, T.A., Myrold, D.D., Bottomley, P.J., 2012. Dynamics of ammonia-oxidizing archaea and bacteria populations and contributions to soil nitrification potentials. *ISME J* 6, 2024–2032.
- Tiedje, J.M., 1994. Denitrifiers. In: Weaver et al. *Methods of Soil Analysis: Part 2- Microbiological and biochemical properties*, 5. Soil Science Society of America. 245–267.
- Vajrala, N., Martens-Habbena, W., Sayavedra-Soto, L.A., Schauer, A., Bottomley, P.J., Stahl, D.A., Arp, D.J., 2013. Hydroxylamine as an intermediate in ammonia oxidation by globally abundant marine archaea. *Proceedings of the National Academy of Sciences* 110, 1006–1011.
- Venterea, R.T., 2007. Nitrite-driven nitrous oxide production under aerobic soil conditions: kinetics and biochemical controls. *Global Change Biology*. 13, 1798–1809.

- Venterea, R.T., Clough, T.J., Coulter, J.A., Breuillin-Sessoms, F., Wang, P., Sadowsky, M.J., 2015. Ammonium sorption and ammonia inhibition of nitrite-oxidizing bacteria explain contrasting soil N₂O production. *Scientific Reports* 5, doi: doi:10.1038/srep12153.
- Walker, C.B., de la Torre, J.R., Klotz, M.G., Urakawa, H., Pinel, N., Arp, D.J., Brochier-Armanet, C., Chain, P.S.G., Chan, P.P., Gollabgir, A., Hemp, J., Hügler, M., Karr, E.A., Könneke, M., Shin, M., Lawton, T.J., Lowe, T., Martens-Habbena, W., Sayavedra-Soto, L.A., Lang, D., Sievert, S.M., Rosenzweig, A.C., Manning, G., Stahl, D.A., 2010. *Nitrosopumilus maritimus* genome reveals unique mechanisms for nitrification and autotrophy in globally distributed marine crenarchaea. *Proceedings of the National Academy of Sciences* 107, 8818–8823.
- Wang, B., Zhao, J., Guo, Z., Ma, J., Xu, H., Jia, Z., 2015. Differential contributions of ammonia oxidizers and nitrite oxidizers to nitrification in four paddy soils. *ISME J* 9, 1062–1075.
- Wessén, E., Nyberg, K., Jansson, J.K., Hallin, S., 2010. Responses of bacterial and archaeal ammonia oxidizers to soil organic and fertilizer amendments under long-term management. *Applied Soil Ecology* 45, 193–200.
- Wrage, N., Velthof, G., van Beusichem, M., Oenema, O., 2001. Role of nitrifier denitrification in the production of nitrous oxide. *Soil Biology and Biochemistry*. 33, 1723–1732.
- Zhu-Barker, X., Cavazos, A.R., Ostrom, N.E., Horwath, W.R., Glass, J.B., 2015. The importance of abiotic reactions for nitrous oxide production. *Biogeochemistry* 126, 251–267.
- Zhu, X., Burger, M., Doane, T.A., Horwath, W.R., 2013. Ammonia oxidation pathways and nitrifier denitrification are significant sources of N₂O and NO under low oxygen availability. *Proceedings of the National Academy of Sciences* 110, 6328-6333.

Table 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	AOA ^a	AOB ^a	($\text{NO}_2^-/\text{NO}_2^-$	Total ^a	AOA ^a	AOB ^a	($\text{NO}_2^-/\text{NO}_2^-$
	(-oxygen)	(+oxygen)	(oxygen sensitive)	+ NO_3^-)% ^b	(-oxygen)	(+oxygen)	(oxygen sensitive)	+ 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 $\mu\text{mol NO}_2^- + \text{NO}_3^-$ accumulated g^{-1} soil d^{-1}

^a Nitrification potential activities for Total (AOA+AOB activity, -oxygen), AOA activity (+oxygen) and AOB activity (oxygen sensitive).

^b Percentage of total $\text{NO}_2^- + \text{NO}_3^-$ accumulated

Table 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/($\text{NO}_2^- + \text{NO}_3^-$ -N))^{*}.

	Total nitrification activity (-oxygen) [‡]		AOA dependent activity (+oxygen) ^{‡§}		AOB dependent activity (oxygen 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 $\text{NO}_2^- + \text{NO}_3^-$ -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: Kinetic parameters of N₂O production derived from the regression analysis of the relationship between NO₂⁻ concentrations and N₂O 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	Pendleton	0.62(0.07) ^{***}	0.30(0.07) ^{***}	0.82	<0.0001
(-octyne)	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	Pendleton	0.15(0.03) ^{**}	0.02(0.02) ^{ns}	0.54	0.0040
(+octyne)	Madras	0.05(0.01) [*]	0.02(0.03) ^{ns}	0.57	0.0004

^a V_{max} values given as nmol N₂O g⁻¹ soil d⁻¹.

^b K_m values given as μmol NO₂⁻ 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 (+octyne) activity was excluded.

Figure 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 (-oxygen) represents total AOA+AOB nitrification activity, panel B (+oxygen) represents AOA activity. Error bars represent the standard deviation of the mean ($n=4$).

Figure 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 oxygen, 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 ($p < 0.05$) between $+\text{NH}_4^+$ and $-\text{NH}_4^+$ treatments. Different upper case letters represent differences between $+\text{NO}_2^-$ and $-\text{NO}_2^-$ treatments within each NH_4^+ treatment. Error bars represent the standard deviation of the mean ($n=4$).

Figure 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 ($p < 0.05$) between +NOB and -NOB treatment. Error bars represent the standard deviation of the mean ($n=4$).

Figure 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 5: Relationship between accumulated NO_2^- concentration and N_2O production rate. Dark circles represent total AOB + AOA activity (-oxygen), and open circles represent AOA (+oxygen) activity. Panel A, Pendleton; Panel B, Madras; Panel C, Klamath. Dashed lines represent non-linear regression fit for total N_2O production rate, and solid lines represent non-linear regression fit for AOA-dependent (+oxygen) N_2O production. Regression analysis for +oxygen (AOA) data from Klamath was non-significant.

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5