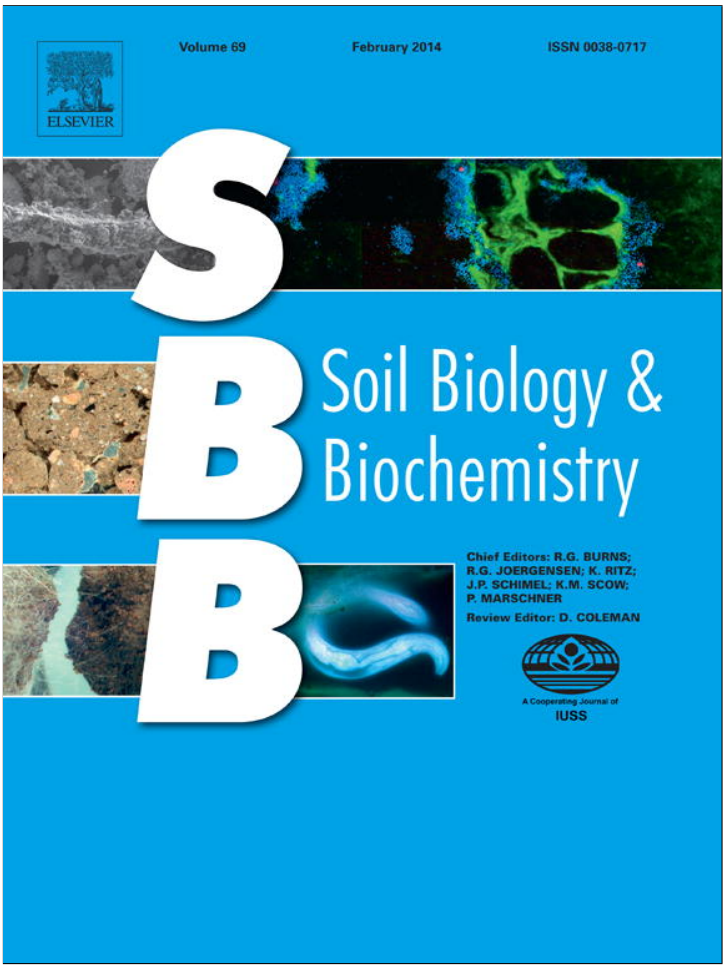

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Substrate and nutrient limitation of ammonia-oxidizing bacteria and archaea in temperate forest soil

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ABSTRACT

Ammonia-oxidizing microbes control the rate-limiting step of nitrification, a critical ecosystem process, which affects retention and mobility of nitrogen in soil ecosystems. This study investigated substrate (NH_4^+) and nutrient (K and P) limitation of ammonia-oxidizing bacteria (AOB) and ammonia-oxidizing archaea (AOA) in temperate forest soils at Coweeta Hydrologic Laboratory, a long-term ecological research site in western North Carolina, USA. We investigated substrate and nutrient limitation by amending soils with either ammonium or a nutrient solution containing P and K, then assessing the growth of these organisms during *in situ* soil incubations. We found substantial growth of both AOA and AOB during all incubations including unamended control incubations. Our results demonstrate that substrate availability limits nitrification by AOB and that high levels of substrate addition inhibit the growth of AOA in these soils. We found no evidence for nutrient limitation of AOB, though nutrient addition indirectly stimulated nitrification by AOB through increased nitrogen mineralization. Our data did suggest nutrient limitation by AOA, though it is unclear whether AOA significantly contribute to ammonia oxidation in this system. Furthermore, we show that AOB are responsible for the majority of ammonia oxidation in high substrate, high nutrient conditions.

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1. Introduction

Photoautotrophic organisms, such as plants, respond to nutrient addition by increasing rates of carbon fixation through photosynthesis, often resulting in increased growth rates (Chapin et al., 1987). The effect of fertilization on rates of carbon fixation by chemoautotrophic organisms is harder to predict however, since these organisms acquire energy for carbon fixation by the oxidation of reduced inorganic compounds. For example, ammonia-oxidizing archaea (AOA) and bacteria (AOB), which perform the rate-limiting step of nitrification, primarily use ammonium as the substrate for energy acquisition in support of chemoautotrophic growth. This energy-based demand on the inorganic nitrogen (N) pool may affect the rate at which AOA and AOB acquire other major soil nutrients, such as phosphorous (P) and potassium (K).

AOB were first discovered in the 19th century and thrive under high nutrient conditions in pure culture (Martens-Habbena et al., 2009). AOA were first isolated in pure culture in 2005 (Könneke et al., 2005) and exist in oligotrophic environments such as those found in the open ocean, where they may be responsible for the majority of ammonia oxidation (Martens-Habbena et al., 2009). AOA and AOB also exhibit significantly different ammonia oxidation kinetics in pure culture (Martens-Habbena et al., 2009). Since these organisms control the rate-limiting step of nitrification, a critical process that regulates the mobility of N in soil, understanding the independent effects of substrate and nutrient availability on AOA and AOB activity is key to understanding controls over the nitrification in any environment.

Here we test whether AOA and AOB are substrate-limited by ammonium availability or nutrient-limited by P and K in temperate forest soils. To investigate substrate and nutrient limitation of AOA and AOB, we amended forest soil with either ammonium (NH_4^+) or a nutrient solution containing both P and K. We then measured the growth response of both AOA and AOB to these additions during net nitrification incubations. Growth was assessed by estimating changes in copy number of domain-specific ammonia monooxygenase subunit A (*amoA*) genes. In this paper, we use the term nutrient to refer to elements such as P and K, which ammonia-oxidizing microbes (AOM) only use to meet assimilatory demand; we assume that assimilatory demand for NH_4^+ is low, relative to substrate requirements of these organisms, and therefore consider NH_4^+ only as a substrate for chemoautotrophic growth by AOM. We

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predicted that AOB would exhibit increased growth in response to NH_4^+ additions, while high levels of NH_4^+would inhibit the growth of AOA, as has been shown in culture based studies (e.g. Haztenpichler et al., 2008; Martens-Habbena et al., 2009). We also predicted that ammonia oxidation would exhibit a positive, saturating response to NH_4^+ addition reflective of Michaelis–Menten enzyme kinetics. Finally, we predicted that nutrient amendment would not directly affect ammonia-oxidizing microbes since these organisms should require excess NH_4^+, relative to P and K, to fill both assimilatory and energetic requirements.

2. Materials and methods

2.1. Site description and incubation conditions

This experiment was performed at Coweeta Hydrologic Laboratory, a United States Forest Service research facility and National Science Foundation Long-Term Ecological Research site, in Otto, North Carolina, USA. During the summer of 2011, we excavated approximately 5 kg of soil from a forested reference watershed (Coweeta watershed 18), which has remained undisturbed since 1927 and contains a mix of hardwood tree species (Swank and Vose, 1997). Soil was passed through an ethanol-sterilized 4 mm sieve, to remove small rocks and fine roots, and then mixed to homogenize. The sieved soil was divided into five 1-kg sub-samples, which we separately amended with two levels of ammonium chloride (NH_4Cl) (high and low substrate treatment), two levels of monobasic potassium phosphate (KH_2PO_4) (high and low nutrient treatment), or with distilled water (control treatment).

Previous samples we have taken from Coweeta had a maximum NH_4^+ concentration of 10.54 μg NH_4^+–N/g wet weight of soil. In the low substrate addition treatment, we assumed that the soil contained roughly the same background NH_4^+ concentration, corrected for moisture, and added enough NH_4Cl to double this amount. Similarly, in the high substrate addition treatment we added enough NH_4Cl to increase the ambient NH_4^+ to 10 times the background value. We designed our low nutrient treatment and high nutrient treatment to increase inorganic soil P and K molar concentrations by 1/10th of the amounts that we increased inorganic N concentrations in our low substrate treatment and high substrate treatment soils, respectively. All additions were made as solutions dissolved in 40 mL of distilled water in order to avoid increasing the water content of the soil by more than 10%, based on previous data. Solutions were applied with a spray bottle while soil was mixed by hand. Control treatments were amended with 40 mL of distilled water to control for changes in soil moisture associated with substrate and nutrient amendment.

Following the amendment procedure, we used soil from each treatment to conduct 28-day buried-bag incubations. Buried-bag assays exclude plant roots from affecting soil inorganic N pools thereby allowing NH_4^+ and nitrate (NO_3^-) to accumulate over the course of incubation (Eno, 1960). The rates at which total inorganic nitrogen (TIN) and NO_3^- accumulate during buried-bag incubations were used to estimate respective mineralization and nitrification rates in each bag. To conduct buried-bag assays, we filled Whirl-pak (Nasco, CA, USA) bags from each treatment with approximately 100 g of soil each. Whirl-pak bags are made of polyethylene, which allows for gas exchange, but not water exchange during incubation. Bags were sealed as recommended by the manufacturer. We stored three bags of each treatment at 4 deg. C (day 0 bags) until analysis and incubated 3 replicate bags in the ground, which were excavated for analysis after 28 days (day 28 bags). Incubations were conducted in the same location from which soil was initially collected, and bags from each treatment were randomly distributed in the ground during incubation.

While estimates of net nitrification could be affected by denitrification occurring during buried-bag incubations, this possibility was minimized by both increased aeration when the soil was sieved and the gas permeability of the bags we used. Furthermore, by adding substrate and nutrient solutions with spray bottles while soil was mixed by hand, we assured that there were no large saturated zones, which would serve as hotspots of denitrification. We therefore assume that the magnitude of this flux was small and consistent across treatments and that nitrification represents the major control on the nitrate pools at the end of buried-bag incubations.

2.2. Soil chemical analyses

Soil pH of day 0 samples was estimated by measuring the pH of a 1:2 soil:water slurry using an Orion 3-star benchtop pH meter (Thermo Fisher Scientific, MA, USA) (McLean, 1982). Soil moisture content was estimated by mass loss of a ~10-g subsample of day 0 and day 28 bags after overnight drying at 105 °C. Inorganic N was extracted from day 0 and day 28 samples by suspending 5-g of soil in 50 mL of 2 M KCl, and agitating for 30 min at 250 RPM on an orbital shaker table (Bundy and Meisinger, 1994). Bulk extracts were filtered through pre-leached 11-micron filter paper (Whatman International Ltd, Kent, UK) and then filtered through 0.7-μM glass fiber syringe filters (Tisch Scientific, OH, USA) prior to storage at −20 °C until further analysis. NH_4^+ and NO_3^- concentrations in KCl extractions were measured using a Lachat flow-injection autoanalyzer (Hach company, Loveland, CO, USA), and values were reported as μg N–NH_4^+/g dry weight of soil and μg N–NO_3^-/g dry weight of soil, respectively. TIN was defined as (μg N–NH_4^+ + μg N–NO_3^-)/g dry weight of soil. Net nitrification was calculated for each incubation by subtracting average day 0 NO_3^- for that treatment from day 28 NO_3^- in each bag (Eno, 1960). Similarly, net mineralization was calculated for each incubation by subtracting average day 0 TIN for that treatment from day 28 TIN in each bag (Eno, 1960).

2.3. Available ammonia (NH_3) estimations

Ammonia (NH_3) rather than ammonium (NH_4^+) is thought to be the substrate oxidized by AOB (Suzuki et al., 1974). NH_3 concentrations depend not only on the amount of NH_4^+ in a given environment but also on the pH of that environment. We therefore estimated the amount of NH_3 available in each day 0 bag after addition using day 0 NH_4^+ concentration and day 0 pH data by Equation (1).

$$[NH_3] = \left[NH_4^+\right]\left(10^{(\text{day 0 pH}-9.25)}\right) \quad (1)$$

Equation (1) is based on the Henderson–Hasselbalch equation, and assumes that the p*K*a of NH_3/NH_4^+ is 9.25.

2.4. Soil DNA extraction and quantitative PCR

DNA was extracted from ~0.25 g of soil from Day 0 and Day 28 soil samples, using MOBIO powersoil® DNA isolation kits. The manufacturer's instructions were followed except that DNA was eluted in 100 μL of solution C6 warmed to 55 deg. C to maximize elution efficiency, and an extra ethanol wash step was employed as recommended for soils with high humic content. Quantitative polymerase chain reaction (qPCR) was used in conjunction with AOA and AOB specific primers to estimate AOA and AOB abundance by quantifying gene copy number of ammonia monooxygenase subunit A (*amoA*) genes characteristic for each group. All qPCR reactions were performed in triplicate using a Biorad CFX96 quantitative thermocycler set to read SYBR green fluorescence.

Copy numbers were corrected for initial wet soil weight and soil moisture and reported as *amoA*/g dry weight of soil. Product specificity for both reactions was determined by melting curve analysis in conjunction with gel electrophoresis. AOB *amoA* gene copy number was estimated by amplifying a 491 bp fragment of the AOB *amoA* gene and comparing threshold cycle values of unknown samples to a standard curve generated from serial dilutions of a 491 bp fragment of the *amoA* gene sequence from *Nitrosomonas europea* (McTavish et al., 1993) ligated TA-TOPO cloning vector (Invitrogen life technologies, NY, USA). AOA *amoA* gene copy number was estimated by amplifying a 628 bp fragment of the AOA *amoA* gene and comparing threshold cycle values of unknown samples to a standard curve generated from serial dilutions of a 628 bp fragment with a sequence identical to soil fosmid 54d9 (Treusch et al. 2005) also ligated TA-TOPO cloning vector (Invitrogen life technologies, NY, USA). Primers sets, thermal protocols, master mix recipes, standard curve r^2 values, and standard curve reaction efficiencies for each reaction are summarized in Table 1.

2.5. AOM growth calculations

AOM growth was modeled as exponential growth, solved for number of generations by Equation (2).

$$\text{generations} = \text{Log}_2[(\text{day 28 } amoA/\text{g dw soil})/ \times (\text{avg. day 0 } amoA/\text{g dw soil})] \quad (2)$$

The mean day 0 copy number of *amoA* across treatment bags was used as day 0 *amoA*/g dw soil in equation (2).

2.6. Statistical analyses

Differences among net nitrification, growth of AOA, and growth of AOB were analyzed by one way analysis of variance (ANOVA) and significant differences between pairs of treatments were assessed by Tukey's post-hoc test using R statistical software. The relationships between growth of AOM and nitrification were assessed by linear regression.

3. Results

3.1. General soil characteristics

The soil used was fine-loamy in texture (Knoepp et al., 2008) and was typical of temperate forest soils in that it was acidic (pH = 4.95), had moderate levels of organic carbon (6% by weight), and had low standing stocks of inorganic N (4.79 μg N–NH_4^+/g dw, 0.66 μg N–NO_3^-/g dw). On average, we found 5.3×10^4 copies of AOA-specific *amoA*/g dw soil and 1.9×10^6 copies of AOB-specific *amoA*/g dw of soil prior to incubation. Though our day 0 AOA numbers seem low in comparison to other studies, low numbers of AOA may be typical of some forest soils (e.g. Boyle-Yarwood et al., 2008).

3.2. Effects of substrate addition

We found a significant effect of substrate addition on rates of nitrification (1-way ANOVA; $p < 0.001$) and growth of both AOA (1-way ANOVA; $p = 0.008$) and AOB (1-way ANOVA; $p = 0.004$) (Fig. 1). We found higher rates of nitrification and more AOB growth in the low substrate treatment incubations than in control treatment incubations, while AOA growth was not significantly different between the low substrate treatment and the control treatment. Rates of nitrification and AOB growth in the high substrate treatment did not significantly differ from the control treatment, while growth of AOA was suppressed in the high substrate treatment relative to the control treatment. We also found a significant effect of substrate addition on day 0 soil pH (1-way ANOVA; $p < 0.001$). Both levels of substrate amendment acidified day 0 samples relative to control (Table 2).

3.3. Effects of nutrient addition

We found a significant effect of nutrient addition on rates of nitrification (1-way ANOVA; $p = 0.001$), growth of AOB (1-way ANOVA; $p = 0.002$). Furthermore, we found a marginally-significant effect of nutrient addition on growth of AOA (1-way ANOVA; $p = 0.094$) (Fig. 1). Nitrification rates increased with nutrient amendment, with the highest rates of nitrification occurring in the high nutrient treatment incubations and intermediate rates of nitrification, relative to control treatment samples, occurring in low nutrient treatment incubations. AOB growth followed a similar pattern as rates of nitrification under nutrient addition, while AOA showed marginally higher growth in the high nutrient addition treatment only. We also found a marginally-significant effect of nutrient addition on rates of mineralization (1-way ANOVA; $p = 0.074$), with the lowest mineralization rates occurring in control treatment incubations and the highest rates occurring in high nutrient treatment incubations (Table 2). Nutrient addition slightly increased soil pH by 0.04 units in both high and low nutrient addition treatments relative to control (Table 2) (1-way ANOVA; $p = 0.011$).

Table 1
Details of qPCR reactions used in this experiment.

Gene amplified	AOA *amoA*	AOB *amoA*
Forward Primer	CrenamoA23f (Tourna et al., 2008)	*amoA*-1F (Stephen et al., 1998)
Reverse Primer	CrenamoA616r (Tourna et al., 2008)	*amoA*-2R (Rotthauwe et al., 1997)
Thermal Protocol	Enzyme Activation: 15 min at 95 °C 40 cycles: 1 min at 95 °C, 10 s at 52 °C, 1 min at 72 °C, plate read at 76 °C Final Extension: 10 min at 72 °C Melt curve: 65°C–95 °C	Enzyme Activation: 15 min at 95 °C 40 cycles: 1 min at 95 °C, 1 min at 54 °C, 1 min at 72 °C, plate read at 76 °C Final Extension: 10 min at 72 °C Melt curve: 65°C–95 °C (Leininger et al., 2006)
Master Mix Recipe	5 μL Quantitecht SYBR Green PCR Mix (Qiagen Inc, CA, USA) 1.5 μM forward primer 1.5 μM reverse primer 0.2 mg/mL BSA 1 μL template DNA Nuclease free water to 10 μL	5 μL Quantitecht SYBR Green PCR Mix (Qiagen Inc, CA, USA) 0.5 μM forward primer 0.5 μM reverse primer 0.2 mg/mL BSA 1 μL template DNA Nuclease free water to 10 μL
Standard curve r^2	0.970–0.994	0.985–0.998
Reaction efficiency	90.5%–102.3%	83.9%–89.2%

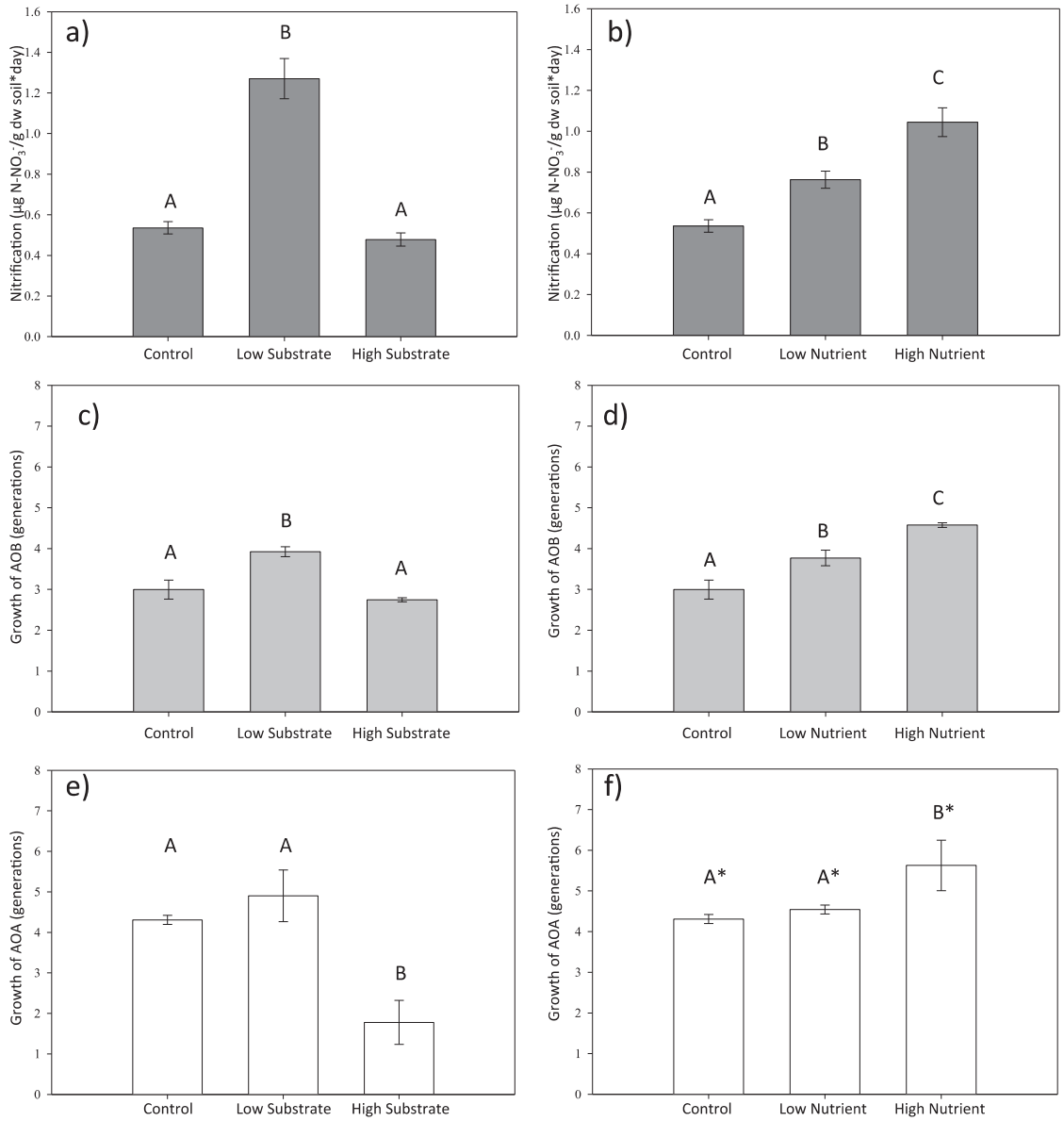

Fig. 1. Effect of substrate and nutrient amendment on net nitrification (a,b; dark gray bars), growth of AOB during the incubation (c,d; light gray bars), and growth of AOA during the incubation (e,f; white bars). Growth of AOA and AOB were calculated as the number of generations that occurred during incubation, assuming exponential growth. Letters above bars represent differences within each group by 1-way ANOVA followed by Tukey's post-hoc test. Astrices indicate $p < 0.1$, all other differences represent $p < 0.05$.

3.4. Relative roles of AOA and AOB

NO_3^- production and the number of copies of AOB *amoA* produced were strongly correlated across treatments (linear regression; $p = 0.002$; $r^2 = 0.53$) (Fig. 2). We also found a significant relationship between NO_3^- production and AOA *amoA* produced during incubations (linear regression; $p = 0.047$; $r^2 = 0.27$), but this result was entirely driven by growth of AOA in the high nutrient treatment; when the high nutrient treatment samples were removed from this analysis, no significant relationship between NO_3^- production and AOA *amoA* produced during incubation was detected.

Table 2
Net N mineralization, day 0 NH_4^+, day 0 pH, and calculated NH_3 concentrations. Values are means for each treatment ± standard error. NH_3 concentrations were calculated based on soil pH and NH_4^+ values, by a derivation of the Henderson–Hasselbach equation, using a p*K*a value of 9.25 for the ionization of NH_4^+/NH_3.

Treatment	Net N Min. (µg DIN/g DW soil*day)	Day 0 NH_4^+ (µg N–NH_4^+/g DW soil)	Day 0 pH	Day 0 NH_3 (ng N–NH_3/g DW soil)
Control	1.14 ± 0.03	4.72 ± 0.46	4.96 ± 0.01	0.24 ± 0.03
Low Substrate	1.53 ± 0.13	17.0 ± 0.31	4.73 ± 0.00	0.52 ± 0.01
High Substrate	1.80 ± 0.16	130 ± 2.30	4.47 ± 0.01	2.15 ± 0.01
Low Nutrient	1.15 ± 0.06	5.66 ± 0.51	5.00 ± 0.01	0.32 ± 0.03
High Nutrient	1.29 ± 0.01	5.47 ± 0.23	4.99 ± 0.01	0.30 ± 0.02

4. Discussion

4.1. Effects of substrate addition

A low level of substrate (i.e. NH_4^+) addition stimulated nitrification as we predicted based upon Michaelis–Menten kinetics. The concurrent stimulation of nitrification and AOB growth along with a lack of stimulation of AOA growth suggest that AOB were responsible for the increased nitrification we observed in response to a low level of substrate amendment. From these observations, we

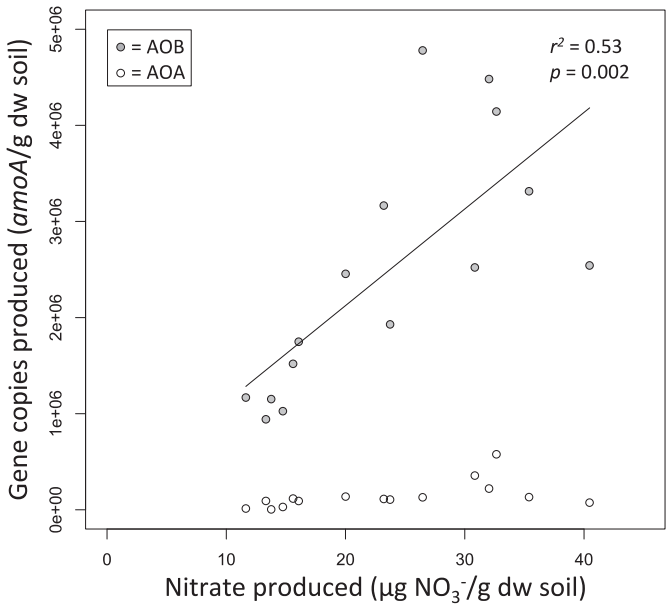

Fig. 2. Relationships between nitrate produced and both gene copies of AOB *amoA* produced (gray circles), and gene copies of AOA *amoA* produced (white circles). Line represents a significant linear regression with equation ($y = 100{,}545x + 113{,}433$).

conclude that AOB are substrate limited in this environment. Though growth of either AOA or AOB has not, to our knowledge, been demonstrated during *in situ* incubations, laboratory studies have demonstrated similar responses of AOB to substrate addition, either by monitoring the growth of these organisms by qPCR (Glaser et al., 2010; Verhamme et al., 2011) or by monitoring carbon fixation with stable isotope probing-based approaches (Jia and Conrad, 2009; Zhang et al., 2010; Pratscher et al., 2011). We found no evidence for substrate limitation of AOA. This observation is consistent with culture-based studies showing that AOA isolates reach their maximum levels of ammonium oxidation under very low nutrient concentrations (Martens-Habbena et al., 2009).

We did not observe stimulation of nitrification or AOB growth in the high substrate treatment, despite evidence for substrate limitation of AOB in the low substrate treatment. This is most-likely due to the 0.49 unit decrease in day 0 soil pH we observed when soils were amended with high levels of NH_4^+ (Table 2). Cultured AOB isolates exhibit reduced growth rates in acidic media, and other studies have shown a generally positive relationship between soil pH and AOB *amoA* transcript abundance in pH-controlled plots (Nicol et al., 2008). Low pH conditions may either directly affect AOB physiology or decrease the availability of ammonia (NH_3), which, rather than NH_4^+, is the actual substrate oxidized by AOB (Suzuki et al., 1974). To understand which of these factors influenced AOB growth in the high substrate treatment, we estimated the concentration of NH_3 across treatments and found that NH_3 concentrations increased by approximately 10× above control in the high substrate treatment (Table 2). Since NH_3 concentrations were not reduced by the effects we discuss here, we conclude that soil pH directly inhibited growth and nitrification by AOB in the high substrate treatment.

AOA growth was inhibited in the high substrate treatment relative to controls. AOA growth inhibition may have been an effect of high substrate concentrations rather than an effect of low pH since AOA have been shown to thrive in low pH conditions (Nicol et al., 2008; Lehtovirta-Morley et al., 2011), but ammonia oxidation activity by AOA isolates can be inhibited at fairly moderate substrate concentrations (e.g. Haztenpichler et al., 2008), consistent with the patterns we observed.

4.2. Effects of nutrient addition

There was a positive relationship between nitrification and nutrient addition during incubations, with the highest rates of nitrification occurring in the high nutrient treatment incubations and intermediate rates of nitrification, relative to control, occurring in low nutrient treatment incubations. As in our substrate addition experiment, growth of AOB mirrored the pattern we observed in nitrification, while growth of AOA did not (Fig. 1). We therefore conclude that AOB were responsible for the increased nitrification we observed at each level of nutrient amendment.

Though our results suggest that AOB are nutrient limited in these soils, there are other mechanisms by which the addition of KH_2PO_4 could have affected the growth of these organisms. For example, nutrient addition increased soil pH very slightly (0.04 units) in both high and low nutrient addition treatments relative to control. Though higher pH values could favor the growth of AOB, possibly through an increase in available soil NH_3, this low level of increase was probably not enough to explain the effects on AOB growth and nitrification. However, the high nutrient addition treatments showed increased N mineralization rates over control, though this result was only marginally significant (1-way ANOVA; $p = 0.078$). Still, the increased availability of substrate could favor ammonia oxidation by AOB in the nutrient addition treatments, as was the case for our substrate addition experiment. Since we can explain increases in nitrification by AOB based on increases in soil pH and mineralization rates associated with nutrient addition, we cannot conclude that AOB are nutrient limited in this environment.

Few studies have directly investigated the effects of nutrient, rather than substrate addition on AOM. Analysis of AOB in long-term fertilization plots showed effects of P and K amendment on AOB community structure (Chu et al., 2007) but not on AOB abundance (Chu et al., 2008). Similarly, P and K addition affected AOB community structure in a microcosm-study investigating stream biofilms (Lage et al., 2010). Two studies show a positive growth response of AOM to nutrient addition; Dodor and Duah-Yentumi (1999) showed that P addition led to growth of soil AOB in a field setting, while de Vet et al. (2012) showed that P addition led to growth of AOB in a flask study inoculated with organisms from nitrifying biofilms at a wastewater treatment plant. However, neither study conclusively demonstrated that increased growth of AOB resulted from direct P uptake by AOB rather than indirect effects of nutrient addition on N mineralization rates or environmental pH as we demonstrate here.

The marginally significant effects of nutrient addition on growth of AOA in the higher nutrient treatment suggest that AOA could be nutrient limited in the soil we tested. The presence of P in our high and low nutrient treatment may be especially relevant to AOA. Though a phosphonate transport system was identified in the genome of *Nitrosopumilus maritimus*, no known carbon-phosphorous lyases or hydrolases have been identified from genomic evidence and phosphonate does not relieve P limitation of *N. maritimus* in culture-based studies (Walker et al., 2010). Furthermore, P concentrations have been shown to drive AOA abundance in estuarine sediments (Sakame, 2012). Free PO_4^{3-} may have stimulated phosphorus assimilation by soil AOA in the incubations we conducted as well.

4.3. Relative roles of AOA and AOB during net nitrification incubations

To understand whether AOA and AOB growth was related to ammonia oxidation, we sought to establish a relationship between net nitrification and growth of AOM across treatments. The strong relationship we observed between NO_3^- produced and the number

of gene copies of AOB *amoA* produced (Fig. 2) suggests that AOB used ammonia oxidation to support growth across treatments. Though we also found a positive relationship between NO_3^- produced and the number of gene copies of AOA *amoA* produced as well, this relationship was entirely driven by the high nutrient treatment, and no significant relationship was evident when high nutrient treatment data were excluded from the analysis. Either AOA were only contributing to increased nitrification in the high nutrient treatment, or they responded to nigh nutrient addition by increasing heterotrophic activity. Since genomic evidence indicates a capacity for mixotrphy in marine AOA (Walker et al., 2010), AOA could be living heterotrophically in the soil we investigated here.

While net nitrification incubations have often been used to assess *in situ* process rates at a variety of sites (e.g. Knoepp and Vose, 2007), we believe that this is the first documentation of AOA and AOB growth during these incubations. It is of note that we detected substantial growth of both AOA and AOB during unamended incubations, a fact that highlights the non-equilibrium nature of these incubations. Furthermore, the change in enzymatic concentrations we documented during unamended incubations shows that these incubations violate the assumption of constant enzyme concentrations necessary for the application of Michaelis–Menten enzymatic kinetics. The qPCR-based approach we employed in this experiment allows researchers to glean additional information about how AOA and AOB contribute to the process of ammonia oxidation during net nitrification incubations, and we encourage its use in future studies on soil AOA and AOB.

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