Environmental Controls on the Diversity, Growth, and Activity of Ammonia-Oxidizing Microorganisms in Temperate Forest Soils

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ABSTRACT

The goal of my dissertation research was to investigate the structure and function of ammonia-oxidizing microbial communities in temperate forest soils. Accomplishing this goal required a hybrid approach: I used modern molecular biology techniques alongside soil biogeochemical measurements and framed my research using ecological theory largely developed in plant systems. All of my field work was done at Coweeta Hydrologic Laboratory, a Forest Service Station and Long Term Ecological Research Site near Otto, NC. Watershed-level land use manipulations have been performed at Coweeta since the 1930s, including clear-cutting, fertilizing, liming, burning, grazing by cattle, and replanting entire watersheds in white pine. While these treatments were originally imposed to assess the effects of land use on water yield, they have resulted in changes in soil characteristics as well. Working at Coweeta has therefore allowed me to sample ammonia-oxidizer communities across a gradient of soil variables, such as pH and nitrogen (N) availability, within the geographically-constrained area of the Coweeta Basin.

First, I used amplicon-based pyrosequencing to independently assess the diversity of ammonia-oxidizing archaea (AOA) and bacteria (AOB) at several sites within Coweeta. I found that AOA and AOB diversity were a function of both resource availability (i.e. N availability) and environmental harshness (i.e. soil pH) in line with general ecological theory developed for plant systems by Tilman and Grime, respectively. Next, I tested whether AOA and AOB were substrate or nutrient limited in this system by adding either N or a nutrient solution containing both potassium and phosphorus to soil incubations and assessing the growth response of AOA and AOB using quantitative polymerase chain reaction (qPCR). I found strong evidence for substrate limitation by AOB and a marginally-significant positive effect of nutrient

addition on growth of AOA. Another intriguing finding from this study was that both AOA and AOB grew during unamended soil incubations. Unamended (buried-bag) incubations have been used to estimate *in situ* rates of nitrification for over 50 years. By measuring the growth of AOA and AOB alongside nitrification during buried-bag incubations, I discovered that AOA are the dominant ammonia-oxidizers in temperate forest soils. However, I found that AOA are much less efficient at using the energy from ammonia oxidation to create biomass than AOB in the forest soils I sampled.

Overall, I found that temperate forest soils contain low abundances of AOA and AOB, with relatively low diversity in both groups. This is especially true for the diversity of AOA, where a single taxon dominated the community at every site. Soil pH and N availability seem to be major selective forces for forest soil ammonia oxidizers, though other nutrients such as potassium and phosphorus may regulate the activity of AOA as well. AOA are most-likely the dominant ammonia oxidizers in temperate forest systems, though this may change with increased disturbance. In a broader sense, I found that ecological theory developed for plant communities was applicable to chemoautotrophic microbes despite the large differences in life history between these groups of organisms.

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Chapter 1: General Introduction

Nitrification, the oxidation of ammonia to nitrate is an essential ecosystem process, which links mineralization and denitrification, thereby acting as a bottle-neck between nitrogen regeneration and the major pathway for nitrogen removal in many ecosystems. Due to a global increase in anthropogenic nitrogen loading in recent decades (Aber et al. 1998, Galloway et al. 2003, Vitousek 1994), processes affecting the retention and removal of nitrogen are of critical importance for terrestrial and aquatic ecosystems alike.

Nitrification is a two-step process consisting of the oxidation of ammonium to nitrite followed by the oxidation of nitrite to nitrate. Both steps of the process are independently controlled by specific groups of chemoautotrophic microorganisms. In soils, the oxidation of ammonium to nitrite is performed by two distinct groups of microorganisms: ammonia-oxidizing bacteria (AOB) and recently-discovered ammonia-oxidizing archaea (AOA) (Venter et al. 2004, Konneke et al. 2005, Treusch et al. 2005). AOB and AOA form a single functional group of aerobic ammonia-oxidizing microorganisms (AOM). Since the oxidation of ammonium to nitrite is considered to be the rate-limiting step of nitrification, environmental factors affecting the abundance, growth, and diversity of AOM may have ramifications at the ecosystem scale.

AOB were first isolated in pure culture by Sergei Winogradsky in 1890, and known genera exist within class Betaproteobacteria (Nitrosomonas and Nitrosospira) and class Gammaproteobacteria (Nitrosococcus) (Madigan et al. 2002). AOB are obligate chemoautotrophs and fix carbon through the Calvin cycle (Madigan et al. 2002). AOB are slow growing organisms, due to the low energy yield of their metabolism, and therefore difficult to study by culture-based techniques (Kowalchuck and Stephen 2001). The advent of molecular methods and development of primers relevant to AOB ammonia monooxygenase subunit A (AOB *amoA*)(Rotthauwe et al. 1997), a gene essential to ammonia oxidation, made the ecology of these organisms much easier to study. Early molecular studies revealed that edaphic variables such as pH (Stephen et al. 1998) and

oxygen availability (Kowalchuck et al. 1998) have significant effects on AOB community structure.

Over a century after the isolation of AOB, the first AOA isolate, *Nitrosopumilus maritimus*, was obtained in pure culture (Konneke et al. 2005). Analysis of the rate kinetics of ammonia oxidation by *N. maritimus* in the laboratory revealed that AOA can outcompete AOB for ammonium at low concentrations (Martens-Habbena et al. 2009), but are inhibited by high concentrations of ammonium used to isolate AOB in enrichment culture. In this way, AOA conform to the paradigm proposed by Valentine (2007), who suggested that life under energy stress is a uniting ecological principal differentiating archaea from bacteria.

Following the discovery of AOA, primers were developed based on AOA *ammonia monooxygenase* genes (AOA *amoA*) (Francis et al. 2005), which allowed researchers to perform environmental studies investigating the full diversity of the ammonia-oxidizing microbes for the first time. The numerical dominance of AOA over AOB was quickly established in both temperate soils (Leininger et al. 2006) and the open ocean (Wuchter et al. 2006). Not only are AOA more prevalent than AOB in these environments but there is speculation that they are responsible for most of the nitrification in oligotrophic systems as well. By comparing laboratory rates of ammonia oxidation with experimentally measured field rates, Martens-Habbena et al. (2009) deduced that AOA are responsible for ammonia oxidation in the open ocean, and the majority of ammonia oxidation in unfertilized soils.

My dissertation work has focused on the structure and function of temperate forest soil AOA and AOB communities, along with certain aspects of AOA and AOB physiology. I used culture-independent techniques such as amplicon-based pyrosequencing and quantitative polymerase chain reaction (qPCR) to investigate naturally-occurring AOA and AOB communities in temperate forest soils. Using these techniques along with primers that target the *amoA* genes of each group, I was able to independently assess AOA and AOB diversity and abundance from the same sample. Furthermore, using *in situ* incubations allowed me to track the growth of these organisms alongside nitrification. Here I present the results of three studies about temperate forest

soil ammonia oxidizers. The first study investigates environmental controls on the diversity of ammonia oxidizers. In the second study, I track AOA and AOB growth in response to the addition of nitrogen and other nutrients in order to understand limitation in these organisms. Finally, I monitor both growth and nitrification during unamended *in situ* incubations in order to assess the relative contributions of AOA and AOB to ammonia oxidation in temperate forest soils.

All of my dissertation work was performed at Coweeta LTER, a long term ecological research site near Otto, North Carolina. Coweeta was originally developed as a hydrologic research laboratory where manipulations were imposed on different watersheds in order to assess the effects of land use on water yield. Though watershed-scale manipulations have not been performed at Coweeta in recent years, past manipulations still affect nitrogen yield from various watersheds (Swank and Vose 1997). Presumably these patterns reflect differential nitrogen processing within the soils in these watersheds. While the work I report in Chapter 3 was performed in a reference watershed at Coweeta, I took advantage of the differences in land use history between watersheds to sample across a gradient of nitrogen availability for the work I present in chapters 2 and 4 of this document. In total, I sampled across 4 watersheds at Coweeta which range in nitrogen content from 0.09-7.09 g Dissolved inorganic nitrogen per hectare of watershed area. A summary of the land use history and nitrogen export from the four watersheds I used during my dissertation is presented in Table 1.

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Table 1. Land use history and nitrogen export from the watersheds at Coweeta used in this study. All data were obtained from Swank and Vose (1997), which contains a more-detailed description of watershed land use history. DIN-N was calculated by adding reported values of NO_3^--N and NH_4^+-N per hectare per watershed.

Watershed	Land use HIstory	DIN-N output per hectare
18	Reference	0.09
7	Clear-cut	0.64
17	Hardwood to pine conversion	1.00
6	Hardwood to grass conversion	7.09

Chapter 2: Environmental Controls on the Diversity of Ammonia-Oxidizing Bacteria and Archaea in Temperate Forest Soil

Abstract

The purpose of this study was to investigate environmental controls on the diversity of ammonia oxidizers in temperate forest soils. I predicted that ammonia oxidizer diversity would be a product of N availability and soil pH, with N availability acting as a resource for ammonia oxidizers, and soil pH acting as a source of environmental stress for these organisms. I used amplicon-based pyrosequencing to measure the diversity of ammonia-oxidizing archaea (AOA) and bacteria (AOB) at several temperate forest sites, which span a gradient in N availability and soil pH. I found that AOA diversity was related to soil ammonium (NH_4^+), while AOB diversity was related to soil ammonia (NH_3). I also found a strong positive relationship between AOB diversity and soil pH. My results show that ecological theory concerning the diversity of plants in response to resource availability and environmental stress may be applicable to microbial communities as well. Furthermore, my results lend evidence to speculation that AOA and AOB may use different substrates for ammonia oxidation, a question still unresolved in the field of microbial ecology.

Introduction

Ammonia oxidation, the rate-limiting step of nitrification, is a microbially-driven process, which affects the fate and mobility of nitrogen (N) in terrestrial ecosystems. Ammonia oxidation is performed by two groups of chemoautotrophic microbes: ammonia-oxidizing bacteria (AOB) and ammonia-oxidizing archaea (AOA). Understanding the factors that control the diversity of AOA and AOB in a given environment is critical to understanding controls over ammonia oxidation as a process. Here I investigate the effects of soil pH and N-availability on the diversity of AOA and AOB communities in temperate forest soils.

AOA and AOB exhibit general differences in both the amount of N they prefer and pH tolerance at the group level. AOB are competitive at high N availability, and a number of studies have shown a positive growth response by soil AOB following N amendment (for example Di et al., 2009, Jia and Conrad 2009, Di et al., 2010, Norman and Barrett *in press*). In contrast to AOB, AOA were first isolated using low N media (Könneke et al., 2005), and *Nitrosopumilus maritimus*, the first cultured AOA isolate, reaches its maximum rate of ammonia oxidation at low ammonium concentrations in pure culture (Martens-Habbena et al., 2009). Also, AOB exhibit a preference for neutral pH conditions, while AOA can thrive in low pH environments: Nicol et al. (2008) found a negative relationship between AOA activity and soil pH in pH-controlled plots, while the opposite pattern was true for AOB in the same soils. Furthermore, AOB isolates do not grow below a pH of ~4.5 in pure culture, while an acidophilic AOA representative was cultured in 2011 (Lehtovirta-Morley et al. 2011).

N and pH tolerance may structure diversity of taxa within the larger groups of AOA and AOB as well. For example, N availability may control AOA and AOB diversity according to the framework proposed by Tilman (1982) in which species diversity is a function of resource availability. According to Tilman's proposed mechanism, low resource availability will select for the oligotrophic subset of the species pool, while high resource availability will allow for dominance by the most competitive subset of the species pool. Tilman predicted that diversity would peak at moderate levels of resource availability due to a lack of these two extremes. Soil pH may control AOA and AOB diversity according to the framework proposed by Grime (1973), in which

species diversity is a consequence of environmental stress. According to this mechanism, high stress environments will select for the subset of species that can tolerate these conditions, while low-stress environments will once again allow for dominance by the most competitive subset of the species pool. As in Tilman's resource availability framework, a peak in species diversity is expected at moderate levels of environmental stress due to a lack of these extremes.

Here I describe the results of a field study in which I measured AOA and AOB diversity across a gradient of N availability and soil pH in temperate forest soils. Though they span a variety of conditions, temperate forest soils tend to be low N, low pH systems, in comparison to other temperate soils. I therefore expected to see more-pronounced effects of N availability on AOA rather than AOB diversity in temperate forest soils, since the resource levels in these systems most-likely span a range of values more relevant to the oligotrophic AOA group than the copiotrophic AOB group. Furthermore, I predicted that AOB diversity will increase with soil pH in temperate forest soils, since these generally acidic systems represent high to intermediate levels of environmental stress for AOB.

Methods

Site Selection and Study Design

This study was completed at Coweeta Hydrologic Laboratory, a US National Forest Service Station and National Science Foundation-sponsored Long Term Ecological Research Site. Coweeta has a humid, temperate climate with mean annual precipitation varying between 1800 mm and 2500 mm depending on elevation (Swank and Vose 1997). I established study sites within four experimental watersheds (WS) at Coweeta, which differ in their land-use history, thereby spanning a range of values of soil pH and substrate availability in temperate forest soils within a geographically-constrained area. Watershed (WS) treatments were as follows: WS 6 was clear-cut and burned in 1958, replanted in grass, limed, and fertilized in 1959, fertilized again in 1965, treated with herbicide in 1966 and 1967, then allowed to regrow; WS 7 was grazed by cattle from

1941-1952 then cable-logged in 1977; WS 17 was cut annually from 1940-1955 then planted in white pine in 1956; WS 18 has been maintained as a reference watershed since 1927 (source: coweeta.uga.edu). I located three sampling sites along stream-to-hillslope transects in each watershed to sample a variety of environmental conditions within in each watershed, and sampled each site during the summer of 2011. Sites were located approximately 5 m, 10 m, and 25 m from streams. At each site, I excavated soil from the top 5 cm, passed it through a 2-mm sieve to remove small rocks and fine roots, placed ~100 g of soil into each of three replicate bags (subsamples), and stored the bags at 4 deg. C until further analysis.

Soil pH and N Availability Measurements

I measured soil moisture, soil pH, and three metrics of N availability, including total percent nitrogen (%N), soil ammonium (NH_4^+) and soil ammonia (NH_3) in each subsample. Moisture content was estimated by measuring the change in mass of 10 g of soil after incubation overnight at 105°C. Soil pH was estimated by measuring the pH of a 1:2 soil:water slurry using a benchtop pH meter (Thermo Fisher Scientific, MA, USA) (McLean 1982). Soil %N was measured using an FlashEA 1112 Series Elemental Analyzer (OI Analytical, College Station TX, USA). Inorganic nitrogen was also extracted from ~5 g dry weight (dw) of day 0 and day 30 soils by adding soil to 100 ml of 2M KCl and shaking at 250 RPM for 30 min (Bundy and Meisinger 1994). Extracts were filtered and NH_4^+ was measured using a Lachat Quickchem flow injection autoanalyzer (Lachat Instruments, Loveland, CO, USA) and reported as μg NH_4^+-N/g dw soil. Finally, I estimated day 0 NH_3/g dw at each site using equation 1, since ammonia (NH_3) rather than ammonium (NH_4^+) is thought to be the substrate oxidized by AOB (Suzuki et al. 1974).

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

AOA and AOB Diversity

I extracted DNA from day 0 soils using a PowerSoil® DNA isolation kit (MO BIO Laboratories Inc., CA, USA) and following the manufacturer's instructions except that DNA was eluted twice with additions of 75 μL of solution C6 each time to maximize extraction efficiency. DNA concentrations were enumerated in each extract using a Nanodrop spectrophotometer (ThermoFisher Scientific, MA, USA), and all extracts had a 260/280 ratio of greater than 1.8, indicating low humic contamination.

AOA and AOB diversity was assessed by targeted pyrosequencing of group-specific ammonia monooxygenase subunit A (*amoA*) genes. Replicate DNA extracts from each site were pooled and these pooled extracts were then frozen and sent to Molecular Research LP (MR DNA, Shallowater, TX, USA) for unidirectional amplicon-based sequencing of group-specific *amoA* genes using primer sets *amoA-1F** and *amoA-2R* for AOB, and *Arch-amoA-1F** and *Arch-amoA-2R* for AOA (*Indicates sequencing primer). PCR reactions used HotStarTaq Plus Master Mix (Qiagen, Valencia, CA) and were inoculated with 10 ng of template DNA. Reaction conditions for both genes were as follows: 94°C for 3 minutes; 33 cycles of: 94°C for 30 seconds, 53°C for 40 seconds, 72°C for 1 minute; 72°C for 5 minutes. Amplicons from each sample were combined at equimolar concentrations, purified using Agencourt Ampure beads (Agencourt Bioscience Corporation, MA, USA), and sequenced according to the manufacturers instructions using a Roche 454 FLX titanium instrument and Roche reagents (Roche Applied Science, IN, USA). AOA sequences were not obtained for one site in WS 6.

I used MOTHUR to filter sequences and estimate diversity (Schloss et al. 2009), following the Schloss lab 454 SOP (Schloss et al. 2011), available online at http://www.mothur.org/wiki/Schloss_SOP. Briefly, the "shhh.flows" function was used to remove low quality sequences; the "trim.seqs" function was used to remove short sequences; the "align.seqs" function was used to align sequences against a representative sequence for each group; the "screen.seqs" function was used to trim sequences to the same length; the "chimera.uchime" function was used to remove chimeric sequences. After these initial filtering steps, one site in WS 18 only contained only 64 sequences, and was removed from further AOB analysis.

Prior to diversity estimation, genetic sequences are typically clustered into operational taxonomic units (OTUs) based on sequence similarity. Studies that estimate bacterial diversity by sequence analysis almost universally use OTU cutoff of 97% sequence similarity to define different taxa of microbes (*sensu* Stackebrandt and Goebel 1994), which correlates with the 70% DNA homology requirement suggested by Wayne et al. (1987). No such universal OTU cutoff has yet been agreed upon for *amoA* genes and even recent studies use OTU cutoffs ranging from 85% (for example, Pester et al. 2013) to 97% (for example, Hou et al. 2013). In this study I estimated diversity based on an OTU cutoff of 94% nucleotide identity, which has been used as a species-level cutoff for functional genes (e.g. Levine et al. 2011) based on the findings of Konstantinidis and Tiedje (2005). Once OTU's were defined, I used the "Get.oturep" function in MOTHUR to obtain a representative sequence for each OTU in the dataset, and screened these sequences against sequences from cultured isolates in the non-redundant protein database using blast (http://ncbi.nim.nih.gov), to insure sequence specificity. All representative sequences matched bacterial and archaeal *amoA* genes as expected.

To estimate AOA and AOB diversity, datasets were subsampled to 1243 sequences (based on the lowest number of sequences remaining at a single site across both AOA and AOB datasets) at each site, and a Shannon diversity index was estimated at each site using the "summary.single" command; the Shannon index (Shannon 1948) accounts for evenness in community structure and can be reliably estimated from microbial sequence data, while richness estimates such as the Chao richness estimator cannot (Haegeman et al. 2013).

Statistical Analyses

To test my predictions about the relationships between N availability and AOA and AOB diversity, I regressed AOA and AOB diversity against three metrics of N availability: soil %N, soil NH_4^+, and soil NH_3. Similarly, I regressed AOA and AOB diversity against soil pH as a metric of environmental harshness. Regression analysis was performed using R statistical software (the R Project, Vienna, Austria). I used

function "lm" to fit either linear or second-degree polynomial functions to my data, since I predicted both linear and hump-shaped relationships between variables.

Results

General Community Characteristics

I found 138 AOA OTUs and 394 AOB OTUs across all sites. However, many of these OTUs were of low abundance; I found single sequence representatives of 272 AOB OTUs and 94 AOA OTUs. Rank abundance curves for AOA and AOB are presented in Figure 1. AOA communities were largely dominated by a single OTU (AOA OTU 1) at each site. AOA OTU 1 comprised 85% of the sequences I detected and ranged in dominance from 67%-99% of the sequences detected at each site. A representative sequence from this OTU showed an 86% sequence identity with the *amoA* gene from *Nitrosotalea devanterra*, an acidophilic AOA isolate first enriched from agricultural soil in 2011 (Lehtovirta-Morley et al. 2011). Several other AOA OTUs were closely related to *N. devanterra* as well, while I also found sequences closely related to *Nitrososphaera viennensis* (~4% of the community), and *Nitrososphaera gargensis* (~0.5% of the community). AOB communities showed greater evenness than AOA communities in the soils I sampled (Figure 1). Nine of the ten most abundant AOB OTUs (over 90% of the sequences across the entire AOB dataset) were identified as being most-similar to sequences from cultured isolates of the genus *Nitrosospira*. The seventh most-abundant AOB OTU, which comprised ~4% of the total sequences across all sites, was most-closely related to *Nitrosovibrio tenuis.*

AOA and AOB Diversity Patterns

In general, AOB communities were more diverse than AOA communities, as represented by Shannon indices (paired t-test, $p<0.05$). I found a hump-shaped relationship between AOA diversity and soil NH_4^+ in the soils I sampled (second degree polynomial regression, $p<0.05$; $r^2=0.53$; Figure 2a). However, AOA diversity was not

significantly related to either %N (Figure 2c) or soil NH_3. (Figure 2e). I also found a significant positive relationship between AOB diversity and soil NH_3 (linear regression, $p<0.05$, $R^2=0.44$, Figure 2d), though I did not find significant relationships between AOB diversity and soil NH_4^+(Figure 2b), or AOB diversity and soil %N (Figure 2f). I also found a positive relationship between AOB diversity and soil pH (linear regression, $p<0.05$, $R^2=0.61$, Figure 3b), but no significant relationship between AOA diversity and soil pH (Figure 3a).

Discussion

I found evidence that both AOA and AOB diversity are driven by resource availability in temperate forest soils. While AOA diversity showed a significant hump-shaped response to soil NH_4^+ levels, a predicted response for plant systems over a resource gradient (Tilman 1982), AOB diversity showed a strong positive response to soil NH_3, and neither AOA or AOB diversity responded to soil %N. The substrate-specific responses of AOA and AOB diversity I outline here could reflect group-level physiological differences in ammonia monoxygenase genes. While Suzuki et al. (1979) showed that AOB use NH_3 as the substrate for oxidation, the substrate of oxidation has not yet been determined for AOA (Hatzenpichler 2012), and several authors has speculated that NH_4^+, rather than NH_3, could be oxidized by AOA (Martens-Habbena and Stahl 2011, Lehtovirta-Morley et al. 2011), though this has not directly been tested to my knowledge. I feel that a direct test of AOA substrate preference would shed further light on my results.

Though AOB diversity showed a strong, possibly saturating, response to soil NH_3, this response could be strongly driven by soil pH, which determines the concentration of NH_3 (Equation 1). Indeed I saw a strong positive relationship between soil pH and AOB diversity as well (Figure 3b). Whether forest soil AOB diversity is driven by the availability of NH_3, *sensu* Tilman (1982) , or the environmental stresses associated with differences in soil pH, *sensu* Grime (1973), cannot be determined from this dataset and is most-likely due to physiological tolerances of AOB. While AOB show low activity in

acidic soils relative to neutral soils (Nicol et al. 2008), authors often speculate that this is due to the decreased availability of NH_3 (Hatzenpichler 2012). However, I have found that decreased pH alone explained growth inhibition of AOB growth during *in situ* field incubations despite an overall increase in the availability of NH_3 (Norman and Barrett *in press*). Therefore, I feel that there is a role for pH regulation of AOB diversity as source of environmental stress rather than a modulator of resource availability in temperate forest soils.

In general, AOA and AOB communities are dominated by relatively few taxa in the soils I sampled. This was especially true for AOA, since AOA OTU 1 made up a 67-99% of every community I sampled. Though a representative sequence from AOA OTU 1 was most-closely related to *N. devanterra*, the level of relatedness was quite low (86%) and an isolated organism from AOA OTU 1 would most-likely be classified as a novel taxon, distinct from *N. devanterra*. It is also unsurprising that AOB communities were dominated by various species of genus *Nitrosospira*, since these organisms tend to be more competitive for ammonium than other AOB (Taylor and Bottomley 2006). The large number of OTUs for which I found only a single sequence indicates that there may a "rare biosphere" of ammonia-oxidizers in temperate forest soils as has been speculated for microbial communities in general (for example Sogin et al. 2006).

Overall, my results highlight the point that ecological theory developed in plant systems may be applicable to microbial systems despite large differences in life history traits such as generation times, genetic exchange, capacity for dormancy and dispersal ability between plants and microbes. Since microbes have unique forms of metabolism, which control critical choke-points in the global carbon, nitrogen and sulphur cycles (among others), testing community ecology theory on microbial systems could give great insight into biogeochemical cycling. This is especially true when researchers use functional gene based approaches to limit the scope of their study to a particular functional group, as I have done here.

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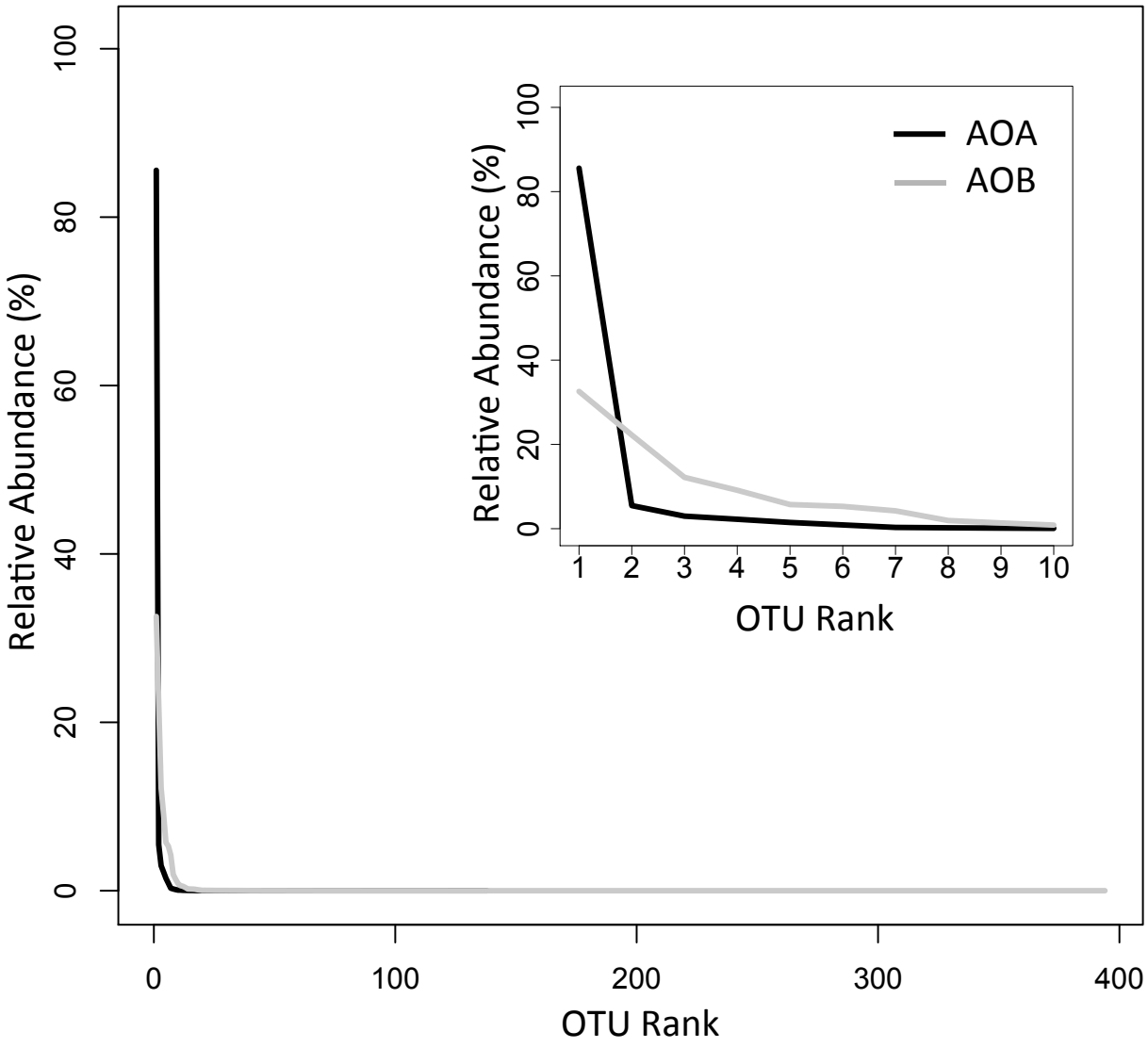

Figure 1. Rank abundance curves for AOA and AOB communities across all sites. Inset represents rank abundance curves for top 10 most abundant OTUs of each group.

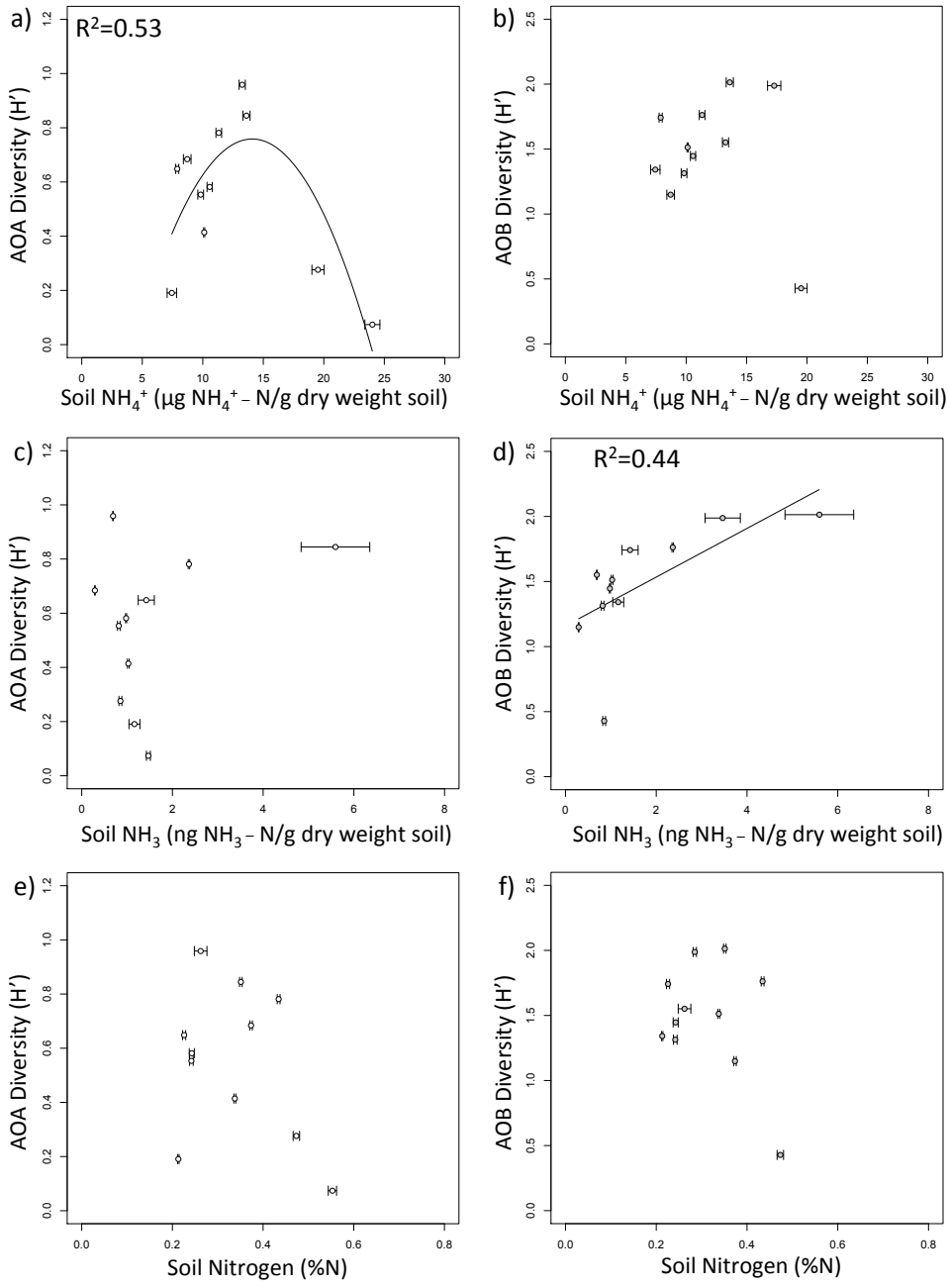

Figure 2. The effects of resource availability on AOA and AOB diversity. Panels a,c, and e show the relationship between AOA diversity and soil NH_4^+, soil NH_3, and soil %N, respectively. Panels b,d, and f show the relationship between AOB diversity and soil NH_4^+, soil NH_3, and soil %N, respectively. Regression results are only shown if $p<0.05$. The line in panel a represents a second degree polynomial regression ($y = -0.0079x^2 + 0.2237x – 0.8171$). The line in panel d represents a linear regression ($y = 0.1872x + 1.159$).

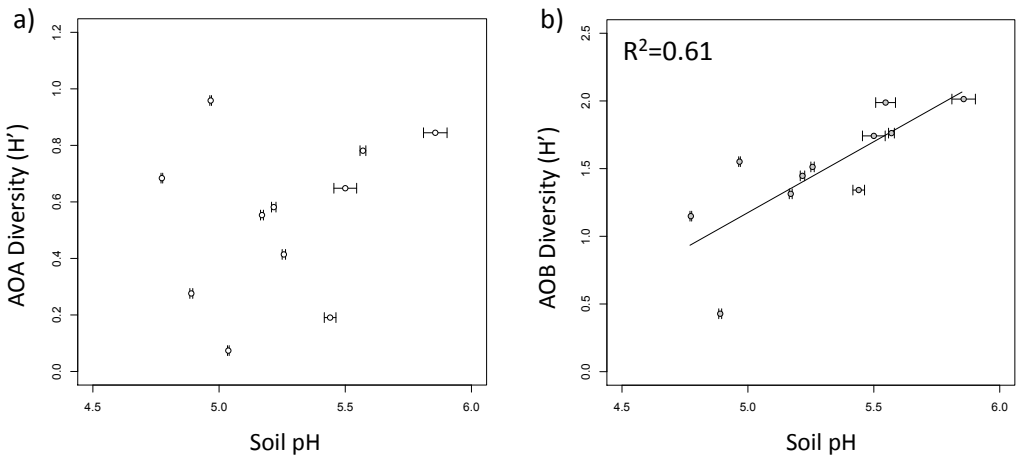

Figure 3. The effects of environmental stress on AOA and AOB diversity. Panel a shows the relationship between AOA diversity and soil pH, which panel b shows the relationship between AOB diversity and soil pH. Regression results are only shown if $p<0.05$. The line in panel b represents a linear regression ($y = 1.048x – 4.065$).

Chapter 3: Substrate and Nutrient Limitation of Ammonia-Oxidizing Bacteria and Archaea in Temperate Forest Soil

Norman, J.S., and Barrett, J.E. 2013. Substrate and nutrient limitation of ammonia-oxidizing bacteria and archaea in temperate forest soil. Soil Biology & Biochemistry *in press.*

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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. I 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. I found substantial growth of both AOA and AOB during all incubations including unamended control incubations. My results demonstrate that substrate availability limits nitrification by AOB and that high levels of substrate addition inhibit the growth of AOA in these soils. I found no evidence for nutrient limitation of AOB, though nutrient addition indirectly stimulated nitrification by AOB through increased nitrogen mineralization. My data did suggest nutrient limitation by AOA, though it is unclear whether AOA significantly contribute to ammonia oxidation in this system. Furthermore, I show that AOB are responsible for the majority of ammonia oxidation in high substrate, high nutrient conditions.

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 I 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, I amended forest soil with either ammonium (NH_4^+) or a nutrient solution containing both P and K. I 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. Here, I use the term nutrient to refer to elements such as P and K, which ammonia-oxidizing microbes (AOM) only use to meet assimilatory demand; I 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. I 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. Hatzenpichler et al. 2008, Martens-Habbena et al. 2009). I also predicted that ammonia oxidation would exhibit a positive, saturating response to NH_4^+ addition reflective of Michaelis-Menten enzyme kinetics. Finally, I 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.

Methods

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, I 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 I 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 I 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, I 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 I added enough NH_4Cl to increase the ambient NH_4^+ to 10 times the background value. I designed my low nutrient treatment and high nutrient

treatment to increase inorganic soil P and K molar concentrations by $1/10^{th}$ of the amounts that I increased inorganic N concentrations in my 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, I 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, I 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. I 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 I used. Furthermore, by adding substrate and nutrient solutions with spray bottles while soil was mixed by hand, I assured that there were no large saturated zones, which would serve as hotspots of denitrification. I 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.

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).

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. 1973). NH_3 concentrations depend not only on the amount of NH_4^+ in a given environment but also on the pH of that environment. I 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.

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

Equation 1 is based on the Henderson-Hasselbalch equation, and assumes that the pKa of NH_3/NH_4^+ is 9.25.

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.

AOM Growth Calculations

AOM growth was modeled as exponential growth, solved for number of generations by equation 2.

(2) generations = Log_2[(day 28 *amoA*/g dw soil)/(avg. day 0 *amoA*/g dw soil)]

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

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.

Results

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 $N\text{-}NH_4^+$/g dw, 0.66 µg $N\text{-}NO_3^-$/g dw). On average, I found 5.3 x 10^4 copies of AOA-specific *amoA*/g dw soil and 1.9 x 10^6 copies of AOB-specific *amoA*/g dw of soil prior to incubation. Though my 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).

Effects of Substrate Addition

I 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; $p=0.004$) (Figure 1). I 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. I 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).

Effects of Nutrient Addition

I 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, I found a marginally-significant effect of nutrient addition on growth of AOA (1-way ANOVA; $p=0.094$) (Figure 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. I 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; $p=0.011$).

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) (Figure 2). I 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.

Discussion

Effects of Substrate Addition

A low level of substrate (i.e. NH_4^+) addition stimulated nitrification as I 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 I observed in response to a low level of substrate amendment. From these observations, I conclude that AOB are substrate limited in this environment. Though growth of either AOA or AOB has not, to my 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). I found no evidence for substrate limitation of AOA. This observation is consistant 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).

I 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 I 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, I estimated the concentration of NH_3 across treatments and found that NH_3 concentrations increased by approximately 10X above control in the high substrate treatment (Table 2). Since NH_3 concentrations were not reduced by the effects I discuss here, I 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. Hatzenpichler et al. 2008), consistent with the patterns I observed.

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 my substrate addition experiment, growth of AOB mirrored the pattern I observed in nitrification, while growth of AOA did not (Figure 1). I therefore conclude that AOB were responsible for the increased nitrification I observed at each level of nutrient amendment.

Though my 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 my substrate addition experiment. Since I can explain increases in nitrification by AOB based on increases in soil pH and mineralization rates associated with nutrient addition, I 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 I 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 I tested. The presence of P in my 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 I conducted as well.

Relative Roles of AOA and AOB During Net Nitrification Incubations

To understand whether AOA and AOB growth was related to ammonia oxidation, I sought to establish a relationship between net nitrification and growth of AOM across treatments. The strong relationship I observed between NO_3^- produced and the number of gene copies of AOB *amoA* produced (Figure 2) suggests that AOB used ammonia oxidation to support growth across treatments. Though I 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 I 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), I believe that this is the first documentation of AOA and AOB growth during these incubations. It is of note that I 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 I 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 I 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 I encourage its use in future studies on soil AOA and AOB.

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Table 1. Details of qPCR reactions used in this experiment.

Gene Amplified	AOA *amoA*	AOB *amoA*
Forward Primer	CrenamoA23f (Tourna et al.,	*amoA*-1F (Stephen et al., 1998)
Reverse Primer	CrenamoA616r (Tourna et al.,	*amoA*-2R (Rotthauwe et al.,
Thermal Protocol	Enzyme Activation: 15 min at 95°C 40 cycles: 1 min at 95°C, 10 sec at 52°C, 1 min at 72°C, plate read at	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
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	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
Standard curve r^2	0.970–0.994	0.985–0.998
Reaction	90.5%-102.3%	83.9%-89.2%

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 pKa 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

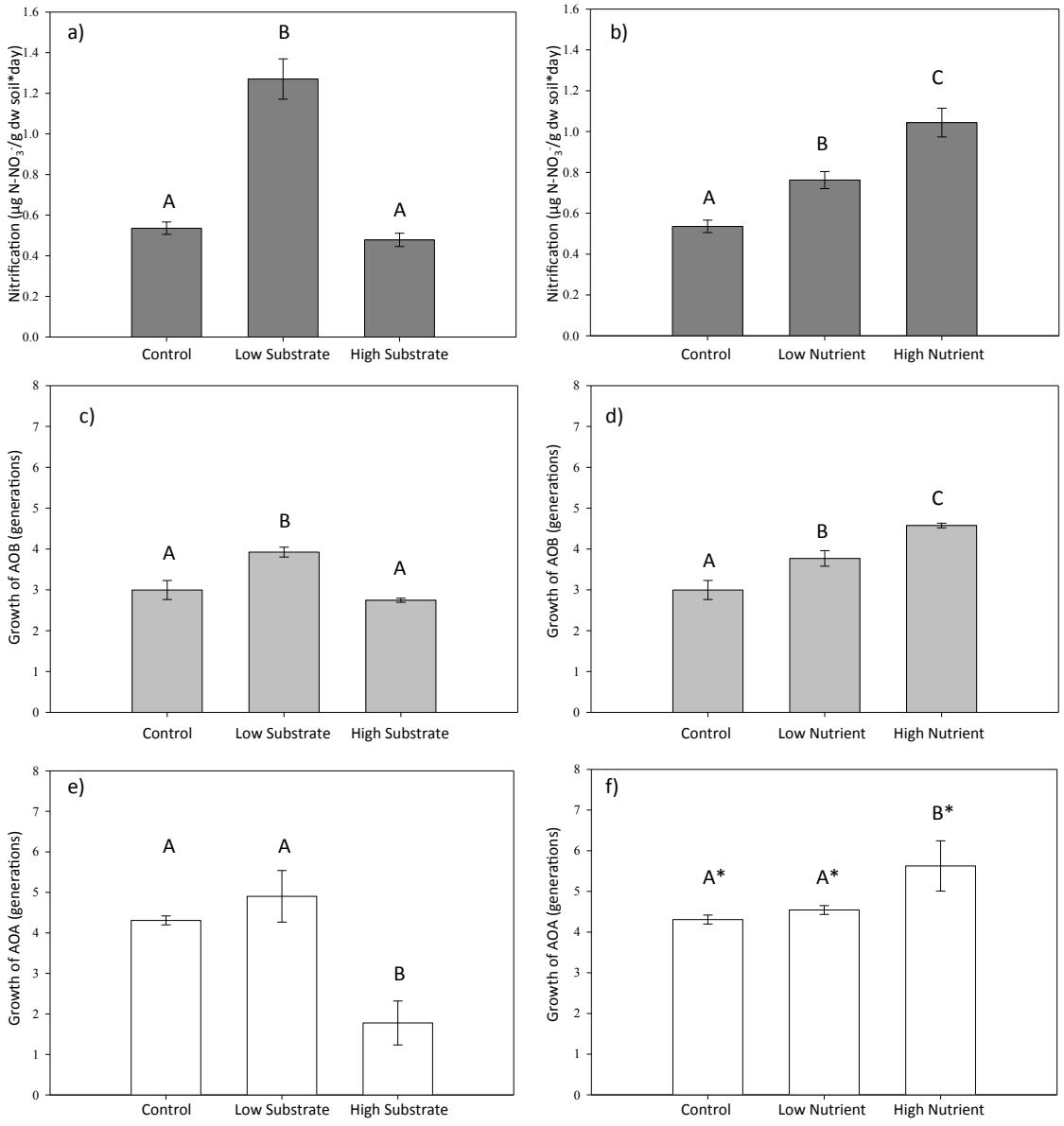

Figure 1. Effects of substrate and nutrient amendment on net nitrification (a,b; dark grey bars), growth of AOB during the incubation (c,d; light grey bars), and growth of AOA during the incubation (e,f; white bars). Growth of AOA and AOB were calculated as the number of generations that occurred during 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 $p<0.05$.

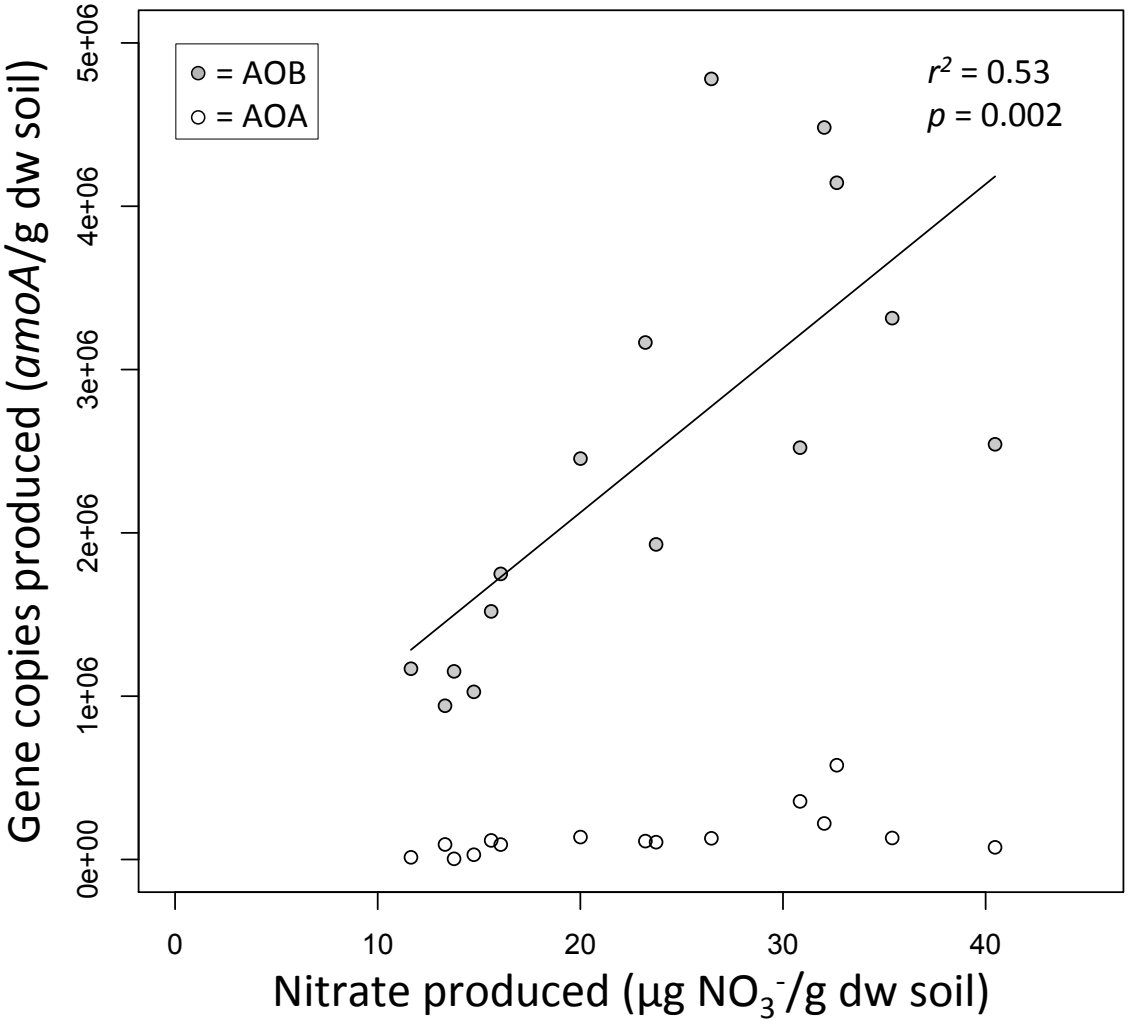

Figure 2. Relationships between nitrate produced and both gene copies of AOB *amoA* produced (grey circles), and gene copies of AOA *amoA* produced (white circles). Line represents a significant linear regression with equation (y = 100545x + 113433).

Chapter 4: The Relative Contributions of Ammonia-Oxidizing Bacteria and Archaea to Ammonia Oxidation in Temperate Forest Soil

Abstract

Nitrification is a biologically-mediated nutrient transformation, which controls the availability of inorganic nitrogen in terrestrial ecosystems. Ammonia oxidation, the rate-limiting step of nitrification, is performed by two groups of chemoautotrophic microbes: ammonia oxidizing archaea (AOA) and bacteria (AOB). The objective of this study was to estimate the relative contributions of AOA and AOB to ammonia oxidation in temperate forest soil. To accomplish this objective, I used quantitative polymerase chain reaction to monitor the growth of AOA and AOB during *in situ* incubations and compared growth values to observed nitrification rates. I found evidence that both groups contribute to ammonia-oxidation in forest soils, though growth of AOA was more highly correlated with net nitrification than growth of AOB. Interestingly, AOA showed much lower rates of production than AOB across the incubations conducted in this study. From these findings, I conclude that AOA may contribute disproportionally to ammonia oxidation in temperate forest soils and that they may be less efficient than AOB at converting carbon fixed by ammonia oxidation into new biomass.

Introduction

In 2005, the first archaeon capable of ammonia oxidation was isolated from a saltwater aquarium (Könneke et al. 2005). Shortly afterwards, ammonia-oxidizing archaea (AOA) were found to coexist with ammonia-oxidizing bacteria (AOB) in a variety of temperate soil systems (Leininger et al. 2006). The widespread abundance of AOA suggests that these organisms may play a critical role in the terrestrial nitrogen cycle. The objective of this study was to estimate the group-level contributions of AOA and AOB to *in situ* ammonia oxidation in temperate forest soils.

Several studies have attempted to estimate ammonia oxidation by AOA and AOB in soil. Generally these studies rely on laboratory incubations where ammonia oxidation is tracked over time and compared to either growth of AOA and AOB as measured by quantitative PCR (qPCR) (e.g. Di et al. 2009, Jia and Conrad 2009, Schauss et al. 2009, Di et al. 2010, Onodera et al. 2010, Zhang et al. 2010, Gubry-rangin et al. 2010), or carbon-fixation by AOA and AOB as measured by stable isotope probing (SIP) (e.g. Jia and Conrad 2009, Zhang et al. 2010, Pratscher et al. 2011, Zhang et al. 2012). While they provide valuable information, generalization from these studies is limited, since the vast majority of these studies were performed with soil from one site. A multisite approach is necessary to estimate system-wide contributions by AOA and AOB to ammonia-oxidation.

Zhang et al. (2012) used a multisite approach to investigate ammonia-oxidation by AOA and AOB in acidic soils. They tracked growth of AOA and AOB along with nitrification during lab incubations of unamended acidic soils from five sites across south China. They found that growth of AOA was positively correlated with nitrification in all five acidic sites, while growth of AOB was either negatively correlated with or unrelated to nitrification at every site. I used a similar approach to investigating ammonia oxidation of AOA and AOB in temperate forest soils with two fundamental differences. First, rather than using laboratory incubations, I conducted buried-bag incubations, in which soils are divided into polyethylene bags and incubated in the ground from which they were collected (*sensu* Eno 1960). This was done to ensure that AOA and AOB were exposed to field-relevant temperature regimes and dissolved oxygen concentrations

during incubation. Secondly, rather than comparing ammonia oxidation to growth of each group at each site, I looked for patterns across sites, in an effort to understand the general contributions of AOA and AOB to ammonia oxidation in temperate forest soils.

I predicted that AOA would be the dominant ammonia oxidizers in temperate forest soil ecosystems, because these soils are generally acidic and contain low levels of ammonium; conditions that have been shown to favor AOA rather than AOB activity (e.g. Nicol et al. 2008, Martens-Habbena et al. 2009). However, both AOA and AOB growth has been demonstrated in unamended forest soil incubations (Norman and Barrett, *in press*), and I therefore predict that there is a role for both groups in ammonia oxidation.

Methods

Site Selection

This study was conducted at Coweeta Hydrologic Laboratory, a Long Term Ecological Research site located in the southern Appalachian Mountains of North Carolina, USA. Coweeta is a humid, temperate forest system, with a history of man-made watershed-level manipulations (Swank and Vose 1997). These variations in land-use history have resulted in large variation in soil chemistry within the Coweeta Basin. Three sampling sites were located along stream-to-hillslope transects within each of four experimental watersheds (WS) at Coweeta, for a total of 12 sampling sites. I sampled within the following watersheds at Coweeta: WS18, a reference watershed that hasn't been disturbed since 1927; WS7, which was clear cut in 1977 using cable-logging; WS6, which was clear-cut, burned, limed, and fertilized from 1965-1967; and WS17, which was clear cut, then replanted in white pine in 1956. For more information on watershed treatments, see Swank and Vose (1997).

Buried-Bag Incubations

At each site, I collected approximately 1 kg of soil from the top 5 cm. The soil was then sieved to remove small rocks and fine roots, and the sieved soil was then divided into Whirl-pak® bags containing ~100 g of soil each. Three replicate bags from each site were stored at 4°C prior to processing and analysis of day 0 soil conditions (day 0 soils), and three replicate bags were incubated in the ground for approximately 1 month (day 30 soils) to estimate net rates of nitrification *sensu* Eno (1960) along with growth of AOA and AOB. Day 30 bags from one site in WS 7 were damaged in transport, and this site (7C) was excluded from further analysis.

Inorganic Nitrogen and DNA Extraction

I measured soil water content and concentrations of NH_4^+ and NO_3^- from each day 0 and day 30 bag. Soil water content was measured as weight loss from 10 g of soil after overnight incubation at 105°C. NH_4^+ and NO_3^- were extracted from soils in 2M KCl (Bundy and Meisinger 1994). Extracts were filtered, and NH_4^+ and NO_3^- were measured using a Lachat Quickchem flow injection autoanalyzer (Lachat Instruments, Loveland, CO, USA). Values were reported as μg NH_4-N/g dry weight (dw) soil, and μg NO_3-N/g dw soil, respectively.

I extracted DNA from each bag of soil using PowerSoil® DNA isolation kits (MO BIO laboratories Inc., CA, USA). Kit protocols were followed, except that the final elution step altered to include two separate additions of 75 μL of solution C6, designed to maximize DNA extraction efficiency. A spectrophotometer (ThermoFisher Scientific, MA, USA) was used to measure the 260/280 ratio of each extract. This ratio exceeded 1.8 in every extract, indicating that DNA was of high quality with low humic contamination.

qPCR Methods

AOA and AOB population sizes were estimated in DNA extracts from day 0 and day 30 samples by amplifying *ammonia monooxygenase subunit A* (*amoA*) genes relevant

to each group using SYBR-green based qPCR with Domain-specific primers, including *amoA-1F* (Stephen et al. 1998) and *amoA-2R* (Rotthauwe et al. 1997) for AOB, and *CrenamoA23f* and *CrenamoA616r* (Tourna et al. 2008) for AOA. All samples were run in triplicate and gene copy number was enumerated in each sample by comparing average threshold cycle values to a standard curve ranging from 10^1-10^6 copies of each gene ligated into a TOPO TA cloning vector (Invitrogen, NY, USA), which was linearized prior to use as a qPCR standard. Standard curve r^2 values ranged between 0.998 and 0.999 for AOB and 0.984 and 0.995 for AOA; standard curve efficiency values ranged between 81% and 85% for AOB and 84% and 89% for AOA. Product specificity was verified by melt curve analysis. 50 μL reactions contained 25 μL of Quantitect SYBR green PCR mix (Qiagen, Venlo, Netherlands), 0.2 mg/mL BSA, and 0.5 μM of each primer for AOB *amoA* enumeration, or 1.5 μM of each primer for AOA *amoA* enumeration. I initially added 50 ng of template DNA in both AOA and AOB reactions, but had to re-run AOB reactions for one site with 250 ng template DNA in order to achieve a detection limit of >10 copies per reaction, and had to re-run AOA reactions for another site with 1.25 ng of template DNA in order to discourage the formation of non-specific PCR products, the presence of which could not be otherwise controlled without unacceptable losses in reaction efficiency. The thermal protocol for both AOA and AOB was 95°C for 15 minutes, followed by 40 cycles of 95°C for 15 seconds, 53°C for 30 seconds, 72°C for 1 minute. All values were reported as either AOA or AOB *amoA*/g dw soil.

Net Nitrate Production and Net Nitrification Calculations

Net nitrate production and net nitrification were estimated for each day 30 bag by equations 1 and 2, respectively.

(1) net nitrate production = day 30 NO_3-N/g dw soil – avg day 0 NO_3-N/g dw soil

(2) net nitrification = (day 30 NO_3-N/g dw soil – avg day 0 NO_3-N /g dw soil)/days in ground

Net nitrate production was reported as μg NO_3-N/g dw soil, while net nitrification was reported as μg NO_3-N/g dw soil*day.

AOA and AOB Growth Calculations

I used day 0 and day 30 qPCR data to calculate three growth metrics for AOA and AOB in each day 30 bag: net *amoA* produced, net cells produced, and net growth carbon (C). Calculations for net AOA *amoA* produced and net AOB *amoA* produced were estimated for each day 30 bag by equations 3 and 4, respectively.

(3) net AOB *amoA* produced = day 30 AOB *amoA*/g dw soil – avg day 0 AOB *amoA*/g dw soil

(4) net AOA *amoA* produced = day 30 AOA *amoA*/g dw soil – avg day 0 AOA *amoA*/g dw soil

Both net AOA *amoA* produced and net AOB *amoA* produced were reported as copies/g dw soil.

While all known AOA isolates have one copy of *amoA* per genome, AOB isolates may have more than one copy of *amoA* per cell, depending on the species (Hatzenpichler 2012). I therefore assumed that net AOA cells produced was equivalent to net AOA *amoA* produced, but a conversion factor had to be identified prior to calculating net AOB cells produced. I used amplicon-based pyrosequencing of group *amoA* genes to identify the most dominant AOB species in day 0 DNA extracts; pyrosequencing and sequence processing methods are discussed elsewhere (Chapter 2). Once sequences were clustered into operational taxonomic units (OTUs) at 94% similarity, representative sequences from each OTU were aligned against the non-redundant protein database using web-based blast x (www.ncbi.nlm.nih.gov). From this analysis, the most-abundant AOB OTU was identified as belonging to the genus *Nitrosospira*. Norton et al. (2008) found that *Nitrosospira multiformis,* the only member of the genus to have its genome sequenced to date, has three copies of *amoA* per cell, and I used this information to convert net AOB

amoA produced into net AOB cells produced. Therefore, net AOB cells produced was calculated as net AOB *amoA* produced divided by three.

Net growth C is an estimate of the cumulative amount of net primary production by each group during incubation. I used published cell volume to biomass conversions, along with cell size calculations, and information from sequence data to convert net cells produced into net growth C for each group. For both groups, I used a cell volume to carbon conversion factor of 2.2×10^{-19} μg C/ μm^3 based on Bratbak (1995). I used size estimates of *Nitrosospira briensis* to estimate AOB cell volume. Watson (1970) found that *N. briensis* cells were tightly coiled spirals, best represented as cylinders 1.5-2.5 μm in length and 0.8-1.0 μm in width. Using an average of these dimensions I calculated an AOB cell volume of 1.27 μm^3. I identified the most dominant AOA species in day 0 DNA extracts, using a procedure identical to the one I used for AOB, and found that the dominant AOA OTU, which comprised ~85% of the sequences sampled, was most closely related to *Nitrosotalea devanaterra*, an archaeal ammonia oxidizer isolated from acidic soil (Lehtovirta-Morley et al. 2011). Lehtovirta-Morley et al. (2011) described *N. devanaterra* cells as straight rods with an average length of 0.89 μm and an average width of 0.89 μm. Once again I used this information to estimate an average AOA cell volume of 0.067 μm^3. Based on these values, I calculated net AOA growth C, and net AOB growth C by equations 5 and 6 respectively.

(5) net AOA growth C = (net AOA cells produced)*(0.067 μm^3/cell)*(2.2×10^{-19} μg C/μm^3)

(6) net AOB growth C = (net AOB cells produced)*(1.27 μm^3/cell)*(2.2×10^{-19} μg C/μm^3)

Statistical Analyses

I assessed the relationships between growth metrics of AOA and AOB and nitrate production by multiple regression using the function "lm" in R statistical software (the R Foundation for Statistical Computing, Vienna, Austria). This was followed by a stepwise

model selection procedure based on Akaike information criterion (AIC) values using the function "stepAIC" in library "MASS." For this analysis, all negative AOA growth values (4 sites total) were converted to zeros since net cell death should not contribute to nitrate production.

Results

I found values of net nitrate produced to range between 0.031 and 38.840 μg NO_3-N g dw soil, which correspond to net nitrification values between 0.001 and 1.339 μg NO_3-N/g dw soil*day, across all sites examined at Coweeta (Figure 1). Values of net AOA *amoA* produced ranged between -1.61×10^6 and 8.17×10^6 copies/g dry weight of soil. These values corresponded to net AOA growth C ranging from -0.024 to 0.120 μg C/g dw soil. I found values of net AOB *amoA* produced ranging between 2.42×10^4 and 6.79×10^6 copies/g dry weight of soil, which corresponded to values of between 8.065×10^3 and 2.26×10^6 cells/g dw soil for AOB cells produced and of 0.0023 to 0.633 μg C/g dw soil for AOB growth C. AOA cells produced, AOA growth C, AOB cells produced, and AOA and AOB growth C are presented for each site in Figure 1.

Regression models, which included both AOA and AOB growth metrics, explained observed values of net nitrate produced better than models that included growth of either group alone, as determined by stepwise model selection based on AIC scores. The equations of these models are presented below (equations 7-9) where z represents net nitrate produced.

(7) $z = 2.45 \times 10^{-6}$(net AOA *amoA* prod.) + 2.88×10^{-6}(net AOB *amoA* prod.) + 8.10

(8) $z = 2.45 \times 10^{-6}$(net AOA cells prod.) + 8.65×10^{-6}(net AOB cells prod.) + 8.10

(9) z = 166.5(net AOA growth C) + 30.9(AOB growth C) + 8.10

Since all three AOA and AOB growth metrics presented here are multipliers of one another, all three models are the same level of significance (P=0.044), and have the same coefficient of determination (R^2=0.54). I present all three models here so I can

discuss the significance of model coefficients. It is important to note that while the relationships observed between variables I report here were created by replacing negative AOA growth values with zeros, these relationships are also statistically significant without doing this. The relationship between AOA biomass C, AOB biomass C, and net nitrate produced, represented by equation 9, is presented in Figure 2. My model selection procedure also showed that models including only AOA growth metrics better explained observed values of net nitrate produced than those including only AOB growth metrics.

Discussion

Genomic evidence indicates that AOA have the capacity for heterotrophic growth (Walker et al. 2010), and the relevance of AOA to the process of ammonia-oxidation has therefore been questioned. Some authors even refer to these organisms as ammonia-oxidase encoding archaea (AEA) rather than ammonia-oxidizing archaea i.e., AOA (Hatzenpichler 2012). However, SIP-based studies have shown that AOA fix carbon in conjunction with ammonia oxidation in a variety of environments including agricultural soils (Zhang et al. 2010, Pratscher et al. 2011) and a variety of acidic soils (Zhang et al. 2012). I found evidence that both AOA and AOB contribute to ammonia oxidation in temperate forest soils by estimating the growth of both AOA and AOB during buried-bag incubations across multiple sites. The use of *in situ* incubations of unamended soils in this study lends field relevance to these results as well. Furthermore, I found that AOA growth alone was significantly correlated with net nitrate produced while AOB growth was not, suggesting that AOA may be greater drivers of ammonia oxidation in these temperate forest soils than AOB.

Though growth of AOA is on par with growth of AOB in terms of cell counts (Figures 1a and b), AOA exhibit far less growth than AOB in terms of carbon accruing to biomass (Figure 1c). It is therefore surprising that AOA, rather than AOB, seem to drive ammonia oxidation in this system. One explanation for this discrepancy could be that biomass production in AOB is tied to something other than ammonia oxidation (*i.e.* forest soil AOB could be living on organic carbon). However, an alternative explanation

revolves around group-level differences in growth efficiency of AOA and AOB. To investigate this explanation, I calculated growth efficiency metrics for AOA and AOB from equations 7, 8, and 9 as described here:

Equations 7, 8, and 9 have the general form below (equation 10).

(10) net nitrate produced = A(AOA growth metric) + B(AOB growth metric) + C

Coefficients A and B in this general formula essentially show how much ammonia must be oxidized for each unit of growth of AOA and AOB respectively; thus these coefficients can be considered as growth efficiency metrics. I have summarized these metrics in Table 1. Comparing growth efficiency metrics across groups reveals some interesting patterns. The efficiency metrics derived from equation 8 show that temperate forest soil AOB must oxidize approximately three times as much ammonia as AOA to produce one new cell. This suggests that AOB are more efficient than AOA, but this interpretation fails to take into account the differences in carbon content of AOA and AOB cells. The growth efficiency metrics derived from equation 9 show that AOA must oxidize over 5 times as much ammonia than AOB to add one μg of C into biomass. In fact, converting the growth efficiency metrics obtained from equation 9 into molar ratios shows that forest soil AOA must oxidize ~167 moles of ammonia for every mole of C they gain in biomass, while forest soil AOB only oxidize ~31 moles of ammonia for every molecule of carbon they gain in biomass (Table 1). These differences in growth efficiency provide a mechanism by which AOA can drive the process of ammonia oxidation in temperate forest soils without exhibiting a large growth response as measured by units of carbon (Figure 1c). Furthermore, the high growth efficiency of AOB may explain their ability to avoid competitive exclusion by AOA in temperate forest soils, since AOA should outcompete AOB for access to ammonium at the low concentrations found in this system based on enzyme kinetics alone (Martens-Habbena et al. 2009).

These results suggest that AOA, rather than AOB, are the dominant ammonia-oxidizers in temperate forest soils, though both groups play a role in nitrification. Differences in group-level carbon use efficiency may explain the more significant role

that AOA play in this system. While forest soil AOB seem to be capable of transforming a large amount of carbon fixed by ammonia oxidation directly into growth, AOA are much less efficient and may therefore affect rates of ammonia oxidation far beyond what is suggested by measuring their growth alone. Differences in the amount of ammonia each group must oxidize to fix one unit of carbon could be reflective of group-level differences in ammonia oxidation or carbon fixation pathways. Alternatively, these group-level differences in growth efficiency could simply reflect how AOA and AOB act in response to the low ammonium, acidic conditions found in temperate forest soils.

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Table 1. Growth efficiency metrics for AOA and AOB. NH_4^+ oxidized (oxid.) /*amoA* produced (prod.), NH_4^+ oxid. /cell prod., and NH_4^+ oxid. /growth C, were taken from equations 7, 8, and 9, respectively. Molar NH_4^+ oxid./growth C for each group was calculated from NH_4^+ oxid./growth C, using molar mass conversions for N and C.

Efficiency Metric	AOA	AOB
NH_4^+ oxid./*amoA* prod.	2.45 x 10^{-6} μg NH_4-N/copy	2.88 x 10^{-6} μg NH_4-N/copy
NH_4^+ oxid./cell produced	2.45 x 10^{-6} μg NH_4-N/cell	8.65 x 10^{-6} μg NH_4-N/cell
NH_4^+ oxid./growth C	166.5 μg NH_4-N/μg C	30.9 μg NH_4-N/μg C
Molar NH_4^+ oxid./growth C	142.7 mol NH_4^+/mol C	26.5 mol NH_4^+/mol C

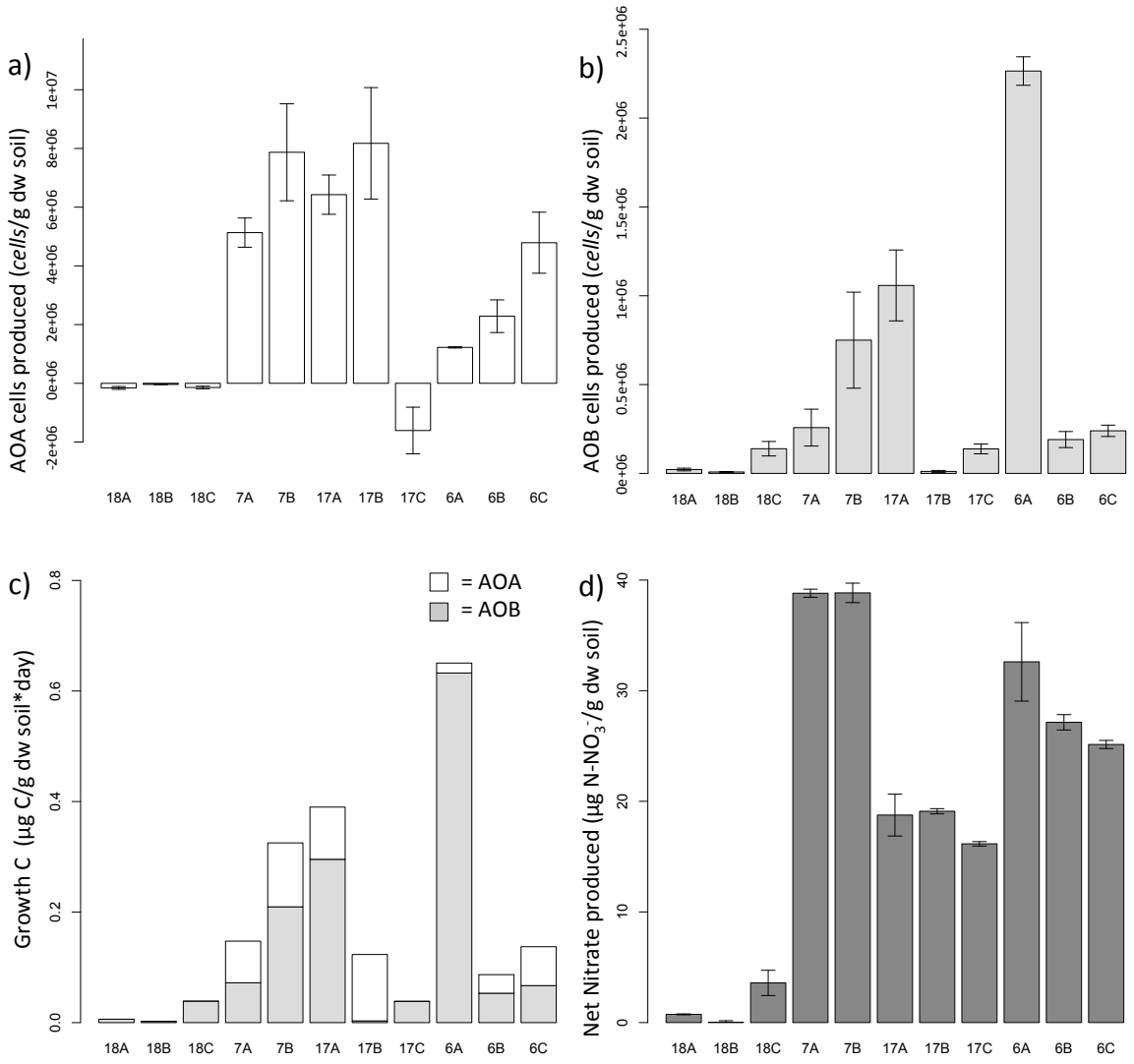

Figure 1. Site specific estimates of AOA and AOB growth and nitrification. All bar heights represent site averages, while error bars in panels a, b, and d represent standard error. Sites are grouped by watershed, and watershed are arranged from lowest N export to highest N export. a) Net AOA cells produced at each site. Negative values represent net cell death. b) Net AOB cells produced at each site. c) Stacked bar graph representing AOA and AOB growth C at each site. Grey portions of bars represent AOB growth C, while white portions of bars represent AOA growth C. d) Net nitrification at each site.

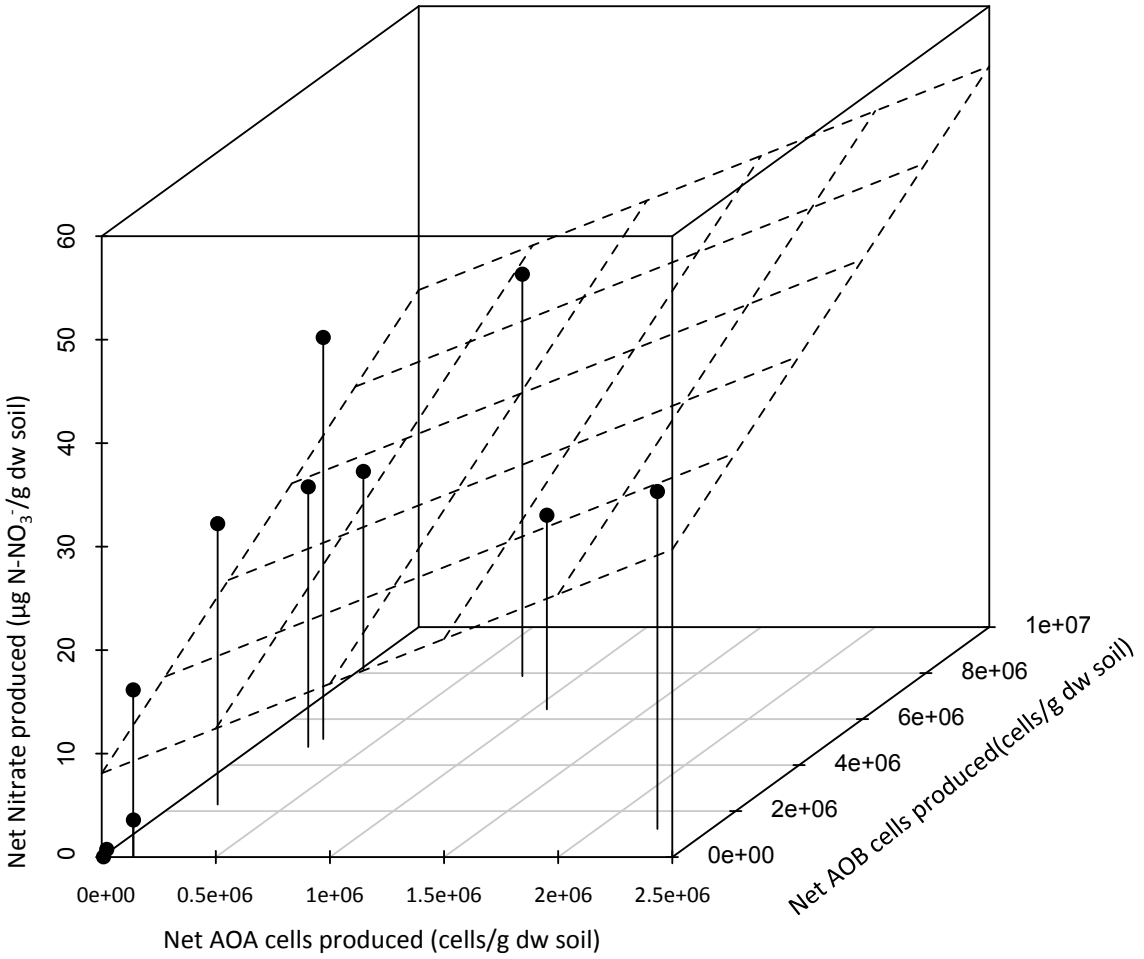

Figure 2. The relationship between average values of net AOA cells produced, net AOB cells produced, and net nitrate produced across 11 temperate forest soil sites. Plane represents a significant multiple regression with the equation $z = 2.447 \times 10^{-6}(x) + 8.650 \times 10^{-6}(y) + 8.101$.

Chapter 5 – Synthesis

In a study that changed long-held conceptions about the microbes involved in the terrestrial nitrogen cycle, Leininger et al. (2006) showed that ammonia-oxidizing archaea (AOA) are numerically-dominant to ammonia-oxidizing bacteria (AOB) in a variety of temperate systems. However, no forested sites were included in this analysis, and forest soil ammonia-oxidizers remained understudied for several years afterwards. Boyle-Yarwood et al. (2008) performed the first analysis of soil AOA and AOB in temperate forest systems, and their results showed that communities in these systems differ from those studied by Leininger et al. (2006). While they found ample AOB in all four temperate forest sites they sampled, Boyle-Yarwood et al. (2008) failed to detect AOA in two temperate forest sites. These findings remain surprising since temperate forest soils are generally acidic low nitrogen systems, both conditions shown to favor the activity of AOA rather than AOB (Nicol et al. 2008, Martens-Habbena et al. 2009).

My dissertation work adds to the growing literature about the structure and activity of ammonia-oxidizing microbial communities in temperate forest soils. Using amplicon-based pyrosequencing, I found that AOA communities are remarkably low in diversity in temperate forest soils (Chapter 2). In fact, 85% of the sequences in my AOA dataset belonged to one uncultured taxon, most-closely related to the acidophilic AOA isolate *Nitrosotalea devanterra* (Lehtovirta-Morley et al. 2011). The AOB communities I sampled were more diverse than the AOA communities I sampled, but 90% of sequences were from the genus *Nitrosospira* (Chapter 2). This result mirrors those for other temperate forest soil studies (e.g. Boyle-Yarwood et al. 2008, Yao et al. 2011, Malchair and Carnol 2012). The diversity of each group seems to be independently controlled by different local-scale edaphic factors (Chapter 2). AOA diversity responds to soil ammonium (NH_4^+), while AOB diversity responds to either soil pH or ammonia (NH_3). Since the concentration of NH_3 depends on soil pH, the independent effects of these two

factors cannot be disentangled from the studies performed here. However, when testing substrate and nutrient limitation of ammonia-oxidizers (Chapter 3), I found AOB growth was inhibited by the pH effects of substrate addition, even when soil NH_3 was increased several fold. Based on this finding, it seems likely that the diversity of AOB is responding to pH in temperate forest systems, rather than the availability of NH_3.

My finding that AOA diversity responds to soil NH_4^+ (Chapter 2) suggests that NH_4^+, rather than NH_3, may be the substrate oxidized by soil AOA. AOB have been shown to oxidize NH_3 rather than NH_4^+ (Suzuki et al. 1974), though a test of this nature has yet to be performed for AOA. In fact, many authors have speculated that that NH_3 may not be the substrate oxidized by AOA (e.g. Martens-Habbena and Stahl 2011, Lehtovirta-Morley et al. 2011), and the use of NH_4^+ as a substrate for oxidation could explain how AOA are able to maintain high activity in low pH environments where the availability of ammonia is limited (Nicol et al. 2008, Lehtovirta-Morley et al. 2011). However, AOA have been shown to hydrolyze urea in acidic soil (Lu and Jia 2013), which could also allow them to survive in the absence of available NH_3, even if NH_3 is actually their substrate for oxidation. Still, several AOB isolates are also capable of urea hydrolysis (Koops et al. 1991) and the activity of AOB has been shown to decrease with soil pH nonetheless (Nicol et al. 2008). Therefore, it seems unlikely that urea provides the mechanism for the survival of AOA in acidic systems including forest soils.

During my dissertation, I also used *in situ* incubations to investigate AOA and AOB growth and ammonia oxidation activity. While several studies have measured growth of soil ammonia oxidizers during lab incubations (e.g. Di et al. 2009, Onodera et al. 2010) and soil biogeochemists have used *in situ* incubations to estimate field rates of nitrification (*sensu* Eno 1960), combining these approaches allowed me to investigate growth of AOA and AOB under field temperature and oxygen regimes. I used this approach to measure AOA and AOB responses to substrate and nutrient addition (Chapter 3) as well as AOA and AOB contributions to ammonia oxidation (Chapter 4). Perhaps unsurprisingly, I found that forest soil AOB are substrate

limited, while AOA were not (Chapter 3). Interestingly, both AOA and AOB show substantial growth during unamended incubations as well. I used the growth of these organisms to estimate the contributions of each group to ammonia oxidation in temperate forest systems (Chapter 4). While several studies have used either growth during lab incubations or stable isotope probing to estimate group-level contributions to ammonia oxidation in a single soil sample (e.g. Zhang et al. 2010, Pratscher et al. 2011), I measured AOA and AOB growth across sites and found that AOA seem to play more of a role than AOB in ammonia oxidation in this system (Chapter 4). Given the dominance of a single AOA taxon in the soils I sampled (Chapter 2), ammonia oxidation at Coweeta could largely result from the activity of one as-of-yet uncultured AOA isolate.

By combining molecular techniques with standard methods of soil analysis including *in situ* incubations, I feel that I have shed light not only on the forces that shape naturally-occurring communities of ammonia-oxidizing microbes but also on the role of these organisms in temperate forest soils. I hope that the approach of monitoring the growth of these organisms during *in situ* incubations will be adopted by other researchers. Culture-based studies of AOA physiology are necessary to inform future microbial ecology investigations into the relative roles of these organisms in natural systems. These should include an investigation of substrate preference of AOA, comparisons of carbon use efficiency between AOA and AOB isolates, and an investigation of the conditions that favor heterotrophic growth by both groups.

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