DRIVERS OF NITROGEN AVAILABILITY, CYCLING, AND DEMAND IN TEMPERATE AND TROPICAL FOREST ECOSYSTEMS

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The nitrogen (N) cycle is series of biogeochemical transformations involving soil microbes and plants that are modulated by environmental conditions. Because N limits primary production, understanding interactions among these factors is central to predicting soil resource availability to primary producers and the global distribution of terrestrial biomass carbon (C). Phosphorus (P), another limiting nutrient, and C, further affect microbial N transformations and N bioavailability through the stoichiometric requirements of plant and microbial biomass. Natural environmental gradients coupled with field manipulations are investigative tools that have helped to elucidate controls on C, N and P interactions, particularly under conditions of environmental change. This work considered the influences of increasing mean annual temperature (MAT) and the relative availabilities of soil N and P on belowground plant activity, microbial functional groups, and N cycling rates in tropical and temperate forests. Past research showed that belowground C flux and soil respiration increased with warming across an elevation/MAT gradient (13-18.2 °C) in a tropical montane wet forest. Across the same gradient, this study found that soil N bioavailability and the abundance of nitrifying archaea also increased with warming, suggesting that MAT drives nitrifier populations and resultant N availability. The present study also showed that warming reduced fine root proliferation into soil microsites enriched with P or a combination of N and P across the gradient. This suggests that fine root growth is more co-limited by N and P at low MAT where bulk

soil N is more limiting than in warm conditions where N is abundant. Additionally, arbuscular mycorrhizal colonization of fine roots increased with warming, possibly indicating that nutrient acquisition is performed by fungal symbionts more than fine roots in warm, N-rich environments. Using a stand fertilization experiment in a northern hardwood forest, this work showed that litter N:P and gross N cycling declined in the presence of elevated P, while microbial biomass N increased with elevated P. The results show that litter chemistry is strongly related to soil N and P limitation, and may control gross N cycling through litter substrate limitation. Together, these studies highlight the complex interplay among temperature, soil P availability, and plant/microbial nutrient uptake that shape the N cycle in different forest ecosystems.

BIOGRAPHICAL SKETCH

Suzanne Pierre was born in Jersey City, New Jersey, to parents Anita and Yonel Pierre, who emigrated to the United States from India and Haiti, respectively. Suzanne grew up with siblings Alexander and Christine in Somerset, NJ. She attended Elizabeth Avenue Elementary School, Sampson G. Smith Intermediate School, and Franklin High School. Suzanne earned a B.A. in Environmental Studies with a minor in Environmental Biology at New York University. While at NYU, Suzanne completed a semester abroad in Madagascar, where she conducted research on tropical seagrass biodiversity. As an undergraduate, Suzanne held research positions at the Center for Biodiversity and Conservation at the American Museum of Natural History and the Cary Institute of Ecosystem Studies. She published independent undergraduate research on urban afforestation and soil nitrous oxide production in New York City parks under the advisement of Dr. Mary Killilea and Dr. Peter Groffman. She entered the Ph.D. program in Ecology and Evolutionary Biology in August 2013 and graduated from the Stable Isotopes in Ecology and Biogeochemistry course at the University of Utah in 2014. Suzanne co-founded the Cornell Diversity Recruitment Weekend program in 2016 and was awarded the Cornell Diversity and Inclusion Change Agent Award in 2017 for this work. Following completion of the Ph.D., Suzanne will accept the University of California President's Postdoctoral Fellowship at UC Berkeley, where she will work in the laboratory of Dr. Todd Dawson.

Dedicated to the unarmed Black Americans killed by police during the duration of this work (2013-2018).

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Many individuals helped support this work and have my deepest gratitude. Jed Sparks of Ecology and Evolutionary Biology was integral in supporting my initial

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Above all, I thank my family and friends, who have been the greatest supporters and sources of light throughout this process, and Samuel Chamberlain, who was by my side throughout this journey.

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CHAPTER 1

AMMONIA OXIDIZER POPOULATIONS VARY WITH NITROGEN CYCLING ACROSS A TROPICAL MONTANE MEAN ANNUAL TEMPERATURE GRADIENT[1]

1.1. Introduction

Ecosystem responses to global change are greatly influenced by the cycling and availability of nutrients. Nitrogen (N) availability often limits primary production and influences terrestrial ecosystem structure and function (Vitousek and Howarth 1991, Thomas *et al*. 2013). Nitrification is a critical step in the N cycle that transforms ammonium (NH_4^+) to nitrate (NO_3^-). Following nitrification, N can be immobilized in microbial biomass, taken up by roots, or lost through hydrologic or gaseous pathways (Kaye and Hart 1997, Vitousek *et al*. 1997). Nitrification is mediated by several microbial functional groups (De Boer and Kowalchuk 2001, Isobe *et al.* 2011). Community composition and relative gene expression (i.e. quantity of RNA transcripts produced per DNA gene copy) of these groups can affect N cycling dynamics (Wilmes and Bond 2006, Blazewicz *et al.* 2013, Bowen *et al*. 2014). Despite evidence that microbial communities regulate N cycling rates (Webster *et al.* 2005, Baldrian *et al.* 2012, Taylor *et al*. 2012, Litchman *et al*. 2015) no research to date has generated a mechanistic understanding of how microbial N cycling responds to environmental change *in situ* (Bisset *et al*. 2013, Sundqvist *et al*. 2013).

Molecular techniques have been useful for identifying and characterizing biogeochemical pathways (Morales and Holben 2011, Schimel 2016). Despite the

[1] Reviewed and accepted for publication as Pierre, S., Hewson, I., Sparks, J. P., Litton, C. M., Giardina, C., Groffman, P. M. & Fahey, T. J. Ammonia oxidizer populations vary with nitrogen cycling across a tropical montane mean annual temperature gradient. *Ecology* **98,** (2017).

advancement of these tools, relationships between microbially driven biogeochemical pathways and drivers such as temperature, moisture, and pH have been minimally explored under field conditions (McCalley *et al*. 2014), and environmental heterogeneity has challenged efforts to isolate individual drivers of biogeochemical cycling (Austin and Sala 2009, Sundqvist *et al*. 2013). Natural covariation among environmental variables across gradients has caused researchers to rely on laboratory and field manipulations (Melillo *et al.* 2002, Horz *et al*. 2004, Bowen *et al*. 2014). Despite insights into how genes control biogeochemical processes (Torsvik and Øverås 2002, Fuhrman 2009, Schimel *et al*. 2016), the isolated roles of environmental drivers in gene-flux linkages in undisturbed settings remain understudied.

Ammonia oxidation (AO), the conversion of ammonia (NH_3) to nitrite (NO_2^-), is the rate limiting step of nitrification. AO is performed by obligate aerobic bacteria (AOB) and archaea (AOA) and is a pH-sensitive metabolic process (Kowalchuck and Stephen 2003, Francis *et al*. 2007). The sensitivity of AO may have important implications for the availability of N to primary producers as well as the mobility of N in soils (Horz *et al.*2002). Ammonia oxidation is regulated by the gene *amoA*, which encodes the ammonia monooxygenase small alpha subunit (Rotthauwe *et al* 1997). The *amoA* gene is ubiquitous in terrestrial and aquatic systems (Rotthauwe *et al*. 1997, Fierer *et al*. 2009), and because it is phylogenetically constrained within the Betaproteobacteria and Crenarchaeota, it is a tractable target in environmental samples (Purkhold *et al*. 2000). Many studies have targeted *amoA* in soils, but most have relied on artificial warming to understand the role of temperature in AO and nitrification regulation via *amoA* (Avrahami *et al.* 2003, Horz *et al*. 2004, Tourna *et al.* 2008,

Osborne *et al*. 2015). The relationship between temperature and microbial AO regulation is not entirely predictable based on variable responses in AOA and AOB abundance and expression across studies (Avrahami and Conrad 2003, Tourna *et al*. 2008). Short-term warming approaches may obscure the importance of long-term, *in situ* differences in mean annual temperature (MAT) that may shape microbial communities and their potential activity (Osborne *et al*. 2015).

Natural environmental gradients have proven useful in understanding ecosystem responses to global change (Vitousek and Matson 1991, Fukami *et al*. 2005, Malhi *et al*. 2010). Well-constrained elevation gradients can provide insight into the direct role of mean annual temperature (MAT) in ecosystem process rates (Litton *et al*. 2011, Giardina *et al*. 2014). In particular, nitrogen transformations are influenced by several environmental characteristics, including climate, plant community composition and edaphic properties (Booth *et al.* 2005, Wallenstein *et al*. 2006, Petersen *et al*. 2012). Environmental gradients allow researchers to isolate responses to one variable of interest (Vitousek *et al.* 1988, Knoepp and Swank 1998, Raich *et al.* 1997, Schuur and Matson 2001, Salinas *et al*. 2011, Girardin *et al*. 2013, Giardina *et al*. 2014), though many gradient studies suffer from confounding covariation among environmental parameters (Malhi *et al*. 2010, Sundqvist *et al*. 2013).

We took advantage of a unique tropical wet montane elevation gradient where abiotic and biotic variables besides MAT remain constant (Litton *et al.* 2011). The gradient features uniform parent material resulting in uniform soil type due to the island's volcanic history (Litton *et al*. 2011). In addition, the isolation of the Hawaiian islands has allowed for limited species diversity and uniform vegetation composition

along the gradient. Soil pH and water content are also uniform across the gradient (Litton *et al*. 2011; Table 1), making this a model ecosystem for comparing microbial processes at different MAT.

We aimed to answer the following questions: Does N availability change systematically across the elevation (MAT) gradient? Do the abundance and expression of AOA and AOB *amoA* in soils vary predictably across MAT? Do *amoA* abundance and expression predict the availability and cycling of N with MAT? Studies at this MAT gradient site have shown that plant belowground carbon (C) allocation increases with MAT (Giardina *et al.* 2014). This suggests that increased rhizosphere C flux with MAT may differentially stimulate microbial activity along the gradient (Kuzyakov *et al.* 2007, Brzostek *et al*. 2013, Finzi *et al.* 2015). Further, litter N loss rates during decomposition increase with MAT along this gradient (Bothwell *et al*. 2014), supporting the notion that N cycling may vary predictably with MAT (Rustad *et al*. 2001, Bai *et al*. 2013). We predicted that the abundance and normalized gene expression (cDNA:DNA) of AOB and AOA *amoA* would increase with MAT given the positive effects of warming on microbial metabolic efficiency (Price *et al.* 2004, Allison *et al*. 2010) and transcriptional activity (Avrahami *et al.* 2003, Tourna *et al*. 2008). We also predicted that increasing MAT would increase the bioavailability of N and rates of potential net nitrification and N mineralization in soils (Melillo *et al.* 2011, Bai *et al*. 2013).

1.2. Methods

1.2.1. Study Site

The elevation gradient is comprised of nine 20 x 20 m plots forming an 800 m elevation gradient that corresponds to a ~5.2°C MAT gradient (13.0 to 18.2°C). This gradient is located on the northeastern slope of the Mauna Kea Volcano on the Island

of Hawaii (Table 1). Seven plots are located in the Hawaii Experimental Tropical Forest (HETF; 19°56′41.3″N, 155°15′44.2″W; 600–1800 m.a.s.l) and two high elevation plots are located in the Hakalau Forest National Wildlife Refuge (HFNWR; 19°50′31.3″N, 155°17′35.2″W; 600–2000 m.a.s.l) (Litton *et al*. 2011). For this study, we sampled from eight of nine plots because of timing limitations. All plots are located within tropical montane wet forests characterized as *Metrosideros polymorpha* Gaudich.–*Acacia koa* A. Gray forests. *M. polymorpha* and *Cheirodendron trigynum* (Gaudich.) A. Heller dominate the canopy and midstory, respectively, across all plots (84-97% of basal area excluding tree ferns), while tree ferns (*Cibotium* spp.) make up ~46% of total stand basal area in all plots (Litton *et al*. 2011).

Soil water balance is relatively constant across all plots because annual precipitation and evapotranspiration declines with increasing elevation (Litton *et al*. 2011). Substrate in all plots is derived from ~20 ky (14–65 ky) weathered tephra (Giardina *et al.* 2014). Soils are moderate to well-drained hydrous, ferrihydritic/amorphic, isothermic/isomesic Acrudoxic Hydrudands of the closely related Akaka, Honokaa, Maile, and Piihonua soil series (Soil Survey Staff 2010). Mean soil pH is 3.9 and varies little with MAT (± 0.3; Table 1). Base saturation and estimated mean cation exchange capacity are 32.4(±10.56) % and 11.9(±2.75) cmol kg^{-1} (measured to 9.5 cm depth), respectively (Litton *et al*. 2011). Mean bulk density across plots is 0.21(±0.07) g cm^{-3} (Litton *et al*. 2011), mean surface organic matter (Oa-Oe horizons) N content is 29.59(±11.09) Mg N ha^{-1} and mean C content is 1499.68 (±1626.17) Mg C ha^{-1}.

PLOT	Elevation (m a.s.l.)	MAT (˚C)	Soil pH	Microbial Biomass N (µg-N g dry soil $^{-1}$ day^{-1})	Soil N Stock (0-10 cm; g N m^{-2})	Soil C Stock (0-10 cm; g C m^{-2})
SPE800	800	18.2	4.1	--	162.6 (75.0)	4066.7 (1382.3)
SPE934	934	17.3	4.2	493.186	291.2 (105.0)	4400.1 (1581.0)
SPE1024	1024	16.5	3.7	407.787	152.5 (88.2)	2562.4 (1248.0)
SPE1116	1116	16.1	3.8	--	157.3 (108.0)	2662.8 (1923.1)
WPL1204	1204	15.5	3.7	368.976	192.1 (102.9)	4139.4 (2577.6)
WPL 1274	1274	15.1	3.9	--	29.0 (4.6)	3674.2 (719.1)
HAK1468	1468	13.8	4.2	354.644	151.6 (12.1)	2958.3 (326.5)
HAK1600	1600	13	4.1	339.448	311.9 (283.4)	5666.0 (5020.1)

Table 1.1: Soil properties measured in eight plots along a mean annual temperature gradient along the northeast slope of the Mauna Kea volcano on the Big Island of Hawai'i, USA. Mean (± SD) values for soil properties from 0-10cm depth encompassing the Oe-Oa horizons. All values presented represent subsamples taken from a pool of five soil cores at each plot. Three plots were excluded from potential net N mineralization and nitrification measurements because representative samples were destroyed in transport. Soils were collected in October 2014 and August 2015.

1.2.2. Nitrogen Bioavailability

Bioavailable inorganic N was measured using Plant Root Simulator (PRS™) resin probes (WesternAg Innovations, Saskatoon, SK). Three pairs of cation and anion-adsorbing probes were buried to 8 cm depth at random locations in the plots to measure total bioavailable NH_4^+ and NO_3^- (Western Ag Innovations, PRS™ probe Operations Manual). Probes were collected after an average of 17 days, cleaned with distilled water to remove soil and shipped to the manufacturer for extraction. Ions adsorbed to the resin probes were analyzed colorometrically and the net rate of nutrient supply is expressed here in mg m^{-2} d^{-1}. Probes were grouped at the plot level and extracted together. Individual values represent the pooled extractions for three probes from each plot (See Fig. 1).

1.2.3. Potential Net Nitrogen Mineralization and Net Nitrification

Total inorganic N (TIN), potential net N mineralization (PM), potential net nitrification (PN) and soil microbial biomass N (used as a proxy for soil microbial biomass; MB) were determined for five of eight MAT gradient plots sampled in this study (Table 1) using the chloroform fumigation-incubation method (Jenkinson and Powlson 1976, Durán *et al*. 2013). Soil extracts from three of the eight plots sampled were destroyed in shipping, thus nitrification and N mineralization data for only five of eight plots are reported here (Table 1). In October 2014, five replicate soil cores (5cm wide x 25cm deep, OiOe-OaA) were removed from randomized locations in eight MAT gradient plots and were stored in plastic bags on ice in the field. Within 2 hours, these cores were taken to the lab and stored at 4˚C for approximately three days until processing.

Cores from each plot were then carefully homogenized by hand to remove roots, woody debris and rocks, and pooled into one sample per plot. From each pooled sample, soil gravimetric water content was determined on a subsample by drying at 60˚C for 5 days. An initial 7 g soil subsample from the homogenized pool was shaken with 30 ml 2M KCl for 1 hour and filtered using Whatman #1 filters. Filtrate was stored at 4˚C. A 10 ± 0.05 g subsample of the homogenized soil from each plot was then fumigated with chloroform in a dessicator for 12h to lyse microbial cells and the fumigated samples were then inoculated with 0.2 ± 0.05 g of fresh soil from the remaining unfumigated homogenized soil. Inoculated samples and 10 ± 0.05 g unfumigated control samples were incubated for 10 days at 16˚C. After incubation, samples were shaken with 10mL of 2M KCl for 2 hours and filtered using Whatman

#1 filters. TIN ($NH_4^+ + NO_3^-$) was quantified from KCl extracts colorimetrically using a flow injection analyzer (Lachat QuickChem 8100). The amount of NH_4^+ released during the 10-day incubation was assumed to be directly proportional to the amount of microbial biomass in the original (unfumigated) sample; however, no correction was applied for the representation of relative microbial biomass N (Durán *et al*. 2013). PM was calculated as the accumulation of TIN over the 10-day incubation of unfumigated samples and PN was calculated as the accumulation of NO_3^- over the 10-day incubation.

1.2.4. Soil Sampling and DNA/RNA Extraction

A 10 g subsample was taken from the homogenized soil pool for each of eight MAT plots in October 2014 for DNA extraction and stored on ice in the field. Within 12 hours of collection, each core from each plot was separately homogenized by hand into bulk samples and a subsample was stored at -22˚C until extraction. DNA was extracted from ~2.3 g soil subsamples using the ZR Soil Microbe DNA Midiprep Kit according to manufacturer instructions (Zymo Research Corporation, Irvine, CA). In August 2015, four spatially independent (~10m separation) soil samples were taken from 0-10cm depth, placed on ice, stored in liquid nitrogen within 1 h of collection, and then at -80˚C in the laboratory until RNA extraction. Soil RNA was extracted from ~200 mg subsamples using the ZR Soil/Fecal RNA MicroPrep kit according to manufacturer's instructions (Zymo Research Corporation, Irvine, CA). cDNA was synthesized in duplicate from RNA samples (diluted to 10% in nuclease free H_2O) using the SuperScript® III First Strand Synthesis System for RT-PCR according to manufacturer's intructions (Life Technologies, Carlsbad, CA).

1.2.5. PCR Amplification and amoA *Sequencing*

PCR amplification of archaeal and bacterial *amoA* genes was conducted using general published primer sequences from Francis *et al*. (2005) and Rotthauwe *et al*. (1997), respectively. Initial PCR reaction volumes were 25 µl (see Appendix 1 for reaction mixture). PCR products were then separated using gel electrophoresis and PCR products were cloned in the laboratory and sequenced at the Cornell Bioinformatics Facility. We confirmed the identities of the *amoA* gene sequences against the non-redundant protein databases at NCBI using the BLASTn algorithm (www.ncbi.nlm.nih.gov/blast, Altschul *et al*. 1997). We then designed qPCR primers and probes around based on recovered sequences from three clades most highly represented in clone libraries.

1.2.6. Quantitative PCR with DNA and cDNA

Quantitative PCR (qPCR, "real time PCR") was performed with DNA and cDNA templates extracted from soils to estimate abundance of *amoA* copies in each plot. Quantification was based on the fluorescent probe SYBR-Gold (Molecular Probes). We used AOB and AOA *amoA* primers designed based on sequences amplified by previously published primers (Rotthauwe *et al*. 1997; Francis *et al*. 2005). Reactions were performed using a StepOnePlusTM Real-Time PCR system (Applied Biosystems). Standards for AOA *amoA* and AOB *amoA* were prepared from synthesized oligonucleotide standards and were analyzed in a serial dilution over eight orders of magnitude to generate a standard curve. StepOnePlus (v.2.3) software

automatically calculated the cycle threshold (C_T) values for all reactions (see Appendix 1) (Applied Biosystems).

1.2.7. Phylogenetic Analysis

Sequences were first trimmed of primers and vector. They were then compared by BLASTn against the non-redundant database at NCBI (Madden 2013). From there, the closest cultivated and uncultivated relatives were imported into the alignment. The alignment itself was performed by ClustalW. The tree was constructed using neighbor joining and UPGMA in ClustalX, where tree topology was bootstrapped with 1,000 iterations. The tree was then viewed and edited in TreeViewX. The tree is rooted to the closest archaeal cultivated representative, *Nitrosopumilus marinus*.

1.2.8. Statistical Analysis

Relationships among MAT, bioavailable NH_4^+ and NO_3^- from resin probes, PM and PN rates from laboratory incubations, and *amoA* abundance and relative expression were determined using Spearman correlation and linear regression models. Diagnostic tests for data conformance to assumptions of normality were performed and data were log transformed as needed. A partial F test was used to compare a full model using all measured variables to predict bioavailable NO_3^- to reduced models to determine the main drivers of NO_3^- availability. Bartlett's test and the Fligner-Killeen test were applied to determine the equality of variances in mean cycle threshold values from qPCR with DNA and cDNA among different *amoA* phylotypes. Analyses of variance were then conducted to determine significant differences among phylotype abundance, expression and response to MAT.

1.3. Results

1.3.1. Soil Nitrogen Availability and Cycling

Soil NO_3^- availability strongly increased with MAT (r^2= 0.75, P=0.0033, Fig. 1a). The estimated rate of total nitrogen (NO_3^- + NH_4^+) bioavailability increased with MAT across the gradient (r^2= 0.42, P=0.047; Fig. 1b), whereas soil NH_4^+ availability was not correlated with MAT (P= 0.26). Potential net N mineralization and PN showed positive but non-significant trends across increasing MAT (Table 1). Potential net N mineralization was positively correlated with TN bioavailability (P=0.041), and PN was not correlated with TN bioavailability.

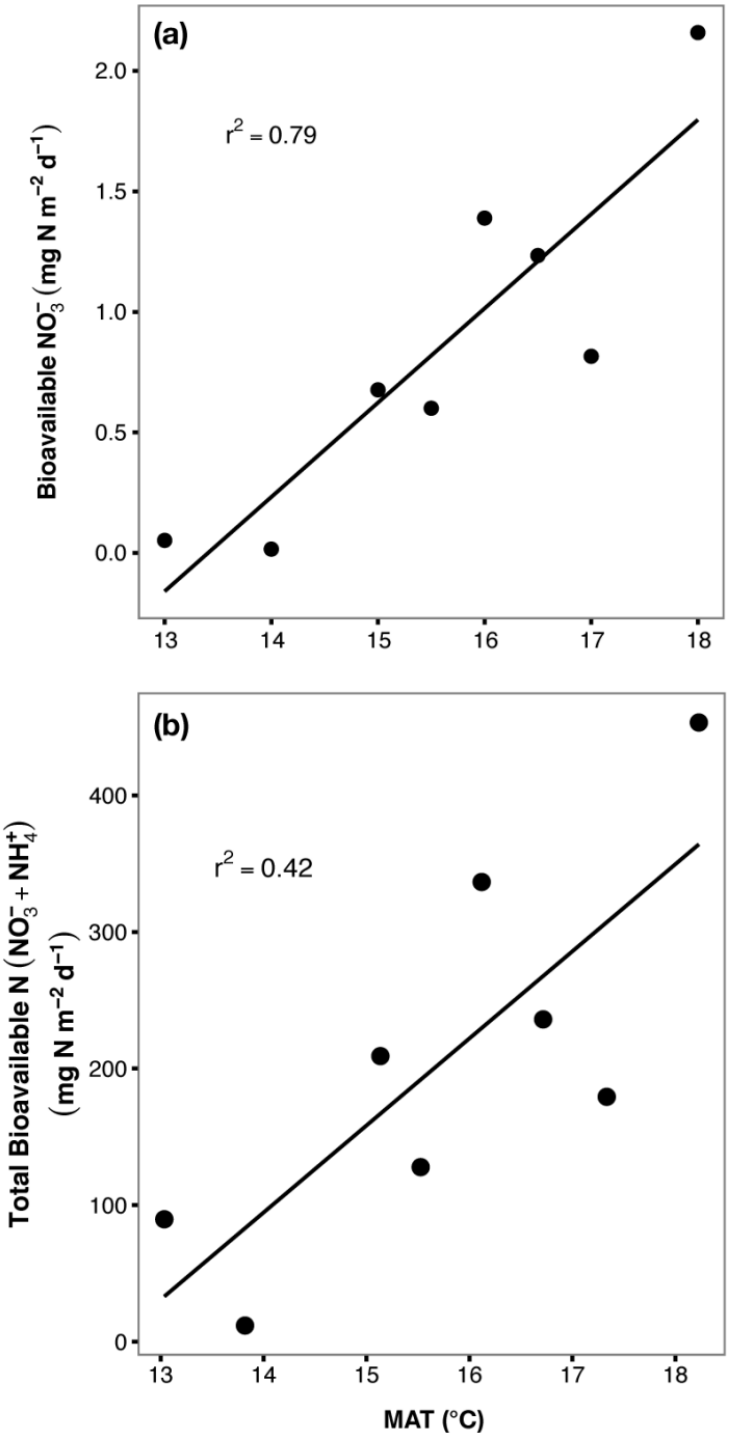

Figure 1.1. The strong, positive relationship between NO_3^- bioavailability and MAT in situ (1.1.a.; r^2= P=0.003) drives the relationship between bioavailable N (NO_3^- + NH_4^+) and MAT across the elevation/mean annual temperature (MAT) gradient (1.1.b.; P = 0.047).

1.3.2. Ammonia Oxidizer Abundance and Expression

The AOA *amoA* gene was present in soils from all the MAT gradient plots whereas the AOB *amoA* gene could only be PCR amplified in a small subset of samples and were found in very negligible quantities via qPCR. Moreover, the ratio of AOA to AOB *amoA* gene abundance did not differ across the gradient. Thus, AOA appear to be the dominant domain of ammonia oxidizers in these tropical wet montane forest soils. The abundance of AOA *amoA* gene copies increased linearly with MAT (P=0.00024, Fig. 2). Abundance of the AOA *amoA* gene was also strongly correlated with total NO_3^- availability (P=0.0071). In contrast, the absolute expression of the AOA *amoA* gene was not correlated with MAT (P=0.106).

We found that rates of PN and normalized *amoA* expression were positively, but not significantly (P=0.194), correlated with MAT across the gradient (r^2=0.53). Further, MAT was not predictive of the normalized expression of AOA *amoA* across the gradient (P=0.573). Additionally, a partial F test revealed that NO_3^- availability was best predicted by a reduced linear model including only the interaction between the MAT and AOA *amoA* abundance variables (P<0.05) This suggests that MAT and AOA *amoA* abundance are the primary drivers of NO_3^- bioavailability.

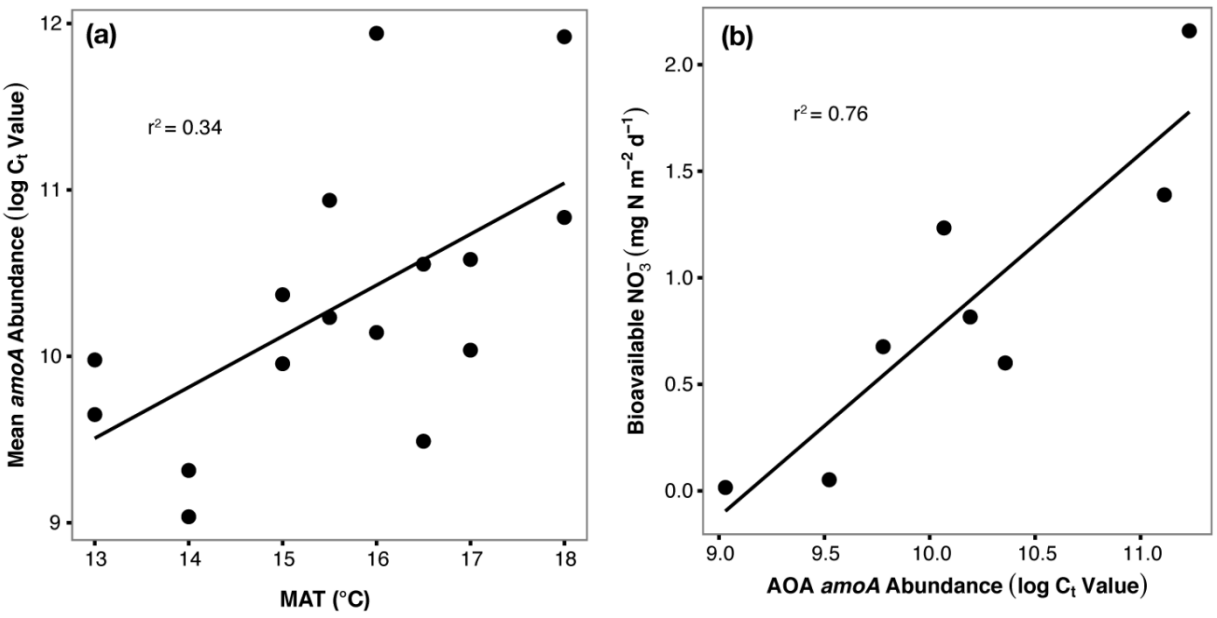

Figure 1.2. Abundance of ammonia oxidizing archaea (AOA) *amoA* gene (log transformed cycle threshold value) is positively correlated with mean annual temperature (MAT) (1.2.a.; P=0.00024, r^2=0.34). AOA *amoA* abundance is also positively correlated with total bioavailable NO_3^- (1.2.b.; P=0.0071, r^2=0.34).

1.3.3. Community Structure

Among the AOA *amoA* sequences detected, three unique clades (described here as Phylotype 1, Phylotype 2, and Phylotype 3) (Fig. 5) were detected. Phylotype 1 and Phylotype 3 were most abundant, and TaqMan qPCR primers and probes were designed around these two major clades and one additional clone to understand abundance across sites. Quantitative PCR with DNA revealed differences (P=0.0019) in DNA abundance among the three dominant phylotypes detected. Only Phylotype 3 showed differences in DNA abundance across MAT (P= 0.0017, r^2 = 0.39). Relative expression among the three phylotypes differed (Fig 3b; P= 0.029).

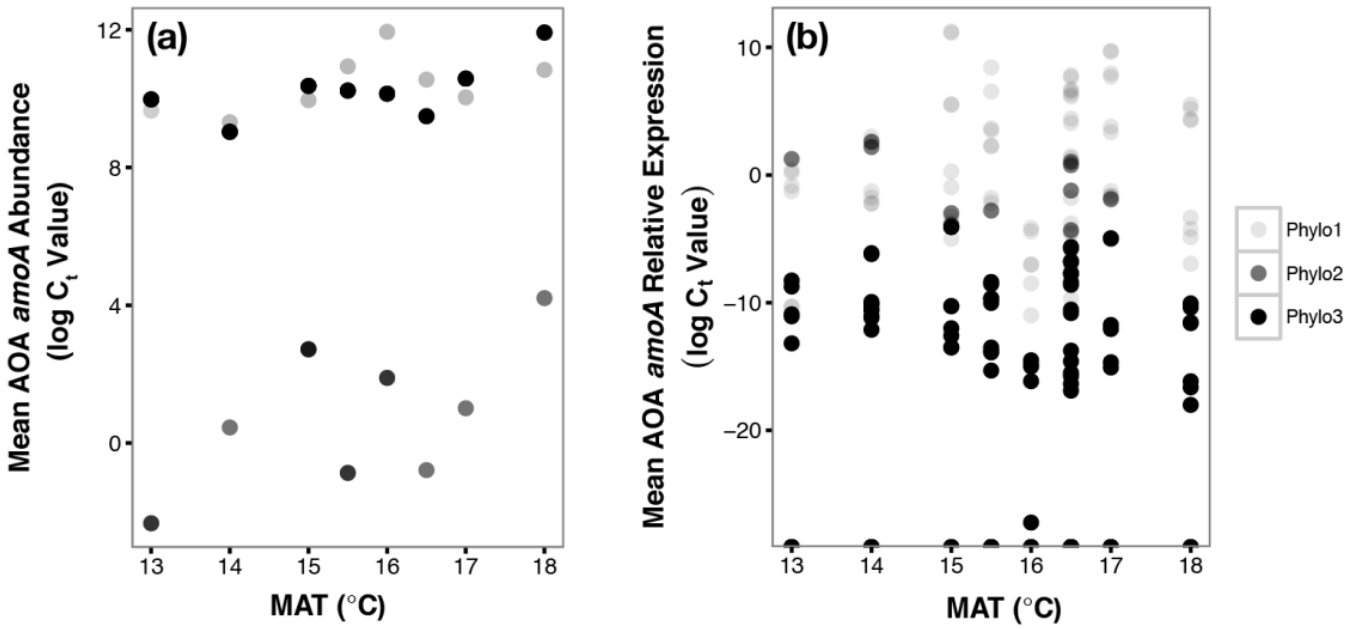

Figure 1.3. Abundance of AOA *amoA* quantified using DNA (3a) and relative expression of AOA *amoA* (cDNA:DNA) (3b). DNA abundances (3a) differ significantly (P=0.0019) among Phylotype 1 (light gray), Phylotype 2 (dark gray), and Phylotype 3 (black). Relative expression (3b) differs significantly (P=0.029) among the three observed phylotypes.

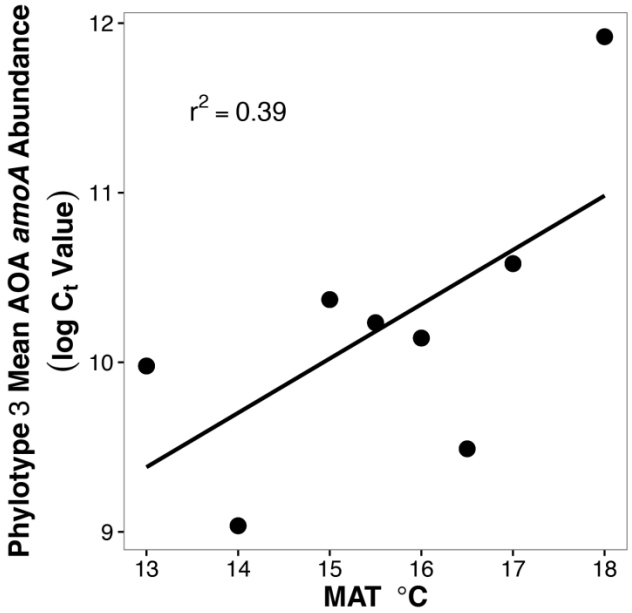

Figure 1.4. Fig. 4: AOA *amoA* in Phylotype 3 showed a significant (P=0.0017), positive relationship to MAT in the field while Phylotypes 1 and 2 did not respond to MAT.

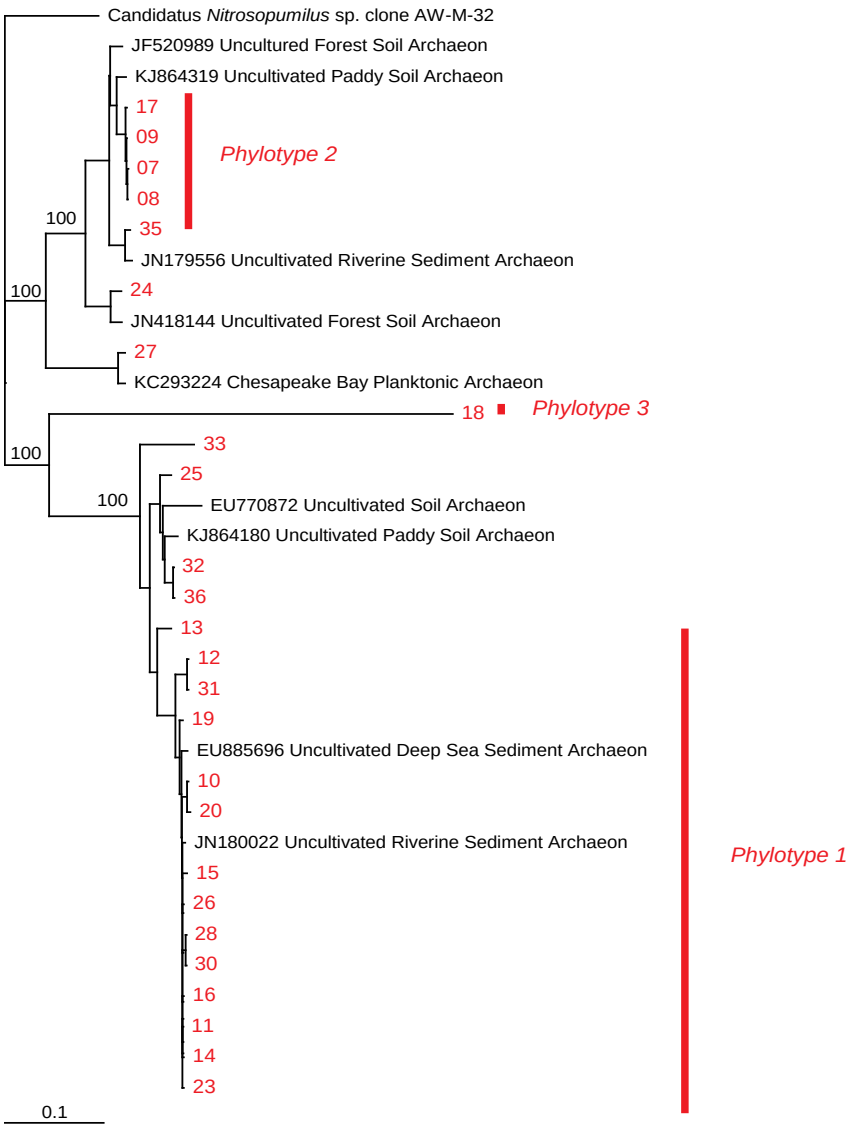

Figure 1.5. Phylogeny of AOA *amoA* sequences from soils sampled at nine sites along the Mauna Kea, Hawaii MAT gradient.

1.4. Discussion

1.4.1. Nitrogen Cycling and Bioavailability Across the MAT Gradient

We found clear evidence that soil N availability increases with MAT in this tropical montane wet forest. In particular, both total inorganic N and nitrate availability increased significantly with MAT (Fig. 1). Patterns of N cycling and bioavailability across natural MAT gradients have been previously investigated

(Martinson *et al*. 2012), but the patterns observed have been inconsistent, likely due to differences among field sites and experimental design. Interactions among MAT, mean annual precipitation (MAP) (Idol *et al*. 2007), vegetation composition (Fissore *et al.* 2008), and soil characteristics (Smith *et al*. 2002), among other variables, can confound results pertaining to the specific effects of MAT. Some previous studies of N cycling across MAT gradients have focused on rates of nitrification (Bohlen *et al.* 2001, Groffman *et al.* 2009, Fisk *et al.* 2010, Martinson *et al.* 2012, Durán *et al*. 2014) but the microbial mechanisms underlying the observed patterns have not been evaluated. The work presented here provides evidence of a microbial mechanism that underlies the observed positive relationships among *in situ* MAT, N bioavailability and N cycling rates.

1.4.2. Ammonia Oxidizer Community Abundance

We hypothesized that bacterial and archaeal ammonia oxidizer abundance and expression of their *amoA* genes would increase linearly with MAT. This hypothesis is predicated on the observations from this gradient that primary productivity, total belowground C allocation and microbial N metabolism all increase with temperature (Litton *et al.* 2011, Giardina *et al*. 2014). N recycling through the detrital food web would thus be expected to increase with temperature, resulting in higher supply of mineral N to nitrifiers. Our findings partially supported this hypothesis. We observed that AOA *amoA* gene abundance was positively related to MAT (Fig. 2a), which, to our knowledge, is the first finding of its kind along a natural elevation gradient. Moreover, *amoA* abundance was strongly correlated with soil nitrate availability. It is

unclear why the relationship between *amoA* abundance and soil PN was weaker than that between *amoA* abundance and nitrate availability, but this reflects the overall weak correlation between MAT, PM and PN. Understanding of this relationship would be improved by future studies including a greater number of sites with unique MAT.

Our results are consistent with previous studies of temperature effects on PN and ammonia oxidizer community dynamics, which have shown that PN responds positively to increasing temperature while AOB abundance decreases and AOA abundance increases (Avrahami and Bohannan 2006). Others have demonstrated that while PN rates, AOA abundance, and AOA community structure changes with increasing temperature, AOB abundance and community structure remain unchanged (Tourna *et al.*2008). When studies have focused only on AOB responses to elevated temperature, results showed no significant response in AOB *amoA* abundance to elevated temperature, and instead revealed stronger correlations between AOB abundance and other environmental variables (Horz *et al*. 2004). These and other temperature manipulation studies suggest that AOA are more responsive to increasing temperature while AOB are less well acclimated to warming and less involved in nitrification as temperatures increase. They further illustrate the variability in results among warming experiments targeting AOA and AOB communities. This variability may result from differences in laboratory and field warming methodologies, which may not capture long-term AOA and AOB community acclimation to MAT and the sustained differences in abundance and structure between these groups across temperature (Ågren and Bosatta 2002). Instead, warming studies may inform more on

the abilities of AOA and AOB communities to adjust to short-term temperature changes, rather than long term climate changes.

In this study, AOB *amoA* gene amplification was only detectable in a small number of soil samples in a subset of gradient plots and were not quantifiable via qPCR. This suggests that AOB are not the dominant group of ammonia oxidizers in this ecosystem. Low or absent AOB copy numbers have been reported for other undisturbed terrestrial (Leininger *et al.* 2006, Adair and Shwarz 2008, Prosser and Nicol 2008, Zhang *et al*. 2012) and aquatic (Bowen *et al*. 2014) environments, and several studies point to AOA as the dominant ammonia oxidizers in most natural environments (Leininger *et al*. 2006). Further, our results are in line with the findings of Tourna *et al*. (2008) which show that AOA, and not AOB, are responsive to changing environmental conditions. Horz *et al*. (2004) showed that experimentally elevated temperature augmented the abundance of AOB, but their community analysis did not target AOA. This may have neglected relevant differences between AOA and AOB community responses to increased temperature (Tourna *et al*. 2008). The dynamics of AOA and AOB abundance and contribution to nitrification are dependent on many environmental drivers (Horz *et al*.2004, Taylor *et al*. 2012); thus, the abundance and dominance of AOA and AOB in nitrification appears to depend on these conditions, potentially leading to niche specialization of AOA and AOB (Erguder *et al.* 2009, Martens-Habenna *et al*. 2009). The negligible amplification of AOB *amoA* copies in soils from the present study suggest that increasing MAT may selectively augment the number of AOA *amoA* copies while AOB abundance does not respond to temperature. Avrahami and Conrad (2003, 2011) and Tourna *et al*. (2008)

similarly showed that responses to increasing temperature lead to shifts in the dominance of AOA versus AOB dominance across *in situ* and incubation temperatures. These results provide further evidence that targeting of AOB alone in the study of soil N cycling may neglect the contribution of AOA to ammonia oxidation.

We observed differences in abundance among three clades of AOA *amoA* in soils across the gradient (Fig. 3a). Previous studies have shown similar responses in ammonia oxidizer community structure with changing temperature (Avrahami and Conrad 2003, Tourna *et al*. 2008, Avrahami and Conrad 2011). We also observed that relative *amoA* expression among the phylotypes differed, with Phylotype 1 displaying the highest levels of expression across the gradient (Fig. 3b). This suggests that expression of this nitrification gene is dominated by one group within the ammonia oxidizer community. The selective growth of AOA and certain AOA phylotypes, as seen with Phylotype 3 at plots with increasingly warm MAT (Fig. 4), has been attributed to differences in maximum specific growth rate and saturation constant of different taxa within the AOA group, in addition to the generally greater sensitivity of AOA to increasing temperature (Tourna *et al*. 2008, Prosser and Nicol 2012, Litchman *et al*. 2015). The positive response of Phylotype 3 abundance to increasing MAT suggests that certain AOA taxa are more sensitive to rising temperature and may be more important targets for trait-specific studies of N cycling and MAT interactions. While our results suggest that AOA abundance may drive N bioavailability across MAT, AOA exhibit mixotrophy, which may allow for changes in AOA abundance

without reflecting changes in ammonia oxidation (Prosser and Nicol 2008, Tourna *et al*. 2011).

1.4.3. Ammonia Oxidizer Gene Expression and N Cycling

We measured the relative expression of AOA and AOB *amoA* genes in soils across the MAT gradient and found no significant relationship between these variables. Contrary to our expectation, this result suggests that MAT may not drive variation in the transcription of AOA *amoA* in soils despite the observed increase in the abundance of AOA *amoA* with MAT (Fig. 2a). This is surprising in light of experimentally demonstrated effects of temperature on AOA *amoA* gene transcription (Tourna *et al*. 2008), which have been explained in terms of higher growth rate at elevated temperatures and selective growth of certain phylogenetic groups of ammonia oxidizers. In this study, the lack of significant response in relative *amoA* expression to increasing MAT, despite the increase in *amoA* abundance with MAT, suggests that *in situ*, MAT may select for population size and structure but not gene expression in AOA. This result may illustrate differences in AOA response to field conditions versus experimental warming. This finding may also support the aforementioned potential for metabolic shifts among mixotrophic AOA as MAT and AOA abundance increase (Prosser and Nicol 2008). The differences in temperature sensitivity among microbial taxa have been well documented (Zogg *et al*. 1997), and are known to determine ammonia oxidizer community structure and resource use efficiency both spatially and temporally under future climate changes (Fierer *et al*. 2009).

1.4.4. Implications for Ecosystem Function

The positive relationships among AOA *amoA* abundance, soil nitrate availability and MAT observed in this study provide strong evidence that MAT is the primary driver of N cycling in this system; however, the causal chain underlying this relationship is not entirely clear. Higher temperatures at lower elevations result in higher forest productivity (Litton *et al.* 2011, Giardina *et al*. 2014), but the maintenance of high rates of production requires a larger nitrogen supply (Treseder and Vitousek 2001, Fernández-Martínez *et al*. 2014). Giardina et al. (2014) demonstrated that belowground carbon flux increases strongly with temperature along this gradient, presumably reflecting increased demand for soil resources in the rhizosphere, including N. Higher temperatures also leads to faster litter decomposition (Bothwell *et al.* 2014), accelerated forest floor turnover (Giardina *et al*. 2014), and a lower ratio of detrital C to total soil C along this gradient (Selmants et al. 2014). Thus, recycling of N through the detrital food web increases with MAT in this system. Our results suggest that this increased N recycling may stimulate AOA abundance and consequently higher nitrification and soil NO_3^- availability at higher temperatures.

Functional gene abundance and biogeochemical process rates are directly related in natural environments (Beman *et al*. 2008, Tourna *et al.* 2008, Yao *et al.* 2011, Bowen *et al*. 2014, Reed *et al*. 2014). Responses of microbial communities to environmental changes at the genetic level are therefore responsible for biogeochemical cycling rates in many ecosystems (Horz *et al.* 2004, Castro *et al*. 2010, Taylor *et al.* 2012, Kelly *et al.* 2013). Despite this, gene mediation of biogeochemical processes is not commonly studied among the components of

ecosystem response to environmental change (Reed *et al*. 2014). Few findings have illustrated how the abundance and expression of functional genes change under different environmental conditions and whether these changes influence ecosystem properties (Horz *et al.* 2004, Tourna *et al.* 2008, Yao *et al.* 2011, Taylor *et al*. 2012).

The next step in this work is to identify sources of N inputs in soils at warmer MAT. As MAT increases, N substrate for ammonia oxidation and other downstream N processes may enter forests through increased biological N fixation (BNF), and numerous studies have illustrated the dominance of BNF in tropical forests (Houlton *et al*. 2008, Vitousek *et al*. 2013). Our observations of a shift in N availability and cycling with MAT may alter the presumed 'steady state' of nutrient limitation in forests (Walker and Syers 1976). This would imply that ecosystems undergoing climate change are moving toward a state of unbalanced limitation between N and phosphorus, where N is increasingly available and primary production is mainly phosphorus limited (Elser *et al*. 2007). Coupled with evidence of higher fluxes of new N in the tropics compared to temperate zones (Vitousek *et al*. 2013), the results of this study suggest that soil AO communities may be acclimated to increased fluxes of new N entering forest systems with increasing MAT. Further studies of the relationship between BNF and ammonia oxidizer community dynamics with increasing MAT will help clarify how forest N demand and ammonia oxidizer dynamics interact with BNF.

1.5. Conclusion

Our study concludes that with all else being equal, ammonia oxidizer populations increase with rising MAT at this site. In tandem, ammonium and nitrate supplies to terrestrial primary producers also increase. Similar patterns have been

observed along elevation gradients in other forest types, suggesting that increasing MAT may be a driver of internal N fluxes in other forest systems (Vitousek *et al.* 1988, Knoepp and Swank 1998, Johnson *et al.* 2000, Liu and Wang 2010, Averill and Finzi 2011, Salinas *et al*. 2011). If soil NH_4^+ supplies increase in the future, we predict from our results that nitrifier populations will continue to increase nitrification rates, thus affecting forest N demand and rates of primary production (Raich *et al.* 1997, Nemani *et al.* 2003, Giardina *et al*. 2014). The responses of different AOA and AOB groups to rising MAT and their impacts on IN availability may hold information about future steady states for forest nutrient limitation. Further, the ability for nitrifier populations to sustain increased nitrification as MAT increases may alter N mobility in soils as MAT increases (Groffman *et al.* 2009, Nemani *et al*. 2003). This study shows that the abundance of ammonia oxidizers in soils may be a useful parameter in predicting future biogeochemical changes in forests under climate change.

SUPPLEMENTARY INFORMATION FOR CHAPTER 1: AMMONIA OXIDIZER POPOULATIONS VARY WITH NITROGEN CYCLING ACROSS A TROPICAL MONTANE MEAN ANNUAL TEMPERATURE

1.S1. *PCR Reaction Mixture*

Each 25 µl PCR reaction included 2.5 µl of reaction buffer, 2.5 µl magnesium chloride ($MgCl_2$), 0.5 µl deoxynucleotides (dNTP), 1 µl forward primer, 1 µl reverse primer, 0.25 µl bovine serum albumin (BSA), 1 µl *Taq* polymerase, and 15.25 µl nuclease-free water.

1.S2. *Complementary DNA Synthesis from RNA*

Each cDNA synthesis reaction had a volume of 23 µL and was comprised of 1 µL RNA template, 1 µL gene-specific primer, 1 µL 10mM dNTP mix, 7 µL DEPC-treated water, 1 µL 10X RT buffer, 4 µL 25 mM $MgCl_2$, 2 µL 0.1 M dithriothreitol, 1 µL RNaseOUT™, and 1 µL SuperScript® III RT. A control assay was performed in duplicate for each RNA sample without SuperScript® III RT. Standard sequences used for AOA *amoA* were: Primer Forward: 5'-STAATGGTCTGGCTTAGACG-3'; Primer Reverse: 5'-GCGGCCATCCATCTGTATGT-3' (Francis *et al*. 2005). Standard sequences used for AOB *amoA* were: Primer Forward: 3'-GGGG TTTCTACTGGTGGT-5'; Primer Reverse: 5'-CCCCTCKGSAAAGCCTTCTTC-3' (Rotthauwe *et al*. 1997).

1.S3. *qPCR Reaction Details and Primer/Probe Sequences*

Each 25µl qPCR reaction was comprised of 2 µl of DNA or cDNA template (1:2 dilution in nuclease-free H_2O), 9.9 µl of nuclease-free H_2O, 0.2 µl of forward and

reverse primer, respectively, and 0.2 µl of TaqMan probe and 12.5 µl of *Taq* polymerase (Applied Biosystems).

Table 1.S1. *Forward and reverse primer sequences and probe sequences for three AOA phylotypes*

Target	**Primer Forward Sequence**	**Primer Reverse Sequence**	**Tm ˚C (F, R)**
AOA *amoA* Phylotype 1	5'-CCCACTATCTGTTCATCGTT-3'	3'-CCAGTCGGTGTAGAAGATGT-5'	45.5, 58.4
P1 Probe	[FAM]GCACACTGCTGACCATAAATGCA[TAM]		62.8
AOA *amoA* Phylotype 2	5'-ATATTGGGCAACAAAGAAGA-3'	3'-CGACATAGACATTCCACACA-5'	55.8
Phylotype 2 Probe	[FAM]TGATCCTAATTGGCGGTGTGC[TAM]		62.6
AOA *amoA* Phylotype 3	5'-TCTCGTACCAGCAATAGGTT -3'	3'-TAATACTCGTTGGCGGTACT-5'	58.4
Phylotype 3 Probe	[FAM]ACGGAAGTGACAGGCCAACC[TAM]		64.5

Table 1.S2. *qPCR reaction details for three AOA phylotypes*

AOA *amoA* Phylotype	**Annealing Temperature**	**R^2 of Standard Curve**	**Reaction Efficiency (%)**	**Limit of Detection (Ct Value)**	**Gene Copies/ µl**
Phylotype 1	55 ˚C	0.98	107.84	23.84	3.01E5
Phylotype 2	55 ˚C	0.98	125.99	18.392	2.96E5
Phylotype 3	56 ˚C	0.99	122.29	16.26	2.56E5

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CHAPTER 2

MEAN ANNUAL TEMPERATURE INFLUENCES FINE ROOT NUTRIENT FORAGING

Introduction

Increasing temperature resulting from greenhouse gas forcing is expected to affect terrestrial primary production (Luyssaert *et al*. 2007) by altering aboveground and belowground carbon (C) allocation (Raich and Nadelhoffer 1989; Litton and Giardina 2008). These temperature-driven changes in C allocation may induce feedbacks that alter the carbon dioxide (CO_2) balance of forest ecosystems (Vogel *et al.* 2008). Belowground C allocation comprises a significant and variable portion of gross primary production (GPP) (Litton *et al.* 2007) and is an important source of C for various belowground processes including the formation of the stable fraction of soil organic matter (SOM) (Kuzyakov and Schneckenberger 2004, Schmidt *et al*. 2011). Changes in C allocation to belowground components in response to temperature is influenced by variation in the availabilities of multiple resources (BassiriRad 2000, Pendall *et al*. 2004, Melillo *et al*. 2011, Reich *et al.* 2014). Plants adjust the proportion of C allocated to aboveground and belowground components in order to overcome resource limitation (Bloom *et al.* 1985, Treseder and Vitousek 2001). There is particular uncertainty about how fine root growth and mycorrhizal colonization of roots, two primary mechanisms for plant nutrient acquisition, are responding to rising mean annual temperature (MAT) in forest ecosystems (Gill and Jackson 2000).

Natural field observations (Ostertag 2001), field fertilization experiments (Haynes and Gower 1995), and modeling exercises (Dybzinski *et al.* 2011) have all shown that increasing soil nutrient availability leads to reduced belowground C allocation and proportional increases in aboveground C allocation (Litton et al. 2007). Fertilization studies show that plants in nutrient-poor soils may allocate a greater proportion of total plant C to fine roots, root exudates, and fungal symbionts, compared to those in nutrient-rich sites (Treseder and Vitousek 2001, Johnson *et al.* 2010, Vicca *et al.* 2012). The degree to which any nutrient limits production is context dependent and can be related to successional stage (Naples and Fisk 2010), age of parent material (Reed *et al.* 2011), and climatic conditions (Vogt *et al.*1995, Heinemeyer and Fitter 2004, Ostonen *et al*. 2011). For example, P may be more limiting in older, highly weathered substrates (Walker and Syers 1976) and N may be more limiting in colder climates (LeBauer and Treseder 2008). Fine root biomass (FRB) and fine root length (FRL) influence the root surface area available for soil exploration and nutrient uptake, and so provide indices of plant demand for nutrients (Berntson *et al.* 1995, Powers *et al.* 2004). Fine root foraging for nutrient-enriched microsites also depends on the ability of roots to elongate and proliferate in response to patchy soil resource distribution (Farley and Fitter 1999, Adams *et al.* 2013). Plant access to nutrients is also predicated on root nutrient transport efficiency, which is affected by association with mycorrhizal fungi, variations in root/mycorrhizal morphology, and nutrient transport (Marschner and Dell 1994). Associations with mycorrhizal fungi are more prevalent under conditions of nutrient scarcity in a range of plant species and ecosystems and work in conjunction with root exudates to

stimulate microbial activity in rhizosphere soils (Dakora and Phillips 2002, Dijkstra *et al.* 2013). Higher mycorrhizal colonization confers a C cost to plants and must therefore significantly relieve nutrient limitation to primary production to be cost-effective (Millard, Sommerkorn and Grelet 2007). Conversely, under strongly nutrient limiting conditions, fertilization can greatly reduce mycorrhizal colonization of fine roots (Giardina *et al.* 2003)

Rates of N mineralization and nitrification increase with temperature in forest soils (Zak *et al.* 1999; Rustad *et al.* 2001), and both inorganic N availability and the microbial genes associated with N cycling also increase with temperature in warming experiments and across natural temperature gradients (Tourna *et al.* 2008, Pierre *et al.* 2017). Increased N cycling and bioavailability with rising temperature have been suggested as potential mechanisms contributing to increased aboveground biomass and soil CO_2 efflux with increasing MAT (Rustad *et al.* 2001, LeBauer and Treseder 2008, Cleveland *et al.* 2011). Giardina *et al.* (2014) used a natural but highly constrained elevation gradient to show that MAT is positively related to both litterfall and total belowground C flux (TBCF), suggesting that increasing N availability with MAT may drive higher stand productivity. High N availability may also lead to greater P limitation with increasing MAT, as the N:P ratio of soil may increase without concomitant increases in P availability.

We investigated the effects of MAT and site-level N bioavailability, where N bioavailability is positively correlated with MAT (Pierre *et al.* 2017), on fine root nutrient foraging across the elevation-based MAT gradient used by Litton *et al.* (2011). This gradient is highly constrained because variables other than temperature

(soil moisture, vegetation, successional stage, soils, geology) are held constant (Litton *et al*. 2011, Giardina *et al*. 2014; Selmants *et al*. 2014). Specifically, we determined how fine root proliferation into N and P enriched microsites responded to the coincident variation in MAT and site fertility. Natural elevation gradients provide an ideal setting to test the role of environmental variation in ecological processes in ways that more accurately represent real ecosystems than manipulation studies that often produce transitional effects and experimental artefacts (Fukami and Wardle 2005, Malhi *et al.* 2010, Sundqvist *et al*. 2013). By leveraging this natural MAT gradient where abiotic and biotic parameters besides MAT are held constant, we were able to isolate the effects of MAT on FRB nutrient foraging. Recognizing that fine root foraging may be primarily constrained by availability of carbon for root growth, we expected an overall increase in root ingrowth with increasing temperature. We also hypothesized that in plots with lower MAT and lower N bioavailability, fine root foraging for N would be greatest. Conversely, we hypothesized that foraging for P would be greatest at sites with high MAT and high N availability (Hendricks *et al.* 1993). Finally, we expected that arbuscular mycorrhizal colonization of tree roots in the fertilized microsites would increase with MAT and N bioavailability, with the greatest mycorrhizal colonization in P-fertilized cores.

Methods

Study Site

The study was conducted in nine 20 x 20 m plots distributed along an 800 m elevation gradient on the northeastern slope of the Mauna Kea Volcano on the Island of Hawaii as described in Litton *et al*. (2011). Seven permanent plots are located in the Hawai'i Experimental Tropical Forest (HETF; 19°56′41.3″N, 155°15′44.2″W; 600–

1800 m.a.s.l) and two plots in the adjacent Hakalau Forest National Wildlife Refuge (HFNWR; 19°50′31.3″N, 155°17′35.2″W; 600–2000 m.a.s.l). All plots are located within tropical montane wet forests classified as *Metrosideros polymorpha* Gaudich.–*Acacia koa* A. Gray forests. *M. polymorpha* and *Cheirodendron trigynum* (Gaudich.) A. Heller dominate the canopy and midstory, respectively, across all plots (84-97% of basal area excluding tree ferns). Additionally, three species of tree ferns (*Cibotium* spp.; midstory) make up an average 46% of stand basal area in these plots (Litton *et al*. 2011).

Plots along the elevation gradient vary in MAT from 13˚C at the highest elevation plot (1600 m.a.s.l.) to 18.2˚C at the lowest elevation plot (800 m.a.s.l.), thus forming a 5.2˚C MAT gradient (Table 1). Soil water balance is relatively constant across all plots due to a compensatory reduction in mean annual precipitation with increasing elevation (Litton *et al*. 2011; Selmants et al. 2014). Substrate in all plots is derived from ~20 ky (14–65 ky) weathered tephra, and soils are moderate to well-drained hydrous, ferrihydritic/amorphic, isothermic/isomesic Acrudoxic Hydrudands of the Akaka, Honokaa, Maile, and Piihonua soil series (Soil Survey Staff 2010). Mean soil pH is 3.9 and base saturation and estimated mean cation exchange capacity are 32.4 % and 11.9 cmol kg^{-1}, respectively (Litton *et al*. 2011). Mean soil (0-10 cm) bulk density and carbon content across the MAT gradient are 0.21 g cm^{-3} and 14%, respectively. Mean soil nitrate (NO_3^-) bioavailability was measured in these plots using ion exchange resins, as detailed by Pierre *et al*. (2017).

Root Ingrowth Core Construction and Sampling

Cores were constructed of plastic mesh netting with 1mm x 1.5 mm holes (open area=50%), 10cm in height and 7.5 cm in diameter. Each core was filled with ~25g of a mixture of a calcined clay plant growth medium, Turface®, and vermiculite matching the average bulk density of 0.21 g cm^{-3} of native soils across the gradient. Each core was sewn closed using nylon thread. Cores were dosed with 100 mL of either deionized water (control), 6.016 g/L urea (CH_4N_2O) (N treatment), 21.69 g/L $Na_3PO_4 \cdot H_2O$ (P treatment), or a combined solution of both solutes at the same concentrations (+N+P treatment). Each treatment was applied with a needle and syringe by evenly injecting and releasing small aliquots totaling 100 mL throughout the media (Raich *et al.* 1994). Cores were covered and air dried in the lab overnight, and then weighed and stored in plastic bags at room temperature until burial.

Between August 25-29, 2015, five replicates of each treatment (Control, +N, +P, +N+P) were buried in each of the nine MAT plots. Cores were deployed in clusters, with each cluster including a core from each of the four treatments and placed at each of the four corners of the plots, with the fifth cluster equidistant between two of the corners on the perimeter of the plot. Each core within a cluster was buried (0-10cm depth) equidistant from the other cores in a square pattern. Collection occurred between November 1-11, 2015, for a mean burial period of 75 days.

Immediately after collection, cores were stored in plastic zip-lock bags and placed in coolers for transportation back to the laboratory where they were stored at ~1.5˚C until root sampling approximately 10 days later. Roots were removed from the media using a fine (<1mm) sieve and forceps and were rinsed using deionized water. When a sufficient quantity of fine (<2mm) roots was present in a core, a subsample

was stored in 50% ethanol for quantification of mycorrhizal colonization (n=37) (Grace and Stribely 1991). The root samples were weighed fresh and then dried at 60˚C for 72h for reweighing to calculate moisture content. Dried root samples were then scanned using a digital scanner and FRL was measured using the WinRhizo software (Régent Inc., QC, Canada).

Mycorrhizal Colonization

Fine root subsamples for mycorrhizal colonization were cleared, stained and scored based on the methods of Brundrett and Abbott (1994). Briefly, roots were cleared in 10% KOH solution and autoclaved on a liquid cycle in 10-minute intervals until roots were cleared of all pigment (~20-30 minutes total), with KOH changed between each autoclave cycle. Roots were then stained in 0.05% Parker Quink blank ink (Parking Pen Products, New Haven, UK) in a 1:1 solution of glycerol and vinegar for ~24 hours. Roots were then cut with scissors into 2 cm segments and mounted parallel to one another. Roots were observed at 200x magnification, with one pass across the upper half and a second pass across the lower half of the slide. At each intersection with a root, an observation was scored for the presence or absence of fungal structures (arbuscules and hyphae, both connected and unconnected to other structures). Total root mycorrhizal colonization was then scored as the percentage of observations where mycorrhizal structures were present. Observations were made by three different observers and reported percent colonization is an average of these three scores.

Statistical Analysis

A linear mixed effects regression model was employed to analyze root ingrowth data. In this model, MAT, fertilization treatment, and mean soil NO_3^- bioavailability were included as fixed effects, and plot and core cluster were random effects. Fine root biomass (FBR), FRL, and specific root length (SRL= FRL/FRB (cm/g)) data were log transformed to satisfy assumptions of normality. Model comparison and selection was performed using F tests. A linear regression of the marginal means (least squares means) of root ingrowth parameters (*i.e.* root length, root mass, SRL) was plotted to evaluate effects of interactions between predictor variables (*i.e.* MAT, NO_3^- bioavailability). The slopes of the regressions were compared using Tukey's method. All statistical analyses were performed in the R environment (R Core Team 2016).

Results

A fixed-effects regression model indicated no FRL response to MAT independent of treatment (P=0.46) (*i.e.* when all treatments were grouped together). Pierre et al. (2017) found that MAT does not account for all variation in N bioavailability, likely due to random effects that could not be controlled for despite the well-constrained gradient design. To handle random effects, we included site NO_3^- bioavailability (as reported by Pierre et al. 2017) as a fixed effect in the mixed effects regression model and included nested random effects which reflected the design of the study. Mean fine root length showed a significant, negative response to increasing MAT (P=0.041) and

to the interaction of MAT and NO_3^- bioavailability (P=0.017) based on the linear mixed effects regression model when treatment effect was not included (Table 1, Model 2). However, the full model, which includes treatment effects, represents the study's experimental design and explains the most variation in FRL response 1compared to more parsimonious models (Table 2.1.; Model 1).

Table 2.1.: Predictors of ln(Fine Root Length) Response			
	Model 1	Model 2	Model 3
MAT	0.057 (0.359)	0.558** (0.264)	-0.039 (0.192)
Bioavailable Nitrate	10.425 (13.27)	23.619** (9.756)	NA
+N Treatment	-6.443 (5.729)	NA	NA
+N+P Treatment	-13.663** (5.838)	NA	NA
+P Treatment	-10.794* (5.716)	NA	NA
MAT: Bioavailable Nitrate	-0.706 (0.856)	-1.567** (0.630)	NA
AIC	735.631	732.344	739.474
*** P<0.01; ** P<0.05; *P<0.1			

Fine root length responded to the +N+P treatment (P= 0.02) and to the interaction of MAT and the +N+P treatment (P=0.023, Table 1, Model 1). We illustrated the predicted FRL response to the interaction of MAT and +N+P using least square means interaction plot, where values are the difference from a null response of zero (Figure 1). This visualization of the +N+P and MAT interaction (Table 1, Model 1) shows that the direction of the FRL response to +N+P microsites across MAT is dependent on ambient soil N bioavailability (Figure 1). Fine root length did not respond to the N, P or control treatments (P>0.05). The FRL response to MAT and treatment is illustrated by the difference from a null response of predicted marginal means of FRL, where the null response equals zero (Fig. 1). Despite the large amount of variability observed in FRL across MAT (r^2=0.02), MAT and +N+P availability were significant predictors of FRL across the gradient, suggesting that root growth in

response to a combination of N and P is influenced by MAT and soil N bioavailability. Neither fine root biomass (FRB) nor SRL (g/cm) of ingrowth responded to MAT or to fertilization treatments (C, N, P, +N+P) in this study.

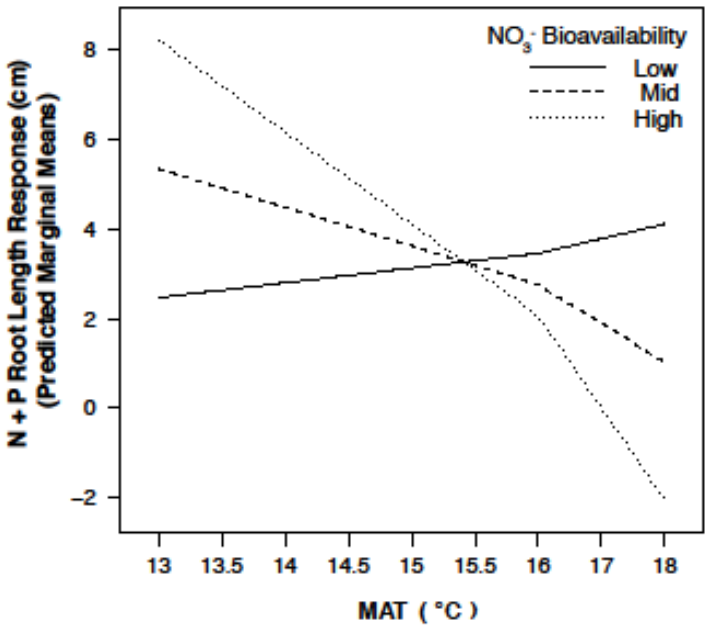

Figure 2.1. A visualization of linear mixed effects model predicted responses of fine root length (FRL; cm; log transformed) to a nitrogen and phosphorus (+N+P) fertilized root ingrowth core treatment across a range of MAT (x axis) and at three levels of NO_3^- bioavailability (lines; mg N m^{-2}; Low=0.25, Mid=0.75, High=1.25). The y-axis denotes the difference in FRL predicted least squares means from a null response, where FRL response equals zero. The root length response to the +N+P treatment is significantly different from other treatments across MAT (P= 0.023) and root length response to +N+P at High, Mid and Low NO_3^- availability responded to MAT (P=0.02).

Arbuscular mycorrhizal colonization of fine root ingrowth increased linearly with MAT (P=0.0022, Figure 2). However, none of the fertilization treatments significantly influenced the percent mycorrhizal colonization observed in fine root ingrowth.

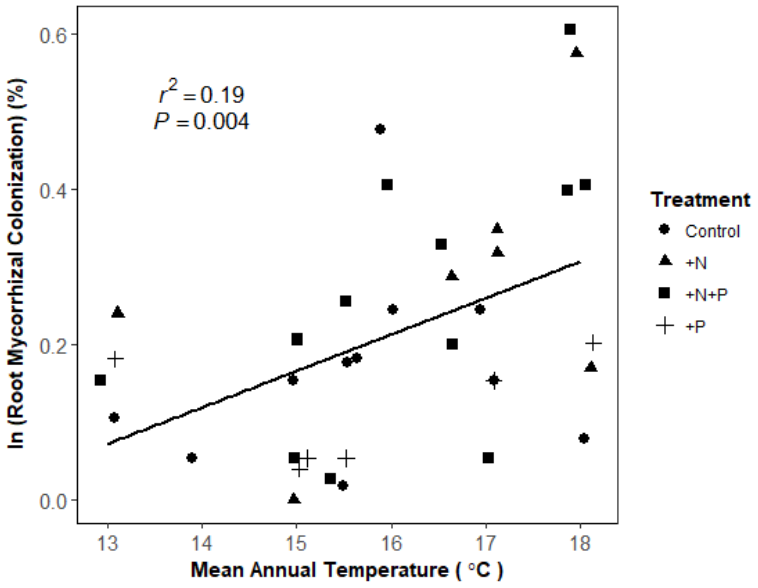

Figure 2.2. Arbuscular mycorrhizal colonization (% of root length colonized, log transformed) of fine roots in fertilized root ingrowth cores increases linearly with mean annual temperature (MAT) (r^2=0.43; P=0.0039). Points represent % mycorrhizal colonization of roots within a single core. Mycorrhizal colonization does not differ across fertilization treatments.

Discussion

Fine Root Responses to Nutrient Availability and MAT

The results of this study suggest that a complex interaction between nutrient availability and MAT influences fine root foraging in nutrient-enriched microsites at the ecosystem scale. The negative response of FRL to the +P and +N+P fertilization treatment with increasing MAT differed slightly from our expectation that increasing N bioavailability with MAT would relieve fine root N demand, shifting root demand to P only. Instead, the stronger foraging response to the combination of N and P than to P alone suggests that co-limitation by both N and P may be a determinant of fine root microsite proliferation at lower MAT.

Co-limitation by N and P has been observed across many terrestrial and aquatic ecosystems, where a simultaneous addition of both nutrients increases primary production above enrichments of either nutrient alone (Vitousek and Farrington 1997, Elser *et al.* 2007). Observed increases in primary production in response to +N+P additions reflect the stoichiometry of photosynthesis and plant growth, but these observations do not shed light on the dynamics of internal plant C partitioning for N and P acquisition. Roots respond dynamically to heterogeneous supplies of potentially growth-limiting resources through internal signaling pathways induced by internal and external cues (Hutchings and de Kroon 1994, Forde and Lorenzo 2001). While diverse root responses to nutrient microsites under different background nutrient conditions have been shown in model plant systems (Drew *et al.* 1973, Zhang and Forde 1998), patterns have not been linked to their ecological causes or consequences (Callaway *et al.* 2003). Primary root initiation and elongation are linked to the immediate presence of these nutrients through root elongation gene expression (Zhang and Forde 1998) and hormone pathways (Drew *et al.* 1973, Le Deunff *et al.* 2016). Root foraging responses to external cues are species-specific and seasonally-dependent (Eissenstat and Caldwell 1988, Kembel and Cahill 2004). These sources of variation were minimized by constant vegetation composition, parent material and soil moisture across the MAT gradient and by the short-term nature of this study. Decreasing +N+P root foraging with naturally increasing MAT suggest that internal plant nutrient status responds directly to an abiotic cue.

Our observations illustrate the interplay between plant nutrition, fine root behavior, and environmental conditions at the ecosystem scale (de Kroon *et al.* 2009).

The plastic responses of roots to external cues (Drew *et al.* 1973, de Kroon *et al.* 2009) appear to vary systematically across a gradient of MAT and bulk soil fertility. By quantifying root foraging dynamics across a natural ecological gradient, this study begins to link the nutrient conditions of tropical forest stands to MAT and soil fertility. We suggest that fine root nutrient foraging, as a direct indicator of individual plant nutritional status (Hodge 2004, Ruffel *et al.* 2011), may be applied as a proxy for nutrient limitation and resource conditions in natural forests stands.

Proximate and ultimate limiting conditions (*sensu* Vitousek *et al*. 2010) are important to distinguish in order to characterize ecosystem responses to abiotic change. Vitousek *et al.* (2010) define proximate and ultimate limiting nutrients as those which influence individual biological process and structure ecosystems, respectively. While this definition serves to contrast short versus long-term responses to additions of different macronutrients, it should also invoke the role of abiotic conditions in determining the availability of nutrients leading to limitation (Raich *et al.* 1997, Vitousek and Farrington 1997, Vitousek *et al.* 2010). By using an environmental gradient where environmental factors other than MAT were constrained, we were able to show the relationship between the bioavailability of an ultimate limiting nutrient, N, and an ultimate limiting condition, MAT (Pierre *et al.* 2017). Our results suggest that N availability due to rising MAT has consequences for fine root foraging behavior. Decreasing fine root foraging for +N+P fertilized ingrowth cores with increasing MAT may indicate how tropical montane wet forests prioritize nutrient acquisition as N becomes less limiting. Unless at least one environmental condition, such as MAT, is highly constrained, plot fertilization studies

may confound the roles of first-order variables such as temperature, pH, substrate age, and precipitation which influence nutrient limitation of forest growth (Vitousek 1984, Lambers *et al.* 2008, Reed *et al*. 2011).

While prior research has shown that temperature influences fine root growth and turnover (Pregitzer *et al.* 2000, Gill and Jackson 2000, Norby and Jackson 2000), and that NO_3^- bioavailability drives fine root proliferation and turnover (Pregitzer *et al.* 1993, Robinson *et al.* 1999), few studies have described their interactive influences (Vogt *et al.* 1995, BassiriRad *et al.* 1993, Leppälammi-Kujansuu *et al.* 2014 b). In our study, MAT and N bioavailability both increase with decreasing elevation, complicating the interpretation of root foraging responses to nutrient microsites. Moreover, C availability for fine root foraging in enriched microsites may increase with temperature due to concomitant increases in C cycling rates (Litton et al. 2011, Giardina *et al.* 2014). However, the results of our statistical model suggest that fine root foraging in +N+P treated microsites significantly decreased with increasing site-level NO_3^- bioavailability at low MAT (P=0.023, Figure 1). One interpretation of this result is that root response to enriched microsites is co-dependent on C availability and site fertility (Robinson *et al.* 2001); in warmer, more productive climates, trees are better able to forage for limiting nutrients (Lynch and Ho 2005). If this interpretation is correct, it would imply that with increasing temperature and constant moisture availability, trees may be better able to overcome nutrient limitation with microsite root foraging as a result of overall increased productivity.

Mycorrhizal Colonization Across MAT

Through symbioses with mycorrhizal fungi, plants can compensate for nutrient limited conditions (Rillig 2004, Treseder 2004, Johnson 2010) at the cost of fixed C to support the association (Lynch and Ho 2005, Hodge *et al.* 2010). The degree of symbiosis with arbuscular mycorrhizal fungi (AMF) is a straightforward proxy for the degree of plant P limitation, though it can also reflect plant productivity (Hawkes *et al.* 2008, Fellbaum *et al.* 2012). We observed increasing root colonization by AMF with increasing MAT (Fig. 2), which may reflect an increase in P limitation at higher N bioavailability at warmer sites (Pierre *et al.* 2017). Arbuscular mycorrhizae generally enhance P acquisition (Rillig 2004, Lambers *et al.* 2008), and provide an advantage to plants in high-NO_3^- environments where P demand is typically higher (Egerton-Warburton and Allen 2000, Bradley *et al.* 2006). Increasing AMF colonization with MAT suggests that MAT impacts the ecological stoichiometry of this tropical montane wet forest by proportionally changing N bioavailability relative to P bioavailability. While we did not measure soil bioavailable P, we can infer from increasing percent AMF colonization that C allocation to mycorrhizae is increasingly prioritized with MAT, as plants can invest between 4 and 20 % of total C budget in the AMF mutualism (Eissenstat *et al.* 1993, Peng *et al.* 1993, Watkins *et al.* 1996).

AMF colonization did not respond to any of the fertilized microsite treatments, suggesting that the AMF mutualism across the gradient is driven by an interaction between productivity and bulk soil fertility, rather than the availability of nutrient-enriched soil microsites (Treseder and Allen 2002). Plants may be able to supply more carbohydrates to support their AM symbionts at higher MAT, diverting C that

would otherwise go to the root apoplast (Fitter 2006). Our results show that AMF symbioses are more responsive to MAT than to nutrient-rich microsites, which is in contrast with our observation of decreasing fine root +N+P microsite foraging with increasing MAT. Taken together, the opposing patterns of fine root microsite foraging and fine root AMF colonization with MAT suggest that fine roots forage in nutrient-rich microsites under N-limited conditions at a lower C cost to the plant, while AMF are more abundant and forage more broadly under N-rich conditions at a greater C cost to plants (Johnson *et al.* 1997). Increasing primary production with MAT, coupled with increasing soil N bioavailability in high-moisture forests, may favor increased AMF colonization to maintain N and P co-limitation.

Implications for Ecosystem Biogeochemistry

Across these tropical montane wet forest plots, a significant root foraging response to N and P enriched microsites suggests that fine root foraging is most strongly influenced by the availability of both N and P. Fine roots apparently forage more actively for patches of available N and P when background nutrient availability is low under cooler climatic conditions. The effect of background N fertility of a site appears to be temperature-dependent in the absence of other ecosystem variation, suggesting that fine root foraging activity depends on the interaction between temperature and N bioavailability. Our results suggest that increasing AM fungal colonization with MAT may be related to previously observed increasing primary production with MAT across this gradient (Giardina *et al.* 2014). Increasing primary production with warming may interact with temperature-driven N availability (Pierre *et al.* 2017) to affect the C cost of mycorrhizal associations (Treseder and Allen 2002).

We hypothesize that more productive lowland tropical plant communities under warmer climatic conditions increase AMF colonization for nutrient acquisition. Further study of the dynamics of total fine root biomass and turnover across gradients of MAT, paired with whole stand fertilization experiments and N availability analyses will help to determine how influential these conditions are to fine root contributions to soil C. These results add to the growing body of research demonstrating the interconnections among increasing MAT, soil nutrient availability, and plant C allocation strategies (Johnson *et al.* 2010, Ostonen *et al.* 2011, Reich *et al.* 2014).

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CHAPTER 3

NITROGEN AND PHOSPHORUS COLIMTATION OF MICROBIAL NITROGEN CYCLING IN NORTHERN HARDWOOD FORESTS

Introduction

Nitrogen (N) and phosphorus (P) are the major elements that limit primary production in most terrestrial environments (Harpole et al. 2011, Reed et al. 2011, Treseder and Vitousek 2001). The availability of these elements for primary production is mediated by soil microbial communities which mobilize N and P using endogenous and exogenous enzymes for energetic processes (Schimel and Weintraub 2003, Allison and Vitousek 2005). When N and P are in balanced supply, their concomitant availability can have positive, synergistic effects on plant growth (Harpole et al. 2011, Fisk et al. 2014, Fay et al. 2015).

The multiple element limitation (MEL) hypothesis anticipates that the growth response of vegetation to the addition of multiple limiting nutrients will be greater than for the addition of individual nutrients (Bloom, Chapin and Mooney 1985, Rastetter et al. 1997). This hypothesis has been tested in forest ecosystems globally, with results showing that ecological context, including litter chemistry and climactic conditions, influence the direction of N and P availability effects on primary production (Ågren et al. 2012, Marklein and Houlton 2012). To overcome limitation by N and P, plants in a wide range of ecosystems have symbioses with microbial communities, such as mycorrhizal fungi and symbiotic N fixers, which can liberate physically and chemically inaccessible N and P (Lambers et al. 2009, Dijkstra et al. 2010, Johnson et al. 2015). Communities of free-living microorganisms, such as

nitrifiers, may also influence the degree of N and P colimitation via their assimilatory and dissimilatory metabolic processes (Lage et al. 2010, Norman and Barrett 2014). In particular, N and P amendments have led to increased rates of N cycling processes either directly, through increased N substrate availability, or indirectly, through an overall increase in ecosystem productivity (Hallin et al. 2009, Ying et al. 2017, Isobe et al. 2018).

The key microbial processes that mediate forest nitrogen limitation are N mineralization and nitrification. Nitrogen mineralization involves the depolymerization of high molecular weight organic polymers (e.g. protein, nucleic acids), derived from litter and soil organic matter (SOM), by extracellular enzymes from saprotrophic microorganisms and soil fauna (Schimel and Bennett 2004). The resulting low molecular weight organic monomers can be assimilated by some plants and microorganisms, or further converted to ammonium (NH_4^+), which enters more readily into solution and is more easily accessible to most plants (Schimel and Bennett 2002, Averill and Finzi 2011). The gross rate of N mineralization represents the total conversion of organic monomers to NH_4^+, while the net N mineralization rate represents the total NH_4^+-N released to the bulk soil after the microbial demand for N (i.e. immobilization in microbial biomass) is met. Because plants in N-poor ecosystems are capable of taking up organic N monomers, mineralization becomes increasingly important with increasing overall soil N availability (Averill and Finzi 2011). Ecosystem N availability (i.e. N concentrations in organic and inorganic forms) also depends upon environmental conditions, such as temperature (Rustad et al. 2001) and soil water content (Paul et al. 2003). Leaf litter chemistry and soil organic matter

content also influence the baseline substrate availability for N mineralization and interact with abiotic conditions to determine the importance of N mineralization relative to other N cycle processes for forest nutrient limitation (Schimel and Bennett 2004, Barrett and Burke 2000, Zechmeister-Boltenstern et al. 2015).

Nitrification is the conversion of ammonia (NH_3) to NO_3^-, a highly mobile bioavailable form of N that is readily leached into groundwater and/or converted into gaseous forms of N, including nitrous oxide (N_2O) or dinitrogen (N_2) (Boring et al. 1988). In nitrification, NH_3 is oxidized to nitrite (NO_2^-) and subsequently to nitrate (NO_3^-) by nitrifying bacteria and archaea (Prosser and Nicol 2008). Assays that measure gross nitrification rates quantify the overall conversion of NH_4^+ to NO_3^-, while net nitrification assays quantify the total extractable remaining in soil after NO_3^- immobilization in microbial biomass (Schimel and Bennett 2004). Neither of these measurements directly quantifies the intermediate steps of nitrification. Assays for gross and net nitrification, as with N mineralization, provide estimates of potential rates in the absence of roots, and are therefore useful as indices of N turnover and availability in soils, but are less useful for calculating ecosystem N budgets (Schimel and Bennett 2004).

Soil microbial metabolism, community composition, and community function interact with multiple element nutrient limitation at both micro and ecosystem scales. For example, Camenzid et al. (2018) showed in a review of tropical forest nutrient addition experiments that microbial abundances and N-cycling processes in soils respond primarily to P addition, with the effect size dependent on forest elevation. However, the ecosystem scale at which this meta-analysis was conducted precluded a

more specific understanding of which N-cycling processes or microbial functional groups respond to fertilization. In a laboratory experiment, Fanin *et al.* (2013) similarly showed sensitivity of microbial biomass stoichiometry and community composition to litter (*i.e.* substrate) P stoichiometry. This and other studies reveal a potential litter-to-microbe pathway by which forest nutrient co-limitation may be microbially mediated (Hofmann *et al*. 2016, Tatariw *et al*. 2018). While some of the biogeochemical mechanisms linking microbial communities and ecosystem stoichiometry have been identified, a process-based understanding of this relationship is lacking, despite the large number of stand-scale multiple nutrient addition studies to date (Vadeboncoeur 2010, Camenzid et al. 2014, 2018).

Foliar nutrient resorption is a primary strategy by which plants acclimate to long-term forest nutrient limitation (Diehl et al. 2003, Manzoni et al. 2010). Foliar resorption efficiency, the percent difference between nutrient content of green leaves before and after abscission, may indicate which nutrients are most limiting to a forest ecosystem, as plants tend to retain elements that are most scarce (Franklin and Ågren 2002, Vergutz et al. 2012, Han et al. 2013). Foliar resorption proficiency is the extent to which a plant reduces the nutrient concentration in senescing leaves (Killingbeck 1996). Resorption proficiency determines the chemistry of substrates that support N mineralization and nitrification in soil, as well as other biotically important variables such as soil pH (Satti et al. 2003, Manzoni et al. 2008). The indirect effects of changing soil nutrient availability, such as soil acidification and mineral weathering, may have stronger influences on nutrient-induced changes in soil microbial processes than the direct effects of nutrient additions (Ramirez et al. 2012).

Recent modeling work by Averill and Waring (2017) suggests a conceptual framework that centers on the role of pH and microbial physiology in determining the effect of N availability on ecosystem C cycling and storage. While their hypothesis mainly concerns the ultimate effects of N availability on soil mineral associated C, their efforts highlight a shortcoming of many nutrient co-limitation studies in forest ecosystems. That is, microbial physiology, a key determinant of microbial function in soils, responds to both direct and indirect effects of nutrient addition, and ultimately influences the resources available for plant growth (van der Heijden et al. 2008). On a coarse scale, processes such as nutrient immobilization in microbial biomass and nitrification mediate the effects of nutrient additions on actual nutrient limitation of plant growth (Zogg et al. 2000, Cotrufo et al. 2015).

This study investigated the long-term (i.e. seven year) effects of multiple element nutrient fertilization on litter chemistry, gross and net rates of soil N mineralization, nitrification, and immobilization. Our main objective was to test a conceptual model in which N and P fertilization effects on litterfall chemistry drive the availability of organic N substrates for mineralization, which in turn affects nitrification. In this framework, we expected that N enrichment leads to reduced foliar N resorption, higher litter N concentrations, and increased N availability for N mineralization and nitrification. Conversely, we expected that P enrichment would increase foliar N resorption, driven by the need to maintain constant N:P ratios during resorption (Gonzalez et al., unpublished data). This increase in N resorption would lead to reduced N in litterfall and lower rates of N mineralization and nitrification.

We expected that co-fertilization with +N+P would relieve limitation by both nutrients, leading to high N and P substrate availability for microbial processes. Specifically, we anticipated higher gross N immobilization in microbial biomass and moderate rates of gross nitrification in +N+P plots (Reed et al. 2012, See et al. 2015). We focused on the soil surface (0-10 cm depth) because we anticipated that this depth would elicit the most rapid response to nutrient additions and changes in litter chemistry. Overall, we expected that fertilization would produce similar responses in gross and net N fluxes; however, patterns in net rates would be less apparent, possibly due to N immobilization and plant uptake processes.

Methods

Study Site

This study was conducted in research plots located in mature (>100 year old) stands in the Jeffers Brook Forest (JBF, 44°02′N, 71°53′W), Bartlett Experimental Forest (BEF; 44°2–4′N, 71°9–19′W), and the Hubbard Brook Experimental Forest (HBEF; 43°56′N, 71°44′W), in the White Mountains of N.H., USA. At each of the five sites, there were four 50 x 50 m plots (plots included in this study: one at JBF, one at HBEF, three at BEF; Table 1). Plots were dominated by sugar maple (Acer saccharum Marsh.), American beech (Fagus grandifolia Ehrh.), and yellow birch (Betula alleghniensis Britton), with occasional white ash (Fraxinus americana L.), white birch (Betula papyrifera Marsh.), and red maple (Acer rubrum L.) (Goswami et al. 2018). Before treatment Jeffers Brook had the highest inherent (i.e. naturally occurring, without nutrient amendment) N mineralization rates, HB had mid-levels of N mineralization, and BEF had the lowest inherent N mineralization (Table 1, Bae et

al. 2015). At each site, each of the four permanent plots was randomly assigned to one of the four treatments: N addition, P addition, N+P addition or control. N was added annually as pelletized NH_4NO_3, P as powdered NaH_2PO_4, at a rate of 30 kg/ha of N and 10 kg/ha of P (Table 1; Fisk et al. 2014).

Site	Year Clearcut	Elevation (m.a.s.l.)	Aspect	Slope (%)	Inherent Fertility	Potential Net N Mineralization ($\mu g\ g^{-1}\ day^{-1}$)	Potential Net Nitrification ($\mu g\ g^{-1}\ day^{-1}$)
C7 (BEF)	1890	440	ENE	5-10	Low	10.16 (± 2.21)	−0.87 (± 0.32)
C8* (BEF)	1883	330	NE	5-35	Low	8.91 (±1.69)	−1.64 (± 0.51)
C9 (BEF)	1890	440	NE	10-35	Low	17.45 (± 3.21)	−0.46 (± 1.37)
HBEF*	1911	500	S	25-35	Mid	17.48 (± 2.70)	1.73 (± 0.58)
JBF*	1915	730	WNW	30-40	High	23.39 (± 3.17)	5.55 (± 1.65)

Note: Aspect codes are SE, southeast; NE, northeast; NNE, north-northeast; NNW, north-northwest; ENE, east-northeast; S, south; WNW, west-northwest

Table 3.1. Characteristics of five experimental sites located in central New Hampshire, USA. Potential net N mineralization and nitrification data, and site inherent fertility categories are from Bae *et al.* (2016). Asterisk (*) indicates sites for which only net N cycling processes were estimated in the present study.

Field Sampling

In June 2016, soil cores (5 cm x 20 cm) were taken from all treated and control plots (n=5 cores/plot x 4 plots/site x 5 sites=100 cores). Gross N cycling processes were only measured in soil samples from sites C7 and C9. The soil core was separated at 10cm depth, and the 0-10 cm depth portions were placed in zip-lock freezer bags and stored in coolers on ice in the field until they were moved to 4°C laboratory storage. All subsequent analyses were performed with soil samples from 0-10cm. Soils were homogenized by hand for removal of roots and rocks, and then sieved and subsampled for all analyses. Volumetric water content was calculated on a dry weight basis from one oven dried subsample from each homogenized soil core.

Microbial Biomass and Potential Net N Cycling

Microbial biomass C and N content were measured using the chloroform fumigation assay (Jenkinson and Powlson 1976, Brookes et al. 1985). Pre-incubation ammonium (NH_4^+) and NO_3^- concentrations were extracted for one 10 g (±0.05 g) subsample by adding 40mL of 2M potassium chloride (KCl), followed by shaking on an orbital shaker for 4 hours, and filtering through a Whatman #2 filter (GE Healthcare). A second set of subsamples (7.5 ±0.05 g) was exposed to chloroform ($CHCl_3$) under vacuum for 24 h and then inoculated with fresh soil (0.2 ±0.5 g) (*i.e.* not treated with $CHCl_3$). Unfumigated "control" subsamples (10 g) and fumigated soils were then placed in glass jars with lids fitted with septa to facilitate sampling of the jar headspace (10 mL). After a 10 day incubation at laboratory temperature (~20°C), headspace gas was sampled through the septa and stored in evacuated glass vials. Soils were then extracted for NH_4^+ and NO_3^- using 2M KCl as described above. The three soil extracts (initial, control, fumigated) for each soil core were analyzed colorometrically (Lachat QuikChem Automated Flow Injection Analysis System) (Lachat, Loveland, CO). Headspace gas was analyzed for CO_2 concentration using an infrared gas analyzer (Li-Cor 6250, Li-Cor, Lincoln, Nebraska, USA). Accumulated CO_2 in the 10 mL headspace of fumigated samples was assumed to be directly proportional to the microbial biomass C of the soil (corrected by a proportionality constant of 0.45). Accumulation of NH_4^+ over 10 days in the fumigated samples was assumed to be proportional to microbial biomass N in samples. The accumulation of NH_4^+ plus NO_3^- in the unfumigated control incubations was used to calculate potential

net N mineralization, and the accumulation of NO_3^- alone in the control incubations was used to estimate potential net nitrification.

Gross N Cycling

Gross rates of N mineralization and nitrification were estimated using an isotope pool dilution assay (Davidson et al. 1991) that quantifies the gross production of NH_4^+ and NO_3^- over a 10-day incubation period using a known quantity of a ^{15}N isotope label. First, a 10 g subsample was added to a 946 cm^3 glass jar. An enriched ^{15}N solution was delivered as a 1mL aliquot using a small (21 G) needle and was distributed evenly throughout the soil sample. The concentration of 99 atom % ^{15}N isotope label to be added to each sample was calculated based on the mean extractable NH_4^+ and NO_3^- concentrations in the 1-10cm depth under each treatment, averaged over 4 years (M. Fisk, unpublished data). This allowed us to achieve approximately 1-3 atom % enrichment of each soil sample. After a 24 h incubation at lab temperature (~20 °C), enriched soil samples were extracted with 40 mL 2 M K_2SO_4 solution. Extracted soil solutions were refrigerated (4°C) for approximately 2-3 weeks before being diffused according to the method of Hart *et al.* (1994). Complete diffusion of NH_4^+ and NO_3^- onto acidified filter paper discs (Whatman # 2) was assumed to be achieved after 10 days at lab temperature. Filter discs were harvested from samples and dried in closed dessicators with an open acid reservoir to trap ambient atmospheric NH_3 that could react with sample discs. Dried sample discs were submitted to the Cornell Stable Isotope Laboratory facility for isotope mass ratio analysis of $^{15}N/^{14}N$.

Data Analysis

This study used plots, which differed in fertilization treatment, as experimental units (n=20). Plots were grouped by site (n=5), which were geographically separate and comprised a gradient in soil fertility (Table 1). We determined differences in net N cycling rates in response to fertilization with linear mixed effects models in which site was treated as a random effect. Analyses were performed in R (R Core Team, 2016) using the lme4 package (Bates, Maechler & Bolker, 2012). Models were selected by comparing the Akaike's Information Criterion (AIC), and model significance was determined using the likelihood ratio test.

Soil core samples for net N cycling rate analyses were collected in groups of 5 per plot (n=100). Soil samples for microbial community analyses were collected in groups of 3 per plot (n=60). All net N cycling laboratory assays were performed in duplicate for each individual soil core, and duplicates were averaged. Net N cycling rates were averaged by fertilization treatment and secondarily grouped by inherent plot fertility (see Table 1). Potential net N cycling rates were determined for all five sites, with equal replication for all 20 plots.

Gross N cycling laboratory assays were performed in duplicate, as for the net rates, but data reported here are unevenly distributed among site and fertilization treatment, due to sample losses in processing. Therefore, evaluation of the responses of gross N cycling processes to fertilization treatments were limited to control, +N and +N +P plots at site C9 (C9C, C9N, C9NP) and +N and +P plots at site C7 (C7N, C7P). These five plot/treatment comparisons were evaluated for pairwise differences using the Tukey method in the ImerTest package in R (R Core Team, 2016). Differences in

gross N cycling rates between fertilized plots were determined by comparing the estimated marginal means using the emmeans package in R.

Results

Litter Chemistry

Long-term fertilization with N resulted in significantly elevated N concentrations in litterfall in fall 2017, while fertilization with P led to significantly elevated P concentrations in litterfall, relative to control plot litter. In particular, stands receiving N alone (+N; P=0.018) or combined N and P (+N+P; P=0.022) had elevated litter N concentrations relative to the control plot while stands receiving P, alone, had elevated P concentrations (P=4.78e-06, Figure 1b). These results support our hypothesis that high availability of N or P in soil would lead to higher concentrations of these nutrients in litterfall. Fertilization with P (with and without N) decreased the N:P ratio of litter compared to control and +N treatments (Figure 2) supporting our hypothesis that P additions would alter litter N:P stoichiometry, potentially due to increased foliar N resorption.

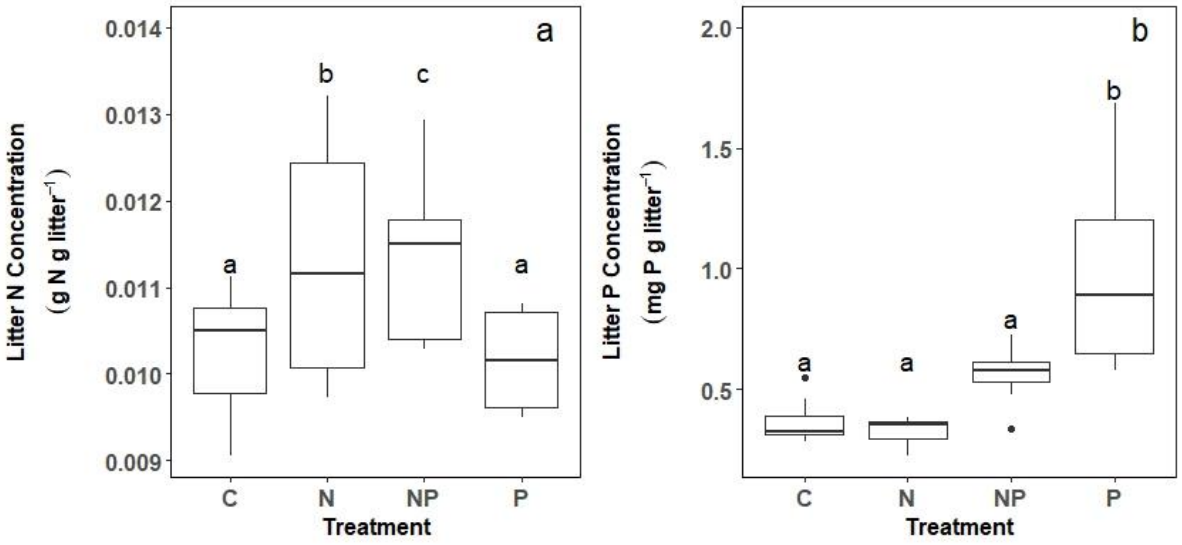

Figure 3.1. The concentration of phosphorus (P; mg P g litter-1; Figure 1a) and nitrogen (N; g N g litter-1; Figure 1b) in leaf litter in response to long-term stand fertilization in the Multiple Element Limitation in Northern Hardwood Experiment (MELNHE). Fertilization treatments are N (NH_4NO_3, 30 kg/ha), P (NaH_2PO_4,10 kg/ha), a combination of N and P (NP, rates as described), and an unfertilized control (C). Litter N concentration was analyzed for all 5 sites (C7, C8, C9, JBO, HBO), while litter P concentration was analyzed in all sites except C9. Data collection and analysis was conducted in October 2017 from 3 litter baskets per fertilized plot. Litter P concentration was highest in response to P fertilization relative to the control (P= 4.78e-06). Litter N concentration increased in N (P=0.018) and NP fertilization (P=0.022). All litter chemistry data were collected and analyzed by M. Fisk. Different letters indicate significant differences between treatments (P<0.05).

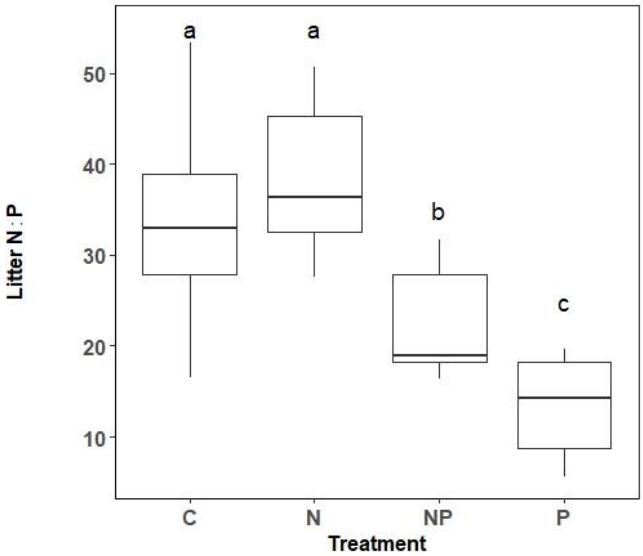

Figure 3.2. The ratio of nitrogen (N; g N g litter^{-1}) to phosphorus (P; mg P g litter^{-1}) to in leaf litter in response to long-term stand fertilization in the Multiple Element Limitation in Northern Hardwood Experiment (MELNHE). Different letters above boxes denote significantly different treatment responses (P<0.05). Data collection and analysis was conducted in August 2017 from 3 litter baskets per fertilized plot. Litter N:P was significantly lower in NP-fertilized plots than control plots

(*P*=0.0044), and was lowest in P-fertilized plots (*P*=2.19e-06). All litter chemistry data was collected and analyzed by M. Fisk.

Net N Cycling

Nitrogen fertilization (+N treatment) led to significantly higher KCl-extractable soil NH_4^+ and NO_3^-, while other treatments had no effect on KCl-extractable inorganic N (*P*= 0.038; Table 2). The +N treatment had lower potential net nitrification rates relative to the control plots (*P*= 0.0073; Table 2). "Low" inherent fertility plots had significantly lower potential net nitrification rates than "mid" and "high" inherent fertility plots (*P* =0.016, data not shown). Inherent fertility and plot fertilization did not have interactive effects on any net N cycling process tested. Microbial biomass N increased significantly in response to the +N+P treatment (*P*=0.0388) and increased marginally in response to the +P treatment (*P* =0.0739) relative to the control plot. Fertilization treatments (+N, +P, +N+P) had no effect on potential net N mineralization or microbial biomass C content (Table 2).

Gross N Cycling

Gross N mineralization differed among fertilized plots within sites C7 and C9. Gross N mineralization rates in plots C9N, C7P, and C9NP were significantly lower than the control (C9C) and C7N (*P* <0.05), but were not different from one another (*P*<0.05; Figure 3a). Gross N mineralization in the N-fertilized plot at site C7 (C7N) was not significantly different from the control (*P*= 0.95). Gross nitrification in the C9NP plot was significantly lower than the N fertilized plots (C7N, C9N; *P*<0.01). Gross nitrification in plot C7P as not significantly different from the control (*P*=0.97; Figure 3b).

Gross NH_4^+ immobilization showed a similar pattern to gross N mineralization. Gross NH_4^+ immobilization was significantly lower in plots C9N, C7P, and C9NP than in the C7N and control (C9C) plots ($P<0.01$; Figure 4a), but were not significantly different from one another ($P>0.05$). Gross NO_3^- immobilization was significantly lower in plots C7P and C9NP than in N-treated plots (C7N, C9N) ($P<0.01$; Figure 4b). Gross NO_3^- immobilization was lower in plot C9NP than the control ($P=0.022$). Gross NO_3^- immobilization did not differ among the N-treated plots (C7N, C9N).

Response Variable	**+N Treatment**	**+P Treatment**	**+N+P Treatment**	**Control**
Total Extractable Inorganic N (µg N g dry soil^{-1})	137.2 (±39.08) **(*P*=0.0029)**	64.25 (±21.22)	63.66 (±13.29)	37.45 (±6.29)
PN Nitrification (µg N g dry soil $^{-1}$ day^{-1})	-7.10 (±1.68) **(*P*=0.0073)**	-3.90 (±1.37)	-0.74 (±1.19)	-2.07 (±0.42)
PN N Mineralization (µg N g dry soil $^{-1}$ day^{-1})	33.50 (±2.52)	42.70 (±4.57)	44.60 (±5.77)	33.63 (±4.32)
Microbial Biomass C (µg C g soil^{-1})	2907.00 (±781.40)	5501.57 (±1478.00)	4319.01 (±1079.00)	2679.08 (±1109.00)
Microbial Biomass N (µg N g soil^{-1})	472.40 (±35.47)	490.90 (±47.94)	510.40 (±49.89) **(*P*=0.0388)**	373.70 (±45.47)

Table 3.2.: Mean (± one standard error of the mean) extractable inorganic N, potential net N mineralization and potential net nitrification in plots fertilized with N (30 kg ha $^{-1}$ NH_4NO_3), P (10 kg ha $^{-1}$ NaH_2PO_4), NP (combined N and P fertilization), and control (C) in June 2016. *P* values reported in bold font indicate significant differences between fertilized and control plots.

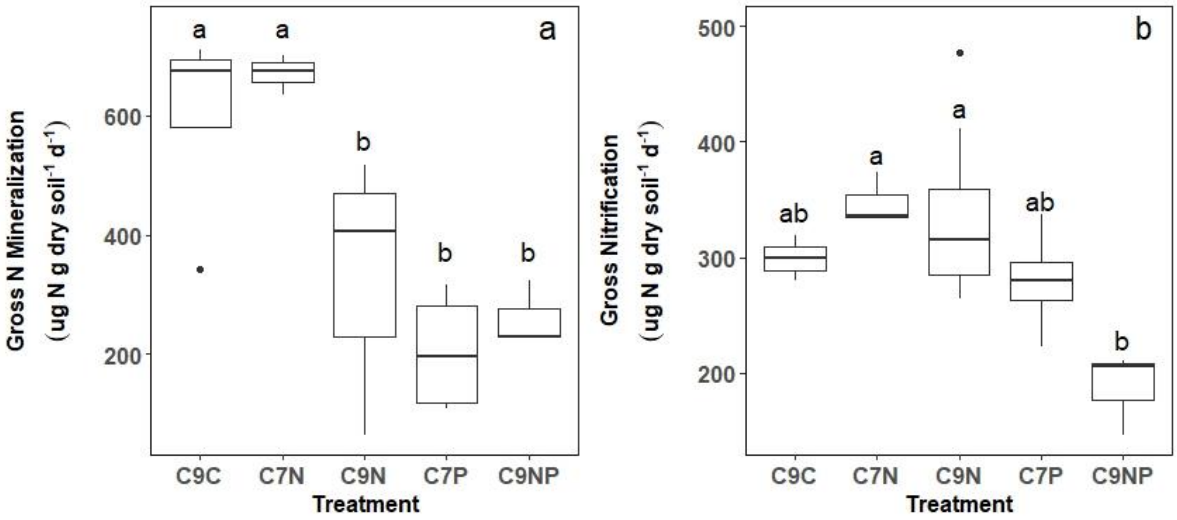

Figure 3.3. Gross N mineralization (3a; µg N g dry soil $^{-1}$ day $^{-1}$) and gross nitrification (3b; µg N g dry soil $^{-1}$ day $^{-1}$) in plots fertilized with +N (C7N, C9N; 30 kg ha $^{-1}$ NH_4NO_3), +P (C7P;10 kg ha $^{-1}$ NaH_2PO_4), +N+P (C9NP; combined N and P fertilization), and unfertilized control (C9C) in June 2016. Sample size is *n*=5-10 cores per treatment, different letters above boxes denote significant differences between treatments (P<0.05).

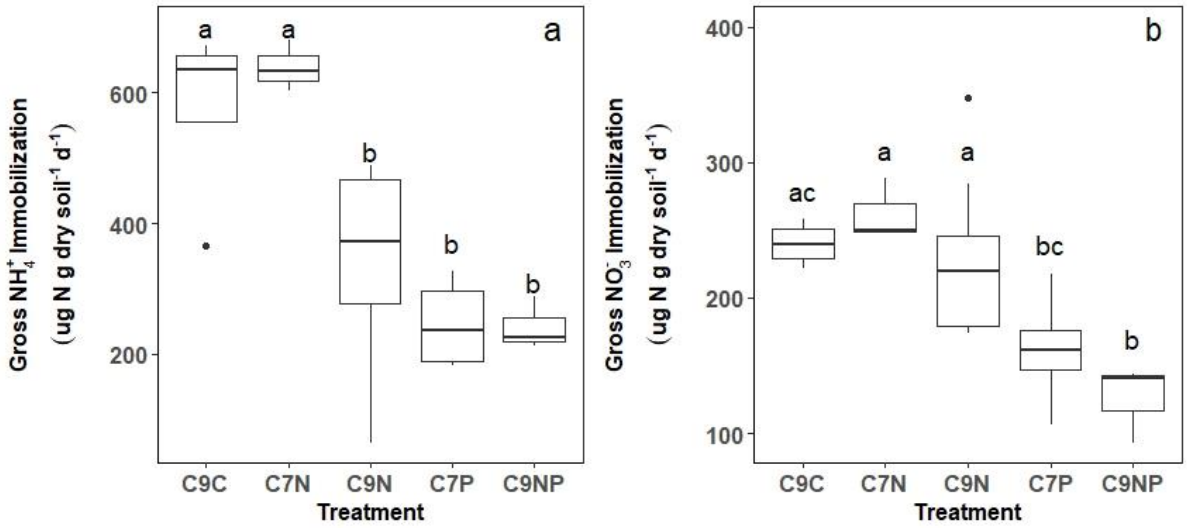

Figure 3.4.: Gross ammonium (NH_4^+; a) and nitrate (NO_3^- ; b) immobilization rates (µg N g dry soil $^{-1}$ day $^{-1}$) in plots fertilized with +N (C7N, C9N; 30 kg ha $^{-1}$ NH_4NO_3), +P (C7P;10 kg ha $^{-1}$ NaH_2PO_4), +N+P (C9NP; combined N and P fertilization), and unfertilized control (C9C) in June 2016. Sample size is *n*=5-10 cores per treatment, different letters above boxes denote significantly differences between treatments (P<0.05).

Discussion

Results suggest that the addition of P, either alone or in combination with N, may suppress gross N mineralization (Figure 3a), gross NH_4^+ immobilization, gross nitrification, and gross NO_3^- immobilization (Figure 4) to varying degrees in soils of these northern hardwood forests. This observation is consistent with past data from these experimental forests which show that greater availability of P relative to N in soils reduces the concentration of N in litterfall (See *et al.* 2015). This may be the driving factor behind the lower gross N mineralization and immobilization rates, possibly due to increased foliar N retranslocation (M Fisk, unpublished data); notably, leaf N:P ratio was greatly reduced in the P addition plots (Figure 1), potentially signaling an imbalance in nutrient supply that suppressed microbial activity. These results support the notion that foliar nutrient resorption efficiency and proficiency may change in response to variation in soil N:P to optimize plant tissue N:P by retaining greater amounts of the most limiting element (Treseder and Vitousek 2001, Enoki and Kawaguchi 2002, See *et al*. 2015). Thus, N and P additions appear to affect rates of N cycling in soil through a complex interaction between internal plant processes, litterfall and microbial process rates (Hobbie 2015, Zechmeister-Boltenstern *et al.* 2015).

Our results concur with those of other studies which show that in N-limited forest ecosystems, foliar resorption is a key mechanism for N retention which may keep more N in organic, rather than mineralized, forms (Laughlin *et al.* 2015, See *et al*. 2015). Below, we discuss how belowground microbial N cycling may interact with aboveground patterns of foliar nutrient resorption under different conditions of

nutrient availability, and how nitrifying microbes in forest soils may be substrate-limited by foliar N resorption under conditions of low soil N:P.

N Mineralization

Lower gross N mineralization rates in plots amended with P (C9P, C7NP) compared to the +N treatment in plot C7N and the control (C9C) (Figure 3a) is consistent with the idea that foliar N and P resorption efficiencies function bidirectionally, depending the level of limitation imposed by N or P (Kozovits *et al.* 2007, Reed *et al.* 2012). Elevated soil N availability in plot C7N led to higher litter N:P, while elevated P availability in plots C9P and C7NP reduced litter N:P (Figure 2). We suggest that the difference in gross N mineralization rates and litter N:P indicates the influence of soil nutrient availability on foliar nutrient resorption, which ultimately affects N substrate availability for microbial N cycling.

A variety of forest N and P fertilization studies have shown high litterfall N and P concentrations following short and long-term +N+P treatments and indicate that litter decomposition and gross N mineralization rates are high when N and P are both available (Jacobson *et al.* 2011, Yuan and Chen 2015). Yet in northern hardwood and conifer forests, fertilization with both N and P can lead to no change or reduced gross N mineralization (Fisk and Fahey 2001), as we observed in the present study. Low gross N mineralization rates under +N+P fertilization may result from changes in microbial C-use efficiency in decomposition. As reduced soil respiration is often observed under elevated soil N (Fisk and Fahey 2001, Bowden *et al.* 2004, Bae *et al.* 2015), we suggest that microbial interactions with litter nutrient C:N:P stoichiometry

may influence the rate of N mineralization in soils (Vitousek *et al*. 1984, McGroddy *et al.* 2004, Bengtsson *et al.* 2003).

We found that microbial biomass N concentrations significantly increased in response to the +N+P treatment (Table 2, $P = 0.0388$), and observed a potential (non-significant) increase in the +P treatment ($P=0.0739$). Since we observed reduced gross N cycling and N immobilization rates under +N+P and +P treatments, increased microbial biomass N concentrations in these treatments may indicate an increase in the residence time of microbial biomass N where P is more abundant. Previous studies have shown that P fertilization in mature temperate forests leads to increased total microbial biomass and increased microbial biomass N (Scheu 1990, Gallardo and Schlesinger 1994). This may be due to P interactions with sorption sites on mineral surfaces, which may release sorped labile C in exchange for P, thereby liberating labile C for microbial metabolism (Mori *et al.* 2018). Higher N retention in biomass (*i.e.* with no significant increase in immobilization rate) due to greater soil C availability could explain the observed increase in microbial biomass N concentration in +N+P and +P fertilized plots. Additionally, soil microbial biomass P and N are correlated globally (Xu *et al.* 2012), which may explain why increased P availability may have reduced N immobilization rates (Figure 4) and N turnover in microbial biomass, resulting in high microbial biomass N concentrations.

Contrary to our expectation, +P and +N+P treatments in plots C9P and C7NP, respectively, did not significantly reduce gross N mineralization compared to the +N treatment in plot C9N. This observation is somewhat challenging to interpret: data shown in Figure 2 support our hypothesis that +N treated plots would exhibit higher

litter N:P than plots fertilized with +N+P or +P, as the N:P of litter declined significantly between +N, +N+P and +P treatments. However, +P and +N+P treatments significantly reduced gross N mineralization compared to the +N treatment in plot C7N (Figure 3a). The difference in the effect of +N fertilization on gross mineralization between plots C7N and C9N appears to be important for interpreting the effect of P amendment. Plots C7N and C9N differ significantly in their inherent (*i.e.* pre-treatment) N availability. Inherent net N mineralization rates were lower in site C7 than in site C9 (Table 1), which suggests that microbial demand for N is inherently lower in site C9. Fertilization of a more N-rich site (*i.e.* C9) likely shifts microbial N consumption to the readily available N fertilizer, leading to reduced gross mineralization of organic N. This proposed relationship between inherent fertility, N amendment and microbial N demand may explain the significantly higher rates of gross N mineralization in plot C7N relative to C9N, and allows for two different interpretations of the effect of N amendment in plot C7N and C9N relative to P amendment in plots C7P and C9NP. Moreover, the difference in N fertilization response between sites C7 and C9 highlights the importance of spatial heterogeneity in soil fertility, even in relatively similar and proximate forest ecosystems such as those at BEF.

In a study of unmanipulated plots within the same forests studied here, See *et al.* (2015) showed that stands with relatively high soil inorganic N stocks exhibited higher rates of foliar P resorption efficiency and proficiency, and this pattern is reflected in other terrestrial ecosystems (Kozovits *et al*. 2007, Reed *et al.* 2007). Similarly, Hofmann *et al*. (2016) showed that P resorption was greatest in forest stands

with low P availability, and lowest where P was highly available. Drivers of soil fertility, including soil age, mean annual temperature, and mean annual precipitation, drive global patterns in foliar nutrient resorption efficiency and proficiency (Vitousek *et al.* Van Heerwaarden *et al.* 2003). Across terrestrial ecosystems, high soil N:P, such as in highly weathered tropical forest soils, is associated with higher P resorption efficiency than in forest soils where N:P is lower (McGroddy *et al.* 2005, Reed *et al.* 2012, Zotz 2004).

Global variation in foliar N and P resorption efficiency and proficiency in relation to soil nutrient availability contributes to ecosystem differences in litter decomposition rates via effects on litter C:N and C:P, where the highest decomposition rate constants are observed at the lowest litter C:N and C:P (Manzoni *et al.* 2010, Van Heerwaarden *et al.* 2003). Evidence suggests parameters of soil microbial community composition, such as fungal to bacterial ratio, ratio of gram-negative to gram-positive bacteria, and the composition of extracellular enzymes produced, are sensitive to litter stoichiometry, but may also modify initial litter chemistry and microbial community composition to make subsequent microbial transformations more efficient (Bray *et al.* 2012, Kaiser 2014, Knelman *et al.* 2014). Substrate limitation of N mineralization is therefore linked to litter C:N and microbial decomposer assemblage traits, such as C-use efficiency and total enzyme activity, at local and global scales (Parton et al. 2007, Sinsabaugh *et al.* 2008, Kang *et al*. 2015).

Nitrification

We found that gross nitrification rates in +N+P and +P fertilized plots (C7P, C9NP) were significantly lower than in +N fertilized (C7N, C9N) and control plots

(Figure 3b). These results differed from our expectations, as well as with past studies that observed increased nitrification in response to N amendment and have explained this as a direct response to the increased NH_4^+ availability (Lu *et al.* 2011). Our results suggest that increasing soil P availability may suppress gross nitrification, possibly by reducing litter N:P (Figure 2) through internal plant N resorption. Consistent with our conclusion that gross N mineralization is suppressed by elevated soil P and reduced litter N concentrations, reduced gross nitrification suggests that P limitation of N mineralization may impose a bottleneck on subsequent N-cycling processes (Zhang *et al.* 2012). Gross nitrification rates did not respond to the +N treatment (Figure 3b), and N addition led to a small decrease in net nitrification (Table 2). This result is consistent with observed suppressive effects of high soil N concentrations and resulting low soil pH on litter decomposition (Knorr *et al.* 2005). We observed that elevated mineral N supply significantly suppressed net nitrification (Table 2), while the suppressive effects of P availability are only apparent in gross N transformations due to the dominance of N immobilization (Figure 4) relative to net N turnover (Table 2) in these stands.

In a study of measured and modeled N cycling process responses to fertilizer amendments, Zhang *et al.* (2012) showed that gross N mineralization and nitrification rates increased under elevated soil P concentrations. They and others suggested that this may indicate microbial biomass P limitation that is alleviated by decreased soil N:P (Saggar *et al*. 2000, Zhang *et al.* 2012). Evidence from Parfitt *et al*. (2005) also shows that increasing soil P availability is reflected by decreased microbial biomass N:P, and is strongly correlated with increasing soil N mineralization rate. Our

interpretation of reduced N cycling rates with elevated P in this study stands in contrast with these and other observations of positive effects of elevated P on N cycling, suggesting that complex mechanisms related to the extent of pre-fertilization ecosystem N and P limitation, N and P cycling within soil food webs (Parfitt *et al.* 2005), duration of fertilization treatment, and other site-specific conditions, may influence the direction of N cycling response to N and P fertilization.

Litter N and P responses to N and P fertilization in this study support the notion that foliar nutrient resorption may be an influential controller of hydrologic and gaseous N losses from soils (Asner *et al*. 2001, Sardans *et al.* 2016, Crowley and Lovett 2017). Our results indicate that net nitrification may be reduced under conditions of high N deposition and consequently high litter N:P (Figure 2), compared to conditions where N and P are equally available (Table 2), possibly curtailing N leaching and gas flux. However, because the response of gross NO_3^- immobilization to +N treatment was not significant, we cannot strongly infer whether or not net export could be a significant fate of NO_3^- in this system. Overall, these outcomes suggest that equivalent colimitation by N and P may reduce the importance of NO_3^- as a bioavailable form of N in northern hardwood forest systems by limiting gross N mineralization and stimulating gross NH_4^+ immobilization. We suggest that high P availability and low litter N:P may bring about N and P co-limitation of nitrification which may also influence ecosystem N retention.

Conclusion

We offer a novel perspective on the interaction between soil nutrient limitation and microbial N cycling. Internal plant nutrient cycling, including foliar N and P resorption as a means of recovering N or P when their availabilities are low, appears to be a significant mechanism by which microbial access to organic N may be limited. While past research has primarily focused on the direct response of microbial N cycling to elevated labile N and P in soils, we have taken advantage of a long-term fertilization experiment in mature hardwood forest stands. By studying the long-term response of forest stands to N, P and a combination of both, we were likely able to see adjustments in litterfall N:P and consequent effects on soils, that may not be apparent in shorter-term treatments. Our results suggest that the relationship between litter chemistry, microbial N mineralization and nitrification is linked to the nutrient requirements of plants, which may be plastic throughout the plant lifespan (Reich *et al*. 1995). Stands in this study also varied in their inherent fertility, which proved to differentially drive nitrogen cycling responses to N fertilization. We recommend that future studies give attention to the effects of N and P fertilization on litter chemistry. By focusing on nutrient resorption efficiency and proficiency as potential drivers of microbial substrate availability, future studies may reveal how microbial biomass stoichiometry in ecosystems with different inherent N and P availability influences soil organic matter stabilization (Cotrufo *et al.* 2013) and forest N retention (Mooshamer *et al.* 2014). Future questions following this work include how microbial functional groups respond to limitation-driven changes in litter chemistry, and how

patterns in internal nutrient cycling, microbial substrate availability, and long-term soil organic matter stabilization may be related across stand age.

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