1 **Title:**

2 Substrate availability drives spatial patterns in richness of ammonia-oxidizing bacteria
3 and archaea in temperate forest soils

4 **Author names and affiliations:**

5 J.S. Norman^{a,b} and J.E. Barrett^a

6 ^a Department of Biological Sciences, Virginia Tech

7 (MC 0406)

8 Derring Hall Room 2125

9 1405 Perry Street

10 Blacksburg, VA 24061

11 ^b Current Address: Department of Plant Biology, Michigan State University

12 612 Wilson Road

13 East Lansing, MI 48824

14 **Author email addresses:**

15 J.S. Norman – jsnorman7@gmail.com

16 J.E. Barrett – jebarre@vt.edu

17 **Corresponding Author:**

18 J.S. Norman

19 Telephone number: (517) 355-4683

20 Fax number: (517) 353-1926

21

Abstract

We sought to investigate the drivers of richness of ammonia-oxidizing bacteria (AOB) and archaea (AOA) in temperate forest soils. We sampled soils across four experimental watersheds in the Coweeta Hydrologic Laboratory, North Carolina USA. These watersheds are geographically close, but vary in soil chemistry due to differences in land use history. While we found a positive relationship between soil pH and AOB richness in the soils we sampled, we provide evidence that this relationship is driven by the effects of soil pH on the availability of NH_3 , which is the substrate that is directly oxidized by AOB. Conversely, AOA richness responded to NH_4^+ , which these organisms may access directly from the environment. Our results provide evidence that substrate availability may be a dominant driver of both AOA and AOB richness at local scales in forest soils.

Main Text

We sought to investigate the drivers of taxonomic richness in communities of forest soil ammonia-oxidizing archaea (AOA) and bacteria (AOB), at the Coweeta Hydrologic Laboratory, an NSF-sponsored Long Term Ecological Research (LTER) site located in North Carolina, USA. By investigating the differential drivers of AOA and AOB richness, we hope to gain insight into the factors that regulate group level activity of these biogeochemically-relevant organisms, which control the rate-limiting step of nitrification, in temperate forest soils. Coweeta has a history of land-use manipulations resulting in variations in soil chemistry and nitrogen export among watersheds (Swank and Vose 1997). Ecological theory developed in plant systems (Tilman 1982) suggests

that there should be a unimodal (i.e., hump-shaped curve) relationship between the availability of a critical resource and taxonomic richness, with a peak in richness occurring at moderate resource levels (Figure 1). The mechanism behind this phenomenon is as follows: low resource environments select for a subset of species that can survive oligotrophic conditions, while high resource environments encourage interspecific competition, thereby selecting for the subset of species that are most competitive for the given resource; the peak in richness at moderate resource levels simply results from a reduction of these forces that operate in extreme resource environments (Tilman 1982). Based on this model, we predicted that the richness of ammonia-oxidizing microbes would be primarily controlled by the availability of ammonia, the substrate that fuels chemoautotrophic growth by AOA and AOB.

Our past work in this system showed that AOB production was enhanced by the addition of NH_4Cl (Norman and Barrett 2014), indicating that Coweeta soils may be considered to have low substrate availability for AOB. We therefore expected to see either a positive or saturating relationship between substrate availability and AOB richness over the range of soil conditions encountered at Coweeta. In contrast, physiological differences enable AOA to thrive at lower substrate availability than those favored by AOB (Martens-Habbena et al. 2009, Gorman-Lewis et al. 2014) and we found no evidence that AOA were substrate-limited in this system (Norman and Barrett 2014), indicating that Coweeta soils may be considered as having high substrate availability for AOA. We therefore expected to find a negative relationship between AOA richness and substrate availability in Coweeta soils.

To investigate the drivers of AOA and AOB richness in temperate forest soils at Coweeta LTER, we located three sampling sites along stream-to-hillslope transects in four experimental watersheds that vary in their nitrogen export and collected ~ 1 kg of soil from the top 5 cm of soil at each site. Once collected, soils were passed through a 2mm sieve to remove small rocks and fine roots, homogenized, and then three replicate subsamples were stored at 4°C until further analysis. Soil variables including moisture/g dry weight (dw), soil pH, NH_4^+ -N/g dw, NO_3^- -N/g dw, %C, %N, were measured as previously described (Norman and Barrett 2014).

AOA and AOB richness were assessed at each site by targeted pyrosequencing of group-specific ammonia monooxygenase subunit A (*amoA*) genes. We extracted DNA from replicate subsamples using PowerSoil® DNA isolation kits (MO BIO laboratories Inc., CA, USA), pooled extracts from each site, and sent pooled extracts to Molecular Research LP (MR DNA, Shallowater, TX, USA) for unidirectional amplicon-based sequencing of group-specific *amoA* genes using primer sets *amoA-IF** (Stephen et al. 1998) and *amoA-2R* (Rotthauwe et al. 1997) for AOB, and *Arch-amoA-IF** and *Arch-amoA-2R* for AOA (Francis *et al.* 2005) (*Indicates sequencing primer). These sequences are available in NCBI's Sequence Read Archive (study accession number SRP046366).

We filtered sequences and estimated richness using MOTHUR (Schloss et al. 2009), following the Schloss lab 454 SOP (Schloss et al. 2011). Operational taxonomic units (OTU) were created with a 97% sequence similarity cutoff, as used in a similar analysis by Hu et al. (2013). AOA and AOB richness was estimated by repeated subsampling to 1243 sequences (based on the lowest number of sequences remaining at a

single site across both AOA and AOB datasets) using the “summary.single” command in MOTHUR. This analyses procedure excluded AOB data from one site, which only contained 64 sequences after initial processing. AOA data was also unavailable from another site due to lack of PCR amplification for sequencing. Though we had 12 sampling sites initially, we therefore only have richness data for 11 sites for each group.

We used the “Get.oturep” command in MOTHUR to obtain a representative sequence for each OTU in the dataset and screened these sequences against sequences cultured isolates in the non-redundant protein database using blast (NCBI) to insure sequence specificity. All representative sequences matched bacterial and archaeal *amoA* genes as expected. The ten most abundant AOB OTUs, which contained ~88% of the processed sequences, most-closely resembled sequences from representatives of the genus *Nitrosospira*. The three most abundant AOA OTUs, which contained 83% of the processed sequences, were most-closely related to *Nitrosotalea devanaterra*, an acidophilic AOA isolate (Lehtovirta-Morley et al. 2011), though the representative sequences from each OTU only shared ~87% sequence similarity with the *amoA* gene of this organism (GenBank accession number JN227489.1).

We found a strong relationship between soil pH and AOB richness in the soils we sampled (Figure 2b), but we propose that resource availability, rather than the physiological effects of high proton concentrations drive this relationship in the following manner: environmental pH strongly affects the ionization of $\text{NH}_4^+/\text{NH}_3$, thereby affecting the availability of NH_3 as described by equation 1 (a modification of the Henderson-Hasselbach equation that assumes a pKa value of 9.25 for the ionization of $\text{NH}_4^+/\text{NH}_3$):

113 (1) $[\text{NH}_3] = [\text{NH}_4^+](10^{(\text{soil pH} - 9.25)})$

114 Since NH_3 , rather than NH_4^+ is thought to be the actual substrate oxidized by AOB
115 (Suzuki et al. 1974), we suspect that the strong relationship between soil pH and AOB
116 richness could actually be explained by the effects of soil pH on the availability of this
117 important resource. We used soil pH and NH_4^+ concentrations to estimate NH_3 levels in
118 the soils we sampled by equation 1, and found a positive saturating relationship between
119 calculated NH_3 concentration and AOB richness consistent with the predicted increase
120 and peak in AOB richness expected at low to moderate resource availability (Figure 2d).

121 If soil pH drives AOB richness through resource availability as we suggest here,
122 we would expect to see an eventual decline in AOB richness along a pH gradient due to
123 interspecific competition for NH_3 if we had encountered higher pH (and therefore higher
124 NH_3) soils in this study. In seeming contrast to this prediction, Hu et al. (2013) found a
125 positive relationship between soil pH and AOB richness when sampling soils that ranged
126 in pH from less than 4 to greater than 8. However, these differing patterns could be
127 explained by differences in sampling depth of soils used in these two studies: while we
128 sampled from the top 5 cm of soil, Hu et al. 2013 sampled from the top 20 cm of soil,
129 thereby covering a much larger oxygen availability gradient in each sample than we did.
130 In high pH soils, NH_3 limitation should be alleviated for AOB, allowing richness to be
131 governed by other limiting resources. If oxygen availability controls AOB richness in
132 high pH soils, then Hu et al. (2013) may have inadvertently sampled an entire resource
133 availability gradient in high pH soils by homogenizing soils from the top 20 cm, resulting
134 in a higher richness of AOB in high pH soils than expected.

While other studies (Hu et al. 2013, Stempfhuber et al. 2015) have reported positive linear or saturating relationships between soil pH and AOA richness in soils, we did not find a significant relationship between AOA richness and soil pH in the soils we sampled (Figure 2a). This could be due to the fact that the soils present at Coweeta do not span a large enough pH gradient to see the effect of pH on AOA richness (e.g. ~1.5 pH units). We did not find a relationship between calculated NH_3 concentration and AOA richness either (Figure 2e). However, we did find a significant unimodal relationship between NH_4^+ and AOA richness in Coweeta soils with a peak in richness at ~14 $\mu\text{g N-NH}_4/\text{g dw soil}$ (Figure 2c) suggesting that NH_4^+ may be the resource that controls AOA richness in this system through the mechanisms outlined in Figure 1. Future work should investigate this relationship further as our sampling design was unintentionally biased towards sites of low to moderate NH_4^+ availability for AOA.

Whether AOA use NH_3 or NH_4^+ as the substrate for oxidation has yet to be tested to our knowledge, a fact pointed out by other researchers as well (Lehtovirta-Morley et al. 2011, Hatzenpichler 2012). Recent work by Gorman-Lewis et al. (2014) shows that the proteinaceous S-layer covering the outer surface of *N. maritimus* contains a high amount of proton-ionizable amino acid residues that may aid in efficient NH_4^+ capture from the environment. If this finding extends to soil AOA isolates, then cell surface chemistry alone could provide a mechanism by which AOA directly access the soil NH_4^+ pool, explaining the resource-specific response of AOA richness to soil NH_4^+ that we show here. Furthermore, we suggest that pH effects on soil AOA richness demonstrated by other authors (Hu et al. 2013, Stempfhuber et al. 2015) could be due to other mechanisms than modulating the availability of NH_3 .

Acknowledgements

The authors would like to thank members of the Virginia Tech Stream Team including Jack Webster and Bobbie Niederlehner for assistance with site selection, manuscript preparation, and nutrient analyses. This work was funded by two NSF grants (DEB #083293; DEB #1210607) and a grant from the Fralin Life Science Institute at Virginia Tech.

References

- Francis, C.A., Roberts, K.J., Beman, J.M., Santoro, A.E., Oakley, B.B. 2005. Ubiquity and diversity of ammonia-oxidizing archaea in water columns and sediments of the ocean. *Proceedings of the National Academy of Sciences* 102, 14683-14688.
- Hatzenpichler, R. 2012. Diversity, Physiology, and Niche Differentiation of Ammonia-Oxidizing Archaea. *Applied and Environmental Microbiology* 78, 7501-7510.
- Hu, H.W., Zhang, L.M., Dai, Y., Di, H.J., He, J.Z. 2013. pH-dependent distribution of soil ammonia oxidizers across a large geographical scale as revealed by high-throughput pyrosequencing. *Journal of Soils and Sediments* 13, 1439-1449.
- Lehtovirta-Morley, L.E., Stoecker, K., Vilcinskas, A., Prosser, J.I., and Nicol, G.W. 2011. Cultivation of an obligate acidophilic ammonia oxidizer from a nitrifying acid soil. *Proceedings of the National Academy of Sciences* 108, 15892-15897.
- Martens-Habbena, W., Berube, P.M., Urakawa, H., de la Torre, J.R., Stahl, D.A. 2009. Ammonia oxidation kinetics determine niche separation of nitrifying Archaea and Bacteria. *Nature* 461, 976-979.

180 Norman, J.S., and Barrett, J.E. 2014. Substrate and nutrient limitation of ammonia-
 181 oxidizing bacteria and archaea in temperate forest soil. *Soil Biology and*
 182 *Biochemistry* 69, 141-146.

183 Rotthauwe, J., Witzel, K., Liesak, W. 1997. The ammonia monooxygenase structural gene
 184 *amoA* as a functional marker: molecular fine-scale analysis of natural ammonia-
 185 oxidizing populations. *Applied and Environmental Microbiology* 63:4704-4712.

186 Schloss, P.D., Westcott, S.L., Ryabin, T., Hall, J.R., Hartmann, M., Hollister, E.B.,
 187 Lesniewski, R.A., Oakley, B.B., Parks, D.H., Robinson, C.J., Sahl, J.W., Stres, B.,
 188 Thallinger, G.G., Van Horn, D.J., Weber, C.F. 2009. Introducing mother: Open-
 189 Source, Platform-Independent, Community-Supported Software for Describing
 190 and Comparing Microbial Communities. *Applied and Environmental*
 191 *Microbiology* 75, 7537-7541.

192 Schloss, P.D., Gevers, D., and Westcott, S.L. 2011. Reducing the Effects of PCR
 193 Amplification and Sequencing Artifacts on 16S rRNA-Based Studies. *PLoS ONE*
 194 6, e27310

195 Stempfhuber, B., Engel, M., Fisher, D., Neskovic-Prit, G., Wubet, T., Schöning, I.,
 196 Gubry-Rangin, C. 2015. pH as a Driver for Ammonia-Oxidizing Archaea in
 197 Forest Soils. *Microbial Ecology* 69, 879-883.

198 Stephen, J.R., Kowalchuk, G.A., Bruns, M.V., McCaig, A.E., Phillips, C.J., Embley,
 199 T.M., Prosser, J.I. 1998. Analysis of β -subgroup proteobacterial ammonia
 200 oxidizer populations in soil by denaturing gradient gel electrophoresis analysis
 201 and hierarchical phylogenetic probing. *Applied and Environmental Microbiology*
 202 64, 2958-2965.

203 Suzuki, I., Dular, U., Kwok, S.C. 1974. Ammonia or ammonium ion as substrate for
204 oxidation by *N. europaea* cells and extracts. Journal of Bacteriology 120, 556-
205 558.

206 Swank, W.T., Vose, J.M. 1997. Long term nitrogen dynamics of Coweeta forested
207 watersheds in the southeastern United States of America. Global Biogeochemical
208 Cycles 11, 657-671.

209 Tilman, D. 1982. Resource Competition and Community Structure. Monographs in
210 Population Biology, Princeton University Press. 296 pp.

Figure 1. Theoretical relationship between taxonomic richness and resource availability (adapted from Tilman et al. 1982).

Figure 2. The effects of soil variables on AOA and AOB richness. Panels a, c, and e show the relationship between AOA richness and soil pH, soil NH₄⁺, and calculated soil NH₃, respectively. Panels b, d, and f show the relationship between AOB richness and soil pH, soil NH₄⁺, and calculated soil NH₃, respectively.