



Short communication

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

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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.

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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 (Fig. 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 favoured by AOB (Martens-Habbena et al., 2009) 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, 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

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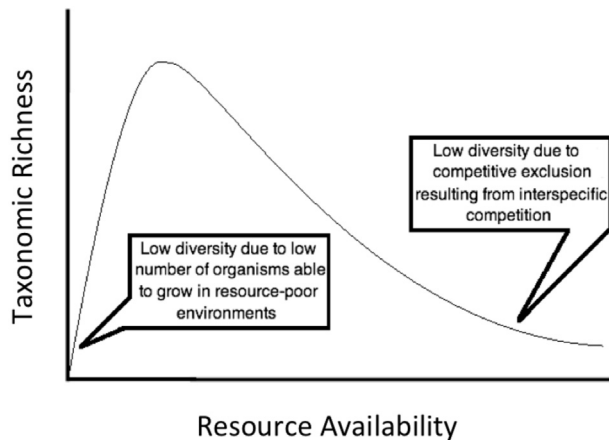


Fig. 1. Theoretical relationship between taxonomic richness and resource availability. Adapted from Tilman (1982).

2 mm 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., Carlsbad, 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-1F** (Stephen et al., 1998) and *amoA-2R* (Rotthauwe et al., 1997) for AOB, and *Arch-amoA-1F** 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 analysis 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. 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 from 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 *Nitrospira*. The three most abundant AOA OTUs, which contained 83% of the processed sequences, were most-closely related to *Nitrosotalea devanaterre*, 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 (Fig. 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–Hasselbalch equation that assumes a pKa value of 9.25 for the ionization of $\text{NH}_4^+/\text{NH}_3$):

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

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

If soil pH drives AOB richness through resource availability as we suggest here, we would expect to see an eventual decline in AOB richness along a pH gradient due to interspecific competition for NH_3 if we had encountered higher pH (and therefore higher NH_3) soils in this study. In seeming contrast to this prediction, Hu et al. (2013) found a positive relationship between soil pH and AOB richness when sampling soils that ranged in pH from less than 4 to greater than 8. However, these differing patterns could be explained by differences in sampling depth of soils used in these two studies: while we sampled from the top 5 cm of soil, Hu et al. (2013) sampled from the top 20 cm of soil, thereby covering a much larger oxygen availability gradient in each sample than we did. In high pH soils, NH_3 limitation should be alleviated for AOB, allowing richness to be governed by other limiting resources. If oxygen availability controls AOB richness in high pH soils, then Hu et al. (2013) may have inadvertently sampled an entire resource availability gradient in high pH soils by homogenizing soils from the top 20 cm, resulting 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 (Fig. 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 (Fig. 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 $\mu\text{g N-NH}_4/\text{g dw soil}$ (Fig. 2c) suggesting that NH_4^+ may be the resource that controls AOA richness in this system through the mechanisms outlined in Fig. 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 *Nitrosopumilus maritimus* contains a high amount of proton-ionizable amino acid residues that may aid in efficient NH_4^+

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