

Short Communication

Abundance and functional importance of complete ammonia-oxidizing bacteria (comammox) versus canonical nitrifiers in temperate forest soils

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ABSTRACT

Forest disturbance increases soil nitrification rates in temperate ecosystems, but little is understood regarding contributions of newly discovered complete ammonia-oxidizing bacteria (i.e., comammox) to this important ecosystem process. We sampled soils across eight historically disturbed and adjacent reference forested watersheds at the Coweeta Hydrologic Laboratory in North Carolina and used qPCR to determine abundance of three groups of nitrifiers: canonical ammonia-oxidizing bacteria (AOB), ammonia-oxidizing archaea (AOA), and comammox bacteria (CAOB). All three groups exhibited significantly higher abundance in disturbed soils, though CAOB were more abundant than either AOA or AOB. Further, CAOB showed similar correlations with soil variables compared with AOB, suggesting similar niche requirements. CAOB were better predictors of soil NO_3^- relative to AOA and AOB, suggesting CAOB are functionally relevant to nitrification in temperate forests.

Nitrification was historically considered to be a two-step process performed by two phylogenetically distinct groups of microorganisms; oxidation of ammonia to nitrite by ammonia-oxidizing bacteria followed by oxidation of nitrite to nitrate by nitrite-oxidizing bacteria (Winogradsky, 1890). However this historical view has changed in recent years, first in 2005 with the discovery of ammonia-oxidizing archaea by Könneke et al., and again in 2015 with the discovery of bacteria capable of complete oxidation of ammonia to nitrate (Daims et al., 2015; van Kessel et al., 2015). These complete ammonia-oxidizing bacteria are members of genus *Nitrospira*, a group previously thought to be only capable of nitrite-oxidation. Following this discovery, complete ammonia-oxidizers (i.e., 'comammox' bacteria) have been found in a variety of habitats such as waters, soils, and sediments (e.g., Xia et al., 2018). Though recent evidence suggests that comammox *Nitrospira* contribute to nitrification in soil (Wang et al., 2019), the abundance and functional relevance of comammox remains largely unknown in soil ecosystems.

Understanding contributions of comammox bacteria to nitrification is particularly relevant in temperate ecosystems of the southeastern US, where forest disturbance (e.g., clear-cut logging, conversion to agriculture) results in long-term increases in soil nitrification rates (e.g., Keiser et al., 2016) and watershed-scale nitrate export (e.g., Webster et al., 2016). These increases in nitrification rates have previously been

attributed to dominance of nitrogen (N)-fixing black locust trees (*Robinia pseudoacacia*) during early forest succession (e.g., Webster et al., 2016), which potentially increased ammonium availability to nitrifiers by reducing N-demand of heterotrophic microorganisms. However, recent evidence suggests that long-term effects of disturbance on soil bacterial and fungal communities may also play a role in persistently altered watershed N-dynamics (Osburn et al., 2019).

To determine abundance and functional relevance of comammox bacteria in these ecosystems, we sampled soils across eight forested watersheds at the Coweeta Hydrologic Laboratory in North Carolina, USA (Fig. S1). Four of the eight watersheds were disturbed ~4–8 decades previously as a result of forest management experiments conducted by the USDA Forest Service (Table S1). Disturbances included clear-cutting, conversion to pine monoculture, conversion to agriculture, and commercial clear-cut cable-logging (Fig. S1, Table S1). Adjacent to each previously disturbed watershed is a reference watershed that has not been manipulated by the Forest Service (Fig. S1, Table S1). Our study watersheds exhibit two soil types: Inceptisols and Ultisols in the Cullasaja-Tuckasegee and Edneyville-Chestnut complexes, respectively (Thomas, 1996). In each watershed, we established six 4 m x 4 m plots evenly spaced along a 200 m transect located 5 m upslope from the stream channel. In June 2018, at the height of the growing season, we used 4 cm diameter PVC corers to sample five soil cores from each plot

Abbreviations: AOB, Ammonia-oxidizing bacteria; AOA, Ammonia-oxidizing archaea; CAOB, Complete ammonia-oxidizing bacteria.

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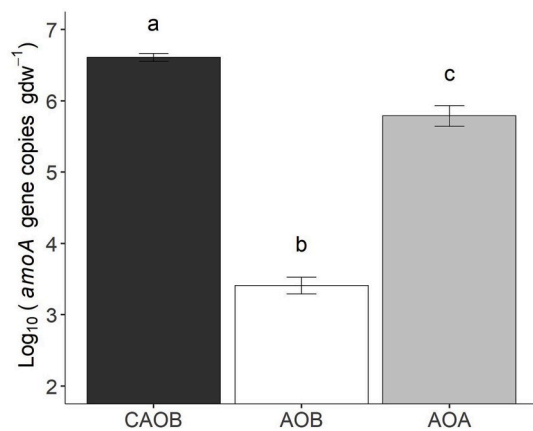


Fig. 1. Abundance of CAOB, AOB, and AOA *amoA* genes averaged across all samples. One-way ANOVA showed significant overall effects of nitrifier group on *amoA* gene abundance ($P < 0.001$). Different letters indicate significant differences in abundance ($P < 0.05$, Tukey's HSD).

(from four plot corners and plot center), sieved them (4 mm), and composited samples by plot, resulting in a total of 48 soil samples (8 watersheds x 6 plots/watershed). Our samples did not include O-horizon material and were taken to 10 cm depth, which generally includes only A-horizon material in our study sites (Knoepp and Swank, 1994). This sampling plan was designed for investigating drivers of soil N-cycling processes, as our watersheds are geographically close, but show large differences in soil properties (e.g., pH, C:N) and microbial-mediated N-cycling due to effects from past disturbance (e.g., Keiser et al., 2016).

We measured soil NH_4^+ , NO_3^- , total dissolved N (TDN), dissolved organic carbon (DOC), total N (TN), and total C (TC) and extracted/quantified DNA using previously described methods (Osburn et al., 2019). All soil physicochemical data are provided in Table S2. We used qPCR to quantify AOA and AOB abundance using the primer pairs Arch-amoAF/Arch-amoAR (Francis et al., 2005) and amoA-1F/amoA-2R (Rotthauwe et al., 1997), respectively. PCR protocols for both AOA and AOB are described in McBride et al. (2019). We quantified comammox AOB (CAOB) abundance using the universal qPCR primers and thermal cycling protocols described by Zhao et al. (2019). All qPCR reactions contained 10 μl Quantitect SYBR green master mix (Qiagen, Valencia, CA, USA), 0.25 μM forward and reverse primer, 2 μl DNA template (~10–40 ng DNA), and nuclease-free H_2O to 20 μl . Standard curves were generated by amplifying serial dilutions of synthesized copies of the target gene sequences and had R^2 values > 0.99 . All qPCR reactions were performed in triplicate and amplification efficiencies ranged from 80 to 90.8%. Amplification specificity was determined using melt curve analysis.

All statistical analyses were performed in R (R Core Development Team, 2017) using the 'lme4' and 'emmeans' packages (Bates et al., 2019; Lenth et al., 2019) and plots were considered independent replicates for statistical analyses. Comparison of log-transformed gene abundance among nitrifier groups using one-way ANOVA followed by pairwise comparisons (Tukey's HSD) revealed large differences, with CAOB exhibiting 2.2-fold higher abundance relative to AOA and

573-fold higher abundance relative to AOB (Fig. 1). Higher abundance of CAOB relative to AOA and AOB has also been shown in high-N environments such as activated sludge, wastewater plants, paddy soils, and some agricultural soils (Li et al., 2019; Liu et al., 2019; Wang et al., 2018; Zhao et al., 2019). Though little information is available for CAOB in forest environments, previous work in a high-N subtropical forest showed AOA more abundant than CAOB (Shi et al., 2018). To our knowledge this is the first report of CAOB dominance relative to other nitrifiers in temperate forests. This result is also consistent with 16S rRNA amplicon sequence data generated from these same samples, which show zero AOB sequences and only four AOA sequences in one sample, while canonical nitrite-oxidizers (i.e., Nitrospirae) were detected in all but one sample and account for as high as 4.4% (average 1%) of all 16S sequences (Osburn et al., 2019). Presumably, some of these nitrite-oxidizers represent CAOB also capable of ammonia-oxidation.

We used Spearman rank correlations to assess relationships between nitrifier groups and soil chemical variables (Table 1). The strong positive correlation of AOB with soil pH reflects well-described niche requirements of AOB (Table 1) (e.g., Prosser and Nicol, 2012), and the positive correlation of AOB with NH_4^+ (Table 1) supports prior studies from Coweeta demonstrating substrate limitation of AOB in these forests (Norman and Barrett, 2014). CAOB show similar correlations with pH and NH_4^+ in our data, potentially indicating similar niche requirements of AOB and CAOB. However, recent studies have suggested higher substrate affinity for CAOB relative to AOB (Kits et al., 2017), which is supported in our data by the lack of negative correlation between CAOB and soil C:N (Table 1), potentially indicating higher competitive ability of CAOB in N-limited environments relative to AOB. In contrast to previous studies investigating environmental correlates of AOA abundance (e.g., Prosser and Nicol, 2012), we found a positive correlation of AOA with soil pH (Table 1), potentially explained by positive effects of disturbance on both soil pH and factors that promote AOA abundance (i.e., NH_4^+) (Osburn et al., 2019).

To determine effects of disturbance on nitrifiers, we used linear mixed effects models with watershed pair as a random effect, which allowed us to account for systematic landscape variation among disturbed-reference watershed pairs (e.g., aspect, elevation). All nitrifier groups exhibited disturbance responses, with 2.9-fold, 5-fold, and 2.2-fold higher abundance in previously disturbed soils for CAOB, AOB, and AOA, respectively (Fig. 2A, B, C). These patterns are consistent with higher net nitrification rates previously reported from these same disturbed and undisturbed watersheds (e.g., Keiser et al., 2016), potentially related to dominance of N-fixing black locust after disturbance in forests of the region (Elliott and Vose, 2011).

Linear regression models showed all nitrifier groups as significant predictors of soil NO_3^- , though CAOB abundance explained more variation in soil NO_3^- than either AOB or AOA ($R^2 = 0.65$ vs .26 and .32, respectively, Fig. 2D, E, F). This apparent coupling of CAOB to soil NO_3^- may reflect higher energy efficiency of CAOB (Costa et al., 2006), which perform both the rate-limiting step (ammonia-oxidation) and the nitrate-producing step (nitrite-oxidation) of nitrification. Additionally, we used model selection with AIC_c to compare models with all possible combinations of nitrifier groups as predictors of soil NO_3^- , selecting the model with the highest AIC_c weight (Table S3). The best-supported model retained CAOB and AOB abundance as predictors, which is

Table 1

Correlations between CAOB, AOB, AOA, and soil chemical variables. Values are Spearman rank correlation coefficients (top) and P -values (bottom). Values in bold represent statistically significant correlations ($P < 0.05$).

	pH	NH_4^+	DOC	TDN	DOC:TDN	DON	TC	TN	C:N
CAOB	.472 .001	.296 .043	-.586 .001	-.291 .047	-.415 .004	-.368 .011	-.253 .087	-.114 .447	-.232 .116
AOB	.515 .001	.385 .012	-.426 .005	-.041 .795	-.581 .001	-.108 .496	.225 .151	.337 .029	-.390 .011
AOA	.377 .009	.187 .209	-.527 .001	-.312 .033	-.301 .040	-.352 .015	-.301 .040	-.167 .261	-.161 .280

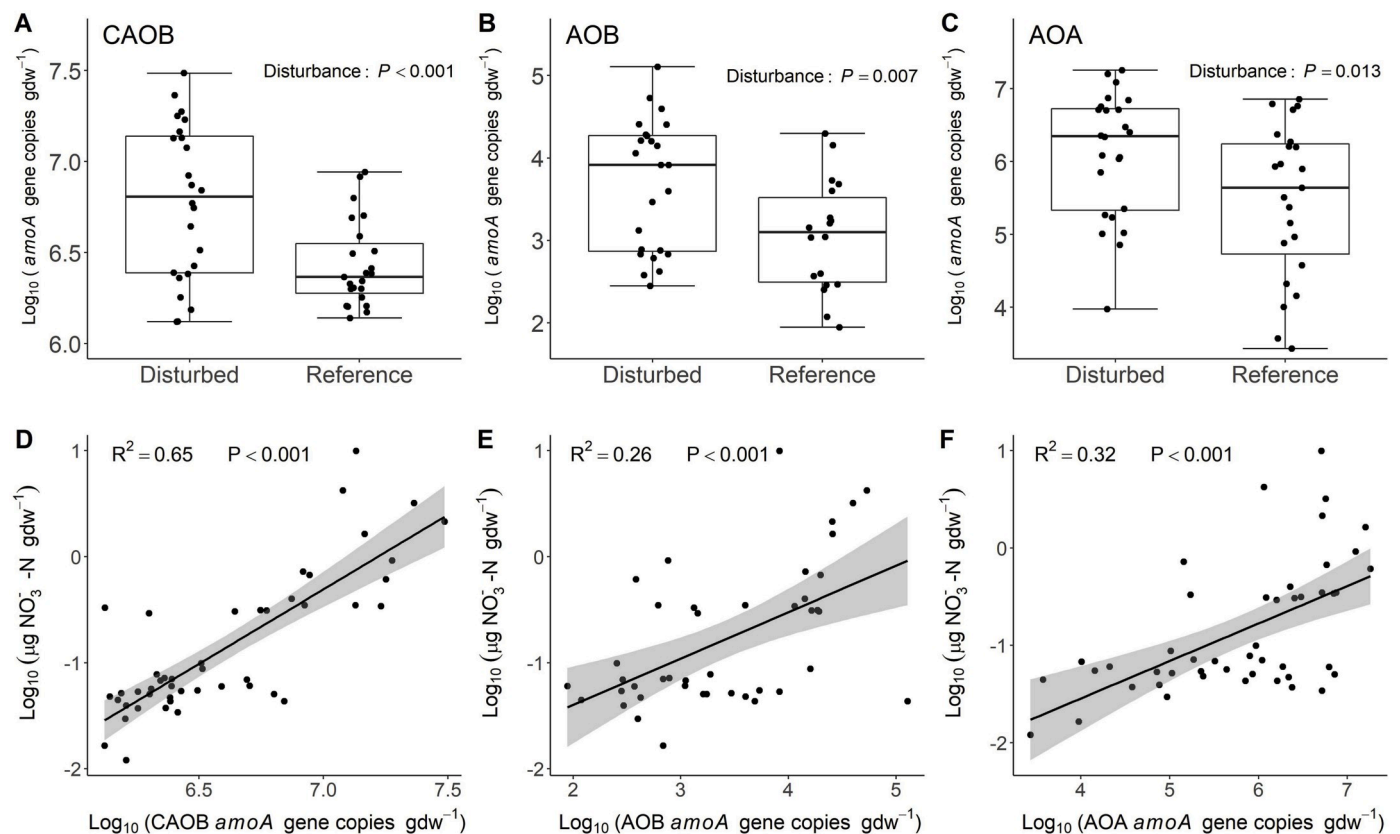


Fig. 2. Disturbance effects on CAOB (A), AOB (B), and AOA (C) *amoA* gene abundance. P -values in A, B, and C are from linear mixed models. Linear regressions of CAOB (D), AOB (E), and AOA (F) versus soil NO_3^- .

consistent with a recent modeling study that suggested AOB are more important than AOA to nitrification in these soils, despite higher abundance of AOA relative to AOB (Lin et al., 2017). Future work should utilize methods to directly attribute nitrification rates to different nitrifier groups to mechanistically test the relationships observed here (e.g., stable isotope probing). However, overall, our results demonstrate that CAOB are more abundant than other ammonia-oxidizers in our soils and strong relationships between CAOB and NO_3^- suggest that CAOB are functionally important to soil N-cycling in temperate forests of the eastern US.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.soilbio.2020.107801>.

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