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## Comment on “A Critical Review on Nitrous Oxide Production by Ammonia-Oxidizing Archaea” by Lan Wu, Xueming Chen, Wei Wei, Yiwen Liu, Dongbo Wang, and Bing-Jie Ni



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The ability of humanity to bring nitrous oxide ( $N_2O$ ) emissions under control hinges on a keen understanding of the mechanisms and processes that cause its formation as well as its natural mitigation (analogous to the methanogen–methanotroph balance that influences methane emissions). Undoubtedly, the activity of nitrifying microorganisms, as exacerbated by large-scale agriculture, land-use change, and fertilizer runoff, is a substantial contributor of nitrous oxide to the atmosphere. However, differences in the metabolic capacity of nitrifying microorganisms dictate how much ammonium-N they can directly release as  $N_2O$ -N within their environmental context, leading to variations in their relative potency. The focus of the review article by Wu et al. (“A Critical Review on Nitrous Oxide Production by Ammonia-Oxidizing Archaea”, [10.1021/acs.est.0c03948](https://doi.org/10.1021/acs.est.0c03948)) misrepresents the current state of experimental and genome-informed knowledge regarding the physiology of nitrifying microorganisms; in particular, the ammonia-oxidizing Thaumarchaeota (AOA), and their contribution to global nitrous oxide emissions.

Many of the pathways and enzymes described in this review are based on hypotheses and even speculations that lack experimental validation, or the authors ascribed published experimental observations incorrectly to activities, pathways, or functions. Furthermore, as documented in the literature, many of the early ideas and speculations in the field have now been refuted and replaced with data-driven evidence from pure laboratory cultures of how AOA oxidize ammonia and contribute to the  $N_2O$  budget in their environment. There is published evidence that AOA contribute equally, and more often less, on a per cell basis, to  $N_2O$  emissions than either the ammonia-oxidizing bacteria (AOB) or the heterotrophic denitrifiers, despite their omnipresent high abundance in oxic environments.

The stated purpose of the review is to synthesize literature that proposes “AOA-driven  $N_2O$  production pathways in combination with enzymology distinction”; however, much of the newer data-centric literature reports substantially different, and even opposite, conclusions to those reported in the review article. Several of the more recent studies were conducted with the intention to narrow down or disprove earlier models and speculations about potential “pathways”. Contrary to the introductory paragraph and Figure 1 of the Wu et al. article, the conclusion of the current literature body reports that (1) there is no experimental evidence that AOA have the capacity to express an enzymatic pathway for the direct conversion of

hydroxylamine ( $NH_2OH$ ) to  $N_2O$ , (2) there is no evidence for a nitroxyl (HNO) intermediate, or nitroxyl oxidoreductase activity, which could participate in the pathway for ammonia oxidation by AOA, and (3) there is no evidence that ammonia-monooxygenase (AMO) can use nitric oxide (NO) as a direct source of reductant for ammonia oxidation. To this date, there is not even experimental evidence for the mechanism that provides needed reductant to AMO in the much more extensively studied AOB or its homologue, particulate methane monooxygenase (pMMO), in methane-oxidizing bacteria.

As importantly, there is ample evidence that a nitrifier denitrification pathway does not exist in AOA. Of the five pathways leading to measurable  $N_2O$  emissions attributed to AOA activity by Wu et al., only two mechanisms have, thus far, been experimentally demonstrated. The first is “hybrid formation of  $N_2O$ ”, wherein one N atom is derived from hydroxylamine (generated by ammonia oxidation), and the other is derived from nitrite, most likely via its reduction to NO by an NO-producing nitrite reductase (experimentally attributed to copper-containing NirK in mesophiles or by a yet to be discovered analogue in thermophilic ammonia-oxidizing Thaumarchaeota). This abiotic reaction between pathway intermediates of ammonia oxidation in combination with transition metals (i.e., Fe, Mn) in the growth medium (or environment) produces negligible  $N_2O$ , and occurs only during active ammonia oxidation when the intermediates are produced at high enough concentrations to react with one another, facilitated by the redox-active transition metals. Whether this transition metal facilitated reaction of hydroxylamine and NO takes place in the cytoplasm or outside of the cell has not been elucidated. Figure 1 in Wu et al. assigned NO a direct role in the oxidation of ammonia, which was presented without experimental support. While several research groups have now independently reported that NO is essential in the ammonia-oxidation pathway of AOA, its precise location and functional role is unknown.<sup>1,2</sup> When NO is removed from AOA cultures using a scavenging compound such as PTIO, ammonia oxidation and nitrite formation cease immediately;

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conversely, AOB continue to oxidize ammonia when the NO scavenger is present, while N<sub>2</sub>O formation ceases, proving that PTIO is actively scavenging NO in AOB that actively perform nitrifier denitrification.<sup>1</sup> These experiments demonstrate a central, but different role of free (i.e., able to be scavenged) NO in the ammonia-oxidation pathway of AOA and AOB downstream of AMO. Whether subsequent oxidation of NO leads to a supply of electrons to the quinone pool and a contribution to increased proton motive force, or directly to reduction of AMO, has not yet been elucidated. Despite speculations beginning in the 1980s, it is not yet clear which inventory could oxidize NO, serve as the cognate quinone-reactive protein, or could engage in a redox interaction with AMO. Furthermore, while an experimentally proven nitrifier denitrification pathway exists for AOB, it does not in AOA. In fact, removal of NO by nitrifier-denitrification by AOA would be disadvantageous; to do so would be a dead end to their growth by consuming an essential intermediate with a competing enzyme—it would be a futile/suicidal activity.

The second proposed mechanism for direct N<sub>2</sub>O formation by AOA is NO reduction to N<sub>2</sub>O, a hypothesis based on a recently reported experiment with a single AOA isolate from soil.<sup>3</sup> The reported activity, catalyzed by a putative cytochrome P450 enzyme, required both oxygen and low pH, which is distinct from and not analogous to the low oxygen high nitrite requirement for stimulating nitrifier denitrification activity by AOB. The relatively extreme environmental conditions for this activity, and that it has not been reported for any other AOA isolate from soils or the marine environment, suggest that the implicated mechanism is not highly conserved in ammonia-oxidizing Thaumarchaeota.

The review article accurately reports that AOA can be present in high numbers in a multitude of ecosystems, yet their ammonia oxidizing activity in soils has usually been demonstrated in correlation with low ammonium availability and is not responsive to inorganic fertilizer amendments. In contrast, the unmitigated detection of N<sub>2</sub>O evolving from terrestrial ecosystems is, in most cases, correlated with high ammonium (N-overload) and hypoxia; conditions which stimulate enzymatic N<sub>2</sub>O formation by both AOB via nitrifier denitrification and heterotrophic denitrifiers.<sup>4</sup> AOA can thus only be minor direct contributors to the N<sub>2</sub>O budget in most agricultural environments. In contrast, the higher ammonia oxidizing activity of AOA in acidic or N-limited arctic soils<sup>5</sup> or any unmanaged ecosystem with low free ammonium content would result in abiotic N<sub>2</sub>O formation through hybrid formation and additionally provide oxidized N-substrates for biotic N<sub>2</sub>O formation by microorganisms other than AOA. In marine ecosystems, which are both oligotrophic and vast, AOA likely contribute substantially to global N<sub>2</sub>O emissions; nevertheless, there is also no experimental evidence for any intracellular mechanism that facilitates N<sub>2</sub>O formation by catalysis. The marine AOA identified thus far are related to *Nitrosopumilus*, which again only contribute to N<sub>2</sub>O formation via abiotic hybrid reactions and not by enzyme catalysis. Wu et al. erroneously interpret the figure from Peng et al.,<sup>6</sup> which they reproduced in Figure 3 and which shows that AOA were more abundant than AOB by ~10- to 150-fold at every depth in that profile, as is usually observed in the ocean. While denitrification contributes to hot spots of N<sub>2</sub>O formation in oxygen minimum zones, the vast expanse of the oxic surface water, the habitat of AOA, represents the major global source for N<sub>2</sub>O (~80%<sup>7</sup>) formation in the oceans. Thus, its

production is mostly likely directly or indirectly attributable to hybrid formation by AOA.

In conclusion, all current experimental evidence supports a low per-cell level contribution by AOA to abiotic N<sub>2</sub>O formation from intermediates or byproducts of their ammonia oxidation pathway. Another indirect contribution of AOA to N<sub>2</sub>O emissions lies in their ammonia oxidizing activity that provides NO and nitrite as substrates for subsequent nitrifying and denitrifying organisms. There is currently no uniform evidence for direct catalysis-based formation of N<sub>2</sub>O by the majority of AOA.

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## Notes

The authors declare no competing financial interest.

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