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Interactive comment on "Isotopomeric characterization of nitrous oxide produced by reaction of enzymes extracted from nitrifying and denitrifying bacteria" *by* T. Yamazaki et al.

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This work represents an important, and until now unprecedented, approach for understanding how nitrous oxide is produced by two distinct microbial pathways – hydroxylamine oxidation (nitrification) and nitric oxide reduction (denitrification). Several weaknesses in interpretation of the data that require correction have already been pointed out by Dr. Ostrom, and I am in full agreement. I would particularly concur with the problematic calculations leading to greater than 100% yield of N2O from NO in Table 3, particularly since the authors argue that this difference was likely from a problem with temperature of the NO preparation. If this is the case, the authors should

re-do the experiments at the appropriate temperatures for accurate comparison. 1) Characterization of purified enzymes. What data did the authors collect to validate that they had purified the correct enzymes? There are two enzymes that can oxidize NH2OH to nitrite in ammonia oxidizing bacteria – HAO and cytochrome P460 (which also requires NO as a substrate). The authors should show some analysis – protein size, absorbance spectra, etc. – that validates purification of the proper homotrimeric enzyme complex that is HAO. 2) I am quite surprised that the authors attained a cell pellet from 70 mL culture of N. oceani! How many cells were present in the culture? How does the density correspond to that of other studies? The reported amount of N2O from ammonia oxidation (Fig. 2) is extremely high for N. oceani, even when compared to other AOB like N. europaea that are known as high producers. Furthermore, it is quite surprising that more N2O would be produced at higher than at lower O2 levels. This is different from other AOB with the exception of a few studies on N2O production by N. europaea cultivated under very high concentrations of nitrite. Since nitrite was initially absent from the cultures, it doesn't make sense that so much N2O would be produced particularly at the given ammonium concentrations. The authors should explain the deviation of this result from other studies of AOB. Is the difference physiological or methodological? And if the authors conclude that the difference is physiological, what is the mechanistic underpinning of this conclusion? 3) How is it that zero nitrite was measured with 10 or 30 micromol NH2OH in assays with HAO? The data in Fig. 1 should be discussed in light of prior studies describing the biochemical and structural details of HAO enzymes. This result, again, is different from others in the literature; hence, it is important to put into context how, based on enzyme structure and biochemistry, N2O is generated by HAO. Most of the key structural and biochemical studies on HAO are not discussed or referenced in this paper. 4) It seems a bit strange to estimate the contribution of NOR to N2O production by N. oceani based on few experiments and then state that the estimations are in line with prior experimental values. The authors go on to state that, using these estimations, that NIR and NOR activities were not enhanced in their experiments. Such statements absolutely require

validation as the regulation and activity of specific enzymes are critical to understanding whether they actually contribute to N2O production in nature. There is a major difference between substrate conversion in vitro by purified enzymes versus substrate conversion through a pathway in vivo. The authors must greatly clarify how they can reasonably and accurately combine in vitro and in vivo data as the calculations in Table 4 essentially refute what is known about experimentally validated results in other AOB strains; that being the relative contribution of NH3-N to the nitrite-N and N2O-N pools by either NH2OH oxidation or nitrite reduction. 4) There is no known enzymatic pathway for N2O production by ammonia-oxidizing archaea. 5) The following reference presents the argument that nitrifier denitrification is not about detoxification of nitrite, but rather is a co-respiratory mechanism to allow AOB to respire under low oxygen levels. Co-respiration of oxygen and alternative electron acceptors, particularly nitrogen oxides, is well characterized in other bacterial (and fungal) lineages. Hence, there IS a defined and referenced physiological purpose for nitrifier denitrification, even though the physiology must be confirmed using a wider spectrum of AOB strains. Reference: Stein, L.Y. 2011. “Heterotrophic nitrification and nitrifier denitrification. In Nitrification. ASM Press, Washington DC. pp. 95-114.

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