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Supplementary Information for

Pathways of N_2O production by marine ammonia-oxidizing archaea determined from dual-isotope labeling

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This PDF file includes:

Supplementary Text

Supplementary Text 1: Compare the $^{45}N_2O$ production ratio observed in the field

Our findings are important for interpretation of isotope labeling patterns observed in isotope tracer experiments in the ocean. When expressed as a fraction of the total ($^{45}N_2O$/ ($^{45}N_2O$ +$^{46}N_2O$)), apparent hybrid formation ranged from 0.24 to 0.85 across the range of NH_4^+:NO_2^- concentration ratios from 0.05 to 10 (*SI Appendix*, Fig. S1). In this experiment $^{45}N_2O$ is truly hybrid, i.e., composed of N from two different sources. $^{46}N_2O$, however, could be partially hybrid, because it might result from the combination of two different compounds, both derived from the initially labeled $^{15}NH_4^+$ (see result from experiment 3 and 5). In a standard isotope tracer experiment performed with natural seawater samples, it is usually assumed that none of the observed $^{46}N_2O$ is hybrid. Thus, the amount of hybrid N_2O might be underestimated if we consider only $^{45}N_2O$. Recent ^{15}N labeling experiments consistently observe a high fraction of $^{45}N_2O$ production (i.e., > 70%) in the world's ocean including the mid-latitude North Atlantic (1), the western North Pacific (2), and the Eastern Tropical South Pacific (3-4), indicating that the hybrid formation is the main source of N_2O in the ocean. A more recent manipulation further found that the $^{45}N_2O$: $^{46}N_2O$ was insensitive to $^{15}NH_4^+$: $^{14}NO_2^-$ ratio when these substrates were experimentally manipulated in the Eastern Tropical North Pacific (5). Currently, we are unable to explain the discrepancy of $^{45}N_2O$: $^{46}N_2O$ production in response to $^{15}NH_4^+$: $^{14}NO_2^-$ ratio between SCM1 culture and the field studies. However, several factors, including the influence of $^{14}NH_4^+$ concentration in the natural environments, the isotope dilution of the tracer substrates, and ammonia oxidation coupled to NO_2^- reduction during the incubation, would also cause deviation of the measured $^{45}N_2O$: $^{46}N_2O$ from the predicted ratio (6). The potential involvement of microbial N_2O production from other members of the microbial community also complicates the interpretation of the observed $^{45}N_2O$: $^{46}N_2O$ in the field. Nevertheless, the field data suggest that further experiments with $^{15}NO_2^-$ tracers or more tests using other marine AOA strains would be useful, but also that interpretation of the hybrid isotope signature in marine samples is not straightforward.

Supplementary Text 2. Using O atom source to deduce NO_2^- production pathways

Based on the result that half of O atoms in NO_2^- produced by marine AOA strain CN25 were sourced from H_2O, a stepwise oxidation of NH_2OH to NO_2^- via NO was proposed as the ammonia oxidation pathway in AOA (7-8). A similar model was also proposed in AOB (9), although the enzyme catalysing the oxidation of NO to NO_2^- remains to be identified. Alternatively, NO_2^- was also hypothesized to be produced by a reaction between NH_2OH and NO that yields two molecules of NO_2^-, in which the NO was produced by NO_2^- reduction (10). In this model, 2/3 of O atoms should be sourced from H_2O and the remaining 1/3 of O atoms from O_2. In our experiment, 63% of O atoms in NO_2^- were sourced from H_2O, which is close to the 2/3 value predicted for NO_2^- produced by NH_2OH and NO. However, it should be noted that any abiotic intracellular O atom exchange between NO_2^- and ^{18}O-H_2O would increase the portion of H_2O as the O source for NO_2^-. For instance, if NO_2^- is produced via NH_2OH oxidation, an intracellular O atom exchange ratio of 26% is

required to explain the ratio observed in our study. On the other hand, O_2 accounted for ~26% of O atoms in NO_2^-, which was lower than the predicted values in both scenarios (50% in NH_2OH oxidation pathway and 33% in NH_2OH plus NO pathway). The low value might be attributed to intracellular O atom exchange, which would reduce the apparent contribution of O_2 to NO_2^-. Incomplete equilibration of $^{18}O_2$ in the headspace with DO in the medium could also lower the contribution of O_2 to NO_2^-, although this seems unlikely. Nevertheless, our results suggest a significantly higher contribution from H_2O than O_2 for the O atoms in NO_2^-. Based on the importance of substrate ratio (NH_4^+: NO_2^-) in determining the N sources for N_2O, it is possible that the relative contribution of different O sources might also vary with substrate ratio.

Supplemental Text 3: Choice of preservative for N_2O and NO_2^- production studies

Mercuric chloride ($HgCl_2$) has been widely used as a preservative to terminate microbial activities due to its high toxicity and high solubility in water (11, 12). In hopes of minimizing the use of this toxic material, and due to concerns about the potential for artefacts of $HgCl_2$ on N_2O production, we compared the effect of $HgCl_2$ and sodium hydroxide (NaOH) on both NO_2^- and N_2O production for three cell treatments and three ^{15}N-labelled substrates (Preservative test in Table S1). Our results suggested that both $HgCl_2$ and NaOH effectively stopped SCM1 activity, and similarly preserved the isotope signal in NO_2^- and N_2O during $^{15}NH_4^+$ labelling incubations (*SI Appendix*, Fig. S3*A-D*). In contrast, N_2O isotopes were differentially affected by the two preservatives in $^{15}NO_2^-$ labelling incubations. $HgCl_2$ resulted in significantly higher $\delta^{15}N$-N_2O than using NaOH in both the viable cell and heat killed cell treatments, indicating the application of $HgCl_2$ induces an artefact of N_2O production from NO_2^- associated pathways (*SI Appendix*, Fig. S3*E, F*). The $^{15}NO_3^-$ labelling experiment was performed only in the viable cell group. There was no significant change in $\delta^{15}N$-N_2O during incubation in either $HgCl_2$ or NaOH treatments, indicating that NO_3^- was not involved in archaeal N_2O production (*SI Appendix*, Fig. S3*G*). Therefore, we concluded that NaOH is a better preservative to study archaeal N_2O production, and NaOH was chosen as the preservative for all further experiments reported here.

Supplementary Figures and Table

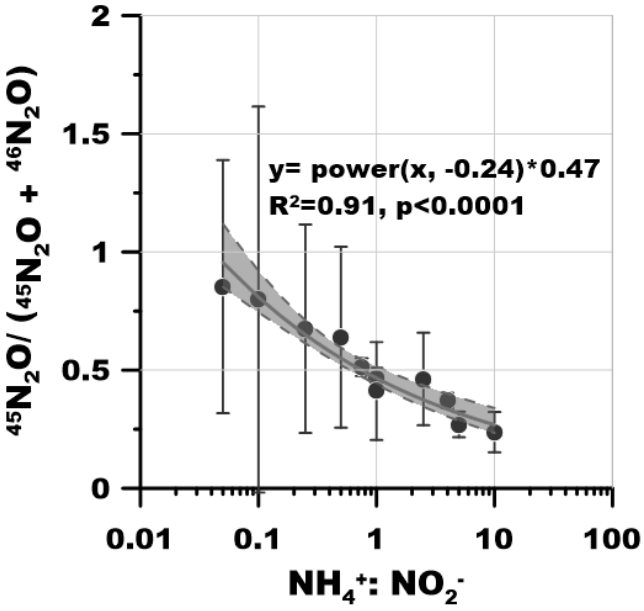

Fig. S1. Fraction of $^{45}N_2O$ to total labeled N_2O production under different $^{15}NH_4^+$: $^{14}NO_2^-$ ratios. Regression between $^{45}N_2O$: ($^{45}N_2O$ + $^{46}N_2O$) production rate against $^{15}NH_4^+$ and $^{14}NO_2^-$ concentration ratio. Error bars represent propagated standard deviation from triplicate samples.

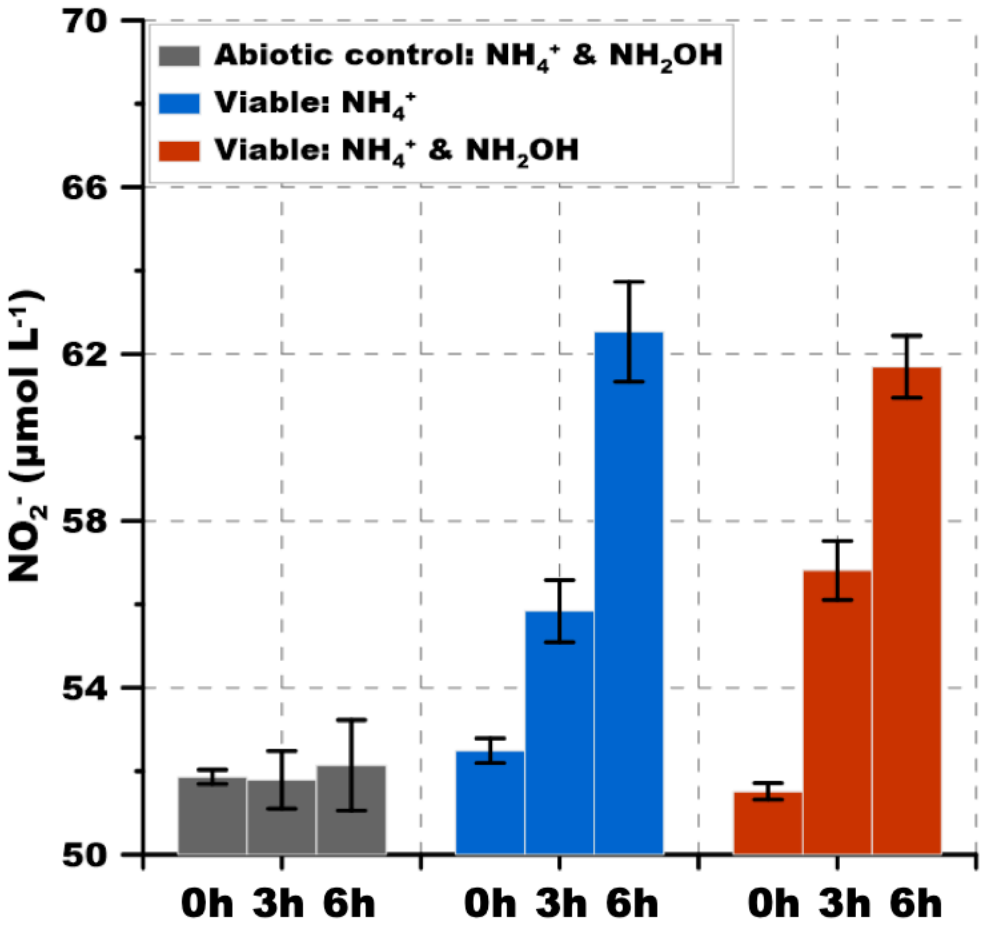

Fig. S2. Impact of NH_2OH on NO_2^- production rate. Gary bars: abiotic control using filtrate with NH_4^+ (50 µmol L^{-1}) and NH_2OH (1 µmol L^{-1}). Blue bars: viable cell with NH_4^+ (50 µmol L^{-1}). Red bars: viable cell with NH_4^+ (50 µmol L^{-1}) and NH_2OH (1 µmol L^{-1}). Error bars represent standard deviation from triplicate samples.

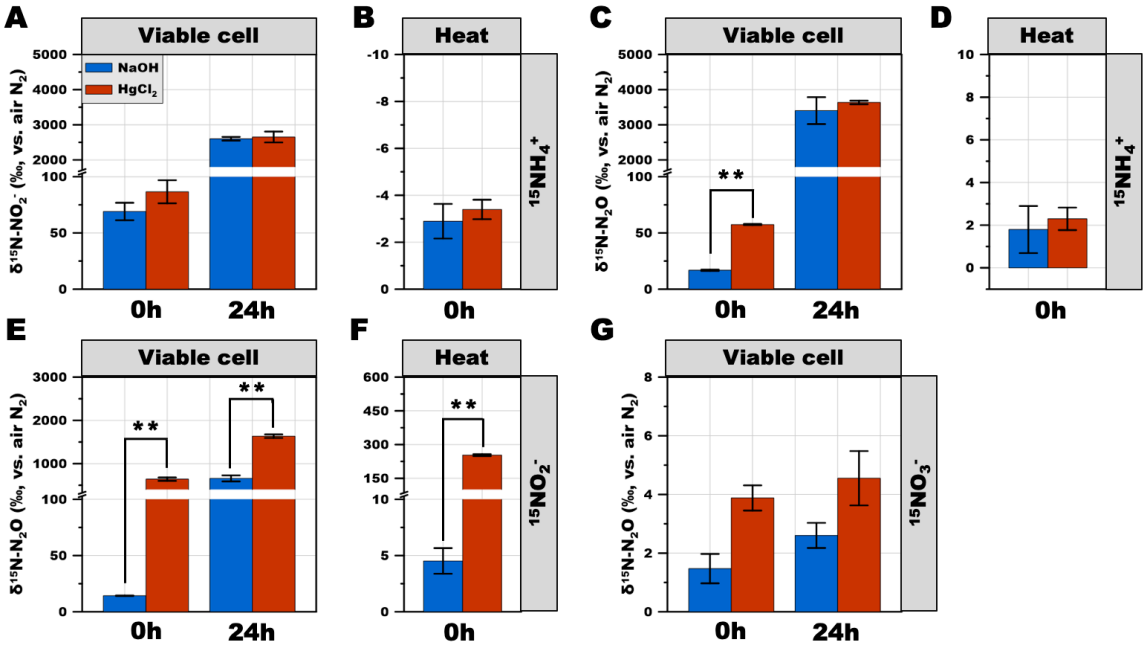

Fig. S3. Preservative Test for the effects of NaOH and $HgCl_2$ on NO_2^- and N_2O production. A-B, $\delta^{15}N$-NO_2^- in cells amended with 50 μmol L^{-1} of $^{15}NH_4^+$. C-D, $\delta^{15}N$-N_2O in cells amended with 50 μmol L^{-1} of $^{15}NH_4^+$. E-F, $\delta^{15}N$-N_2O in cells amend with 40 μmol L^{-1} of $^{15}NO_2^-$. G, Change of $\delta^{15}N$-N_2O during $^{15}NO_3^-$ labeling incubation. The X-axis marks time of adding preservatives after ^{15}N tracer amendment. The $\delta^{15}N$ in NaOH treatment is shown in blue bars and the results of $HgCl_2$ treatment are shown in red bars. Error bars represent standard deviation from triplicate samples. The stars (**) represent significance difference at $p<0.01$ level. Note Y-axes differ in scale.

Table S1. Summary of experimental treatments.

Exp.[1]	Cell treatment	Tracers	Preserve	Target	Time points
Preserv-ative test	1) Viable cells 2) Autoclaved cells	1) $^{15}NH_4^+$: 50 μM 2) $^{15}NO_2^-$: 50 μM 3) $^{15}NO_3^-$: 100 μM	1) $HgCl_2$ 2) NaOH	Determine the best way to terminate incubations and preserve N_2O	0, 24h (for viable cells)
1	1) Viable cells	1) $^{15}NH_4^+$: 0.1, 0.5, 1, 1.5, 2, 5, 8, 10 μM	1) NaOH	Explore the ammonia and N_2O production kinetics	3 timepoints
2	1) Viable cells	1) $^{15}NH_4^+$ + $^{14}NO_2^-$: 1.5 & 15 μM 2) $^{15}NH_4^+$ + $^{14}NO_2^-$: 5 & 5 μM 3) $^{15}NH_4^+$ + $^{14}NO_2^-$: 10 & 1 μM	1) NaOH	Investigate the impact of NH_4^+: NO_2^- on N_2O production	0, 6, 24h
3	1) Viable cells (washed by fresh medium)	1) $^{15}NH_4^+$: 20 μM 2) $^{15}NH_4^+$ + $^{14}NO_2^-$: 20 & 20 μM 3) $^{15}NO_2^-$: 20 μM 4) $^{15}NO_2^-$ + $^{14}NH_4^+$: 20 & 20 μM	1) NaOH	Track the source of N atoms using ^{15}N substrates	0, 24h
4	1) Viable cells 2) Filtrate	1) $^{15}NH_2OH$: 1 μM 2) $^{15}NO_2^-$: 10 μM 3) $^{15}NO_2^-$ + $^{4}NH_2OH$: 10 & 1 μM	1) NaOH	Examine N_2O production from NH_2OH	Time course (0, 1, 3, 6, 12 h)
5	1) Viable cells (washed by fresh medium) 2) Fresh medium	1) $H_2{}^{18}O$, NH_4^+ NO_2^-: 20 & 20 μM 2) $^{18}O_2$, NH_4^+ + NO_2^-: 20 & 20 μM 3) NH_4^+ + $N^{18}O_2^-$: 20 & 20 μM	1) NaOH	1) Track the source of O atoms using ^{18}O substrates 2) Quantify the abiotic O atom exchange rate between H_2O and NO_2^-	Time course (0, 6, 12, 24h) for selected treatments 0, 24h (for the remaining groups)

[1]: All the experiments were carried out in biological triplicates at each timepoint.

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