

Supplemental Information

S1: Cloning and Expressing StrepII-tagged N2OR

As described in the Materials and Methods section 2.1 (*Cloning and culture maintenance*), expression of N2OR from native *S. stutzeri* ZoBell was enhanced via overexpression of the *nosZ* gene inserted into the backbone of the pMVV103 plasmid. Using the primers listed in Table S1, the pMVV103 plasmid, previously found to be compatible with *S. stutzeri* transformations and induction (Venkataraman et al., 2023), was equipped with overhangs to allow Gibson assembly with the native *nosZ* gene (modified to contain the StrepII affinity tag).

Table S1. Primers used in this study

Name	Sequence	Description
N2OR27	GCCTGGAGCCACCCGCAGTTCGAAAAATGActcggtaccaaattccagaaaag	Forward primer to amplify/linearize pMVV103. Adds a 30-nt overlap w/ NosZ-StrepII.
N2OR28	AGTGTTCTTGGAATCTTTGTCGCTCATATGgtatttcccctctttctctagtattaaac	Reverse primer to amplify/linearize pMVV103. Adds a 30 nt-overlap with NosZ (and upstream NdeI sequence).

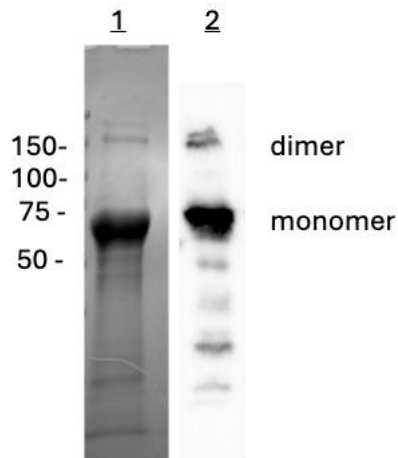


Figure S1: Analysis of Purified Nitrous Oxide Reductase on a 10% SDS-PAGE Gel.

Following size exclusion chromatography, the purity of Strep-tagged N₂OR from *S. stutzeri* was analyzed by SDS-PAGE and Coomassie staining (Lane 1) and western blotting (Lane 2). Strep-tagged N₂OR was probed via western blot using an anti-Strep-Tag II antibody. The positions of protein molecular weight markers are shown on the left (Bio-Rad Precision Plus Protein Dual Color ladder with molecular weights in kDa).

Figure S1 shows SDS-PAGE analysis of overexpressed N₂OR-StrepII after size exclusion chromatography. Coomassie staining and western blot analysis indicated that Strep-tagged N₂OR was the major product in our purifications; however, other contaminating bands were still present (Figure S1). Importantly, size exclusion chromatography removed a heme-containing protein which would interfere with Cu₂ active site characterization. Removal of the heme contaminant was verified by both UV-vis spectrophotometry (Figure 1) and ICP-OES analysis, which confirmed that there was no iron in purified protein samples.

30 **S2: Activation of N₂OR via Pre-incubation**

Purified N₂OR was activated via incubation with methyl viologen and sodium dithionite (see Materials and Methods section 2.4.2 *Indirect activity assays*). Without extended incubation with sodium dithionite-reduced methyl viologen, enzyme activity increased in a manner that would prohibit accurate activity measurements. The data in Table S2 show the activity of N₂OR increasing between technical replicates of the indirect methyl viologen activity assay. The increased activity of N₂OR after incubation with methyl viologen indicates form II N₂OR is present in our protein sample. Following pre-incubation of N₂OR with reductant for 2 hours, as described in Materials and Methods section 2.4.2 *Indirect activity assays*, enzyme activity was stable for 3 hours (Table S3) allowing for consistent, reproducible measurements of N₂O consumption. Figure S2 shows the specific activity of pre-reduced enzyme at varying N₂O concentrations. N₂O concentration was varied by injecting different volumes of N₂O-saturated water into the reaction cuvette, assuming that the concentration of N₂O-saturated water was 25 mM at 25 °C (Weiss and Price, 1980). Note that the maximum specific activity varied across N₂OR preparations, likely due to variable metal loading and/or reductant concentrations during pre-reduction.

Table S2: Form II N₂OR Activation. The activity of purified N₂OR was assessed using the indirect assay described in Materials and Methods section 2.4.2 *Indirect activity assays*, except that N₂OR was not pre-incubated with methyl viologen and sodium dithionite for 2 h. Technical replicates were prepared at the same time, and then N₂O addition and reaction turnover were analyzed approximately 7 minutes apart. Technical replicates 2 and 3 thus were effectively pre-incubated with 0.1 mM sodium dithionite and 0.1 mM methyl viologen for ~7-14 minutes prior to analysis. All reactions were conducted with 5.6 µg of purified Strep-tagged N₂OR and 125 µM N₂O.

Biological replicate	Cu loading (µmol Cu/ µmol monomer)	Technical replicate	Time incubated with methyl viologen (min)	Specific activity (µmol/min/mg N ₂ OR)
1	5.2	1	0	0.07
		2	7	2.05
		3	14	6.96
2	5.47	1	0	5.7
		2	7	10.4
		3	14	19.8
3	5.51	1	0	1.33
		2	7	6.57
		3	14	9.73
4	4.92	1	0	0.04
		2	7	5.09

Table S3: The effect of pre-incubation duration on purified N₂OR activity.

Purified N₂OR was incubated for 0-5 hours with methyl viologen and sodium dithionite as described in Materials and Methods section 2.4.2 *Indirect activity assays*. Specific activity of the enzyme at each hour of incubation was determined.

Pre-reduction time (hours)	Average specific activity ^a ($\mu\text{mol}/\text{min}/\text{mg N}_2\text{OR}$)
0	2.96 $\pm$ N/A ^b
1	55.80 $\pm$ 37.75
2	113.23 $\pm$ 23.94
3	130.79 $\pm$ 4.96
5	102.87 $\pm$ 9.68

^a Average specific activity $\pm$ standard deviation for duplicate indirect activity assay measurements. The enzyme preparation used for this experiment had an average copper loading of 7 Cu/monomer.

^b Initial (no pre-incubation) specific activity was only measured in a single replicate.

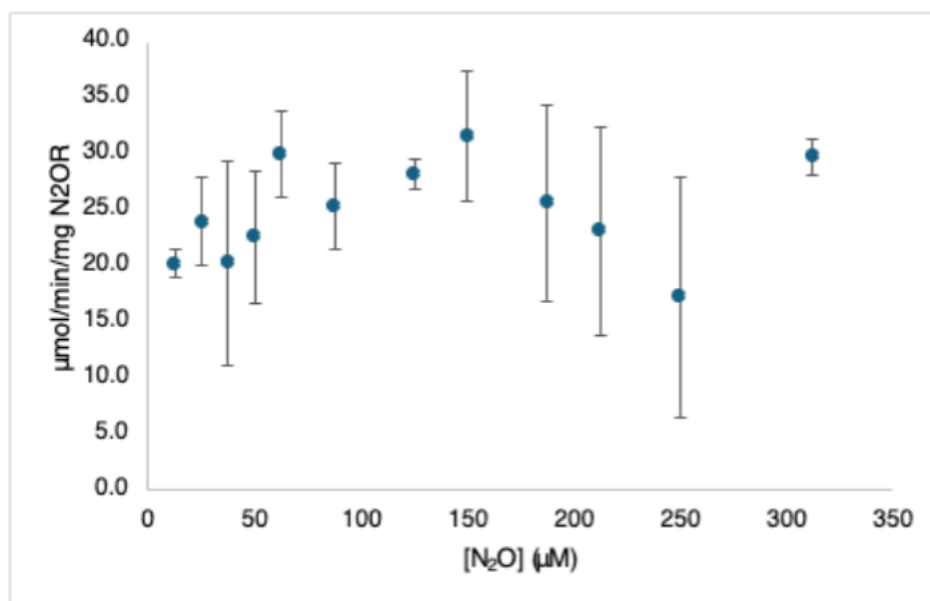


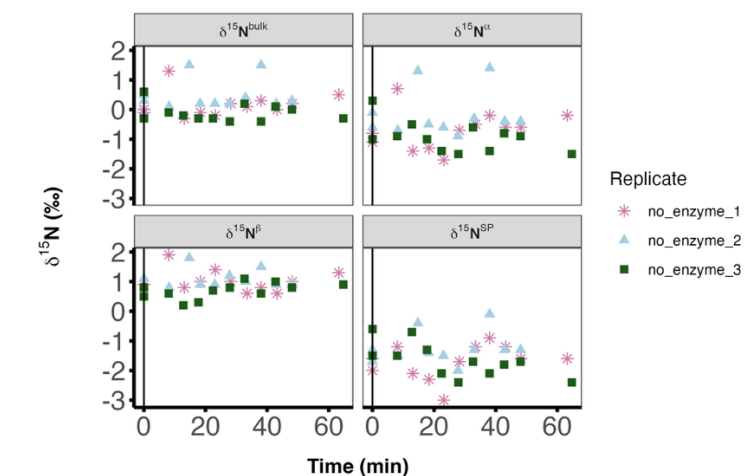
Figure S2: Indirect activity assay of nitrous oxide reductase-catalyzed N₂O reduction.

N₂OR was pre-reduced with methyl viologen as described in Materials and Methods section 2.4.2 *Indirect activity assays*, and activity was analyzed within 2 hours after pre-reduction. Each reaction was initiated by the addition of N₂O-saturated water, and the decrease in 600 nm absorbance was continuously monitored. The initial rate of each reaction was calculated to give specific activity in μmol/min/mg of N₂OR monomer (y axis). Activity at all substrate concentrations was measured in triplicate with 3 individual technical replicates of pre-reduced N₂OR from the same biological purification batch.

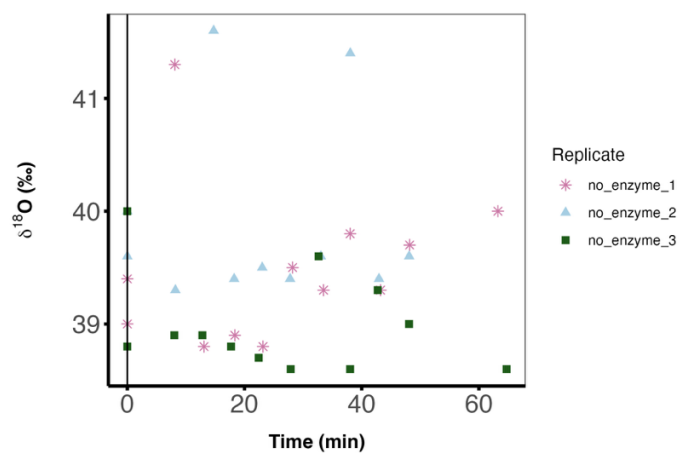
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S3: Isotopic Data



a.



b.

80 **Figure S3: $\delta^{15}\text{N}$ and $\delta^{18}\text{O}$ of N_2O in the absence of N_2OR .**

Control reactions were performed on the same days as reactions with purified N_2OR and were prepared in the same way as reactions with enzyme, except that, for control reactions, anaerobic buffer lacking enzyme was added at time 0. Headspace samples were collected over the course of an hour.

Table S4: δ values for N_2O consumption reactions performed with purified N_2OR in triplicate.

Replicate	Time (min)	N_2O (μmol) ^a	f	$\delta^{18}\text{O}$ (‰)	$\delta^{15}\text{N}^{\text{bulk}}$ (‰)	$\delta^{15}\text{N}^{\alpha}$ (‰)	$\delta^{15}\text{N}^{\beta}$ (‰)	$\delta^{15}\text{N}^{\text{SP}}$ (‰)
N2OR_1	0	4.72	1	39.6	0.2	-0.2	0.6	-0.8
N2OR_1	0	4.72	1	39.9	0.3	0	0.7	-0.7
N2OR_1	8.15	3.36	0.71	43.4	1.5	2.6	0.5	2.1
N2OR_1	13.15	2.73	0.58	47	3.2	4.6	1.7	2.8
N2OR_1	18.22	2.21	0.47	48.9	3.6	6.4	0.9	5.5
N2OR_1	23.25	1.8	0.38	52.1	4.9	8.7	1	7.6
N2OR_1	29.02	1.41	0.3	56.2	6.5	11.6	1.4	10.2
N2OR_1	33.98	1.15	0.24	58.6	7.3	13.1	1.6	11.5
N2OR_1	38.67	0.95	0.2	61.9	8.7	15.6	1.7	14
N2OR_1	43.95	0.76	0.16	66.2	10.3	18.6	1.9	16.7
N2OR_1	48.35	0.63	0.13	68.2	11	20.1	1.9	18.2
N2OR_2	0	4.11	1	39.6	0.2	-0.2	0.5	-0.7
N2OR_2	0	4.11	1	39.5	0.2	-0.4	0.7	-1.1
N2OR_2	8.03	2.98	0.72	43.9	1.9	2.6	1.2	1.5
N2OR_2	13.48	2.39	0.58	47.3	3.2	5.2	1.3	3.9
N2OR_2	18.55	1.95	0.47	51.3	4.8	7.9	1.7	6.3
N2OR_2	23.02	1.63	0.4	54	5.6	10	1.2	8.8
N2OR_2	28.53	1.3	0.32	59.4	8.1	14.2	2	12.2
N2OR_2	33.77	1.06	0.26	63.3	9.6	16.6	2.6	14
N2OR_2	38.9	0.86	0.21	66.5	10.5	19.1	1.8	17.4
N2OR_3	0	4.18	1	39.3	0.2	-0.4	0.8	-1.2
N2OR_3	8.23	2.95	0.71	43.4	1.7	2.2	1.2	1
N2OR_3	13.27	2.38	0.57	46.4	3.1	4.7	1.4	3.3
N2OR_3	18.6	1.9	0.45	48.2	3.3	6	0.6	5.4
N2OR_3	23.85	1.52	0.36	51.4	4.8	7.8	1.7	6.2
N2OR_3	28.83	1.23	0.29	54.3	5.7	10.1	1.4	8.7
N2OR_3	33.5	1.01	0.24	58.3	7.7	13	2.5	10.6
N2OR_3	38.43	0.82	0.2	59.5	7.5	13.9	1.1	12.8
N2OR_3	43.53	0.66	0.16	64.6	10	17.6	2.3	15.3
N2OR_3	48.53	0.54	0.13	66.1	10	18.7	1.2	17.5

^a For replicates 1-2, the initial amount of N_2O (μmol) is the average of two initial measurements (sampled at timepoint 0).

Table S5: δ values for N_2O in control reactions without enzyme, performed in triplicate.

Replicate	Time (min)	N_2O (μmol) ^a	f	$\delta^{18}\text{O}$ (‰)	$\delta^{15}\text{N}^{\text{bulk}}$ (‰)	$\delta^{15}\text{N}^{\alpha}$ (‰)	$\delta^{15}\text{N}^{\beta}$ (‰)	$\delta^{15}\text{N}^{\text{SP}}$ (‰)
no_enzyme_1	0	4.43	1	39.4	0	-0.8	0.9	-1.6
no_enzyme_1	0	4.43	1	39	-0.1	-1.1	0.9	-2
no_enzyme_1	8.1	4.51	1.02	41.3	1.3	0.7	1.9	-1.2
no_enzyme_1	13.08	4.38	0.99	38.8	-0.3	-1.4	0.8	-2.1
no_enzyme_1	18.35	3.87	0.87	38.9	-0.1	-1.3	1	-2.3
no_enzyme_1	23.15	4.08	0.92	38.8	-0.2	-1.7	1.4	-3
no_enzyme_1	28.2	4.39	0.99	39.5	0.2	-0.7	1	-1.7
no_enzyme_1	33.48	4.3	0.97	39.3	0.1	-0.5	0.6	-1.2
no_enzyme_1	38.03	4.43	1	39.8	0.3	-0.2	0.8	-0.9
no_enzyme_1	43.22	4.42	1	39.3	0	-0.6	0.6	-1.2
no_enzyme_1	48.18	4.39	0.99	39.7	0.2	-0.6	1	-1.6
no_enzyme_1	63.25	4.64	1.05	40	0.5	-0.2	1.3	-1.6
no_enzyme_2	0	3.97	1	39.6	0.3	-0.6	1.1	-1.7
no_enzyme_2	0	3.97	1	40	0.5	-0.1	1.1	-1.3
no_enzyme_2	8.2	3.91	0.99	39.3	0.1	-0.7	0.8	-1.5
no_enzyme_2	14.73	3.86	0.97	41.6	1.5	1.3	1.8	-0.4
no_enzyme_2	18.23	4.13	1.04	39.4	0.2	-0.5	0.9	-1.4
no_enzyme_2	23.02	4.1	1.03	39.5	0.2	-0.6	0.9	-1.5
no_enzyme_2	27.78	3.96	1	39.4	0.2	-0.9	1.2	-2
no_enzyme_2	33.02	3.86	0.97	39.6	0.4	-0.3	1	-1.3
no_enzyme_2	38.05	3.84	0.97	41.4	1.5	1.4	1.5	-0.1
no_enzyme_2	42.95	3.68	0.93	39.4	0.2	-0.4	0.9	-1.3
no_enzyme_2	48.1	3.49	0.88	39.6	0.3	-0.4	0.9	-1.3
no_enzyme_3	0	3.22	1	38.8	-0.3	-1	0.5	-1.5
no_enzyme_3	0	3.22	1	40	0.6	0.3	0.8	-0.6
no_enzyme_3	8.02	3.06	0.95	38.9	-0.1	-0.9	0.6	-1.5
no_enzyme_3	12.8	3.42	1.06	38.9	-0.2	-0.5	0.2	-0.7
no_enzyme_3	17.7	3.63	1.13	38.8	-0.3	-1	0.3	-1.3
no_enzyme_3	22.42	3.61	1.12	38.7	-0.3	-1.4	0.7	-2.1
no_enzyme_3	27.88	3.69	1.15	38.6	-0.4	-1.5	0.8	-2.4
no_enzyme_3	32.63	3.66	1.14	39.6	0.2	-0.6	1.1	-1.7
no_enzyme_3	38.03	3.5	1.09	38.6	-0.4	-1.4	0.6	-2.1
no_enzyme_3	42.73	4.21	1.31	39.3	0.1	-0.8	1	-1.8
no_enzyme_3	48.07	4.04	1.26	39	0	-0.9	0.8	-1.7
no_enzyme_3	64.73	4.32	1.34	38.6	-0.3	-1.5	0.9	-2.4

^a For no-enzyme control reactions, the amount of N_2O in the reaction bottle at the GC-IRMS sampling timepoints was assumed to be the same as the amount of N_2O measured by GC-ECD in the samples taken immediately before each GC-IRMS sample. The initial amount of N_2O (μmol) is the average of two initial measurements (sampled at timepoint 0).

Table S6. Comparison of ε and KIE values calculated for Mariotti's approximation of the Rayleigh equation (all data vs early-reaction data) and the ε and KIE values calculated for the Rayleigh model without approximation (all data).

Model/Data	Measurement	R ^{2a}	δ_{s0} measured (‰) ^b	δ_{s0} calculated (‰) ^c	ε (‰) ^d	KIE ^d
Mariotti's Approximation of the Rayleigh Eq. (all data)	¹⁵ N ^{bulk}	0.95	0.2 ± 0.1	0.1 ± 0.3	-5.4 ± 0.2	1.0054 ± 2e-04
	¹⁵ N ^α	0.96	-0.4 ± 0.1	-0.5 ± 0.5	-10.1 ± 0.4	1.0102 ± 4e-04
	¹⁵ N ^β	0.46	0.8 ± 0.1	0.8 ± 0.1	-0.6 ± 0.1	1.0006 ± 1e-04
	¹⁵ N ^{SP}	0.96	-1.2 ± 0.1	-1.3 ± 0.4	-9.5 ± 0.4	1.0096 ± 4e-04
	¹⁸ O	0.96	39.5 ± 0.1	39.3 ± 0.6	-14.2 ± 0.6	1.0144 ± 6e-04
Mariotti's Approximation of the Rayleigh Eq. (f > 0.4)	¹⁵ N ^{bulk}	0.94	0.2 ± 0.1	0.2 ± 0.2	-4.9 ± 0.4	1.005 ± 4e-04
	¹⁵ N ^α	0.97	-0.4 ± 0.1	-0.3 ± 0.2	-9.2 ± 0.5	1.0092 ± 5e-04
	¹⁵ N ^β	0.3	0.8 ± 0.1	0.7 ± 0.2	-0.8 ± 0.3	1.0008 ± 3e-04
	¹⁵ N ^{SP}	0.96	-1.2 ± 0.1	-1 ± 0.2	-8.4 ± 0.5	1.0085 ± 5e-04
	¹⁸ O	0.96	39.5 ± 0.1	39.5 ± 0.4	-13 ± 0.8	1.0132 ± 8e-04
Rayleigh Equation without Approximation (all data)	¹⁵ N ^{bulk}	0.95	0.2 ± 0.1	NA	-5.4 ± 0.2	1.0054 ± 2e-04
	¹⁵ N ^α	0.96	-0.4 ± 0.1	NA	-10 ± 0.4	1.0101 ± 4e-04
	¹⁵ N ^β	0.46	0.8 ± 0.1	NA	-0.6 ± 0.1	1.0006 ± 1e-04
	¹⁵ N ^{SP}	0.96	-1.2 ± 0.1	NA	-9.5 ± 0.4	1.0095 ± 4e-04
	¹⁸ O	0.96	39.5 ± 0.1	NA	-13.5 ± 0.5	1.0137 ± 5e-04

^a $R^2 = [1 - (SSR/SST)]$, where SSR is the residual sum of squares and SST is the total sum of squares.

^b Average initial δ value (δ_{s0}) ± standard error for all three replicates with N₂OR and all three control replicates without enzyme.

^c Intercept of Mariotti's approximation of the Rayleigh equation ± standard error.

^d ε or KIE value ± standard error.

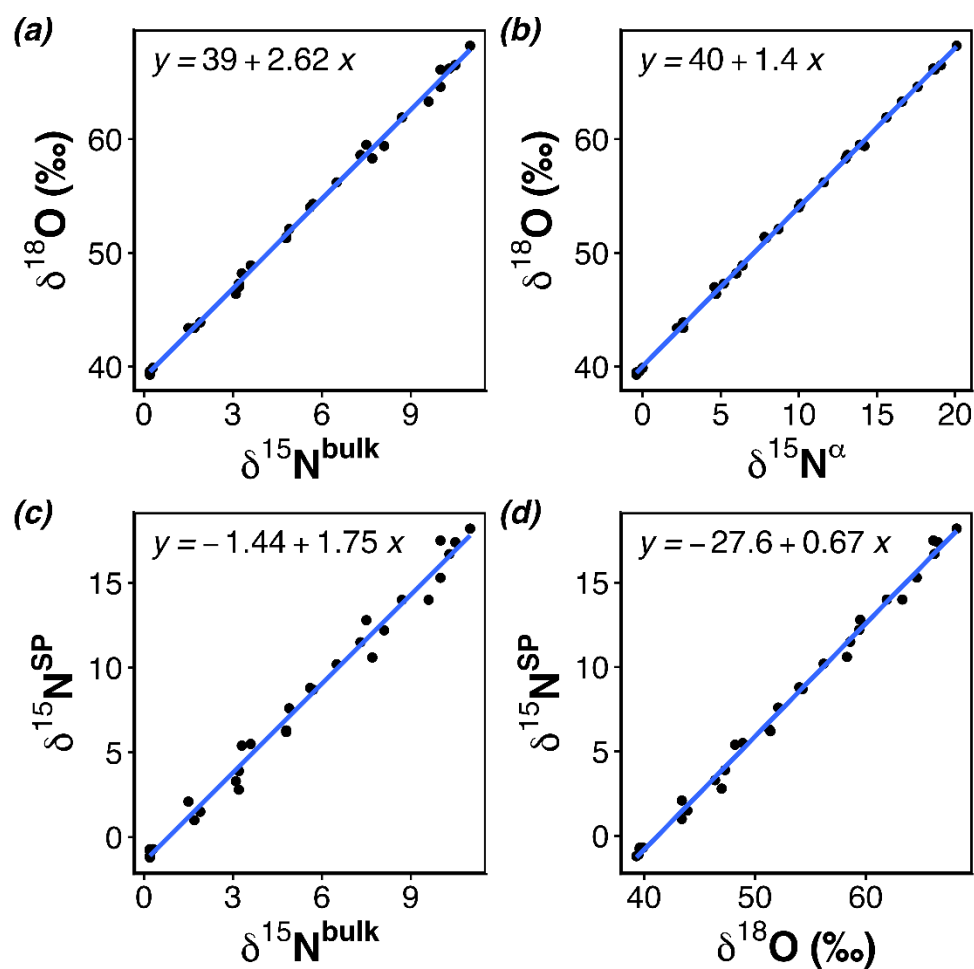


Figure S4: Dual isotope plots for oxygen and nitrogen δ values in residual N_2O during N_2O reduction by purified N_2OR .

a) $\delta^{18}\text{O}$ vs $\delta^{15}\text{N}^{\text{bulk}}$ and b) $\delta^{18}\text{O}$ vs $\delta^{15}\text{N}^{\alpha}$ c) $\delta^{15}\text{N}^{\text{SP}}$ vs $\delta^{15}\text{N}^{\text{bulk}}$ d) $\delta^{15}\text{N}^{\text{SP}}$ vs $\delta^{18}\text{O}$ measured during a one-hour N_2O consumption assay. The data from three replicates were combined. A linear fit of the data is shown in each plot.

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Table S7: N_2O reduction line δ/δ slopes for the various isotope maps shown in Figure S4.

Plot	Purified N_2OR
$\delta^{18}\text{O}/\delta^{15}\text{N}^{\text{bulk}}$	2.62
$\delta^{18}\text{O}/\delta^{15}\text{N}^{\alpha}$	1.40
$\delta^{15}\text{N}^{\text{SP}}/\delta^{15}\text{N}^{\text{bulk}}$	1.75
$\delta^{15}\text{N}^{\text{SP}}/\delta^{18}\text{O}$	0.67

110 **References**

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