



Determination of the Kinetic Isotope Effects for Nitrous Oxide Reduction by Purified Form II Nitrous Oxide Reductase

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Abstract. Mitigating the release of the greenhouse gas nitrous oxide (N₂O) is a major focus of sustainable agricultural practices. Currently, application of nitrogen-based fertilizers to the field drives significant microbial N₂O production. Understanding nitrogen flux through microbial metabolic processes, such as nitrification and denitrification, under different soil conditions is key to tracking sources of N₂O emissions in agricultural soils. Stable isotopes are a powerful tool for tracing sources and sinks of N₂O because isotopic preferences (*e.g.*, isotope effects) of key enzymes in nitrogen cycling pathways allow for the differentiation of microbial sources of N₂O. Ascertaining source contributions of microbial N₂O production can be complicated, however, by the consumption of N₂O by nitrous oxide reductase (N₂OR), which itself exhibits isotopic preferences that influence the isotopic composition of the remaining N₂O. To help provide the isotopic parameters required to quantify and distinguish sources and sinks of N₂O, we determined the kinetic isotope effects (KIEs) for N₂O consumption by purified form II N₂OR (resting state: Cu_Z^{*}) from *Stutzerimonas stutzeri*. The enrichment factors (ϵ) were $-5.4 \pm 0.2\%$, $-10.0 \pm 0.4\%$, $-0.6 \pm 0.1\%$, $-9.5 \pm 0.4\%$, and $-13.5 \pm 0.5\%$ for $\epsilon^{15}\text{N}^{\text{bulk}}$, $\epsilon^{15}\text{N}^{\alpha}$, $\epsilon^{15}\text{N}^{\beta}$, $\epsilon^{15}\text{N}^{\text{SP}}$, and $\epsilon^{18}\text{O}$, respectively. Thus, the kinetic isotope effects were normal for bulk $\delta^{15}\text{N}$ (1.0054 ± 0.0002), $\delta^{15}\text{N}^{\alpha}$ (1.0101 ± 0.0004), and $\delta^{15}\text{N}^{\text{SP}}$ (1.0095 ± 0.0004), and $\delta^{18}\text{O}$ (1.0137 ± 0.0005). The KIE for $\delta^{15}\text{N}^{\beta}$ was 1.0006 ± 0.0001 , indicating there is essentially no isotopic preference for this position. These data improve our understanding of the isotopic fractionation associated with enzymatic N₂O consumption, which will inform modeling of nitrogen flux pathways leading to N₂O emissions in soil microbial communities.



1 Introduction

N_2O is a greenhouse gas with a global warming potential ~300 times greater than carbon dioxide on a 100-year time scale (Solomon et al., 2007; Stocker et al., 2013). Additionally, N_2O poses a serious concern for stratospheric ozone depletion (Ravishankara et al., 2009). Anthropogenic sources account for 40% of global N_2O emissions, with a large portion coming from agricultural soils (Solomon et al., 2007; Stocker et al., 2013; Tian et al., 2020). The invention of the Haber-Bosch process and the subsequent implementation of nitrogen-based fertilizers in agricultural practices has increased atmospheric N_2O emissions by increasing inorganic nitrogen available for microbial metabolism in soil. Therefore, N_2O emission mitigation strategies typically focus on soil nitrogen management and decreasing the release of N_2O from agricultural practices. One barrier to mitigating N_2O emissions, however, has been fully understanding the sources and sinks of N_2O .

Biotic N_2O reduction in soil is an ideal target for mitigation of N_2O emissions (Caldeira et al., 2004; Ferousi et al., 2020). While there are many sources of N_2O in the soil, the most important of which are nitrification and denitrification, N_2O reduction serves as the only N_2O sink. Although the two-electron reduction of N_2O to N_2 is thermodynamically favorable, the relatively high activation energy of this reaction largely precludes abiotic N_2O consumption to make enzyme-catalyzed N_2O consumption the dominant process in soil systems (Ferousi et al., 2020). Nitrous oxide reductase (N_2OR), a copper-dependent enzyme, is the only enzyme known to reduce nitrous oxide as its primary function. The family of N_2OR proteins, encoded by the *nosZ* gene, is divided into Clade I, Clade II, and the recently identified Clade III (He et al., 2025; Orellana et al., 2014; Sanford et al., 2012). Although all three clades appear to be widespread in the soil, the conditions under which Clade II and Clade III N_2OR are ecologically significant are not yet fully defined (He et al., 2025; Shan et al., 2021). Clade I N_2OR is more commonly found in bacteria which encode genes for the complete denitrification pathway (Shan et al., 2021). Clade I N_2OR also appears to be more highly expressed than Clade II N_2OR in agricultural soil under anoxic, high nitrate conditions (Coyotzi et al., 2017). Essentially all biochemical studies of purified N_2OR have been performed with Clade I N_2OR . In particular, *Stutzerimonas stutzeri* (formerly *Pseudomonas stutzeri*) (Lalucat et al., 2022) has been a model species for the study of N_2OR since the discovery of this enzyme in the 1980s (Matsubara et al., 1982; Matsubara and Zumft, 1982; Zumft and Matsubara, 1982).

N_2OR is a functional dimer with two copper centers per monomer, Cu_A and Cu_Z , that appear to be conserved across all three clades (He et al., 2025; Sanford et al., 2012). Crystal structures of Clade I N_2OR show that



the monomers are arranged in a head-to-tail orientation which places the Cu_A site from one monomer 10 Å from the Cu_Z site of the second monomer, creating two catalytic units per dimeric protein (Pomowski et al., 2011). Cu_A is a binuclear copper center that transfers electrons to the Cu_Z active site (Dell'Acqua et al., 2012; Schneider and Einsle, 2016). Cu_Z is composed of a unique tetranuclear μ₄-sulfido-bridged copper center and is likely the site of N₂O binding and reduction (Pauleta et al., 2013; Pomowski et al., 2011; Rasmussen et al., 2000).

The Cu_Z site in purified N₂OR is typically in one of two spectroscopically distinct forms, Cu_Z [4Cu:2S] (form I) or Cu_Z* [4Cu:S] (form II) (Riester et al., 1989; Zhang et al., 2019). As-isolated, form I N₂OR is generally purple while form II N₂OR is usually pink. In both form I and II, one sulfide ligates all four copper ions while the second sulfide in the Cu_Z site ligates two of the copper ions from the Cu_Z center (Brown et al., 2000; Haltia et al., 2003; Paraskevopoulos et al., 2006; Pomowski et al., 2011, 2024; Zhang et al., 2019). Recently, it has also been proposed that the form II site may be a [4Cu:2S] center where one of the Cu-S bonds for the second sulfide has been lost due to a shift in the sulfide's position leading to a lower occupancy of this atom at the bridging position between two copper atoms (Carreira et al., 2020; Pomowski et al., 2024; Zhang et al., 2021). Many purifications show a mixture of the two Cu_Z forms, the ratio of which appears to be influenced by the amount of oxygen present during purification. Form I is dominant in anaerobic purifications and has been isolated and characterized in *S. stutzeri*, *Pseudomonas denitrificans*, *Achromobacter cycloclastes*, and *Marinobacter nauticus* (formerly *Pseudomonas nauticus* or *Marinobacter hydrocarbonoclasticus*) (Johnston et al., 2014; Pauleta et al., 2013; Snyder and Hollocher, 1987). The fraction of form II increases when N₂OR is purified aerobically and has been studied in *Pseudomonas pantotrophus* and *M. nauticus* (Dell'Acqua et al., 2012; Pauleta et al., 2013; Rasmussen et al., 2002). Form II has also been observed in *S. stutzeri* N₂OR following both native expression (Coyle et al., 1985) and heterologous expression in *Escherichia coli* (*E. coli*) (Zhang et al., 2019), although the majority of studies on *S. stutzeri* N₂OR have been conducted with form I.

The two forms of the Cu_Z site in purified N₂OR have markedly different activities. The activity measured for purified form I N₂OR is very low, suggesting that any catalytic mechanism involving the redox states accessible for this form may be too slow to be physiologically relevant *in vivo* (Coyle et al., 1985; Dell'Acqua et al., 2008; Johnston et al., 2014; Zhang et al., 2019). Similarly, the as-isolated form II of N₂OR is in a [1Cu²⁺-3Cu⁺] state (Cu_Z*) with essentially no measurable activity. Incubation of form II N₂OR with reduced benzyl or methyl viologen, however, converts this copper center to a redox-active [4Cu⁺] state, causing activity to increase rapidly, *e.g.*, from



7.89 U/mg to 61.7 U/mg (with benzyl viologen) or 124.6 U/mg (with methyl viologen) (Chan et al., 2004), rates
 95 much more realistic for physiological needs (Chan et al., 2004; Ghosh et al., 2003; Johnson et al., 2016; Johnston et
 al., 2014, 2017; Pauleta et al., 2013; Rasmussen et al., 2002). The maximum rate for activated *M. nauticus* form II
 N₂OR is 6 orders of magnitude faster than *M. nauticus* form I N₂OR, indicating that form II N₂OR dominates in
 activity assays when pre-incubated with reduced methyl/benzyl viologen (Johnston et al., 2014). Overall, the
 physiological relevance of each form is still debated, and investigations into the differences in the catalytic
 100 mechanisms for form I and form II N₂OR are ongoing (Carreira et al., 2020; Pauleta et al., 2013; Pomowski et al.,
 2024; Zhang et al., 2021).

Stable isotope measurements are useful for studying the catalytic mechanisms of enzymes and for tracing
 sources and sinks of environmentally relevant compounds. Such studies are particularly useful when applied to N₂O,
 which has non-exchangeable central (α) and distal (β) nitrogen atoms. Different sources of N₂O can be distinguished
 105 due to the unique patterns of enrichment or depletion of the heavy isotope (¹⁵N or ¹⁸O) at each position in N₂O.
 These differences in isotopic fractionation are driven by the unique catalytic mechanisms of the enzymes from
 different microbial N₂O production pathways. Quantification of each source's contribution to N₂O emissions is often
 performed by comparing the isotopic signature of N₂O from field measurements to the signatures measured for each
 isolated process (e.g., N₂O production in a pure culture or from a purified enzyme). For example, site preference
 110 ($\delta^{15}\text{N}^{\text{SP}}$), the difference between $\delta^{15}\text{N}^{\alpha}$ and $\delta^{15}\text{N}^{\beta}$, can be used to distinguish N₂O produced by bacterial
 denitrification from N₂O produced by nitrification or fungal denitrification (Ostrom et al., 2007; Yu et al., 2020).
 Additionally, kinetic isotope effects, defined as the rate constant for the light isotope divided by the rate constant for
 the heavy isotope, can be used to constrain the impact of each enzymatic step in a specific pathway or to distinguish
 between enzymatic pathways.

115 An important and sometimes overlooked factor in N₂O source apportionment is N₂O consumption by
 N₂OR. Consumption by N₂OR will affect the isotopic signature of the residual (emitted) N₂O. Indeed, *in vivo*
 isotopic studies of denitrification in both pure cultures and in soil mesocosms indicated that the remaining N₂O
 became enriched at both the N ^{α} and O positions as N₂O was reduced to N₂ (Ostrom et al., 2007; Toyoda et al.,
 2017). Determining the kinetic isotope effects of N₂O reduction from *in vivo* measurements, however, can be
 120 difficult as the upstream steps in the denitrification pathway and the diffusion of N₂O into and out of cells can
 convolute the isotope effects associated with N₂O reduction. To ascertain the isotopic fractionation of N₂O due to



N₂O reduction, we quantified the kinetic isotope effects for N₂O reduction by purified form II N₂OR. Here we report the kinetic isotope effects (KIEs) for purified N₂O reductase for the first time. Form II N₂OR was chosen for these studies due to its high, stable turnover rate after incubation with dithionite-reduced methyl viologen and because it is hypothesized to be (or at least mimic) the physiologically relevant form of N₂OR (Ghosh et al., 2003; Johnston et al., 2014, 2017; Pauleta et al., 2013). These data will help improve quantification of N₂O reduction in the field and provide an important starting point for comparing the mechanisms of *in vitro* and *in vivo* N₂O consumption.

2 Materials and Methods

2.1 Cloning and culture maintenance

Stutzerimonas stutzeri ZoBell (ATCC 14405, formerly *Pseudomonas stutzeri* ZoBell) was cultured and maintained in Luria Broth (LB) at 37 °C (supplemented with 50 µg/mL kanamycin (Kan50) when necessary for plasmid selection/maintenance).

The plasmid pTet-NosZ-StrepII, which contains the Strep-tagged *nosZ* gene from *S. stutzeri* ZoBell under the control of the inducible pTet promoter, was constructed as follows. The pMVV103 plasmid (Addgene) (Venkataraman et al., 2023) encoding the pTet promoter and the TetR repressor protein was amplified and linearized by PCR using primers that excluded the sequence for superfolder GFP (see Table S1). The *S. stutzeri* ZoBell *nosZ* gene containing a C-terminal StrepII tag (Twist Biosciences) was cloned into the linearized pMVV103 backbone via Gibson assembly. The entire non-purified Gibson assembly reaction (~30 µL) was transformed into chemically competent DH5α *E. coli* via heat shock, and successful transformants were selected using LB Kan50. Purified pTet-NosZ-StrepII (5 µL of 20-30 ng/µL DNA) was transformed into *S. stutzeri* ZoBell cells by electroporation following the protocol from Venkataraman et al., 2023. The sequence of the entire plasmid (isolated both from DH5α *E. coli* and *S. stutzeri*) was verified by Oxford nanopore long-read sequencing (Plasmidsaurus). A portion (~37%) of the plasmid DNA isolated from *S. stutzeri* was present as dimers and higher-order concatemers.

2.2 Protein expression and purification

S. stutzeri ZoBell containing the pTet-NosZ-StrepII plasmid was streaked onto LB Kan50 plates (2% agar) and grown at 37 °C for 48 h. Single colonies were picked and grown in 10 mL LB Kan50 at 37 °C while shaking at 180 rpm for 16-20 h. Subsequent growths were performed in modified Asparagine Citrate medium (15.1 mM L-asparagine, 23.8 mM trisodium citrate dihydrate, 14.7 mM potassium phosphate monobasic, 8.11 mM magnesium



sulfate heptahydrate, 0.68 mM calcium chloride dihydrate, 11.8 mM sodium nitrate) containing Kan50 and 15 μ M
 150 CuCl_2 (Charnock et al., 2000; Coyle et al., 1985). Starter cultures (100 mL in 125 mL wide-mouth Erlenmeyer
 flasks) were inoculated with 1 mL of overnight culture, capped loosely with foil, and allowed to grow for 16 h at
 30 °C and 180 rpm to an optical density at 600 nm (OD_{600}) of approximately 1. Each 100-mL starter culture was
 used to inoculate a 1-L culture (in a 2-liter, wide-mouth Erlenmeyer flask capped loosely with foil) at a starting
 OD_{600} of 0.1. The 1-L cultures were incubated at 30 °C and 180 rpm until an OD_{600} of 0.5-0.8 was reached.
 155 Expression of Strep-tagged N_2OR (NosZ) from pTet-NosZ-StrepII was induced with 4.7 mL of 214 μ M
 anhydrotetracycline (aTc) (final concentration 1 μ M), and expression of the native *nos* operon (which includes
 proteins needed for N_2OR maturation) was induced with 10 mL of 20% sodium nitrate (*i.e.*, an additional 0.2%
 sodium nitrate was added the growth medium). Cultures were incubated at 30 °C and 120 rpm for 18 h and then
 harvested by centrifugation at 16,994 x g at 4 °C for 10 min. The cell pellet was flash frozen in liquid nitrogen and
 160 stored at -80 °C until purification.

All protein purification steps were performed at 4 °C. The buffer solutions were kept cold while the pH was
 adjusted. Cell pellets from 4 L of culture were combined to produce 12-15 g of cells (wet cell weight) and
 resuspended in 15 mL of Tris buffered saline (TBS, 100 mM Tris-HCl, pH 8.0, 150 mM NaCl). Cell suspensions
 were lysed 3 times via French press at 1,000 psi. The lysate was centrifuged at 100,000 x g for 1 h, and the
 165 supernatant was filtered through a 0.45 μ m syringe filter. Filtered lysate was diluted to 40 mL with TBS and
 immediately used for affinity purification to prevent protease degradation.

N_2OR was purified at 4 °C using a 5 mL Strep-Tactin XT affinity column (Cytiva, 2-5024-001). Lysate was
 applied to the column at a flow rate of 0.5 mL/min, followed by a wash with 12 column volumes of TBS at a flow
 rate of 1 mL/min. Strep-tagged N_2OR was eluted at a flow rate of 0.5 mL/min using 5 column volumes of 50 mM
 170 biotin dissolved in TBS. Elution of N_2OR was detected by monitoring UV-Vis absorbance at 280, 550, and 600 nm.
 Elution fractions with high absorbance at these wavelengths were collected and stored at 4 °C for further
 purification. Eluted protein was concentrated in a 10 kDa ultra centrifugal filter centrifuged at 3,000 rpm in 5-min
 intervals, with mixing between centrifugations to prevent precipitation. Concentrated protein (0.45 mL per run) was
 applied to a 24-mL size exclusion chromatography column (Superdex 200 Increase SEC column, Cytiva 28990944)
 175 equilibrated with TBS. The UV-Vis absorbance of the protein eluate was monitored at 280, 550, and 600 nm, and
 fractions containing N_2OR with high absorbance at 550 nm and 600 nm were collected and concentrated as



described above. Purified protein was stored for up to 4 days at 4 °C. For longer storage, N₂OR was flash frozen using liquid nitrogen and stored at –80 °C.

2.3 N₂OR characterization

180 2.3.1 SDS-PAGE and western blot analysis

S. stutzeri lysate and purified protein samples were combined with equal volumes of 2x Laemmli SDS sample buffer (100 mM Tris-HCl pH 6.8, 200 mM dithiothreitol, 4% SDS, 0.02% bromophenol blue, 20% glycerol). Samples were boiled at 100 °C for 10 min, and any precipitate was pelleted by centrifugation at 21,130 x g for 10 min. 20 µL of each sample was loaded onto a 10% SDS-PAGE gel alongside 7-10 µL of Precision Plus Protein Dual Color
 185 Standards (Bio-Rad, 1610374). SDS-PAGE gels were imaged via Bio-Rad imager following Coomassie staining (20 min, room temperature) and overnight destaining.

For western blot analysis, proteins were transferred to a polyvinylidene difluoride membrane at 60 V for 2 h at 4 °C. Membranes were blocked with 3% BSA prior to incubation with Strep-tag II Antibody HRP conjugate (Millipore, 71591-3) at 4 °C for 1 h. Antibody binding was detected using SuperSignal West Pico PLUS
 190 Chemiluminescent Substrate (Thermo Scientific, 34577).

2.3.2 Protein concentration and monomeric molecular weight determination

Protein concentration was determined using the theoretical extinction coefficient for reduced, monomeric Strep-tagged N₂OR ($\epsilon_{280} = 76,320 \text{ M}^{-1} \text{ cm}^{-1}$) calculated using the EMBOSS Pepstats tool (Rice et al., 2000). The molecular weight of intact, monomeric N₂OR was determined by electrospray ionization mass spectrometry using a Waters
 195 Xevo G2-XS quadrupole time of flight mass spectrometer interfaced with a Waters Acquity ultra-performance liquid chromatograph (UPLC) at the Mass Spectrometry and Metabolomics Core at Michigan State University (East Lansing, MI).

2.3.3 ICP-OES

Copper loading of purified N₂OR was measured using inductively coupled plasma – optical emission spectroscopy
 200 (ICP-OES) (Agilent 5800). Samples for ICP-OES were prepared by diluting purified N₂OR in 2% (v/v) nitric acid, with a minimum estimated copper concentration of 0.1 ppm in a 3 mL sample (assuming a conservative 4:1 Cu/monomer molar ratio). Each sample was analyzed three times (three technical replicates) by ICP-OES. Copper concentrations in purified protein samples were calculated from a standard curve prepared using CuCl₂ standards at concentrations ranging from 0.05 ppm to 5 ppm.



205 2.3.4 Optical spectra

Metalation of the Cu_A and Cu_Z/Cu_Z* sites (*i.e.*, form I and form II Cu_Z) was assessed in an anaerobic chamber (Coy Laboratory Products) by optical spectroscopy (350-850 nm) (Jenway 7200) using deoxygenated protein samples and buffers (see below). Purified N₂OR (~30 μM) was oxidized by sequential additions of potassium ferricyanide, blanking between additions to remove any absorbance interference from ferricyanide. The Cu_A site was reduced via sequential additions of sodium ascorbate. Finally, the Cu_Z site was reduced by sequential additions of sodium dithionite. Absorption peaks were compared to literature values (Carreira et al., 2020; Coyle et al., 1985; Farrar et al., 1998; Pauleta et al., 2013; Riestner et al., 1989; Zhang et al., 2019).

2.4 Enzymatic assays

2.4.1 Deoxygenating solutions and purified enzyme samples

215 Solutions and purified N₂OR samples were deoxygenated in an anaerobic chamber (Coy Laboratory Products) with the atmosphere maintained at ~98% N₂, ~2% H₂. A small volume of concentrated protein (~0.2 mL) was deoxygenated via three intervals of mixing the sample by pipetting 50 times every 5 min, with the sample tube left open in between mixing to allow for equilibration with the gas in the anaerobic chamber. Solutions were deoxygenated by bubbling with ultra-high purity (UHP) N₂, followed by stirring overnight in the anaerobic chamber.

220 2.4.2 Indirect activity assays

Specific activity of N₂OR was measured by following the oxidation of methyl viologen (Kristjansson and Hollocher, 1980) monitored via optical spectroscopy using an HP 8453 UV-Vis spectrophotometer set to Kinetics mode. To increase enzymatic activity, deoxygenated N₂OR (0.01-0.3 μg/μL) was incubated with 0.14 mM methyl viologen and 0.14 mM sodium dithionite for 2 h at room temperature in the anaerobic chamber. Then, 5 μg of pre-reduced N₂OR was added to a 3-mL screw-cap cuvette containing 2 mL of 0.1 mM methyl viologen and 0.1 mM sodium dithionite in anaerobic 10 mM potassium phosphate buffer at pH 8.5 (Zhang et al., 2019). N₂O-saturated water was prepared by bubbling deoxygenated, anaerobic water in a sealed 56 mL serum bottle with N₂O gas (atomic absorption grade) at a high flow rate for 2 h. To sparge the water with N₂O, a stainless-steel inlet needle was inserted through the bottle's septum into the water while an outlet needle was inserted through the septum into the headspace. The stability of reduced methyl viologen in the reaction cuvette was assessed by monitoring the absorbance at 600 nm for 2 min to ensure that no oxidation occurred without substrate. To initiate the reaction, N₂O-saturated water was added to the reaction cuvette via gastight syringe at volumes of 0.5 μL to 50 μL, followed by



fast inversion of the cuvette. Reaction progress was monitored at 600 nm for 5 min. All reactions were performed in triplicate for each substrate concentration. Specific activity was calculated from the initial slope of each reaction using the extinction coefficient for reduced methyl viologen ($\epsilon_{600} = 13.7 \text{ mM}^{-1} \text{ cm}^{-1}$) (Campeciño et al., 2020; Watanabe and Honda, 1982) and a 2:1 stoichiometric ratio of reduced methyl viologen to N_2O . The solubility of N_2O -saturated water was assumed to be 25 mM at 25 °C (Weiss and Price, 1980).

2.4.3 Gas chromatography activity assays

Gas chromatography assays were used to directly measure consumption of N_2O gas by N_2OR . A 160-mL serum bottle was sealed in an anaerobic chamber (Coy Laboratory Products; ~98% N_2 , ~2% H_2) and then sparged with N_2O gas (atomic absorption grade) for 1 h. Following sparging, the exit needle was removed first to slightly overpressure the bottle. A separate 160-mL reaction bottle was prepared by adding 5.6 mL of 1.39 mM methyl viologen and 1.39 mM sodium dithionite in deoxygenated 10 mM potassium phosphate buffer at pH 8.5. The reaction bottle was sealed and removed from the anaerobic chamber, and 73 μL of N_2O gas (approximately 4 μmol) was transferred from the serum bottle filled with pure N_2O to the reaction bottle using a gastight syringe. The reaction bottle was shaken for 2 h to allow the N_2O gas to equilibrate with the buffer. After the 2-h incubation, the initial N_2O concentration and isotopic composition were determined by sampling the headspace of the reaction bottle in duplicate for gas chromatography - electron capture detector (GC-ECD) analysis (0.1 mL) and gas chromatography - isotope ratio mass spectrometry (GC-IRMS) measurements (0.5 mL).

In a separate vial, purified N_2OR (16 $\mu\text{g/mL}$) was pre-reduced by incubating the deoxygenated enzyme with 0.14 mM methyl viologen and 0.14 mM sodium dithionite for 2 h as described above. Pre-reduced N_2OR (5 mL) was then transferred to a 15-mL serum bottle, and the bottle was sealed before being removed from the anaerobic chamber. The reaction was initiated by the addition of 70 μg (4.4 mL) of pre-reduced enzyme to the reaction bottle using a gastight syringe for a final volume of 10 mL of solution and final reductant concentrations of 0.84 mM methyl viologen and 0.84 mM sodium dithionite. Excess reductant concentrations were used to drive the N_2O reduction to completion. Control reactions without enzyme were initiated by adding an equivalent volume of anaerobic buffer with 0.14 mM methyl viologen and 0.14 mM sodium dithionite to reaction bottles with N_2O . After enzyme (or buffer) addition (timepoint zero), the reaction bottle's headspace was sampled for analysis by GC-ECD and then for GC-IRMS every 5 min for 45 min using gastight syringes. Final GC-ECD samples were collected after 60 min of reaction. Prior to sampling, syringes were flushed with UHP N_2 , and a volume of pure UHP N_2 gas equal



to the sample volume was added to the reaction bottle to keep the reaction bottle at atmospheric pressure. Samples for GC-ECD analysis (0.1 mL or 0.3 mL) were transferred to 12-mL Exetainer vials sparged with UHP N₂. Samples for GC-IRMS analysis (0.5 mL to 5.0 mL) were transferred to 38-mL serum bottles (sealed in the anaerobic chamber). For GC-IRMS samples, the gas in the syringe was allowed to equilibrate with the gas in the reaction
 265 bottle for 2 min prior to sample removal. The reaction bottle was shaken vigorously on a rotating platform between sample collection time points to ensure N₂O diffusion between the headspace and solution did not limit the overall reaction rate. Additionally, during GC-IRMS sample collection, the reaction bottle was shaken more slowly on a rocking platform (VWR Standard Analog Rocker) while the syringe inserted through the septum was held in place, allowing us to maintain constant shaking during the 2-min sample equilibration. All reactions were performed at 22-
 270 23.5 °C. Reactions with enzyme were performed in triplicate using the same batch of purified enzyme. No-enzyme control reactions were also performed in triplicate.

2.4.4 Gas chromatography measurements

GC-ECD analysis was used to determine N₂O concentrations using the samples of headspace collected from reaction bottles (see above). Prior to GC-ECD analysis, each 12-mL Exetainer vial containing 0.1-0.3 mL of reaction bottle
 275 headspace was over-pressured by the addition of 2 mL of UHP N₂ with a gastight syringe. One mL of gas from the Exetainer vial was injected into a gas chromatograph (GC-2014 Shimadzu) equipped with an autoinjector (AOC-6000 PLUS PAL RSI System) and an electron capture detector (ECD) (Shimadzu). GC-ECD method settings included argon carrier gas at a flow rate of 15.56 mL/min, with a 1 m HayeSep T column, a GC oven temperature of 80 °C and ECD temperature of 325 °C. Sample analysis time was 20 min. N₂O standards (0.453-6.730 ppm) were
 280 prepared by diluting pure N₂O (atomic absorption grade) with UHP N₂. N₂O concentrations were calculated from the integration of the N₂O peak at 10.8 min using a standard curve prepared by linear or polynomial regression of known N₂O standards ($R^2 = 0.989-0.995$). Calculation of total N₂O in the reaction bottle accounted for the N₂O removed during previous sampling for GC-ECD.

For all reaction bottles with and without enzyme, samples for IRMS were collected alongside the GC-ECD
 285 samples. The concentrations determined by GC-ECD were used to ascertain N₂O concentrations in the samples taken for GC-IRMS (see below). Isotope values from N₂O reactions and no-enzyme controls were analyzed using an Elementar Greenhouse gas analyzer equipped with a Nafion water trap, an EAS Carbosorber CO₂ trap, and a Restek Q-Bond Plot 30 m 0.32 mm column coupled to an IRMS (isoprime precisION). IRMS ion source settings



were optimized for precisions better than the following: 0.3‰ for $\delta^{15}\text{N}$, 0.5‰ for $\delta^{15}\text{N}^{\alpha}$, 0.5‰ for $\delta^{15}\text{N}^{\beta}$, 0.3‰ for
 290 $\delta^{18}\text{O}$, and 0.8‰ for $\delta^{15}\text{N}^{\text{SP}}$. The mass/charge (m/z) ratios used to calculate δ values of N_2O for $^{15}\text{N}^{\text{bulk}}$, $^{15}\text{N}^{\alpha}$, and ^{18}O
 were 45:44, 31:30, and 46:44, respectively. Raw δ values were corrected using the δ values measured for N_2O
 standards prepared from 2 distinct N_2O gas tanks (B and C) of known isotopic composition by performing multiple
 regression with JMP statistical software to adjust for compression and linearity effects. The standards from tanks B
 and C were analyzed at two different N_2O concentrations (5 nmol and 33 nmol) bracketing the expected sample
 295 concentrations, and the regression model was validated using a mixture of tanks B and C at an intermediate
 concentration. Tanks B and C were calibrated against USGS51 (1.32‰ $\delta^{15}\text{N}$, 0.48‰ $\delta^{15}\text{N}^{\alpha}$, 2.15 $\delta^{15}\text{N}^{\beta}$, 41.23‰
 $\delta^{18}\text{O}$, -1.67‰ $\delta^{15}\text{N}^{\text{SP}}$) and USGS52 (0.44‰ $\delta^{15}\text{N}$, 13.52‰ $\delta^{15}\text{N}^{\alpha}$, -12.64‰ $\delta^{15}\text{N}^{\beta}$, 40.64‰ $\delta^{18}\text{O}$, 26.15‰ $\delta^{15}\text{N}^{\text{SP}}$)
 (Ostrom et al., 2018). Tank B contained δ values of -0.7‰ $\delta^{15}\text{N}$, 11.5‰ $\delta^{15}\text{N}^{\alpha}$, -12.9‰ $\delta^{15}\text{N}^{\beta}$, 40.2‰ $\delta^{18}\text{O}$, and tank
 C contained δ values of -0.8‰ $\delta^{15}\text{N}$, -1.1‰ $\delta^{15}\text{N}^{\alpha}$, -0.4‰ $\delta^{15}\text{N}^{\beta}$, 39.2‰ $\delta^{18}\text{O}$. Tanks B and C harbored N_2O with
 300 $\delta^{15}\text{N}^{\text{SP}}$ values of 24.4‰ and -0.7‰, respectively. Standards prepared from tanks B and/or C were analyzed daily to
 verify instrument stability. All isotope values are reported with respect to the following international standards:
 atmospheric N_2 (air) for nitrogen isotope values, and Vienna Standard Mean Ocean Water (VSMOW) for oxygen
 isotope values.

2.5 Calculations

305 2.5.1 Calculating f

The fraction of substrate remaining, f , is defined as follows:

$$f = \frac{n_s}{n_{s0}} \quad (1)$$

where n_s is μmol of N_2O at time t and n_{s0} is the initial μmol of N_2O in a given reaction bottle. Because n_{s0} varied
 slightly among reaction bottles (presumably due to pressure variations for the injected N_2O), the values of n_{s0} and n_s
 310 were calculated separately for each reaction bottle. Each n_{s0} value was determined by averaging the amount of N_2O
 measured by GC-ECD prior to reaction initiation (measured in duplicate for each reaction bottle). The values of n_s
 for the IRMS samples from reaction bottles with enzyme were calculated using an exponential decay model with the
 following general form:

$$n_s = n_{s0} e^{-kt} \quad (2)$$

315 Equation (2) describes N_2O consumption as a first-order process with respect to N_2O , where k is the rate constant in
 min^{-1} . The value of k was determined for each reaction bottle by fitting Eq. (2) to the amounts of N_2O (μmol)



measured by GC-ECD (n_s) and corresponding GC-ECD sampling times (t) (minutes) via nonlinear least squares regression following the method described by Baty et al., with starting values of $n_{s0} = 4$ and $k = 0.04$ (Baty et al., 2015). The final value of k determined by fitting the GC-ECD data to the exponential decay model (Eq. (2)) was then combined with the value of n_{s0} measured by GC-ECD to determine n_s for each IRMS sample taken at time t . The value of n_{s0} was also predicted by nonlinear least squares regression, but the fitted n_{s0} values underestimated the measured values and thus were not used in the final calculations. After the f values for each individual replicate with enzyme were calculated, normalizing the amount of N_2O in each bottle to the fraction of substrate remaining, the isotopic data from all three replicates were pooled. The pooled data were used to calculate isotope effects ($\epsilon_{p/s}$, $\alpha_{p/s}$, and KIE).

2.5.2 Isotope nomenclature and equations

An isotopic ratio (R) refers to the ratio of the abundance of heavy isotope to the abundance of light isotope.

$$R = \frac{\text{heavy isotope}}{\text{light isotope}} \quad (3)$$

In this study, the isotopic ratios of the nitrogen atoms ($^{15}N/^{14}N$) and oxygen atom ($^{18}O/^{16}O$) in N_2O were measured by GC-IRMS as described above. These isotopic ratios are reported as per mil (‰) δ values

$$\delta_{\text{sample}} = \left(\frac{R_{\text{sample}}}{R_{\text{standard}}} - 1 \right) \times 1000 \quad (4)$$

where R_{sample} is the isotope ratio of the sample and R_{standard} is the isotope ratio of an international reference material. For nitrogen isotopes, R_{standard} is the isotopic ratio ($^{15}N/^{14}N$) of atmospheric N_2 (air) (0.0036765) (Junk and Svec, 1958; Skrzypek and Dunn, 2020), and for oxygen isotopes, R_{standard} is the isotopic ratio ($^{18}O/^{16}O$) for VSMOW (0.00200520) (Skrzypek and Dunn, 2020; Werner and Brand, 2001).

We define the isotopic fractionation factor, $\alpha_{p/s}$, for an irreversible chemical reaction converting substrate (s) to product (p) in a closed system following the convention of Mariotti, et al., 1981

$$\alpha_{p/s} = \frac{R_{pi}}{R_s} \quad (5)$$

where R_{pi} refers to the isotopic ratio of instantaneously produced product and R_s refers to the isotopic ratio of remaining substrate at the same instant (Hayes, 2004; Mariotti et al., 1981). When the fractionation factor is defined in this way, it is equal to the inverse of the kinetic isotope effect (KIE) (Mariotti et al., 1981). KIE is defined as the ratio of the rate constant of the light isotope to the rate constant of the heavy isotope ($KIE = k_L/k_H$).

Both $\alpha_{p/s}$ and KIE are calculated using the per mil enrichment factor, $\epsilon_{p/s}$.



$$\varepsilon_{p/s} = (\alpha_{p/s} - 1) \times 1000 \quad (6)$$

345 $KIE = \frac{1}{\alpha_{p/s}} \quad (7)$

For experiments where the substrate δ values (δ_s) are monitored over time (as opposed to the product δ values), $\varepsilon_{p/s}$ is typically calculated using Mariotti's approximation of the Rayleigh equation for substrate:

$$\delta_s = \delta_{s0} + \varepsilon_{p/s} \times \ln(f) \quad (8)$$

In Eq. (8), δ_s is the substrate δ value at time t , δ_{s0} is the initial substrate δ value (at $t = 0$), and f is the fraction of substrate remaining at time t (Eq. 1). Linear regression of δ_s on $\ln(f)$ yields $\varepsilon_{p/s}$ as the slope and δ_{s0} as the intercept. This version of the Rayleigh equation is most accurate when the abundance of the rare isotope is much smaller than that of the non-rare isotope and the values of δ_{s0} and $\varepsilon_{p/s}$ are relatively small (*i.e.*, relatively close to 0‰) (Mariotti et al., 1981). For our studies, which were performed with unlabeled N_2O , the first assumption was met. The δ_{s0} and ε values for ^{18}O - N_2O were large enough, however, to call the second assumption into question. Therefore, we compared three different methods for calculating $\varepsilon_{p/s}$.

- 1) Calculate $\varepsilon_{p/s}$ using Mariotti's approximation of the Rayleigh equation (Eq. (8)) and all of our available isotopic data ($f = 1$ to $f = 0.13$).
- 2) Calculate $\varepsilon_{p/s}$ using Mariotti's approximation of the Rayleigh equation (Eq. (8)), using only δ values from the earlier part of the reaction ($f = 1$ to $f = 0.4$). Hayes (2004) noted that the error introduced by Eq. (8) increases significantly after ~60% of substrate has been consumed, so excluding data where $f < 0.4$ may minimize this error (Hayes, 2004).
- 3) Calculate $\varepsilon_{p/s}$ using the Rayleigh model without approximation using all of our available isotopic data ($f = 1$ to $f = 0.13$) (Eq. (9)) (Hayes, 2004).

$$\ln\left(\frac{R_s}{R_{s0}}\right) = \varepsilon_{p/s} \times \ln\left(f \times \frac{1 + R_{s0}}{1 + R_s}\right) \quad (9)$$

365 In Eq. (9), R_s is the isotopic ratio of remaining substrate at time t , R_{s0} is the isotopic ratio of initial substrate, and $\varepsilon_{p/s}$ is in the dimensionless form instead of the per mil form. (To convert $\varepsilon_{p/s}$ to the per mil form, dimensionless $\varepsilon_{p/s}$ is multiplied by 1000). Like Eq. (8), Eq. (9) is a linear model where $\varepsilon_{p/s}$ is the slope, allowing $\varepsilon_{p/s}$ to be determined by linear regression of $\ln(R_s/R_{s0})$ on $\ln[f^*(1+R_{s0})/(1+R_s)]$. Unlike Eq. (8), the value of R_{s0} must be known independently to determine $\varepsilon_{p/s}$ using Eq. (9). In our case, R_{s0} was easily determined by averaging the δ_{s0} values measured for N_2O from all six reaction bottles (three replicates with enzyme and three control replicates without enzyme).



Site preference ($\delta^{15}\text{N}^{\text{SP}}$) for N_2O is defined as the difference between $\delta^{15}\text{N}^{\alpha}$ and $\delta^{15}\text{N}^{\beta}$.

$$\delta^{15}\text{N}^{\text{SP}} = \delta^{15}\text{N}^{\alpha} - \delta^{15}\text{N}^{\beta} \quad (10)$$

2.5.3 Modeling and figures

Modeling was performed with R statistical software (R Core Team, 2025), and plots were produced using ggplot2 (Wickham, 2016). Microsoft Copilot was used to prepare the initial version of the R script used to plot Fig. 4 and Fig. S4. This code was subsequently validated and modified as appropriate.

3 Results

3.1 Purification and identification

The *nos* gene cluster contains extensive assembly and maturation machinery (in the form of *nosRZDFYL* for *S. stutzeri*) that is necessary for efficient metalation of N_2OR in both heterologous and native expression systems (Honisch and Zumft, 2003; Viebrock and Zumft, 1988; Wunsch et al., 2003; Zhang et al., 2019). Production of N_2OR was accomplished via overexpression of a Strep-II tagged *nosZ* gene in *S. stutzeri*, taking advantage of native maturation factors to metalate the enzyme with supplemental copper from the media (see Materials and Methods). Purification by size exclusion chromatography indicated that the majority of purified N_2OR was dimeric, as expected for a functional dimer. In SDS-PAGE gels and western blots, N_2OR consistently appeared as three bands: a major band between the molecular weight markers at 50 and 75 kDa (monomer, ~67 kDa) and two minor bands between the markers at 150 and 250 kDa (dimer, ~134 kDa) (Fig. S1).

Mass spectrometry analysis indicated that the majority of N_2OR in our purified sample had a monomeric molecular weight of $66,806 \pm 7$ Da (average of three biological replicates $\pm$ instrument error). This molecular weight is consistent with the expected molecular weight of 66,801 Da for Strep-tagged *S. stutzeri* N_2OR with an N-terminal pyroglutamic acid. A minor fraction of untagged N_2OR was also present, indicating that a small portion of native, untagged N_2OR dimerized with Strep-tagged N_2OR .

3.2 N_2OR active site characterization

The N_2OR dimer contains 2 copper sites, a dinuclear Cu_A site involved in electron transfer and a tetranuclear Cu_Z site, which require proper metal incorporation/coordination for enzymatic activity (Matsubara et al., 1982). *In vitro* activity has been observed in preparations of N_2OR with an average of at least 4 Cu/monomer (Zhang et al., 2019). Isolated protein was analyzed by ICP-OES for metal content (Table 1). Similar to heterologous overexpression of



N₂OR in *E. coli* (Zhang et al., 2019), N₂OR was not fully metalated by our expression system. Copper incorporation varied by growth (Table S2). The preparation of N₂OR used for isotopic analysis averaged 5.23 Cu/monomer of protein (Table 1).

Optical spectra of as-isolated, oxidized, and reduced N₂OR were analyzed to confirm proper copper coordination and to discern the form(s) of Cu_Z isolated (Fig. 1). In our preparations, the color of air-oxidized N₂OR isolated from *S. stutzeri* (blue, purple, or pink) varied across biological replicates, a variation which seemed to be at least partly dependent on the degree to which the Cu_A center was oxidized in each preparation (Pomowski et al., 2024). Fully oxidized Cu_A [Cu^{1.5+}-Cu^{1.5+}] has absorbance maxima at ~480, ~540, and ~800 nm, contributing to the pink/purple color of oxidized N₂OR (Pauleta et al., 2013). In the sample used for isotopic analyses, as-isolated N₂OR was purple and had absorbance maxima at 496, 537, 640, and 784 nm (Fig. 1). Upon ferricyanide addition, the peaks at 496, 537, and 784 nm increased, indicating that the Cu_A site was partially oxidized when purified and was fully oxidized by ferricyanide. Fully oxidized N₂OR had an $\epsilon_{538\text{nm}}$ of 5 mM⁻¹•cm⁻¹, matching the reported extinction coefficient for aerobically purified *S. stutzeri* N₂OR from *E. coli* (Zhang et al., 2019). Overall, the ferricyanide-oxidized spectrum for our Strep-tagged *S. stutzeri* N₂OR resembles other air-oxidized N₂OR preparations which are known to be predominantly form II N₂OR (Coyle et al., 1985; Paraskevopoulos et al., 2006).

The Cu_A site can be selectively reduced from the mixed valence state to the [Cu¹⁺-Cu¹⁺] state with ascorbate (a mild reductant), causing the Cu_A absorbance peaks to disappear and revealing the peaks associated with Cu_Z and/or Cu_Z* (Pauleta et al., 2013). For form I (Cu_Z) N₂OR, reduction of Cu_A reveals two absorbance peaks for the Cu_Z [4Cu:2S] site at ~552 nm and ~660 nm (Pomowski et al., 2011; Zhang et al., 2019). The form II Cu_Z site, on the other hand, is isolated in an oxidized state [1Cu²⁺-3Cu⁺] known as Cu_Z* characterized by one absorbance band at ~650 nm that is unaffected by the addition of ascorbate or dithionite (Coyle et al., 1985; Johnston et al., 2014; Zhang et al., 2019). When sodium ascorbate was added to our purified N₂OR, the peaks at 485, 536, and 784 nm decreased, and the peak at 636 nm became more prominent, indicating that the Cu_A site was reduced (Fig. 1, maroon spectrum). Note that for the ascorbate-reduced spectrum shown in Fig. 1, N₂OR still showed a slight absorbance at ~480 and ~780 nm, indicating the Cu_A center was not fully reduced in this experiment. Thus, the small peak at 536 nm that remained in our ascorbate-reduced spectrum was likely due to a combination of absorbance from the Cu_Z site and the residual mixed-valent Cu_A site. The band at 636 nm in our ascorbate-reduced spectrum could be due to absorbance from the reduced Cu_Z site or the oxidized (form II) Cu_Z* site. However, the prominence of this



absorbance in the air-oxidized, the ferricyanide-oxidized, and the ascorbate-reduced samples suggests that it predominantly arises from the Cu_Z^* site (which is not reduced by ascorbate).

Prolonged incubation with dithionite shifts the Cu_Z (form II) absorbance to a single band at ~ 650 nm (Zhang et al., 2019) or ~ 640 nm (Prudêncio et al., 2000). In our experiments, reduction with sodium dithionite revealed a single peak at 640 nm as the Cu_A absorbance maxima at 536 nm and 485 nm disappeared. The broadening of the 640 nm peak in the 640 – 680 nm region is likely due to absorbance contributions from the reduced Cu_Z (form I) center overlapping with the Cu_Z^* form (form II), resulting in a red shift in the absorbance. Thus, our reduced spectra indicate that our aerobic purification process isolated a mixture of form I (Cu_Z) and form II (Cu_Z^*) N_2OR , a well-documented phenomenon for this enzyme (Coyle et al., 1985; Riester et al., 1989).

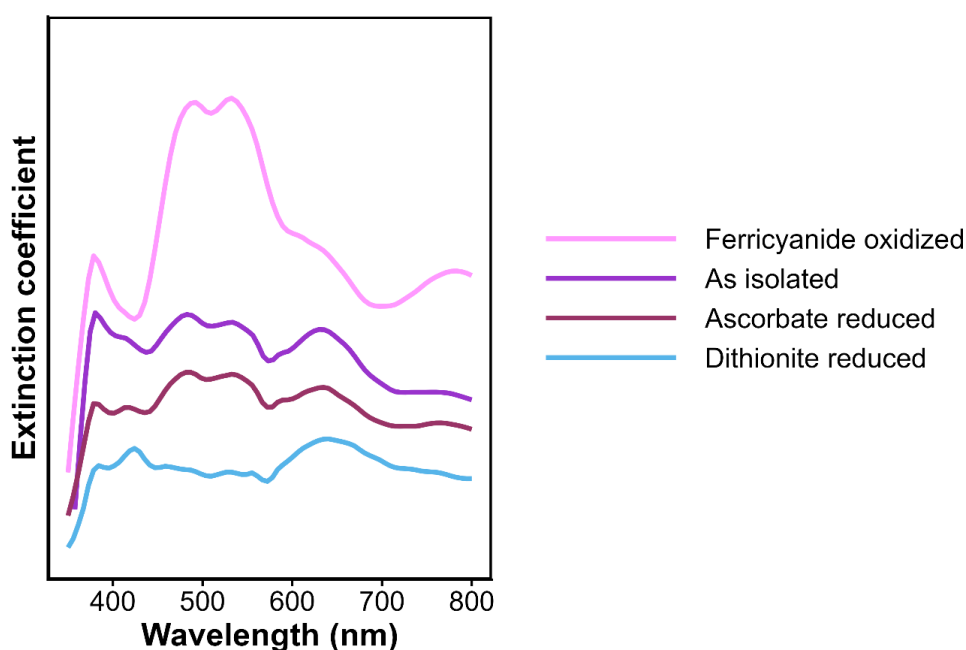


Figure 1: Stacked optical spectra of N_2OR at different oxidation states.

Purified N_2OR ($31 \mu\text{M}$) was analyzed to assess its Cu_A , Cu_Z , and Cu_Z^* content by UV-Vis spectroscopy. Spectra were collected for potassium ferricyanide-oxidized (pink), as-isolated (air-oxidized, purple), sodium ascorbate-reduced (maroon), and sodium dithionite-reduced (blue) samples. The spectra are stacked to highlight the differences in the spectra as N_2OR was increasingly oxidized (most reduced on the bottom). See Results section 3.2 for specific absorbance maxima.



Table 1: Characteristics of purified *S. stutzeri* N₂OR used for isotopic studies.

$\mu\text{mol Cu}/\mu\text{mol monomer}^a$	Ferricyanide-oxidized peaks (nm) ^b	Ascorbate-reduced peaks (nm) ^b	Dithionite-reduced peaks (nm) ^b	Specific activity ^c
5.23 ± 0.11	496, 537, 640, 784	485, 536, 636	640	28.2 ± 1.3

^a Metal content was analyzed in triplicate by ICP-OES for copper at 327 nm. Results are reported as average $\mu\text{mol Cu}$ per μmol of N₂OR monomer.

^b UV-Vis absorbance maxima were determined using the spectra in Fig. 1, which shows the optical absorbance of N₂OR when oxidized with ferricyanide, then reduced with sodium ascorbate, and finally reduced by sodium dithionite.

^c Specific activity was obtained from methyl viologen oxidation assays performed at an aqueous N₂O concentration of 125 μM . Specific activity is reported as $\mu\text{mol}/\text{min}/\text{mg}$ of N₂OR monomer.

3.3 Enzyme activity

N₂OR specific activity was measured indirectly using the oxidation of methyl viologen. Reaction progress was monitored by the disappearance of the 600 nm absorbance peak of reduced methyl viologen as N₂OR reduces N₂O and in turn oxidizes methyl viologen. As expected, specific activity of purified N₂OR was highly dependent on the extent of copper loading; N₂OR preparations with higher Cu/monomer molar ratios had higher specific activity (data not shown). Normalization of specific activity to Cu loading accounted for the majority of differences in specific activity across biological replicates (data not shown). Figure S2 shows the average turnover rate of *S. stutzeri* purified N₂OR containing 5.23 Cu/monomer at various N₂O concentrations. The Michaelis-Menten plots from different biological replicates showed that N₂OR has maximum activity once substrate concentration reaches approximately 100 μM . To compare different reaction conditions, specific activity was measured at a substrate concentration of 125 μM N₂O (Table 1, Fig. S2).

Specific activity increased rapidly with incubation time in reduced methyl viologen (Table S2 and S3), similar to reports in the literature for form II N₂OR from *M. nauticus* (Ghosh et al., 2003; Johnston et al., 2014; Prudêncio et al., 2000). Enzymatic rates for *S. stutzeri* N₂OR were found to plateau at 2 h of incubation with methyl viologen and decline after 5 h (Table S3). Without pre-incubation, the enzymatic rate was found to be 3.0 $\mu\text{mol}/\text{min}/\text{mg}$ N₂OR, about $\sim 1/38^{\text{th}}$ of the maximum activity of pre-incubated N₂OR at the same concentration of substrate (Table S3). This substantial increase in specific activity after incubation with dithionite-reduced methyl viologen is attributed to Cu₂* activation, strongly suggesting that the majority of the activity we observed was due to form II N₂OR (Chan et al., 2004; Johnston et al., 2014; Prudêncio et al., 2000). All subsequent enzyme activity assays were performed with N₂OR that had been pre-reduced for 2 h to obtain efficient, reproducible turnover. Using pre-reduced enzyme, specific activity was measured over a range of N₂O concentrations (Fig. S2), revealing a



specific activity of 28.2 ± 1.3 $\mu\text{mol}/\text{min}/\text{mg}$ N_2OR monomer at 125 μM N_2O for the purified N_2OR used for isotopic studies, which had ~ 5 μmol copper/ μmol monomer (Table 1). These rates are similar to published rates for pre-reduced form II N_2OR ($23 - 55$ $\mu\text{mol}/\text{min}/\text{mg}$ N_2OR) in preparations with a mixture of form I (Cu_Z) and form II (Cu_Z^*) N_2OR (Chan et al., 2004; Johnston et al., 2014; Prudêncio et al., 2000).

3.4 Gas chromatography kinetics

Enzymatic N_2O consumption was monitored directly by GC-ECD and GC-IRMS as described above in the Materials and Methods section. With a three-fold excess of reductant present, pre-reduced N_2OR consumed nearly all (91-92%) of the N_2O present in the reaction bottle within an hour (Fig. 2). Enzymatic N_2O consumption was highly reproducible, showing exponential decay of N_2O with a rate constant of 0.041 ± 0.001 min^{-1} . In contrast, no decrease in N_2O concentrations was observed for any of the three control reactions without enzyme (Fig. 2, “No Enzyme”).

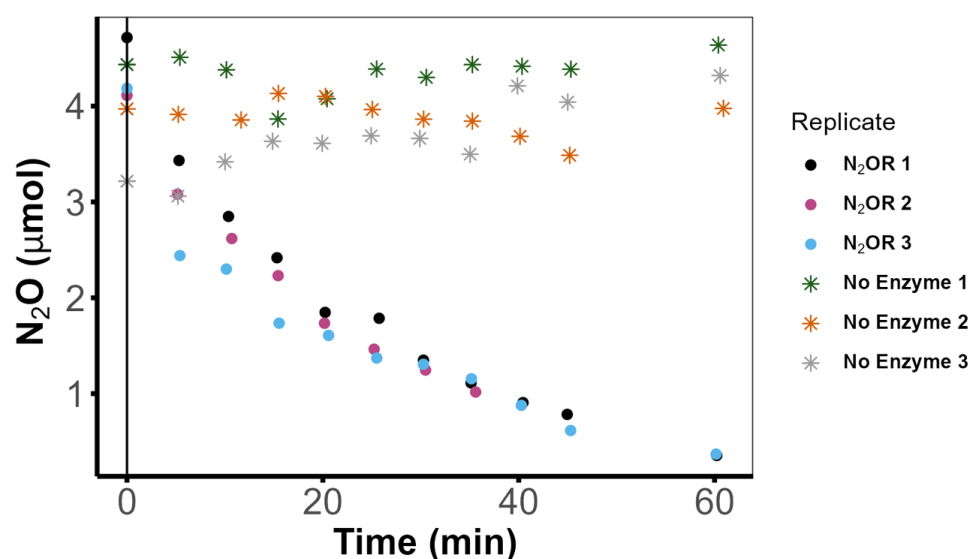


Figure 2: Rate of N_2O consumption by N_2OR measured directly by gas chromatography.

Direct consumption of N_2O (μmol) was monitored by GC-ECD in triplicate for both reactions with enzyme (filled circles) and no-enzyme controls (stars). Purified N_2OR was pre-reduced by incubating with dithionite-reduced methyl viologen as described in the Materials and Methods section, and the activity assay was initiated after 2 h of pre-reduction. The average initial N_2O concentration for each replicate is denoted by the black bar at timepoint 0. All reaction bottles were continuously shaken throughout the reaction.



3.5 Isotope data

495 Headspace samples from the N₂OR reactions were also analyzed by GC-IRMS to measure $\delta^{15}\text{N}$ and $\delta^{18}\text{O}$ in N₂O. All studies were performed with natural abundance N₂O. The initial $\delta^{15}\text{N}$, $\delta^{15}\text{N}^{\alpha}$, $\delta^{15}\text{N}^{\beta}$, $\delta^{15}\text{N}^{\text{SP}}$, and $\delta^{18}\text{O}$ values for N₂O were consistent across all six reaction bottles (three with enzyme and three without enzyme), averaging $0.2 \pm 0.1\%$, $-0.4 \pm 0.1\%$, $0.8 \pm 0.1\%$, $-1.2 \pm 0.1\%$, and $39.5 \pm 0.1\%$, respectively (Table 2). The δ values for the no-enzyme control reactions did not change over the one-hour reaction time course (Fig. S3, Table S5). In contrast, 500 isotopic fractionation was observed in reactions containing N₂OR (Fig. 3), where N₂O consumption was nearly complete within an hour. Thus, we were able to measure δ values for almost the entirety of the N₂O reduction reaction, from 100% N₂O ($f = 1$) at the start of the reaction to ~13% N₂O remaining ($f = 0.13$) at the final IRMS timepoint (48-49 min).

Typically, light isotopes react faster than heavy isotopes when the rate is limited by a bond cleavage step. 505 Thus, at the end of a reaction it is common to see enrichment of the heavy isotope (*e.g.*, ^{15}N) in the remaining reactant. During N₂O reduction *in vitro* where reductant is in excess, breaking the N-O bond is likely the rate-limiting step. Thus, we expect that ^{14}N and ^{16}O will react faster, causing the remaining N₂O to be enriched in ^{15}N and ^{18}O at the end of the reaction. The extent of this isotope effect can be quantified by calculating the isotopic enrichment factor (ϵ , typically expressed in per mil) (Eq. (6)) and/or the kinetic isotope effect (KIE) (Eq. (7)). For a 510 normal isotope effect (where the light isotope reacts faster), ϵ is negative and the KIE is greater than 1.

Rayleigh plots are used to linearize isotopic data according to the Rayleigh model and calculate ϵ . Mariotti's approximation of the Rayleigh model (Eq. (8)) is widely used for these calculations. Because we had accurate measurements of initial substrate isotopic values (δ_{s0}), however, we were also able to perform these calculations without approximation (Eq. (9)). Like Mariotti's approximation of the Rayleigh equation (Eq. (8)) 515 (Mariotti et al., 1981), where $x = \ln(f)$, reaction progress goes from right to left in Rayleigh plots without approximation (as f goes from 1 to 0). Calculations with the Rayleigh models with and without approximation yield similar ϵ values (Table 2, Table S6) for bulk $\delta^{15}\text{N}$, $\delta^{15}\text{N}^{\alpha}$, $\delta^{15}\text{N}^{\beta}$, and $\delta^{15}\text{N}^{\text{SP}}$ in N₂O. However, the results of the two models differed for oxygen ($\delta^{18}\text{O}_{\text{-N}_2\text{O}}$). Due to the larger δ_{s0} value of oxygen, Mariotti's approximation of the Rayleigh equation (Eq. (8)) yields $\epsilon^{18}\text{O}$ values that deviate from and are less accurate than using the Rayleigh 520 equation without approximation (Eq. (9)). Therefore, all enrichment factors (ϵ) were calculated using the Rayleigh model without approximation.



Enrichment factors (ϵ) for the Rayleigh model without approximation (Eq. (9)) were calculated using linear regression of $\ln(R_s/R_{s0})$ on $\ln[f^*(1+R_{s0})/(1+R_s)]$. The slope of this type of Rayleigh plot (Fig. 3c and 3d) yields the dimensionless form of ϵ , which can be converted to the per mil form by multiplying by 1000. These ϵ values were converted to the fractionation factor ($\alpha_{p/s}$) and KIE (see Eq. (6) and Eq. (7)). All enrichment factors (ϵ) were calculated using this approach (*i.e.*, the Rayleigh model without approximation, Eq. (9)).

For all reactions, $\delta^{18}\text{O}$, $\delta^{15}\text{N}^a$, $\delta^{15}\text{N}^b$, $\delta^{15}\text{N}^{\text{bulk}}$, and site preference ($\delta^{15}\text{N}^{\text{SP}}$) were measured. All δ values were plotted against the fraction of substrate reduced/consumed ($1-f$) (Fig. 3a and 3b). These plots present the data with reactions progressing from left to right, a more intuitive format than the Rayleigh plots, where the x axis is $\ln[f^*(1+R_{s0})/(1+R_s)]$ (Fig. 3c and 3d). However, none of the ($1-f$) plots were used for calculations.

The $\delta^{15}\text{N}^{\text{bulk}}$ values are plotted against the fraction of substrate reduced/consumed ($1-f$) in Fig. 3a (gray circles), showing N_2O reacting with N_2O in a left to right progression, with the isotope ratios in the remaining N_2O being measured directly by IRMS. As the reaction progressed, bulk ^{15}N increased, indicating a normal isotope effect for N_2O consumption by N_2O . In Fig. 3c, the $\delta^{15}\text{N}$ values have been converted to the corresponding R values (*i.e.*, $R(^{15}\text{N}/^{14}\text{N})$) which are then used to calculate $\ln(R_s/R_{s0})$ (y values) and $\ln[f^*(1+R_{s0})/(1+R_s)]$ (x values) used for the Rayleigh model without approximation (Eq. (9)). The slope of plot 3c for bulk ^{15}N is -0.0054 ± 0.0002 , which when converted to the per mil form yields an $\epsilon^{15}\text{N}^{\text{bulk}}$ value of $-5.4 \pm 0.2\text{‰}$. This ϵ value corresponds to a bulk KIE of 1.0054 ± 0.0002 . Thus, bulk N in N_2O is subject to a modest normal isotope effect during N_2O consumption by N_2O .

Similar to bulk N, $\delta^{15}\text{N}^a$ (the central atom in N_2O) was measured directly and showed an enrichment in ^{15}N as substrate concentration approached zero (Fig. 3a). Without approximations, $^{15}\text{N}^a$ has an ϵ value of $-10.0 \pm 0.4\text{‰}$ and a KIE of 1.0101 ± 0.0004 and thus has a normal isotope effect (Table 2).

$\delta^{15}\text{N}^b$ was calculated from $\delta^{15}\text{N}^{\text{bulk}}$ and $\delta^{15}\text{N}^a$, following the assumption that bulk $\delta^{15}\text{N}$ is equal to the average of $\delta^{15}\text{N}^a$ and $\delta^{15}\text{N}^b$ [$\delta^{15}\text{N}^{\text{bulk}} = 0.5 * (\delta^{15}\text{N}^a + \delta^{15}\text{N}^b)$], causing $\delta^{15}\text{N}^{\text{bulk}}$ to fall in-between $\delta^{15}\text{N}^a$ and $\delta^{15}\text{N}^b$. The $\delta^{15}\text{N}^b$ values increased only slightly as N_2O was consumed. While this increase may indicate that N^b displays a very small normal isotope effect, the ϵ value for $^{15}\text{N}^b$ ($-0.6 \pm 0.1\text{‰}$) was barely above the standard deviation for $\delta^{15}\text{N}^b$ values (0.5‰), and thus it cannot be unambiguously determined if its KIE of 1.0006 ± 0.0001 is a true isotope effect.



Site preference ($\delta^{15}\text{N}^{\text{SP}}$) is equal to $\delta^{15}\text{N}^{\beta}$ subtracted from $\delta^{15}\text{N}^{\alpha}$ (Eq. (10)). In Fig. 3a, $\delta^{15}\text{N}^{\text{SP}}$ increases as
 550 the reaction progresses (purple stars). This is due to the fact that the isotope effect for N^{β} is, at most, very small, so
 $\delta^{15}\text{N}^{\beta}$ values change very little during the entire reaction. Conversely, the isotope effect for N^{α} is larger, causing the
 $\delta^{15}\text{N}^{\alpha}$ values to increase as the reaction progresses. $\delta^{15}\text{N}^{\text{SP}}$ shows a normal isotope effect with an ϵ value of $-9.5 \pm$
 0.4% and a KIE of 1.0095 ± 0.0004 (Fig. 3c, Table 2). Note that $\epsilon^{15}\text{N}^{\text{SP}}$ was determined via linear regression of
 $\delta^{15}\text{N}^{\text{SP}}$ values, which results in a 0.1% discrepancy for $\epsilon^{15}\text{N}^{\text{SP}}$ compared to the value determined by subtracting $\epsilon^{15}\text{N}^{\beta}$
 555 from $\epsilon^{15}\text{N}^{\alpha}$ due to a rounding error. Similar small deviations in $\epsilon^{15}\text{N}^{\text{SP}}$ have been previously reported in other studies
 (Ostrom et al., 2007; Well and Flessa, 2008).

Fractionation of the oxygen atom was also monitored by IRMS, which showed that $\delta^{18}\text{O}$ values increased
 with reaction progress (Fig. 3b). The $\delta^{18}\text{O}$ enrichment factor, $\epsilon^{18}\text{O}$, was calculated as $-13.5 \pm 0.5\%$. The KIE for
 oxygen was calculated as 1.0137 ± 0.0005 . Thus, the ^{18}O KIE value is normal and slightly larger than the KIE value
 560 for $^{15}\text{N}^{\alpha}$.

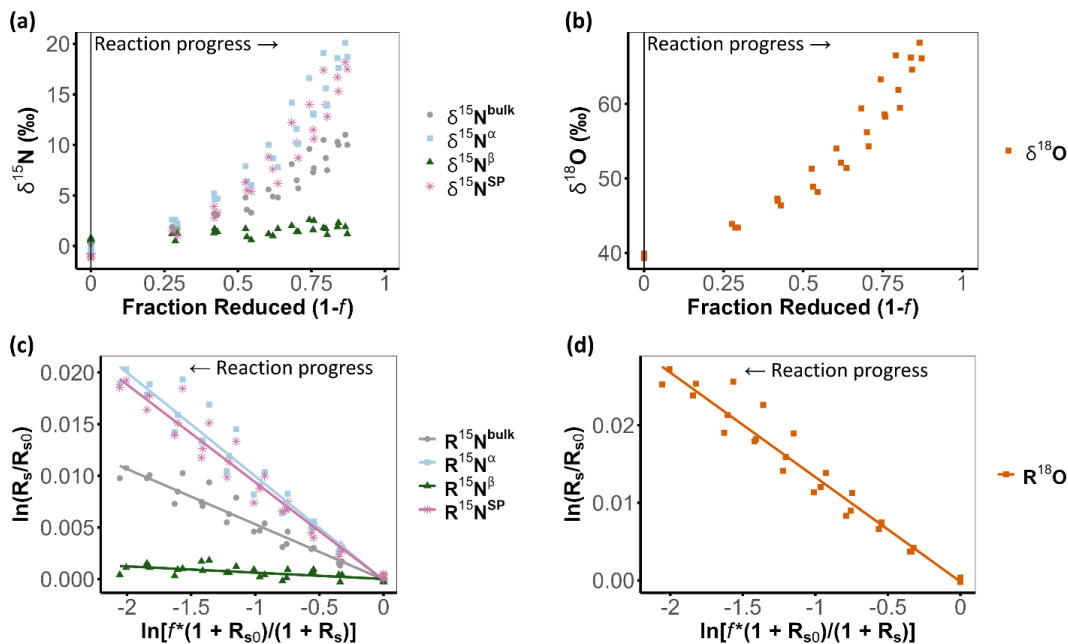


Figure 3: Isotopic enrichment of ^{15}N and ^{18}O for N_2O consumption by N_2OR .

Panels a and b show δ values in ‰ for N_2O over the course of the one-hour reaction. Panel a: $\delta^{15}\text{N}^{\text{bulk}}$, $\delta^{15}\text{N}^{\alpha}$, $\delta^{15}\text{N}^{\beta}$, and site preference ($\delta^{15}\text{N}^{\text{SP}}$). Panel b: $\delta^{18}\text{O}$. These δ values were used to calculate corresponding R values (*i.e.*, $R(^{15}\text{N}/^{14}\text{N})$) which were then used to calculate $\ln(R_s/R_{s0})$ (y values) and $\ln[f^*(1+R_{s0})/(1+R_s)]$ (x values) used for the Rayleigh model without approximation (panels c and d). In panels c and d, reaction progress is depicted right to left. The preparation of N_2O used for GC-IRMS data had a maximum specific activity of $28.2 \pm 1.3 \mu\text{mol}/\text{min}/\text{mg}$ N_2OR and copper loading of $5.23 \pm 0.11 \text{ Cu}/\text{monomer}$ (Table 1).

Table 2: Measured initial substrate δ values, calculated isotopic enrichment factors (ϵ), and calculated kinetic isotope effects (KIEs) for N_2O reduction by purified N_2OR .

Measurement	R^2 ^a	$\delta_{s0} \pm \text{se}$ (‰) ^b	$\epsilon \pm \text{se}$ (‰) ^c	KIE $\pm \text{se}^c$
$\delta^{15}\text{N}^{\text{bulk}}$	0.95	0.2 ± 0.1	-5.4 ± 0.2	$1.0054 \pm 2\text{e-}04$
$\delta^{15}\text{N}^{\alpha}$	0.96	-0.4 ± 0.1	-10.0 ± 0.4	$1.0101 \pm 4\text{e-}04$
$\delta^{15}\text{N}^{\beta}$	0.46	0.8 ± 0.1	-0.6 ± 0.1	$1.0006 \pm 1\text{e-}04$
$\delta^{15}\text{N}^{\text{SP}}$	0.96	-1.2 ± 0.1	-9.5 ± 0.4	$1.0095 \pm 4\text{e-}04$
$\delta^{18}\text{O}$	0.96	39.5 ± 0.1	-13.5 ± 0.5	$1.0137 \pm 5\text{e-}04$

^a $R^2 = [1 - (\text{SSR}/\text{SST})]$, where SSR is the residual sum of squares and SST is the total sum of squares. $\delta^{15}\text{N}^{\beta}$ has a low R^2 value as a result of the very small slope for this Rayleigh plot.

^b Average initial δ value (δ_{s0}) $\pm$ standard error (se) measured by IRMS for all three replicates with N_2OR and all three control replicates without enzyme.

^c ϵ or KIE value $\pm$ standard error (se). ϵ values were calculated without approximation using Eq. (9).



580 4 Discussion

Accurate source apportionment for N₂O emissions requires precisely modeling the relative contribution of various N₂O sources and the extent of N₂O reduction. In the soil, nitrification, bacterial denitrification, nitrifier-denitrification, and fungal denitrification can all contribute to the N₂O pool (Butterbach-Bahl et al., 2013; Zhu et al., 2013). Each biosynthetic process yields N₂O with a characteristic isotopic composition. The final isotopic composition of the N₂O pool depends on the fraction of N₂O contributed by each source. For example, N₂O produced by bacterial denitrification (or nitrifier-denitrification) typically has the lowest $\delta^{15}\text{N}^{\text{SP}}$ values, and mixing N₂O from bacterial denitrification with N₂O from other sources (e.g., fungal denitrification or nitrification) increases the final $\delta^{15}\text{N}^{\text{SP}}$ value in the N₂O pool. N₂O reduction to N₂ can also alter the isotopic composition of the N₂O pool, however, often leading to enrichment of $^{15}\text{N}^{\alpha}$ and ^{18}O in the residual N₂O pool and increasing $\delta^{15}\text{N}^{\text{SP}}$. If N₂O reduction is not properly taken into account, this enrichment of heavy isotopes and increase in $\delta^{15}\text{N}^{\text{SP}}$ could skew N₂O source tracing, leading to an underestimation of the contribution from bacterial denitrification if $\delta^{15}\text{N}^{\text{SP}}$ is used as the only tracer (Ostrom et al., 2007). Thus, accurate KIEs for N₂O reduction are critical for source tracing. While the KIEs associated with N₂O reduction have been previously measured *in vivo*, these studies were complicated by the fact that the observed KIEs were affected both by diffusion of N₂O across the cell membrane, and, in the case of soil incubations, diffusion of N₂O into/out of anaerobic microsites in the soil. These factors likely contributed to the somewhat broad range of reported KIEs for N₂O reduction in soil incubations (Yu et al., 2020). Here, we determined the KIEs for N₂O reduction by a purified N₂OR, providing clarity on the isotopic fractionation that can be attributed to the enzyme-catalyzed reaction.

4.1 KIEs for purified N₂OR

An intrinsic KIE refers to the isotope effect of a specific reaction step when it is fully unmasked. Measured isotope effects are referred to as observed KIEs (KIE_{obs}), which often reflect a combination of chemical and physical processes that can mask the intrinsic KIE of a specific step (Northrop, 1998; O'Leary, 1989). For an enzyme-catalyzed reaction, any step from substrate binding up to and including the first irreversible step can affect KIE_{obs} (Berti, 1999; Northrop, 1998; O'Leary, 1989). The higher the energetic barrier is for either a chemical step (e.g., bond formation or cleavage) or a non-chemical step (e.g., diffusion into the active site/substrate binding), the greater its influence on the observed KIE (O'Leary, 1989; Northrop, 1998). In addition to substrate binding, examples of non-chemical steps relevant for KIE_{obs} measured for N₂O reduction include dissolution of gaseous N₂O into water



and, for *in vivo* experiments, N₂O diffusion into/out of a cell or anaerobic microsite in the soil (Jinuntuya-Nortman et al., 2008; Ostrom et al., 2007). Because the isotope effects for non-chemical steps are typically smaller than the isotope effects for chemical steps, as processes like diffusion become more rate-limiting, they tend to mask the intrinsic KIEs for the chemical step(s) in enzymatic catalysis. These masking effects typically cause observed KIEs for chemical steps to be closer to 1 (smaller) than their unmasked intrinsic KIE counterparts.

Our experimental design was a closed system with only purified N₂OR, mimicking near-perfect Rayleigh parameters as no simultaneous N₂O production pathways or competing N₂O consumption pathways were present. To minimize the rate-limiting effects of N₂O dissolution and diffusion on the observed KIEs in our experiment, reaction bottles were constantly shaken to increase the rates of these processes. As discussed above, the KIEs for these non-enzymatic steps are likely much smaller than the KIEs for enzymatic N₂O reduction. The KIEs for N₂O diffusion through water have not been measured but are assumed to be close to 1, similar to the KIE measured for CO₂ diffusion in water (Jähne et al., 1987; Jinuntuya-Nortman et al., 2008; Richter et al., 2006). Similarly, the KIEs associated with invasion of N₂O from the atmosphere into water (which includes both dissolution and diffusion away from the air-liquid interface) have been determined to be 1.0008 for oxygen (resulting in a slight enrichment of N₂¹⁸O in the gaseous phase) and 1.0000 for bulk nitrogen (no isotope effect) (Inoue and Mook, 1994). In our study, as N₂O was consumed, we detected more significant enrichment of both ¹⁸O and ¹⁵N^{bulk} in gaseous N₂O, corresponding to much larger KIEs for oxygen (1.0137 ± 0.0005) and bulk nitrogen (1.0054 ± 0.0002). These fairly large KIEs indicate that any masking effects from N₂O dissolution or diffusion in the aqueous phase were minimal. Furthermore, because we used purified enzyme, there was no diffusion of N₂O across cell membranes or into/out of anaerobic microsites, meaning the KIEs we measured should be as close as possible to the true, unmasked KIEs for N₂OR-catalyzed N₂O reduction. Thus, our observed KIEs are largely determined by the intrinsic KIEs for enzyme-dependent steps, including N-O bond cleavage and, possibly, N₂O binding within the N₂OR active site.

We compared enrichment factors (ε) and KIEs for N₂O reduction calculated from the Rayleigh model with and without approximations. Due to the large δ₅₀ value of oxygen and the slightly larger ε¹⁸O value (Table 2, Table S6), Mariotti's approximations in the Rayleigh model (Mariotti et al., 1981) led to an ε¹⁸O value that does not match the enrichment factor calculated for the model without approximations (Eq. (9)). This discrepancy between models led to our decision to calculate enrichment factor values without approximations for N₂O reduction by isolated N₂OR. Furthermore, we suggest that for any experiment where the true starting isotopic composition of the



substrate can be measured, results from the Rayleigh model with and without approximation should be compared to verify that the approximated model is not introducing unnecessary error.

4.2 *Cuz form*

In this study, we determined the kinetic isotope effects for activated form II N₂O reductase. While the optical absorbance spectra of our purified enzyme suggest that a mixture of form I N₂OR and form II N₂OR (Cu_z* resting state) was present (Fig. 1), the pre-reduction conditions we used for our activity assays only activate form II N₂OR (Johnston et al., 2014). Due to the much faster turnover of the activated form II N₂OR, it is reasonable to assume that any form I activity is negligible in our measurements. Thus, our results indicate that during turnover for form II N₂OR, the oxygen atom and the central N atom in N₂O (N^α) are subject to normal KIEs. These results are consistent with N-O bond cleavage being one of the rate-determining steps during N₂O reduction by N₂OR, as this step is expected to produce normal KIEs for N^α and the oxygen atom in N₂O (Schmidt et al., 2004; Yung and Miller, 1997).

Interestingly, the KIEs reported for N₂O reduction *in vivo* are in close agreement with the KIEs we observe for purified, activated form II N₂OR. In particular, our KIEs for isolated *S. stutzeri* N₂OR compare remarkably well to those reported for an axenic culture of *S. stutzeri* (Ostrom et al., 2007) except for the ε¹⁵N^{SP} value (see section 4.3 below). While it is tempting to conclude that the active form of N₂OR *in vivo* must therefore be similar to activated form II N₂OR, in the absence of any reported KIEs for form I N₂OR, this conclusion is far from definitive. Future work is needed to determine the KIEs for form I N₂OR and to determine the theoretical isotope effects for the rate-limiting step(s) involving N₂O for each form of the enzyme.

4.3 *Comparison to enrichment factors for in vivo N₂O reduction*

Similar to our results for N₂O reduction by purified N₂OR, *in vivo* studies of N₂O reduction show enrichment of ¹⁵N^α and ¹⁸O in the remaining N₂O. For example, N₂O reduction has been studied in an axenic culture of *S. stutzeri*, with values for ε¹⁵N^{bulk}, ε¹⁸O, ε¹⁵N^α, ε¹⁵N^β, and ε¹⁵N^{SP} reported as -4.1‰, -10.9‰, -6.6‰, -1.6‰, and -5.0‰, respectively (Ostrom et al., 2007). The values for ε¹⁵N^{bulk} and ε¹⁸O are very similar to the enrichment factors we measured for purified *S. stutzeri* N₂OR, -5.4‰ and -13.5‰, respectively. Our ε¹⁵N^β value, -0.6‰, is slightly smaller (closer to 0‰) than the value reported for the pure culture while our ε¹⁵N^α value is slightly larger at -10.0‰. Thus, our measured ε¹⁵N^{SP} value, -9.5‰, is roughly double the value reported for *S. stutzeri* N₂O reduction *in vivo* (Ostrom et al., 2007). The isotope effects for N₂O reduction in axenic cultures have also been measured for two other species, *Pseudomonas denitrificans* (Ostrom et al., 2007) and *Paracoccus denitrificans* (Barford et al., 1999),



which both also have clade I N₂ORs. The enrichment factors reported in these studies align well with the enrichment factors we measured for purified N₂OR, although the $\epsilon^{15}\text{N}^{\text{SP}}$ value for *Ps. denitrificans*, -6.8‰, is also slightly smaller (less negative) than our *in vitro* value. Only $\epsilon^{15}\text{N}^{\text{bulk}}$ was reported for *Pa. denitrificans* (Barford et al., 1999). As the enrichment factors for the *in vivo* study of *S. stutzeri* and *Ps. denitrificans* were determined using fewer data points than we collected for our experiment with purified N₂OR, it is unclear if this discrepancy in $\epsilon^{15}\text{N}^{\text{SP}}$ is an experimental artifact or a bona fide difference between the *in vitro* and *in vivo* systems.

Yu and colleagues (Yu et al., 2020) recently compiled characteristic enrichment factors for N₂O reduction via a comprehensive examination of isotopic data published for soil incubation studies where N₂O was either added to the headspace (Jinuntuya-Nortman et al., 2008; Menyailo and Hungate, 2006; Ostrom et al., 2007; Well and Flessa, 2008) or generated in denitrifying microsites in the soil via denitrification of nitrate (Lewicka-Szczębałak et al., 2014, 2015). Typical ranges for N₂O reduction were -11.0 to -1.8‰ for $\epsilon^{15}\text{N}^{\text{bulk}}$, -25.1 to -5.1‰ for $\epsilon^{18}\text{O}$, and -8.2 to -2.9‰ for $\epsilon^{15}\text{N}^{\text{SP}}$ (Yu et al., 2020). The $\epsilon^{15}\text{N}^{\text{bulk}}$ and $\epsilon^{18}\text{O}$ values we measured for purified N₂OR fall well within the ranges reported for these *in vivo* studies. The general trend in $\delta^{15}\text{N}^{\text{SP}}$ values, with $\delta^{15}\text{N}^{\text{SP}}$ increasing as N₂O consumption increases, is also consistent with our *in vitro* results, although our $\epsilon^{15}\text{N}^{\text{SP}}$ value of -9.5‰ is slightly larger (*i.e.*, more negative) than the largest $\epsilon^{15}\text{N}^{\text{SP}}$ values reported for *in vivo* soil studies. Overall, the similarity between the isotopic fractionation patterns observed *in vitro* and *in vivo* confirms that the isotopic signature for N₂O reduction *in vivo* is largely driven by N₂OR. The range of enrichment factors observed for soil incubation studies (~9‰ for $\epsilon^{15}\text{N}^{\text{bulk}}$, 20‰ for $\epsilon^{18}\text{O}$, and ~5‰ for $\epsilon^{15}\text{N}^{\text{SP}}$), however, indicates that other processes may also be influencing the net isotope effects observed in the soil.

N₂O diffusion into or out of anaerobic cells/microsites in soil can decrease the absolute value of observed enrichment factors for N₂O reduction. As discussed above, the net isotope effects for N₂O reduction include both diffusive and enzymatic fractionation factors. In our experimental setup with purified N₂OR, the main non-enzymatic processes present were invasion of gaseous N₂O into water and diffusion of dissolved N₂O through water, which should both have very small enrichment factors (Inoue and Mook, 1994; Jähne et al., 1987; Jinuntuya-Nortman et al., 2008; Richter et al., 2006). For soil incubation studies, additional diffusion processes can also contribute to the net enrichment factor. Theoretical calculations for N₂O diffusion from air-filled pore space to the atmosphere have predicted values of -4.43‰ for $\epsilon^{15}\text{N}^{\text{bulk}}$, 0‰ for $\epsilon^{15}\text{N}^{\text{SP}}$, and -8.72‰ for $\epsilon^{18}\text{O}$, values which



correspond well with experimental measurements (Well and Flessa, 2008; Yu et al., 2020). While the kinetic isotope effects for N₂O diffusion across cell membranes have not been determined experimentally, it is reasonable to assume that these effects are also normal and have ϵ values close to 0‰ (Jinuntuya-Nortman et al., 2008; Yamagishi et al., 2007). Thus, diffusive enrichment factors nearly always have smaller absolute values than enzymatic enrichment factors for N₂O reduction, with the possible exception of $\epsilon^{15}\text{N}^{\beta}$ as the *in vitro* value of bacterial N₂O reduction is very close to 0‰ (Table 2). Furthermore, if there is an appreciable KIE for N₂O diffusion, the isotopocules that diffuse to a new location can be expected to be depleted in heavy isotopes (resulting in a normal KIE and negative ϵ).

The precise effects of diffusion on the net ϵ values for N₂O reduction *in vivo* depend on the primary route of N₂O diffusion and the relative rates of diffusion versus N₂O reduction. There are two main routes for N₂O during N₂O reduction: N₂O can partially escape prior to N₂O reduction when produced in a denitrifying cell or microsite, or N₂O from the atmosphere can diffuse into the cell/microsite and then be reduced (Lewicka-Szczebak et al., 2014; Ostrom et al., 2007). In the first scenario, N₂O diffusion out will yield an inverse KIE (positive ϵ value), but the net KIE will generally still be normal (negative ϵ value) (Lewicka-Szczebak et al., 2014, 2015). In the second scenario, both N₂O diffusion into a cell/microsite and N₂O reduction will have normal KIEs (negative ϵ values) (Lewicka-Szczebak et al., 2014). In this case, the net enrichment factor will approach the smaller (less negative) diffusive ϵ value as diffusion becomes more rate-limiting (Jinuntuya-Nortman et al., 2008; Lewicka-Szczebak et al., 2015; Ostrom et al., 2007; Toyoda et al., 2017). Thus, regardless of the dominant route of N₂O diffusion in the soil, the combination of diffusive and enzymatic enrichment factors will yield net ϵ values that are more positive than the intrinsic ϵ values for N₂OR (Lewicka-Szczebak et al., 2014). Indeed, under conditions where N₂O diffusion is expected to be more rate-limiting in soil mesocosms, $\epsilon^{15}\text{N}^{\text{bulk}}$ and $\epsilon^{18}\text{O}$ decrease (Jinuntuya-Nortman et al., 2008; Lewicka-Szczebak et al., 2015; Well and Flessa, 2008). Thus, diffusion of N₂O between denitrifying microsites and the atmosphere could explain why some of the $\epsilon^{15}\text{N}^{\text{bulk}}$ and $\epsilon^{18}\text{O}$ values observed *in vivo* are less negative than the values we measured using purified N₂OR (Lewicka-Szczebak et al., 2014).

In theory, the net $\epsilon^{15}\text{N}^{\text{SP}}$ value for N₂O reduction should also approach the diffusive $\epsilon^{15}\text{N}^{\text{SP}}$ value of 0‰ as diffusion becomes more rate-limiting. Interestingly, it is harder to pinpoint this trend in soil incubation studies. In one study where the correlations between the net rate constant for N₂O reduction and the net $\epsilon^{15}\text{N}^{\text{bulk}}$ and $\epsilon^{18}\text{O}$ values were fairly weak, a similarly weak correlation was observed between rate constant and $\epsilon^{15}\text{N}^{\text{SP}}$, suggesting that $\epsilon^{15}\text{N}^{\text{SP}}$



became more negative as diffusivity increased (became less rate-limiting) (Well and Flessa, 2008). In contrast,
 720 another study revealed no apparent correlation between rate constant and $\epsilon^{15}\text{N}^{\text{SP}}$ even though rate constant and the
 other enrichment factors for N_2O reduction were well-correlated (Jinuntuya-Nortman et al., 2008). Despite the lack
 of a consistent trend between the magnitude of $\epsilon^{15}\text{N}^{\text{SP}}$ and the importance of diffusive fractionation *in vivo*, it is
 possible (perhaps even plausible) that diffusion is more rate-limiting in soil incubations than in our shaking *in vitro*
 experiments, where there was no diffusion into cells/anaerobic microsites. Thus, the effects of diffusive fractionation
 725 could explain why the absolute values determined for $\epsilon^{15}\text{N}^{\text{SP}}$ *in vivo* are less negative than the value we determined
in vitro.

Besides diffusion, other factors may also contribute to decreasing the magnitude of *in vivo* ϵ values for N_2O
 reduction relative to the ϵ values we measured for purified N_2OR . For experiments where N_2O production and
 consumption are measured simultaneously, complete consumption of N_2O in some denitrifying microsites, resulting
 730 in the loss of enriched N_2O isotopologues from these sites, could result in smaller (less negative) $\epsilon^{15}\text{N}^{\text{SP}}$ values, as
 well as smaller $\epsilon^{15}\text{N}^{\text{bulk}}$ and $\epsilon^{18}\text{O}$ values (Lewicka-Szczebak et al., 2015). This loss of isotopic data from some
 microsites is most pronounced when the denitrification product ratio $r_{\text{N}_2\text{O}}$ [$r_{\text{N}_2\text{O}} = \text{N}_2\text{O}/(\text{N}_2\text{O} + \text{N}_2)$] is below 0.1 (*i.e.*,
 when > 90% of produced N_2O is consumed (Lewicka-Szczebak et al., 2015)). Although the accepted range of
 typical $\epsilon^{15}\text{N}^{\text{SP}}$ values excludes data where the product ratio is less than 0.1 (Yu et al., 2020), the extent of N_2O
 735 reduction could vary across denitrifying microsites in the soil, potentially leading to microsites with extremely low
 product ratios that could skew net enrichment factors. Additionally, as discussed below, inter-clade (or inter-species)
 variations in N_2OR -driven N_2O consumption could result in net ϵ values for the N_2O -reducing microbial community
 that are slightly smaller than the values we determined for purified *S. stutzeri* N_2OR .

The origin of the net isotope effects observed *in vivo* with absolute values that are larger than our *in vitro*
 740 values cannot be explained by diffusion. One possible explanation for these discrepancies could be that intrinsic
 enrichment factors for N_2OR vary slightly among the diverse N_2OR -containing bacterial species present in the soil.
 While all known N_2O reductases have conserved Cu_A and Cu_Z sites (He et al., 2025; Sanford et al., 2012),
 suggesting the catalytic mechanism is similar for all three N_2OR clades, subtle inter-clade variations in the
 mechanism or kinetics of N_2O consumption (Suenaga et al., 2018; Yoon et al., 2016) could lead to variability in
 745 N_2OR -driven isotopic signatures. Additionally, if any nitrogenase activity is present in the soil community, this
 could lead to a more negative $\epsilon^{15}\text{N}^{\text{bulk}}$ value, as nitrogenase can also reduce N_2O to N_2 , and the enrichment factor for



this side-reaction can be as large as -39.0‰ (Yamazaki et al., 1987). Finally, if the oxygen atom in N₂O can exchange with water during N₂O reduction, this exchange could potentially influence the net $\epsilon^{18}\text{O}$ measured for N₂O reduction in the soil. As N₂O reduction is essentially irreversible at ambient temperature and pressure (Ferousi et al., 2020), however, it is unclear if O-atom exchange can occur at any significant level during this reaction. Future work is needed to address these potential causes for variations in net isotope effects during N₂O reduction in the soil.

4.4 Comparison of in vivo and in vitro N₂O reduction lines

Isotope mapping, also known as dual isotope plotting, is a semi-quantitative method for simultaneously tracing sources of N₂O and determining the amount of N₂O that is reduced to N₂ in complex systems like soil (Ibraim et al., 2019; Lewicka-Szczebak et al., 2020; Menyailo and Hungate, 2006). For two-dimensional (dual) isotope maps, two $\delta_{\text{N}_2\text{O}}$ values (e.g., $\delta^{15}\text{N}^{\text{SP}}$ and $\delta^{18}\text{O}$) that have been normalized to the isotopic composition of the appropriate precursor (e.g., $\delta^{15}\text{N}_{\text{NO}_3^-}$) or water ($\delta^{18}\text{O}_{\text{H}_2\text{O}}$) are plotted against each other, and boxes that represent the characteristic isotopic ranges for each N₂O source (endmember) can be plotted alongside experimental data (Lewicka-Szczebak et al., 2020; Yu et al., 2020). A mixing line drawn between two endmembers represents all possible combinations of N₂O from those two endmembers. For example, the mixing line between heterotrophic bacterial denitrification (bD) and fungal denitrification (fD) starts at 100% bD, 0% fD (Fig. 4; bottom left) and ends at 0% bD, 100% fD (top right). Similarly, an N₂O reduction line depicts the change in δ values as the fraction of consumed N₂O (f or $r_{\text{N}_2\text{O}}$) increases. The N₂O reduction line starts at 0% N₂O consumption (bottom left, $r_{\text{N}_2\text{O}} = 0$) and ends at 100% N₂O consumption (top right, $r_{\text{N}_2\text{O}} = 1$) (Fig. 4). If N₂O reduction occurs, the isotopic composition of the remaining N₂O is altered, shifting δ (or $\delta^{15}\text{N}^{\text{SP}}$) values along the slope of the reduction line (Toyoda et al., 2011; Ostrom et al 2007). Qualitatively, N₂O reduction and mixing N₂O from bD with N₂O from other sources (e.g., fD or nitrification (Ni)) have similar effects, as both processes increase the final $\delta^{15}\text{N}^{\text{SP}}$ and $\delta^{18}\text{O}$ values in the N₂O pool (Fig. 4). The slope of the reduction line, however, is typically distinct enough from the average mixing line slopes to distinguish between mixing and reduction (Lewicka-Szczebak et al., 2017; Yu et al., 2020). Thus, using a mixing line and the reduction line, the fraction of N₂O contributed by two sources can be estimated by assuming one of two scenarios: either N₂O from bD is reduced first and then mixes with N₂O from a second source, or mixing occurs first, followed by N₂O reduction (Lewicka-Szczebak et al., 2017). The success of this source partitioning method depends on the assumption that the slope of the reduction line (e.g., $\epsilon^{15}\text{N}^{\text{SP}}/\epsilon^{18}\text{O}$) is distinct from the mixing line slopes and remains relatively stable across varying levels of N₂O consumption (Lewicka-Szczebak et al., 2017).



775 To further compare the isotopic signature of our purified enzyme to *in vivo* N₂O reduction, we generated isotope maps using our data for N₂O reduction by purified form II N₂OR and calculated the slopes of the associated reduction lines (Fig. S4, Table 3, Table S7). The slope of a reduction line is typically determined by calculating the ratio of enrichment factors for N₂O reduction, *e.g.*, $\epsilon^{15}\text{N}^{\text{SP}}/\epsilon^{18}\text{O}$ (Ostrom et al., 2007; Yu et al., 2020). Because only δ values are typically measured during field studies, we also determined the reduction line slopes via linear regression of the corresponding δ values for N₂O reduction (*e.g.*, $\delta^{15}\text{N}^{\text{SP}}$ versus $\delta^{18}\text{O}$). As expected (Ostrom et al., 2007), both approaches yielded very similar ratios (Fig. 4, Fig. S4, Table 3, Table S7). Three types of dual isotope plots ($\delta^{15}\text{N}^{\text{SP}}/\delta^{18}\text{O}$, $\delta^{15}\text{N}^{\text{SP}}/\delta^{15}\text{N}$, and $\delta^{18}\text{O}/\delta^{15}\text{N}$) have been evaluated for accuracy in quantifying $r_{\text{N}_2\text{O}}$ (Lewicka-Szczebak et al., 2020). Although the $\delta^{15}\text{N}^{\text{SP}}/\delta^{18}\text{O}$ plot has emerged as the most reliable approach (Lewicka-Szczebak et al., 2020), we provide an overview of the reduction line slopes determined *in vitro* for all three types of plots below.

785 The $\epsilon^{15}\text{N}^{\text{SP}}/\epsilon^{18}\text{O}$ and $\epsilon^{15}\text{N}^{\text{SP}}/\epsilon^{15}\text{N}$ slopes we determined for N₂O reduction by purified N₂OR were 0.70 and 1.76, respectively (Fig. 4, Table 3). While both values fall within the corresponding range of reduction line slopes reported for soil incubation studies, they are higher than the median values ($\epsilon^{15}\text{N}^{\text{SP}}/\epsilon^{18}\text{O} = 0.36$; $\epsilon^{15}\text{N}^{\text{SP}}/\epsilon^{15}\text{N} = 0.77$) that are accepted as characteristic of N₂O reduction (Table 3) (Lewicka-Szczebak et al., 2017; Yu et al., 2020). Additionally, the $\epsilon^{15}\text{N}^{\text{SP}}/\epsilon^{18}\text{O}$ and $\epsilon^{15}\text{N}^{\text{SP}}/\epsilon^{15}\text{N}$ slopes for N₂O reduction in an axenic *S. stutzeri* culture were lower 790 (0.46 and 1.22, respectively) than the reduction line slopes determined *in vitro* (Table 3). Thus, we observed a larger fractionation of $\delta^{15}\text{N}^{\text{SP}}$ relative to $\delta^{18}\text{O}$ or $\delta^{15}\text{N}$ than is generally observed *in vivo*. This increase in $\epsilon^{15}\text{N}^{\text{SP}}$ relative to $\epsilon^{18}\text{O}$ or $\epsilon^{15}\text{N}$ is driven by the larger absolute value of $\epsilon^{15}\text{N}^{\text{SP}}$ we measured *in vitro*, coupled with *in vitro* $\epsilon^{18}\text{O}$ or $\epsilon^{15}\text{N}$ values that are more similar to the typical *in vivo* values (Yu et al., 2020). As discussed above, the discrepancies between *in vitro* and *in vivo* enrichment factors for N₂O reduction likely arise from a combination of factors, 795 including species-specific variations in enzymatic $\epsilon^{18}\text{O}$, $\epsilon^{15}\text{N}$, or $\epsilon^{15}\text{N}^{\text{SP}}$ in soil mesocosms; diffusive fractionation effects on net ϵ ; and, for the axenic *S. stutzeri* culture, a potentially poor model fit for the $\delta^{15}\text{N}^{\text{SP}}$ data (Ostrom et al., 2007).

The $\epsilon^{18}\text{O}/\epsilon^{15}\text{N}$ slope we determined for N₂O reduction by purified N₂OR was 2.50. This *in vitro* value is very similar to the $\epsilon^{18}\text{O}/\epsilon^{15}\text{N}$ slope of 2.66 determined for N₂O reduction in a pure *S. stutzeri* culture (Ostrom et al., 800 2007) and is slightly higher than the median reduction line slope of 2.25 from soil incubation studies (Yu et al., 2020). In contrast, the theoretically calculated and experimentally measured $\epsilon^{18}\text{O}/\epsilon^{15}\text{N}$ values for N₂O diffusion are lower than 2 (1.96 and 1.49, respectively) (Well and Flessa, 2008). It has thus been hypothesized that an $\epsilon^{18}\text{O}/\epsilon^{15}\text{N}$



ratio of ~2.5 is largely driven by enzymatic fractionation during N₂O reduction, while as diffusion becomes more rate-limiting, $\epsilon^{18}\text{O}/\epsilon^{15}\text{N}$ decreases (Lewicka-Szczebak et al., 2015). Our data for N₂O reduction in an *in vitro* system where the influence of diffusion was minimized supports this general tenet.

Overall, our data suggest that the $\delta^{15}\text{N}^{\text{SP}}/\delta^{18}\text{O}$, $\delta^{15}\text{N}^{\text{SP}}/\delta^{15}\text{N}$, and $\delta^{18}\text{O}/\delta^{15}\text{N}$ ratios for residual N₂O can increase in systems where the net $\epsilon^{15}\text{N}^{\text{SP}}$ value for N₂O reduction is more negative than average and/or N₂O₂-driven enzymatic fractionation is more rate-limiting than diffusive fractionation. An increase in any of these ratios during N₂O reduction will shift the reduction line slope closer to the mixing line slopes used to model mixing between N₂O from bacterial denitrification and other endmembers (*i.e.*, nitrification or fungal denitrification) (Yu et al., 2020), potentially skewing $r_{\text{N}_2\text{O}}$ estimation and source tracing. While the enrichment factors for N₂O reduction, especially $\epsilon^{15}\text{N}^{\text{SP}}$, appear to be fairly consistent across the soil types and corresponding microbial communities studied thus far (Jinuntuya-Nortman et al., 2008; Lewicka-Szczebak et al., 2014, 2015, 2017; Menyailo and Hungate, 2006; Ostrom et al., 2007; Well and Flessa, 2008; Yu et al., 2020), our data underscores the fact that these net enrichment factors (and reduction line slopes) depend on experimental conditions. For any systems where the effects of diffusion or the community of active N₂O reducers are expected to differ from previous studies, it would be best to experimentally validate the net isotope effects associated with N₂O reduction.

Table 3: N₂O reduction line slopes for various isotope maps.

Plots	Purified N ₂ O ₂	Axenic <i>S. stutzeri</i> ^a	Soil incubations ^b
$\epsilon^{15}\text{N}^{\text{SP}}/\epsilon^{18}\text{O}$	0.70	0.46	0.36
$\epsilon^{15}\text{N}^{\text{SP}}/\epsilon^{15}\text{N}^{\text{bulk}}$	1.76	1.22	0.77
$\epsilon^{18}\text{O}/\epsilon^{15}\text{N}^{\text{bulk}}$	2.50	2.66	2.25

^a Reported slopes for isotope maps from an axenic culture of *S. stutzeri* (Ostrom et al. 2007).

^b Median values of typical N₂O reduction line slopes for controlled soil incubations. Median values were calculated in the literature review by Yu et al. (2020).

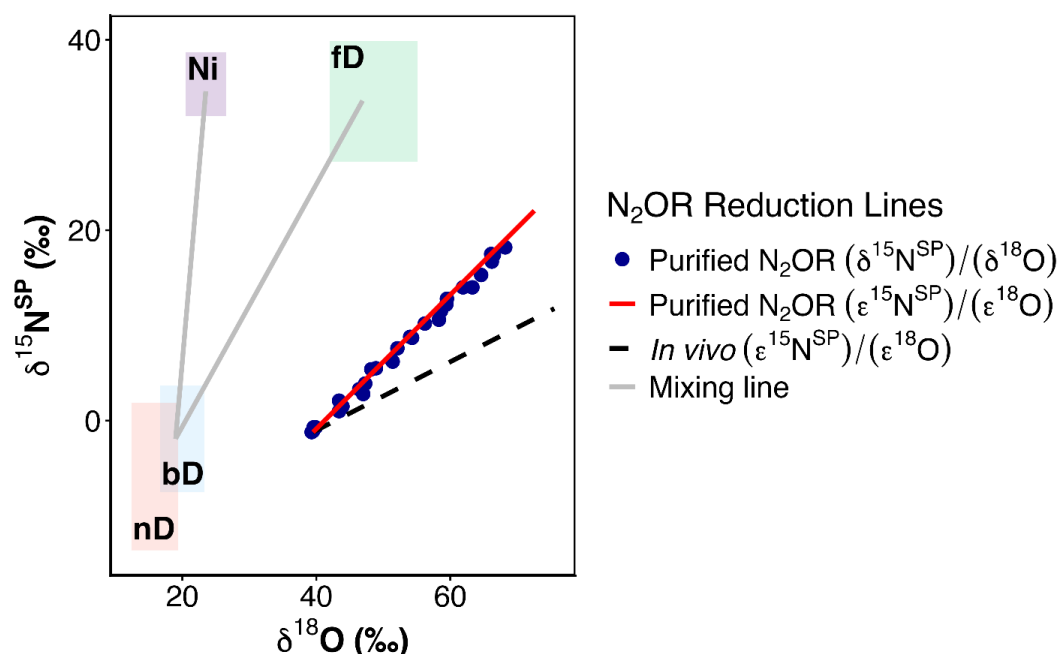


Figure 4: Dual isotope plot comparison of N₂O reduction lines determined for N₂O reduction *in vitro* by purified N₂OR (this study) and *in vivo* (adapted from Yu et al., 2020).

Literature values for $\delta^{15}\text{N}^{\text{SP}}$ and $\delta^{18}\text{O}$ were used to map typical ranges for bacterial denitrification (blue box, bD), fungal denitrification (green box, fD), nitrifier denitrification (red box, nD), and nitrification (purple box, Ni) as reported by Yu et al., 2020. Endmember boxes are connected by mixing lines. The endmember $\delta^{18}\text{O}$ values were normalized to the following theoretical values for the appropriate precursor: $\delta^{18}\text{O}_{\text{H}_2\text{O}} = 0\text{‰}$ for bD, fD, and nD, and $\delta^{18}\text{O}_{\text{O}_2} = 23.5\text{‰}$ for Ni. The $\delta^{15}\text{N}^{\text{SP}}$ values do not need to be normalized. The ratio of enrichment factors for N₂O reduction, $\epsilon^{15}\text{N}^{\text{SP}}/\epsilon^{18}\text{O}$, was used as the slope for the reduction lines. The ϵ values determined in this study were used for the reduction line for purified N₂OR (red line), while the median $\epsilon^{15}\text{N}^{\text{SP}}/\epsilon^{18}\text{O}$ value for soil incubation studies from the literature was used for the *in vivo* reduction line (black dashed line) (Yu et al., 2020). Both reduction lines start (bottom left, 0% N₂O reduced) at the initial $\delta^{15}\text{N}^{\text{SP}}$ and $\delta^{18}\text{O}$ values for the pure, isotopically characterized N₂O gas added to the headspace in this study. The $\delta^{15}\text{N}^{\text{SP}}$ and $\delta^{18}\text{O}$ values measured for N₂O reduction in this study were also plotted against each other (blue dots) for comparison.

5 Conclusion

This study provides a benchmark for observed KIEs for N₂O reduction by form II N₂OR. Enrichment factors of the purified enzyme are highly similar to those measured in *in vivo* studies for all but $\epsilon^{15}\text{N}^{\text{SP}}$, which is slightly more negative *in vitro* than *in vivo*. The similarities in isotope effects observed between pure cultures and purified enzyme allow for the speculation that the active form of the N₂OR enzyme *in vivo* resembles activated form II N₂OR.

However, before a definitive conclusion on the physiologically relevant enzyme form can be made, further studies are needed to allow for a comparison of the KIEs for form I and form II N₂OR, as well as computational modeling



of the KIEs for each proposed mechanism. Additionally, as site preference is commonly used to estimate source partitioning of N₂O emissions, our data suggest that proper consideration of experimental conditions is imperative for evaluating the endmember contributions to the N₂O pool as well as the activity of N₂OR in the field. Modeling isotope effects from N₂O diffusion through soil microcosms, as well as across cell membranes, would enhance the data presented here and ultimately enable improved N₂O emission mitigation strategies.

Code and Data Availability

All GC-ECD and GC-IRMS data used in this study and the code used to analyze these data are available at <https://doi.org/10.5281/zenodo.23091156> (Ellis and Rivett, 2026).

Author Contributions

L.R.E. and E.D.R. designed and carried out experiments. E.D.R. wrote the code for analyzing all isotope data. J.J.M. provided equipment and supervision. E.L.H. provided funding, equipment, and supervision. L.R.E. prepared the manuscript with significant contributions from E.D.R. All authors contributed to the editing of the manuscript.

Competing Interests

The authors declare that they have no conflict of interest.

Acknowledgements

We thank Hasand Gandhi for all of his help with gas chromatography instrumentation and input on sample handling. We also acknowledge and thank him for collecting data for all GC-IRMS samples in this study. We also thank Lin Zhang and the Oliver Einsle group for their advice on experimental methods for native N₂OR expression in *S. stutzeri* and for providing their recipe for AC growth medium. We thank Dr. Maya Venkataraman (Pfleger Lab, University of Wisconsin-Madison) for helpful discussion on cloning and transforming inducible vectors into *S. stutzeri*.



Financial Statement

This material is based upon work at the Great Lakes Bioenergy Research Center supported by the U.S. Department
 870 of Energy, Office of Science, Biological and Environmental Research under Award Number DE-SC0018409.

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