This manuscript represents a valuable contribution to N_2O stable isotope science. It provides a suitable approach for including appropriate mass balance considerations in applying the Rayleigh model to position-specific N isotope effects, and points towards a more comprehensive framework for position-specific isotope effects in a broader array of molecules. The level of clarity and detail with which the assumptions of this model are explained, including how they extend and differ from those of the conventional Rayleigh model, is very welcome. Also, the creation and evaluation of the simulated datasets provides great value in showing the strengths and limitations of the Rayleigh model at fitting natural data with error and variations, even beyond the specific application to the Extended Rayleigh model.

Response: Thank you for your thorough reading of our manuscript, and we are happy to hear that you found it to be a valuable contribution to the field.

However, given its complexity it is hard to follow in places. Any way that the calculations described in the Methods could be better connected with their outcomes in the Results would be welcome, as would better integration between figures illustrating various datasets and the outcome of calculations based on them. Furthermore, perhaps details regarding plotting and calculations that apply throughout could be consolidated and mentioned only once. Sections of the methods, especially Section 2.8 could also be streamlined without eliminating any information, which would improve readability.

Response: We have revised the first two paragraphs of the Results section to highlight the connection between the data shown in Fig. 1 and the results of calculations with the Expanded Rayleigh model (see below). We also added references to the appropriate figure(s) in other parts of the Methods and Results sections (Section 2.6, L232; Table 3 title; Results Section 3.2, L446).

Manuscript changes (Section 3.1, starting at L397, *new text italicized*): "To demonstrate that the standard Rayleigh model produces inaccurate results for the individual nitrogen atoms in N_2O, idealized, error-free datasets were simulated representing different scenarios with varying combinations of KIEs for N^{α} and N^{β} (Fig. 1, Table 1). *Because these data were simulated assuming that the fractions of ^{15}N and ^{14}N apportioned to each position remain constant (*i.e.*, constant ρ and τ), the distance between $\delta^{15}N^{\alpha}$ and $\delta^{15}N^{\beta}$ over the course of each reaction is constant, and the rates of change of $\delta^{15}N^{\alpha}$, $\delta^{15}N^{\beta}$, and $\delta^{15}N^{bulk}$ with respect to reaction progress (1-f) are essentially equal (Fig. 1). Thus, when the standard Rayleigh model (Eq. 2) is applied to each dataset, the slopes (ε) of $\delta^{15}N^{\alpha}$, $\delta^{15}N^{\beta}$, and $\delta^{15}N^{bulk}$ against [-flnf/(1-f)] for each dataset are all approximately equal, as are the corresponding KIE $^{15}N^{bulk}$, KIE $^{15}N^{\alpha}$, and KIE $^{15}N^{\beta}$ values (Table 3).* While the standard Rayleigh KIE $^{15}N^{bulk}$ values match the actual KIE $^{15}N^{bulk}$ values, the KIE $^{15}N^{\alpha}$ and KIE $^{15}N^{\beta}$ values determined using the standard Rayleigh approach differ significantly from the actual KIEs calculated from simulation input values (Table 3). In each of the five simulated reactions (Datasets 1-5), the isotopic preference for N^{α} differs from that of N^{β}, which can be verified visually by noting that the $\delta^{15}N^{\alpha}$ values are significantly different than the $\delta^{15}N^{\beta}$ values throughout each reaction (Fig. 1). However, in all five cases, the standard Rayleigh model produces KIE values for N^{α} and N^{β} that are approximately equal, highlighting the fact that the standard Rayleigh model inaccurately quantifies ^{15}N apportionment between N^{α} and N^{β}. *(If*

KIE $^{15}N^{\alpha}$ were equal to KIE $^{15}N^{\beta}$, the curves for $\delta^{15}N^{\alpha}$, $\delta^{15}N^{\beta}$, and $\delta^{15}N^{bulk}$ shown in Fig. 1 would all be on top of each other.)

… For example, Dataset 1 represents a scenario where there is no isotopic preference at the α position, meaning instantaneous $\delta^{15}N^{\alpha}$ ($\delta^{15}N^{\alpha i}$) is always equal to $\delta^{15}N^{s}$, *which can be verified visually by noting that accumulated $\delta^{15}N^{\alpha} \approx \delta^{15}N^{s}$ at the start of the reaction (Fig. 1)*."

Manuscript changes (Section 2.6, starting at L232): "Idealized, error-free $\delta^{15}N^{\alpha}$ and $\delta^{15}N^{\beta}$ values *(shown in Fig. 1)* were simulated for the values of ρ listed in Table 1 by calculating $\delta^{15}N^{\alpha}$ and $\delta^{15}N^{\beta}$ using Eq. (27) and Eq. (28) (Tables S4-S8)."

Manuscript change (Table 3 title): "Table 3. KIE values for N^{bulk}, N^{α}, and N^{β} calculated directly from simulation input values (Actual) or from the standard Rayleigh model or Expanded Rayleigh model *applied to error-free simulated datasets*."

Manuscript change (Results Section 3.2, starting at L446): "To test the robustness of the Expanded Rayleigh model, we applied this model to simulated data with error at varying levels of size and skewness (Table 2, *Fig. 3, and Figs. S1-S4*) and averaged the results from 1000 simulations for each error category (Table 4, Tables S9-S13)."

We have also streamlined the Methods section (including Section 2.8) by consolidating descriptions of calculations and plotting (all done with R) into one new sub-section (Section 2.10). The rest of Section 2.8, however, describes the generation of datasets with simulated error. Our error simulations are designed for a unique system with a gaseous reactant and product (NO and N_2O) and must include error calculations for $\delta^{15}N^{\alpha}$ and $\delta^{15}N^{\beta}$, unlike the study our method is based on (Scott et al., 2004). Additionally, the simulations have to be described in sufficient detail for these calculations to be reproducible. For these reasons, we feel that the brief explanation of the error levels and skewness values in Section 2.8 is warranted.

Scott, K. M., Lu, X., Cavanaugh, C. M., and Liu, J. S.: Optimal methods for estimating kinetic isotope effects from different forms of the Rayleigh distillation equation, Geochim Cosmochim Ac, 68, 433-442, 10.1016/s0016-7037(03)00459-9, 2004.

Manuscript changes: "*2.10 Modeling, statistical analysis, and figures*

Modeling and statistical analyses of simulated and experimental δ values were performed with R statistical software (R Core Team, 2022), and figures were produced with ggplot2 (Wickham, 2016). To determine ρ for the Expanded Rayleigh model, nonlinear least squares regression was performed as previously described (Baty et al., 2015) using a starting ρ value of 0.5.

For datasets with simulated error, random numbers representing simulated error were generated using the rsn function from the skew-normal distribution package (Azzalini, 2023), and skewness was calculated with the moments package (Komsta and Novomestky, 2022). In the moments package, skewness is defined as $(1/n)*\Sigma((x - \bar{x})/s)^3$, where n is the sample size, $\bar{x}$ is the sample mean, and s is the sample standard deviation (Hippel, 2011).

For previously published experimental data, a linear model was used to determine if SP, ρ, or τ varied as a function of [-flnf/(1-f)]."

Azzalini, A.: The R package 'sn': The Skew-Normal and Related Distributions such as the Skew-t and the SUN (version 2.1.1). URL http://azzalini.stat.unipd.it/SN/,https://cran.r-project.org/package=sn [code], 2023.

Baty, F., Ritz, C., Charles, S., Brutsche, M., Flandrois, J.-P., and Delignette-Muller, M.-L.: A Toolbox for Nonlinear Regression in R: The Package nlstools, J Stat Softw, 66, 1 - 21, 10.18637/jss.v066.i05, 2015.

Hippel, P. v.: Skewness, in: International Encyclopedia of Statistical Science, edited by: Lovric, M., Springer Berlin Heidelberg, Berlin, Heidelberg, 1340-1342, 10.1007/978-3-642-04898-2_525, 2011.

Komsta, L. and Novomestky, F.: moments: Moments, Cumulants, Skewness, Kurtosis and Related Tests. R package version 0.14.1, https://CRAN.R-project.org/package=moments. [code], 2022.

R Core Team: R: A language and environment for statistical computing, R Foundation for Statistical Computing, Vienna, Austria, https://www.R-project.org/. [code], 2022.

Wickham, H.: ggplot2: Elegant Graphics for Data Analysis, Springer-Verlag New York. [code], 2016.

The inclusion of both KIE and ε throughout the manuscript also introduces some potential for confusion. This is mitigated somewhat by inclusion of 'normal' and 'inverse' in reference to various isotope effects in the text, but perhaps it would be clearer to note the alternative definitions but choose a single parameter to report throughout the text. Relatedly, I think that the definition of a given at line 60 would be better referenced to Mariotti et al. (1981) or another source, and the cited reference (Bigeleisen and Wolfsberg, 1958) would be more suitable for the definition of KIE (and the overall concept).

Response: Throughout most of the manuscript, we focus on KIE values, which make it easier to distinguish between normal and inverse isotope effects. For example, Tables 3-6 exclusively list KIE values. However, the simulated datasets were calculated using pre-determined values of $\varepsilon_{N\text{-}bulk}$, and $\varepsilon_{N\text{-}bulk}$ values are frequently reported in the literature on microbial N_2O production. Therefore, we have included some reference $\varepsilon_{N\text{-}bulk}$ values to help the reader compare our data to previously reported values. We have added the corresponding KIE values wherever ε values are listed (*e.g.*, by adding a new footnote for Table 1) so that all of the data within our manuscript can be readily compared.

Manuscript change (Table 1 footnote): "[a] *Note that an ε value of -20‰ corresponds to a KIE of 1.0204 and an ε value of +20‰ corresponds to a KIE of 0.9804.*"

We have also replaced the reference at line 60 with the Mariotti et al. (1981) reference. Bigeleisen and Wolfsberg, 1958 is already cited for the general definition of KIE in section 2.2.

Regarding the fungal P450 NOR case study, it was not entirely clear to me why the variation in fractionation over the course of the experiment requires that the Expanded

Rayleigh model was applied only to subsets of the dataset. Doing so also limits the comparability of these outcomes to the results of the Standard Rayleigh model.

Response: The value of ρ for the P450 data increased from 0.5039 to 0.5075 during the course of the reaction. Our nonlinear models (Eq. 29 or Eq. 30) for determining ρ, however, assume that ρ is constant. Using nonlinear regression to determine ρ when ρ changes linearly yields a value of ρ in between the more extreme values observed at the start and end of the reaction (0.5057 ± 0.0004) and therefore does not represent the data from the overall reaction very well. However, ρ can be calculated for each individual observation without nonlinear regression using Eq. (23) ($\rho = {}^{15}N^{\alpha}/{}^{15}N^{bulk}$), giving us a way to estimate position-specific KIEs for each timepoint. We chose to list the average KIEs for the beginning and end of the observed extent of reaction to provide an estimate for the range of KIEs for N^{α} and N^{β}. Even though the new model does not fit this dataset optimally, probably due to a complex combination of equilibrium isotope effects and kinetic isotope effects, as we discussed in the manuscript (L630-635), the new model provides KIE estimates that are more accurate than those previously reported using the standard Rayleigh model, and we feel it is important to publish these improved estimates.

Manuscript changes (Section 2.9): "*Using nonlinear regression to determine ρ in this case yields a value of ρ in between the more extreme values of ρ observed at the start and end of the reaction and does not represent the data from the overall reaction very well.*"

Response: You bring up an important point about comparing the results of the two models. To make it easier to compare the results of the standard and Expanded Rayleigh models for the P450 NOR data, we have added the results of using the same approach for the standard Rayleigh model to Table 6. That is, $\varepsilon_{N\text{-}bulk}$ was calculated for each observation without linear regression by solving Eq. (2) for ε. This approach effectively determines the slope (ε) between the y-intercept of a Rayleigh plot and the δ value from one individual timepoint. (Note that the y-intercept must still be determined by linear regression of the entire dataset because it is technically challenging to measure this value when the substrate is NO.) Because this approach still relies on determining the slope of a Rayleigh plot, the results are very similar to the normal application of the standard Rayleigh model: Average early KIE ${}^{15}N^{\alpha} = 1.0130 \pm 0.0031$; average late KIE ${}^{15}N^{\alpha} = 1.0121 \pm 0.0029$; average early KIE ${}^{15}N^{\beta} = 0.9687 \pm 0030$; average late KIE ${}^{15}N^{\beta} = 0.9698 \pm 0.0018$. Thus, this method of applying the standard Rayleigh model also inaccurately predicts the types of isotope effects at N^{α} and N^{β}.

Manuscript change: "*(Note that applying the standard Rayleigh model to individual observations and averaging KIEs for the early and later parts of the reaction yields similar results to applying the standard Rayleigh model to the entire reaction (Table 6, Table S17); see SI for details.)*"

Table 6. Comparison of standard Rayleigh and Expanded Rayleigh KIE values ± standard error for N_2O production from NO by purified *Histoplasma capsulatum* (fungal) P450 NOR [calculated using previously published isotopic data (Yang et al., 2014)].

Model	Extent of reaction (range of f)		KIE $^{15}N^{bulk}$ [a]	KIE $^{15}N^{\alpha}$ [a]	KIE $^{15}N^{\beta}$ [a]
Standard Rayleigh [b]	All	0.42-0.87	0.9910 ± 0.0014	1.0127 ± 0.0030	0.9694 ± 0.0022
Standard Rayleigh [c]	Early	0.77-0.81	0.9908 ± 0.0013	1.0130 ± 0.0031	0.9687 ± 0.0030
Standard Rayleigh [c]	Late	0.47-0.52	0.9909 ± 0.0015	1.0121 ± 0.0029	0.9698 ± 0.0018
Expanded Rayleigh [d]	Early	0.77-0.81	0.9910 ± 0.0014	0.9823 ± 0.0016	0.9998 ± 0.0015
Expanded Rayleigh [d]	Late	0.47-0.52	0.9910 ± 0.0014	0.9781 ± 0.0016	1.0041 ± 0.0013

[a] Average value ± standard deviation

[b] KIE values were calculated from $\varepsilon_{N\text{-bulk}}$, $\varepsilon_{N\text{-}\alpha}$, or $\varepsilon_{N\text{-}\beta}$ values obtained via linear regression of $\delta^{15}N^{bulk}$, $\delta^{15}N^{\alpha}$, or $\delta^{15}N^{\beta}$ against [-flnf/(1-f)]). The standard Rayleigh model values presented here differ slightly from the previously published values (Yang et al., 2014) due to our exclusion of the earliest observation(s) from each replicate *(i.e.*, observations with the highest values of f were excluded).

[c] For the standard Rayleigh model applied to individual observations, $\varepsilon_{N\text{-bulk}}$, $\varepsilon_{N\text{-}\alpha}$, or $\varepsilon_{N\text{-}\beta}$ values were determined using Eq. (S24); the y-intercept listed in that equation corresponds to the y-intercept of $\delta^{15}N^{bulk}$, $\delta^{15}N^{\alpha}$, or $\delta^{15}N^{\beta}$ against [-flnf/(1-f)] (determined by linear regression of the data from each replicate). KIE values for six (early) or seven (late) individual observations were pooled and averaged.

[d] For the Expanded Rayleigh model applied to individual observations, bulk values ($\alpha_{N\text{-bulk}}$, $\varepsilon_{N\text{-bulk}}$, and KIE $^{15}N^{bulk}$) were determined with the standard Rayleigh approach. ρ was calculated for each observation using Eq. (23) ($\rho = {}^{15}N^{\alpha}/{}^{15}N^{bulk}$), and τ was determined for every step of the reaction using Eq. (24) ($\tau = {}^{14}N^{\alpha}/{}^{14}N^{bulk}$). Then $\alpha_{N\text{-}\alpha}$ and $\alpha_{N\text{-}\beta}$ were calculated for each individual observation with Eq. (21) or Eq. (22) and converted to KIE values using Eq. (10). KIE $^{15}N^{\alpha}$ and KIE $^{15}N^{\beta}$ values for six (early) or seven (late) individual observations were pooled and averaged.

Manuscript changes (SI): "*Calculation of ε values for individual observations from previously published P450 NOR data*

The data for N_2O production by purified P450 NOR is unusual because plots of $\delta^{15}N^{\alpha}$ and $\delta^{15}N^{\beta}$ against [-flnf/(1-f)] form divergent lines instead of being roughly parallel (Yang et al., 2014). Thus, ρ is not constant, and using nonlinear least squares regression to predict a "constant" value of ρ that fits the entire dataset (i.e., the normal application of the Expanded Rayleigh model), is not appropriate. Therefore, as outlined in the Section 2.9 of the main paper, we calculated ρ for each observation to determine KIE $^{15}N^{\alpha}$ and KIE $^{15}N^{\beta}$ values for each timepoint. Applying the Expanded Rayleigh model to individual observations yielded KIE $^{15}N^{\alpha}$ and KIE $^{15}N^{\beta}$ values that are more accurate than values calculated by applying the standard Rayleigh model to the entire dataset, indicating that the Expanded Rayleigh model outperforms the standard Rayleigh model even when ρ is not constant. To verify that this improved performance was due to the difference between the two models and not due to application of the

Expanded Rayleigh model to individual observations instead of the entire dataset, we also applied the standard Rayleigh model to individual observations.

To apply the standard Rayleigh model to individual observations, $\varepsilon_{N\text{-}bulk}$, $\varepsilon_{N\text{-}\alpha}$, and $\varepsilon_{N\text{-}\beta}$ were calculated for each timepoint without linear regression by solving Eq. (2) for ε

$$\varepsilon_{p/s} = \frac{\delta^{15}N^{p} - (y\ intercept)}{-\frac{fln(f)}{1-f}} \qquad (S24)$$

where $\delta^{15}N^{p}$ represents $\delta^{15}N^{\alpha}$, $\delta^{15}N^{\beta}$, or $\delta^{15}N^{bulk}$ and (y-intercept) is the intercept of $\delta^{15}N^{\alpha}$, $\delta^{15}N^{\beta}$, or $\delta^{15}N^{bulk}$ plotted against [-flnf/(1-f)]. For $\delta^{15}N^{bulk}$, the y-intercept is the initial δ value of the substrate, $\delta^{15}N^{s0}$. While $\delta^{15}N^{s0}$ could theoretically be measured, this value was not measured for this dataset. For $\delta^{15}N^{\alpha}$ and $\delta^{15}N^{\beta}$, the y-intercept doesn't have an analogous physical interpretation and thus cannot be measured directly. Therefore, the value of each y-intercept was determined via linear regression of the appropriate δ value against [-flnf/(1-f)]. Linear regression was performed separately for each replicate (13 observations/replicate). The appropriate y-intercept value was then used to calculate ε for each timepoint using the specific δ value and f value from one observation. As shown in Table S17, the KIEs calculated by applying the standard Rayleigh model to each individual observation are very similar to the KIEs calculated by applying the standard Rayleigh model to all the observations from one replicate."

For the NH_2OH oxidation case study, even after looking in the supporting information and the original Sutka et al. (2006) paper, it was not clear to me what the initial value of substrate was, or exactly how the extent of reaction *f* was calculated from the information provided.

Response: The initial amount of substrate was 3 μmol (0.3 mL of 0.01 M NH_2OH was added to the culture) (Sutka et al., 2006). Values of f (fraction of substrate remaining) were calculated by dividing remaining μmol of substrate by initial μmol of substrate. Remaining μmol of substrate was calculated by converting N_2O concentration (μM) to μmol of N_2O and subtracting twice this value from the initial μmol of substrate. We have rewritten the SI text to make this more clear.

Manuscript changes (SI): "*In this experiment, 0.3 mL of 0.01 M NH_2OH (*i.e.*, 3 μmol of NH_2OH) was added to a 25 mL culture tube containing 2 mL of suspended cells (Sutka et al., 2006). Values of f were calculated by dividing μmol of NH_2OH remaining (N^s) by the initial amount of NH_2OH (N^{s0}) (Eq. (S20))*.

$$f = \frac{N^s}{N^{s0}} \qquad (S20)$$

*As noted above, N^{s0} = 3 μmol. N^s was calculated by subtracting μmol of NH_2OH consumed (*i.e.*, twice the number of μmol of N_2O produced) from N^{s0} (Eq. S21).*

$$N^s = N^{s0} - 2 * (c_{N_2O} * v_{headspace}) \qquad (S21)$$

As shown in Eq. (21), to convert from N_2O concentration in µM (c_{N2O}) to µmol N_2O, N_2O concentration was multiplied by headspace volume ($v_{headspace}$ = 0.0227 L)."

Sutka, R. L., Ostrom, N. E., Ostrom, P. H., Breznak, J. A., Gandhi, H., Pitt, A. J., and Li, F.: Distinguishing nitrous oxide production from nitrification and denitrification on the basis of isotopomer abundances, Appl Environ Microb, 72, 638-644, 10.1128/aem.72.1.638-644.2006, 2006.