Biogeosciences
Discussions

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Interactive comment on "Estimation of isotopologue variation of N$_2$O during denitrification by *Pseudomonas aureofaciens* and *Pseudomonas chlororaphis*: Implications for N$_2$O source apportionment" *by* Joshua A. Haslun et al.

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*The page numbers of the reviewer's responses link to the original document. The page numbers of manuscript changes refer the line numbers in the "track changed" document.

1. I understand the authors' reluctance to over-interpret the δ18O data given the fact that O isotope exchange between intermediates (notably NO2-) and water are known to occur. However, I do feel that more attention could be given to the δ18O data. Certainly,

no new experiments are needed (though parallel experiments in 18O labeled water would be insightful), but I am left wondering whether the authors too quickly neglect the consideration of these data by suggesting water O exchange plays such a large role in the data? More to the point, I wonder how the co-evolving δ15N and δ18O might be used to provide more insight, for example relating to carbon substrate concentrations and types? Is there any more information to be gained about water O isotope exchange and thereby possibly the turnover of intermediate pools by closer consideration of these data in a more 'linked' fashion? Where there coherent trends in the δ15N vs δ18O that could be revealing? Also, were concentrations of NO2- measured during the sampling – in an effort to better constrain pool sizes of reaction intermediates? Even if the isotopic composition of NO2- was unknown – it might be useful for shedding light on variations of η18O.

Response: To address the consideration that water O exchange plays a role in the data we graphically examined the covariation between δ18O/δ15N vs. –flnf/(1-f) as well as the covariation between δ18O and δ15N. Both covariation plots indicated that the relationship between δ18O and δ15N were similar among treatments and replicates. Therefore, no additional information can be gleaned by discussing the relationship between O and N isotope values. Additionally, the fact that we observed a kinetic isotope effect for δ18O suggests that there is little exchange with H2O in the reaction vessels. We were unable to measure the concentration of NO2- in reaction vessels for two important reasons. First, sampling for NO2- and N2O would have added an incredible degree of complexity to the experiment, which could have led to inaccuracies and artefacts in the data. Second, additional punctures of the septa could contribute to N2O leakage into and out of the reaction vessels. Sampling this way would have doubled the number of punctures and thus increased the probability for N2O loss.

Manuscript Changes: P9 L6 – L8 – "Additionally visual inspection of the co-variation between δ18O and δ15N indicated similar trends among treatments and species, and the observed kinetic isotope effect for δ18O suggests that there is little exchange with

H2O in the reaction vessels (Figure 1)."

2. Overall, I would appreciate a bit more insight on why the different carbon substrates might contribute to differential expression of net isotope effects. For example, how are citrate and succinate utilized by these two closely related organisms? Can the authors explain (even speculatively) about how these different carbon substrates might act to regulate expression of net isotope effects? This is an exciting and burgeoning avenue of research for microbial-isotope systematics across many elemental systems – and this study provides a unique perspective for denitrification, in particular. In general not enough attention was given to this result. Different carbon substrates were chosen – in part to explore such metabolic differences. What is the reader to learn from the experimental results using different carbon?

Response: We agree that understanding the influence of carbon-source on η is important and timely. In fact, that was an initial objective of our research; however this objective became difficult to address when we observed that η for $\delta15N$ and $\delta18O$ of N2O was not constant across the extent of the reaction. The fact that η changes as the reaction progresses makes it difficult to statistically quantify (i.e. the sample size at a given point in the reaction) if the difference in η between treatments occurred as a function of substrate. Moreover, we do not know which of the diffusive or enzymatic steps are controlling η at a given extent of the reaction. To address the question regarding the influence of carbon-substrate on bulk isotope values, we will need to perform a detailed study that quantifies the isotope effects of the many nitrogen intermediates of denitrification simultaneously, a significant amount of work and therefore a study of its own.

Manuscript Changes: For the reasons outlined above, we do not feel that manuscript changes are necessary to respond to the comment.

3. The authors note that the N2O site preference is constant among treatments yet distinct between the two bacterial strains investigated. Towards offering some explanation

for this distinction, they correctly suggest that the NOR step is the most critical (combination of two NO molecules to form N2O). However, it is unclear to me in this context how the fraction of NO remaining behind in the cell relates to the site preference (L 14). Site preference is conceptually thought to be the result of the combination of two NO molecules and to reflect the chemical (enzymatic) mechanisms by which this reaction occurs and is therefore agnostic to the composition of the precursor pool. As such – it is unclear to me how the NO precursor pool size (which may relate to its N isotopic composition) can play any role in the determination of site preference. Furthermore, it is stated that the N isotopic composition of the alpha and beta positions in the N2O molecule are 'factors related to site preference' – which makes little sense since these are exactly how site preference is calculated in the first place. Perhaps the authors are referring to the alpha and beta positions represented in the NO precursor molecules – which makes sense but should be clarified. Indeed if there is an argument to be made that the NO pool size somehow influences the partitioning among NO molecules destined for the alpha position from those destined for the beta position, this would be interesting and valuable to develop. At present, however, I am missing the point of this part of the discussion.

Response: We addressed the issues above by altering the text that contributed to the lack of clarity. Please see the manuscript changes below.

Manuscript Changes: P10 L8 -P11 L11 - "In contrast to the results we observed for δ15N and δ18O, isotopic discrimination was not evident for SP regardless of treatment (Figure 2). Instead, SP was constant during the course of the reaction. This finding is consistent with pure culture studies of nitrification and denitrification across multiple species (Frame and Casciotti, 2010; Sutka et al., 2003, 2006; Toyoda et al., 2005). The differences we observed in SP between species, however, is likely to relate to the factors that control SP. Unlike the case for bulk isotopes, SP is determined during a single reaction, the reduction of NO to N2O (Toyoda et al., 2005). Thus, as N2O reduction does not occur in P. aureofaciens or P. chlororaphis, SP is only influenced

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by nitric oxide reductase (NOR) activity and diffusion of NO or N2O into or out of the cell. As SP is the difference between the δ15N value of two N atoms that rely on the same NO substrate, SP is not dependent upon the isotopic composition of the initial substrate (Toyoda et al., 2005; Sutka et al., 2006). The observation that SP remained constant during bacterial denitrification, even though the extent of the reaction varied, (e.g. Sutka et al., 2006) suggests that the expressed fractionation for the α and β N atoms during NO reduction were the same. If so, then one hypothesis is that f can vary markedly and SP will be constant. However, during production of N2O by pure fungal cytochrome P450 NOR enzyme, distinct fractionation factors for the α and β N atoms were observed and it was proposed that observations of constant SP values during production by fungi were the result of f, or the internal pool size of NO, being held relatively constant during cellular metabolism (Yang et al., 2014). We observed a minor but significant different in SP between two species of Pseudomonas sp. during N2O production that is consistent with a difference in the internal pool size of NO within the cell. The abundance of NO within the cell will depend on its production, reduction, and losses due to diffusion into or out of the cell, all of which could vary between species. We do not know, for example, the degree to which the rate of NO production intrinsically differs between the cd1-type NIR of P. chlororaphis and copper containing NIR of P. aureofaciens or how gene expression may alter these rates. We posit that small differences in SP between and even within species in our study and others may relate to the size of the NO pool available to NOR."

4. Figure 1. It would be helpful to know the composition of starting NO3-. Or alternatively are the Y-axes meant to reflect the difference between the starting NO3- and the product N2O? Figure 1 and 2 – while 'no positive values were calculated' – the distribution spills over into positive values in the upper panels of Figure 1 and all panels of Figure 2. It seems like the distribution was 'trimmed' for lower panels in Figure 1. I think some attention could be paid to addressing these differences – both in the text and in the figure caption. In particular – is there any reason to disregard positive values? Is the generation of a positive value in this context mathematically impossible? Figures 1

and 2 – are the tally marks meant to illustrate the distribution for each set of treatments (e.g., are there different color tally marks?). If so, I'm not sure I can distinguish among the different colors. It might be helpful to break out the different treatments and 'stack' the tally marks on top of one another?

Response: We added the isotopic composition of the nitrate source to the text. See below for the manuscript changes. We felt that it would be helpful to review the basis of the generation of the density distributions from our estimates of η in order to address the reviewer's concerns regarding the density distributions. As stated in the text, we applied Gaussian kernel density estimation to determine the density distribution of η predicted to occur across the full extent of the reactions. Kernel density estimation is a non-parametric method of determining the probability density function of a random continuous variable. The kernel applies a density function to each point of data. A probability density function is then created by adding up the sum of functions for each of the supplied points and dividing by the number of data. The output is an estimate of the relative densities of values across the range of η. For example, the density distribution predicts that for P. aureofaciens provided 10 mM citrate, a η value of -100 ‰ would occur relatively infrequently. The horizontal arrow on the x-axis of the graphs indicates that a value of large magnitude, such as the η = -100 ‰ previously described would be observed at the beginning of the reaction. The tick marks are the η values estimated from the derivative of the non-linear model. These η values were used to construct the density distribution. These tick marks allow one to compare the estimated η values to the estimated density distribution. This discussion is reflected by changes in the text outlined below. The reviewer's argument regarding positive values is important. If we examine the curves in figure 1, we see that each curve has a negative slope over the course of the reaction, indicative of a normal isotope effect when the x-axis variable is –flnf/(1-f). If we draw our attention to the δ15N isotope values for P. chlororaphis supplied with 10 mM citrate, we note that the left-hand side of the curve is approaching an asymptote with a slope approaching 0. Transition to a positive slope would require that δ15N isotope values became more negative. This would indicate that an inverse

isotope effect is contributing fractionation, something that is not supported by our data. Moreover, because P. aureofaciens and P. chlororaphis do not produce nitrous oxide reductase it is unlikely that an inverse isotope effect would be observed. Therefore, we have deliberately decided to cut off the density distributions at $\eta = 0$ ‰ and have revised figure 2 and 3 accordingly.

Manuscript Changes: P4 L11 – "The δ15N and δ18O of the NO3- source was 5.4 ‰ and 24.4 ‰ respectively." P6 L2-4 – "Values of "a" affect the y-intercept with larger values contributing to increased prediction of the final isotope value of the reaction. Values of "b" affect the rate of change of the isotope values particularly at the beginning of the reaction. Larger values of "b" result in a more gradual rate of change, whereas as lower values of "b" increase the initial slope. P6 L12-16 – "We used kernel density estimation to illustrate the density distribution (DD) of η across the extent of the reaction observed. Kernel density estimation is a non-parametric method of determining the probability density function of a random continuous variable. Probability density functions were determined with a Gaussian smoothing kernel from 50 equally spaced estimates of η spanning the complete extent of the reaction (i.e. f = 0 to 1). The bandwidth was set to 1 for each density estimate." P7 L3 - L6 – "Values of η15N greater than 0 were not observed. Such values would indicate that an inverse isotope effect is contributing fractionation, something that is not indicated by our data. Moreover, because P. aureofaciens and P. chlororaphis do not produce nitrous oxide reductase it is unlikely that an inverse isotope effect would be observed. Therefore, we have deliberately decided to cut off the density distributions at $\eta = 0$ ‰."" P16-21 - The lines as well as tick marks for figures 2 and 3 have been changed to make comparisons of the treatments easier. The x-axes have been changed to reflect that η values greater than zero were not observed in our reactions. The term PDDs in text and in figure legends has been changed to density distributions (DD) in text to reflect the previous in text changes."

Specific Comments-

P1 L19: Somewhat awkward to use this expression for the Rayleigh accumulated prod-

uct without having definitions for the terms. Consider using 'accumulated product expression' instead perhaps?

Response: We will change this to "the extent of the reaction." for the abstract. We believe that there is some virtue in stating the fraction of the accumulated product for the rest of the manuscript.

Manuscript Change: P1 L20-21 – Changed the expression to "the extent of the reaction".

P2 L10: "include"

Response: Changed "includes" to "include" as recommended.

Manuscript Change: P2 L15 – "includes" changed to "include"

P5 L28: please define "HSD"

Response: We remove HSD and include the full term in text.

Manuscript Change: P6 L22 – Changed "HSD" to "honest significant difference test"

P5 L14: I realize that the coefficient 'b' is a simple fitting parameter, but I am wondering if any sort of 'meaning' is discernible behind the absolute value of this coefficient? Can it be conceptualized as relating to some tangible aspect of the system?

Response: The coefficient "b" affects the rate of change of the isotope value for a given non-linear function closer to the onset of the reaction. Increased values of "b" produce a more gradual rate of change, whereas lower "b" values increase the rate of change of isotope values producing a very steep initial slope. We have included text to explain this effect.

Manuscript Change: P6 L3-4 - "Values of "a" affect the y-intercept with larger values contributing to increased prediction of the final isotope value of the reaction. Values of "b" affect the rate of change of the isotope values particularly at the beginning of

the reaction. Larger values of "b" result in a more gradual rate of change, whereas as lower values of "b" increase the initial slope."

Please also note the supplement to this comment:
https://www.biogeosciences-discuss.net/bg-2017-463/bg-2017-463-AC1-supplement.pdf
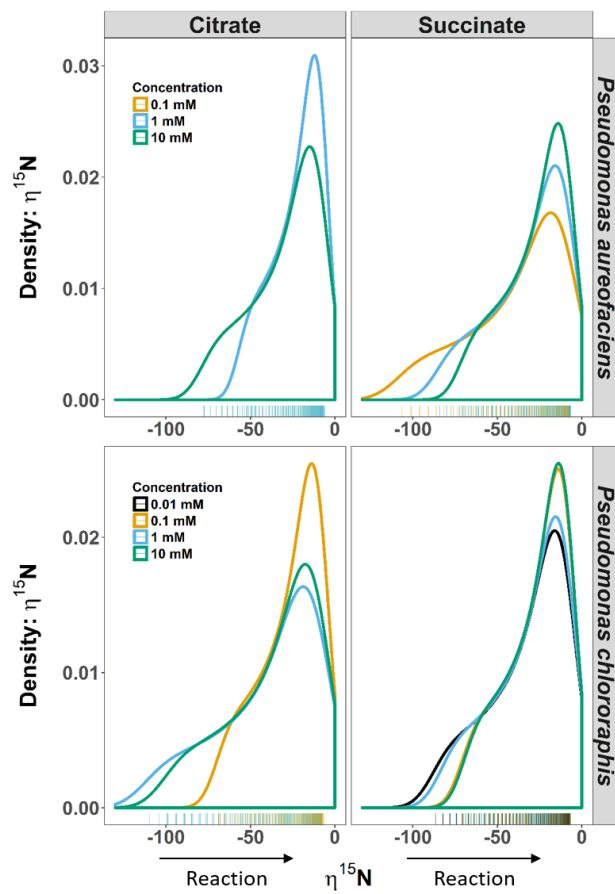
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Fig. 1. Density distributions (DD) of δ15N net isotope effects (η)
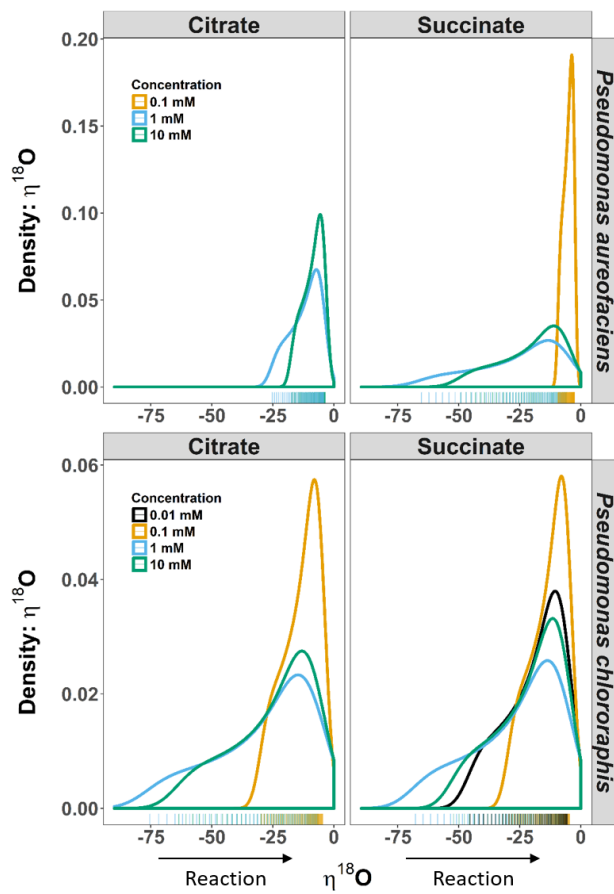
Fig. 2. Density distributions (DD) of $\delta18O$ net isotope effects (η)