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Interactive comment on "Estimation of isotopologue variation of N_2O during denitrification by *Pseudomonas aureofaciens* and *Pseudomonas chlororaphis*: Implications for N_2O source apportionment" *by* Joshua A. Haslun et al.

Anonymous Referee #1

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Summary

The authors present a well-conducted study of N2O production and isotope fractionation by two cultures of denitrifying bacteria during growth on two different carbon substrates. The authors measure the N and O isotopic composition, as well as the N2O intramolecular site preference, during production of N2O in an effort to better understand the underlying mechanisms that regulate its isotopic composition in the context of supply and type of organic carbon substrate and the multi-step process of denitrification. Because production of N2O during denitrification is a multi-step processes,

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the authors observe marked variations in the N and O composition of product N2O over reaction progress. Because shifts in the apparent or net isotope effect during the course of the reaction implicitly violate the Rayleigh model for characterization of closed-system isotope dynamics, the authors adapt a clever new approach for determining the net isotope effects from the experimental data using an exponential function. Further, using probability density distributions, the authors illustrate how the net N and O isotope effects are interpreted to change over reaction progress for different species and carbon substrate types and concentrations. Importantly it is also demonstrated that N2O site preference remains a robust reflector of formation process – albeit with some notable inter-specific differences which remain enigmatic.

The paper is very well written, clear, concise, timely and insightful. I found it easy to read and appreciated the novel approach for determination of the net isotope effects and their probability density distributions. In particular this paper sheds important light on the fact that the isotopic composition of N2O (or any multi-step reaction intermediate, for that matter) can evolve in response to non-steady state reaction conditions, the build up of intermediate pools and other physiological controls on microbial metabolisms. I recommend publication of this manuscript after consideration of my comments detailed below.

General Comments:

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

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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.

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?

3. P9. 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 –

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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.

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?

Specific Comments:

P1 Ln 19 – Somewhat awkward to use this expression for the Rayleigh accumulated product without having definitions for the terms. Consider using 'accumulated product expression' instead perhaps?

P2 L10 – "include"

P5 L28 please define "HSD"

P5 L14 I realize that the coefficient 'b' is a simple fitting parameter, but I am wondering

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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?

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