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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.

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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 not the corrected final version.

1. The novel approach to calculate the "net isotope effect" (Æđ) is not very intuitive; therefore it should be rationalized how and why y = b x ebx (formula 5, Page 5 L14) is equivalent to δ15N-N2O – δ15N-NO3- the standard approach to calculate the net

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isotope effect. In addition, results of the standard approach and the novel approach should be compared and discussed in the manuscript.

Response: The reviewer refers to the Δ as the common approach to calculate a NIE (η). Capital delta is an approximation of the η, the slope of the Rayleigh plot, and therefore additional error is associated with this particular calculation of η (please see O'Neil 1986). Rayleigh models have been traditionally applied to describe isotope effects including η. The curvilinear behaviour of our data demonstrates that we violate the linearity, a critical assumption of the application of Rayleigh models to the estimation of isotopic fractionation. Moreover, a single value for η cannot describe our data. Thus, we provide an equation for η that allows one to obtain an estimate of the isotope effect at any point in the reaction, rather than producing a single value that would fall short of describing our curvilinear system. O'Neil, J.R. (1986) Theoretical and experimental aspects of isotopic fractionation. In Stable Isotopes in High Temperature Geological Processes (eds. J.W. Valley, H.P. Taylor, Jr., and J.R. O'Neil) Rev. Mineral., 16, 1-40

Manuscript Changes: We feel that the response above addresses the concerns of the reviewer.

Specific Comments-

P1 L17: It should be mentioned here and elsewhere in the text that both bacterial strains lack the enzyme for N2O reduction as this might not be known by every reader of the manuscript.

Response: We made changes in the text on page 1 to address the reviewer's concerns.

Manuscript Change: P1 L17-18 – The sentence "Pseudomonas aureofaciens and P. chlororaphis lack the gene nitrous oxide reductase, NosZ, and therefore N2O is the terminal product of the reduction of NO3-." has been included. Additionally, the sentence at the end of the introduction, P3 L6, addresses the lack of N2O reductase in the two species utilized in this study.

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P4 L5: The isotopic composition of the NaNO3 should be given.

Response: We included the requested values in text.

Manuscript Change: P4 L11 - “The δ15N and δ18O of the NO3- source was 5.4‰ and 24.4‰ respectively.”

P4 L15: Which gas volume or range of volumes was sampled into the serum bottles?

Response: We addressed this issue in text. Please see the changes below.

Manuscript Change: P4 L20-22 - “Headspace samples between 200 μL and 500 μL of each of the 3 cultures were injected into 60 ml serum bottles (one per culture) that had been sparged with UHP N2 for 15 min, and stored for isotope analysis. Each bottle contained between 5 nmols and 15 nmols of N2O for isotopic analysis.”

P4 L26: The isotopic composition of the standards used for IRMS analysis of δ15Nα, δ15Nβ, δ15N and δ18O-N2O should be given.

Response: We have included a paragraph in the methods section that provides the values of standards used for analysis.

Manuscript Changes: P4 L28- P5 L2 - “Our internal laboratory pure N2O tank standard (MSU Tank B) was isotopically characterized by analysis relative to the USGS51 and USGS52 reference materials (https://isotopes.usgs.gov/lab/referencematerials.html). Following the guidelines proposed by Coplen (2011), we report here the isotope values of the reference materials as well as our internal laboratory standard. The δ15N, δ18O, δ15Nα, δ15Nβ, and SP values of USGS51 and USGS52 are 1.32 ‰ 41.23 ‰ 0.48 ‰ 2.15 ‰ and -1.67 ‰ and 0.44 ‰ 40.64, 13.52, -12.64 ‰ and 26.15 ‰ respectively. The δ15N, δ18O, δ15Nα, δ15Nβ, and SP values of reference MSU Tank C are -0.9 ‰ 0.7 ‰ -2.6 ‰ 39.6 ‰ and 3.4 ‰ respectively. The δ15N, δ18O, δ15Nα, δ15Nβ, and SP values of the isotope standard MSU Tank B are -0.5 ‰ 11.13 ‰ -12.2 ‰ 40.8 ‰ and 23.3 ‰ respectively. All nitrogen isotope values are reported with respect to the international Air-N2 standard, and all oxygen isotope values with respect to VSMOW.”

P4 L31: How was f determined? From the amount of substrate provided minus the cumulative amount of N2O produced? Please add the respective information here.

Response: We have included our mathematical approach to determining f.

Manuscript Change: P5 L23-24 - “The fraction of substrate remaining was determined by dividing twice the amount of N2O produced by the total amount of nitrate added, and then subtracting this quantity from 1.”

P6 section 3.1 and 3.2: Please provide results for Æð15N and Æð18O using the standard approach to calculate net isotope effects (e.g. δ15N-N2O – δ15N-NO3-).

Response: Please see the response to Reviewer #1’s general comments 1 and 4 for an explanation of the changes made to the manuscript.

Manuscript Change: Please see the previous manuscript changes made as recommended in the response above.

P7 L5: One experiment with P. aureofaciens and succinate at 1 mM yields N2O with low SP similar to P. chlororaphis. How can this be explained or is it just an outlier?

Response: We have changed the text to make the interpretation of our results reflect the reviewer’s concerns.

Manuscript Change: P7 L30-32 – The text has been changed to read “In four of the treatments, the average SP of P. chlororaphis denitrification was lower than that of P. aureofaciens. This resulted in a difference of 4.1‰ between the average SP of P. chlororaphis and P. aureofaciens.”

P7 L12 – 21: This section, the application of the Rayleigh model, should be preferably placed in the introductory or method section.

Response: As recommended by Reviewer #2, we have moved the application of the Rayleigh model to the method section. Please see the text and below for the precise changes.

Manuscript Changes: P5 L12-22 - “According to convention (Mariotti et al., 1981), the magnitude of the isotopic fractionation factor (α) for a single unidirectional reaction is defined by the rate constants of the light (k1) and heavy (k2) isotopically substituted compounds: α=k_2/k_1 . (3) Further, the isotopic enrichment factor, ε, is defined as ε=(α-1)$\times$1000, (4) and can be estimated from the slope of the linear relationship described by the Rayleigh model: δˆ15 N_p=δˆ15 N_so-ε_(p/s) [((flnf))/((1-f))]; (5) where δ15Np is the isotope value of the accumulated product, δ15Nso is the isotope value of the initial substrate, ε is the fractionation factor, and f is the fraction of substrate remaining (Mariotti et al., 1981).”

P9 L14: The statement that SP depends on δ15Nα and δ15Nβ is trivial and could be omitted. The differences in SP between Ps. aureofaciens and Ps. chlororaphis are attributed to differences in the NO pool; which experiment could be used to check this hypothesis?

Response: We have changed the text to reflect that these values are used to calculate SP rather than SP being related to them. As far as designing an experiment to determine if SP is affected by the NO pool, this is an inherently difficult problem, which arises from the difficulty in determining the concentration of NO available to the bacterium within the periplasmic space. We are currently beginning to examine NO reduction during enzymatic reduction both in vitro and in vivo.

Manuscript Changes: See the response for Reviewer #1 comment 3; P10 L8 – P11 L11.

Page 8 (discussion): Temporal resolved data of N2O isotopic composition as used in this study could be preferably be collected using an online technique, e.g. laser spectroscopy – Please comment.

Response: While online techniques such as off-axis integrated cavity output spectroscopy (OA-ICOS) and cavity ring down spectroscopy (CRDS) offer the capability to temporally resolve changes in N2O isotopomer values, the techniques have unique

and challenges inherent to the technology to overcome. For instance, many obstacles remain that constrain the field, mesocosm, and in vitro application of OA-ICOS and CRDS including production and standardization of reference gases spanning a range of SP values and N2O concentrations, as well as the standardization of methods for data analysis. Among other labs, our group is currently working to address challenges such as these as on-line measurements promise rapid continuous field-based and mesocosm data necessary for managing and monitoring N2O flux at local, ecosystem, and global scales. However, these topics have been reviewed and discusses in other peer-reviewed journals (Ostrom and Ostrom 2017). Therefore we feel that including such information detracts from the overall message we present in this manuscript. Ostrom, N.E., Ostrom, P.H., 2017. Mining the isotopic complexity of nitrous oxide: a review of challenges and opportunities. Biogeochemistry, 132:3, 359-372.

Manuscript Changes: Based upon the response above we do not feel that an in text response to the comment is necessary.

Please also note the supplement to this comment:
https://www.biogeosciences-discuss.net/bg-2017-463/bg-2017-463-AC2-supplement.pdf

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