Author's response:

We thank both reviewers for their comments and suggestions, which we addressed in the following individually. Reviewers' comments are given in black, while authors' responses are in red and changes in the manuscript in red italic.

Review #2

Summary

This study presents an examination of N_2O production by two denitrifying bacterial strains lacking nitrous reductase. The study tests several of the assumptions about the nature of N_2O production signals deduced from isotopes of N_2O in a number of useful experimental contexts, including both closed and open type systems and using both laser based and mass spectrometric analytical tools. By analyzing the product N_2O continuously over the course of nitrate consumption, the authors demonstrate that N_2O site preference is not constant, but rather shifts over the course of the culture's consumption of nitrate. The authors also examine the nature of O isotopes in product N_2O, demonstrating that growth status (exponential vs stationary phase) and growth conditions (closed bottle vs gas flushed system) have strong impacts on the degree of O atom exchange with water and hence the resulting $d^{18}O$ of the N_2O. While the study largely examines these two cultures in the context of their utility in the very popular denitrifier method, they demonstrate that these large shifts and deviations do not appear when following the classic method protocols. Rather, their results more importantly point to a wider range of expected N_2O compositions from processes (such as denitrification) that stem from physiological changes in the bacteria themselves – dynamics that may easily occur under a wide range of natural settings. This type of exploration of microbial physiological state and its impact on isotope expression is critical and sorely lacking in our current understanding of isotope dynamics and its extension to field studies. The study does a great job of demonstrating impacts of growth states and conditions on the physiological expression of isotope effects.

Overall, the paper is a very thorough and interesting exploration of microbial metabolic products and how they are influenced by different growth conditions. This study has important implications for environmental studies and delves into relevant aspects that are understudied in studies of environmental systems.

No 24): A final note - the paper could probably be shortened by ~10% without any loss of content. There are some repetitive aspects throughout.

We thank the reviewer for this suggestion. We have carefully reviewed the manuscript to reduce unnecessary repetition and improve conciseness where possible. However, we have retained the overall level of detail, as we believe it is important for communicating the methodological approach and the interpretation of the results.

Major Comments

No 25): My biggest question/concern is regarding the use of a closed system accumulated product framework for interpreting the 'gas-flushed' experiments (described in Section 2.2.3). Figure 2B shows that N_2O levels in the bottle were transient (e.g., continuously flushed). If N_2O really is continuously swept out of the culture and analyzed by QCLDAS, then these measurements would represent point measurements of the 'instantaneous product.' I am

confused about how the Rayleigh accumulated product approach is applied to this open type of analytical system. Perhaps the authors are mathematically 'accumulating' the N_2O in a mass balance manner, such that the evolving N_2O composition truly does represent the integrated accumulating product N_2O. But if so, then this was lost on me and should be clarified. If this is not the case then I am confused about what is being shown in Figure 3A. The authors clearly understand the dynamics here as they describe 'conventional accumulated product' calculations in other portions of the paper. If they truly are measuring the instantaneous product, then the regression of $d^{15}N_{bulk}$ of N_2O should be linearized against -ln(f). If the instantaneous product is regressed against (-f * ln (f))/(1-f), the plot will look quasi-linear (as in Figure 3A), but the slope would not be equivalent to the isotope effect in this case. It would be much higher. Note that in the individual experiments shown (13 – 19) in the Appendix B – the curvature of $d^{15}N$ is very clear toward the end – as would be expected. As such, I'm not sure the isotope effects reported on P13 L9-13 are correct. But it is also possible I have misunderstood. Either way I think there needs to be some clarity here.

We thank the reviewer for carefully identifying this point. The gas-flushed system indeed measures the instantaneous N_2O leaving the reactor. However, the Rayleigh analysis is not based directly on these individual QCLAS measurements. Instead, cumulative N_2O production was reconstructed by integrating the FTIR-derived N_2O flux over time using the known carrier-gas flow. The cumulative N_2O production was then used in a mass balance approach to estimate the remaining nitrate fraction (*f*) and calculate the isotopic composition of the cumulative N_2O product pool. Accordingly, the results presented in Fig. 3A represent the accumulated product pool rather than instantaneous QCLAS measurements plotted against the Rayleigh model coordinate.

We agree that this distinction was not sufficiently clear in the original manuscript. Although the cumulative-product formalism is described in Sect. 2.2.3, we recognize that it should also be reiterated where the Rayleigh analysis is presented. We have therefore clarified this point at the beginning of Sect. 3.2 and in the legend of Fig. 3 by explicitly stating that the reported ε values are derived from the reconstructed cumulative N_2O product pool using the cumulative-product formulation of Mariotti et al. (1981, https://doi.org/ 10.1007/bf02374138) and Sutka et al. (2006, https://10.1128/aem.72.1.638-644.2006) (see also our response to Reviewer #1, Comment 16).

No 26): How widely has the notion of NirS and NirK imparting differing O atom exchange dynamics really been assumed and/or applied in other studies? I understand that this is an important outcome of your study, but don't necessarily feel that this distinction between NirS and NirK is truly 'canonical' as described in the paper numerous times. I would recommend toning this down a little bit maybe?

We thank the reviewer for this helpful comment. We agree that we overstated how firmly established the distinction between NirK- and NirS-bearing denitrifiers is by referring to it as "canonical" throughout the manuscript. Although the literature has repeatedly proposed that NirK and NirS differ in nitrite-water oxygen atom exchange behaviour, we agree that this should be regarded as a widely discussed hypothesis rather than a universally accepted paradigm. We have therefore revised the terminology throughout the manuscript while retaining our central conclusion that oxygen atom exchange is not determined solely by nitrite reductase identity.

Modifications made:

1. Section 3.5 (P23 L36): Replaced *canonical* with *characteristic* and revised the text to refer to $\delta^{18}O–N_2O$ signatures commonly associated with NirK and NirS.

2. Introduction: Revised (P2 L38) "*This contrast has led to the widespread assumption...*" to "*This contrast has often been interpreted as evidence that NirS and NirK differ systematically in their influence on $\delta^{18}O$–N_2O expression (Kool et al., 2007; Guber et al., 2022).*"
3. Introduction: Revised (P3 L31) "*...including the assumed NirK/NirS control on oxygen atom exchange with water...*" to "*...including the proposed relationship between nitrite reductase identity (NirK/NirS) and oxygen atom exchange with water...*"

No 27): Did the GasMet FTIR Analyzer ever see any NO? Were there any variations in NO dynamics that could be connected with accumulation of intermediates or dynamics of N_2O SP, for example? P16 L8.

We thank the reviewer for this interesting question. Although the Gasmet FTIR analyzer was configured to monitor NO, measured concentrations remained at or below the instrument detection limit (0.13 ppm) throughout the experiments. Consequently, we were unable to obtain sufficiently resolved NO time series to evaluate relationships between transient NO accumulation, intermediate dynamics, and the observed SP evolution. Such measurements would indeed be valuable, particularly in light of the proposed involvement of alternative NO reductases, and represent an interesting direction for future work.

No 28): Is there any speculation about how these growth states and conditions could be impacting intracellular pH (which of course has a big impact on abiotic NO_2^- -water equilibration rates)? With respect to nitrite-water exchange in general – is this considered to be abiotic or enzyme-catalyzed and why?

We thank the reviewer for this insightful comment. This point substantially overlaps with Reviewer #1, Comment 19. We have therefore expanded the Discussion to include a mechanistic explanation based on differences in nitrite residence time between active-growth and resuspension conditions. We also note that additional factors, including pH-dependent exchange kinetics, may contribute but cannot be resolved with the current data set, and therefore warrant future investigation (see also our response to Reviewer #1, Comment 19).

I enjoyed the discussion about alternative NOR enzymes that could be involved!

Minor Comments

No 29): P1 L23: perhaps use 'suggesting' instead of 'indicating?'

Agreed. "Suggesting" is probably more appropriate throughout when discussing mechanistic interpretations.

No 30): P1 L35: "physiological state and metabolic context" – could you be a little bit more specific? In the paper this is explored in more detail obviously, but it could be helpful to mention what specific aspects are being investigated.

We agree that the phrase "physiological state and metabolic context" is rather general in the abstract. We have therefore revised the sentence to provide more specific examples, by explicitly referring to differences between active-growth and stationary-phase resuspension conditions and associated differences in nitrite turnover.

We have added to the sentence (P1 L36): "*[...], including active growth versus stationary-phase resuspension and differences in nitrite turnover.*"

No 31): P7 L11: extraneous period

Corrected.

No 32): P10 L 29: Do the low yields imply that the culture died? NO_3^- was not completely consumed? Or some other product accumulated? Were the cultures not happy with the continuous N2 bubbling?

We do not think that the lower N_2O yields imply a loss of culture viability. Rather, they indicate that N_2O was not produced quantitatively from the supplied nitrate. The missing fraction may reflect incomplete nitrate consumption and/or the formation of other reduced nitrogen products that were not quantified in this study. This also represents a limitation of the Rayleigh mass balance approach. In the gas-flushed setup, the residual nitrate fraction (*f*) is reconstructed from cumulative N_2O production relative to the initial nitrate addition, rather than from direct measurements of residual nitrate. Consequently, *f* represents an N_2O-based approximation rather than a direct determination of the remaining nitrate. We have clarified this limitation in the manuscript. Although we cannot exclude the possibility that continuous N_2 stripping imposed some physiological stress on the cultures, we have no experimental evidence to evaluate this possibility.

We have added two more elements in Section 3.1:

4. P11 L14: *"Accordingly, cumulative N_2O yields indicated efficient denitrification (Fig. 2A)., although they varied between incubations, indicating that N_2O was not always the sole nitrogen oxide produced (Appendix B, Fig. B1).*"
5. P11 L19: *"These results demonstrate that both incubation approaches supported efficient denitrification and provided well-constrained systems for isotopic interpretation.*"

No 33): Figure 2: what is the timescale? Days?

Yes, days. We have added the missing information to the figure's legend.

No 34): P17 L10: I think I would disagree that there was comparatively 'little sensitivity' in the P. chlororaphis culture, they still shifted by 10-20 ‰.

We agree that the wording "comparatively little sensitivity" understated the observed response of *P. chlororaphis*. Although the shifts in $\delta^{18}O–N_2O$ were substantially smaller than those observed for *P. aureofaciens*, they still amounted to approximately 10–20 ‰ between incubation modes. We have therefore revised the text to state that *P. chlororaphis* exhibited a less pronounced response to incubation configuration while maintaining consistently elevated $\delta^{18}O–N_2O$ values.

We modified the text as (P16 L13): "*These contrasts indicate a strong incubation-mode sensitivity in P. aureofaciens, whereas P. chlororaphis maintained consistently elevated $\delta^{18}O–N_2O$ values with a less pronounced response to incubation configuration.*"

No 35): Figure 6: For the GF cultures – are these endpoint measurements – or mass integrated $d^{18}O$ values? For oxygen isotopes of N_2O, does this actually matter? In theory, all else being constant, the $d^{18}O$ of N_2O should hold steady (and it doesn't as noted in P. aureofaciens Fig 3C). But here in Figure 6 the 'final' values of N_2O are indicated (single time

point) and compared with closed batch values – which are integrated values of the entire reaction. It feels a bit like comparing apples to oranges, so to speak. Still useful, but there are nuanced differences.

We thank the reviewer for pointing out this distinction. The gas-flushed values shown in Fig. 6A represent the final $\delta^{18}O–N_2O$ values measured at the end of the time-resolved incubations, whereas the closed-batch values represent the isotopic composition of the accumulated N_2O pool. Thus, the comparison is not between mathematically equivalent quantities. Nevertheless, we believe it remains informative because it illustrates the contrasting $\delta^{18}O–N_2O$ signatures produced under the two incubation modes. To avoid confusion, we have clarified in the figure legend that the gas-flushed values represent endpoint measurements from the time-resolved experiments, whereas the closed-batch values represent the accumulated N2O product pool.

No 36): P18 L12: What is the value used for this branching effect and how was it determined (35‰)? Note it might be useful to remind readers that there are three individual branching effects that impact N_2O (branching during NO_3^- reduction, during NO_2^- reduction and during NO reduction). Also has been referred to as "O atom abstraction" isotope effects. For example P19 L19 – "the intercept approximates the sum of the nitrite-water equilibrium isotope effect and the branching isotope effect." – here I think we are considering the 2nd and 3rd downstream branching isotope effects, rather than the 1st effect at NO_3^- reduction. Some clarification would be good for the reader.

We thank the reviewer for this helpful comment. This point substantially overlaps with Reviewer #1, Comment 17, and we agree that the oxygen isotope accounting underlying our conceptual framework should be stated more explicitly. We have therefore revised the manuscript to clarify that the expected $\delta^{18}O–N_2O$ values reflect the combined oxygen isotope effects associated with nitrate reduction to nitrite (approximately 25–30 ‰) and the branching isotope effect during NO_2^- reduction to N_2O (approximately 10–15 ‰), following Casciotti et al. (2002, http://doi.org/10.1021/ac020113w) and Casciotti, et al., 2007 (https://doi.org/10.1021/ac061598). We have also revised the terminology throughout the manuscript to explicitly distinguish these two contributions and updated the figure legend accordingly (see also our response to Reviewer #1, Comment 17).

No 37): P18 L36 - unnecessary comma after 'values.'

Removed.

No 38): Figure 9 - I'm not sure this figure is critical for the paper if space is required. I feel like the point is already well made in the corresponding text.

We thank the reviewer for this suggestion. We agree that the key conclusions are presented in the text. However, we have chosen to retain Fig. 9 because it provides a concise visual synthesis of the study's main conceptual findings and integrates the relationships between physiological state, oxygen atom exchange netween nitrite and water, and isotopocule signatures. We believe this overview complements the detailed discussion and helps readers interpret the broader implications of our results.

No 39): P23 L20: perhaps again use 'suggest' transient activity instead of 'indicate'?

Changed.