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 #1

Dear authors,

This is a very valuable manuscript indicating the possible experimental artefacts in defining the isotope effects associated with different microbial pathways. It contains important new findings which provide better understanding of the observed variability in the isotope values typical for various denitrification pathways. Your in-depth experimental approach utilizing different incubation strategies unravel so far unknown mechanisms that govern the isotope signature of N2O.

No 1): The manuscript is mostly well structured and clear, I have only a few suggestions for structure enhancements. I am missing a data table with the original measured values, that could be placed in the supplementary information or as a repository file.

We have added a supplementary Excel file containing the datasets used to generate Figs. 3–9, including the individual time-resolved QCLAS measurements and the summary datasets used for the remaining figures. The data availability statement has been updated accordingly.

No 2): I think that very important point in this work is the responsible formulation of conclusions and assessment of applicability of these new results into the general definition of isotope characterisation of N2O denitrification pathways. How should we interpret these results? These are just 2 bacterial strains showing some unusual isotope values in very artificial experimental conditions, which are very distinct from natural environmental studies, where we deal with lower substrate availability, lower fluxes, microbes interactions, process limitations. The current ranges for N2O isotope mixing endmember for denitrification are based on controlled incubation experiments favouring denitrification, where quite homogenous isotope values have been reported for SP and d18O values (Toyoda et al., 2017, Lewicka-Szczebak et al, 2014, 2017, Yu et al., 2020). After figuring out that in natural soil incubations we mostly deal with near complete O-isotope exchange (Lewicka-Szczebak et al., 2016, Kool et al., 2007), indeed later pure culture studies showed large variations in this exchange between various microbial strains (Rohe et al., 2017, Haslun et al., 2018), however still maintaining differences between bacterial and fungal denitrification. Current findings arise further questions on this issue, but are the new values representative for typical conditions or are rather an experimental artefact? These new values must be surely taken into account by conducting experiments but do they have direct impact in the N2O analyses in natural soil and water environments?

The authors agree that the conclusions of this study should be framed carefully and that the implications for environmental isotope applications require a balanced discussion. We do not suggest that the elevated SP or variable $\delta^{18}O$ values observed here necessarily dominate under field conditions. Rather, our results demonstrate that physiological state represents an additional source of variability that should be considered when interpreting isotope signatures of N_2O derived from denitrification. Accordingly, we do not advocate replacing isotopic signatures currently applied for identification with a single much broader interval of delta values. Instead, our findings support interpreting and, where

appropriate, refining endmember ranges within the physiological and environmental context in which they were established. To better emphasize this point, we have added clarifying statements to Sect. 3.5 and the Conclusions.

We agree that laboratory incubation experiments cannot fully reproduce the complexity of natural environments, which is true for most, if not all, pure-culture studies that have contributed to the current understanding of N_2O isotope signatures. However, we do not agree that this limits their mechanistic relevance of our findings. The currently accepted denitrification endmembers are themselves derived largely from laboratory incubations and pure-culture studies. Our study demonstrates that even within this controlled experimental framework, isotopic expression varies substantially with physiological state and experimental design, indicating that endmember signatures are conditional rather than universal. Moreover, not all environmentally relevant N_2O-producing systems are characterized by low substrate availability and process limitation; examples include wastewater treatment systems, agricultural urine patches, and other N-rich environments. We therefore consider it plausible that the greater physiological and environmental complexity of natural systems may give rise to an even broader diversity of N_2O isotopic signatures. However, evaluating this hypothesis will require dedicated environmental studies.

To objectify results of our study we added some clarifying statements:

1. End Section 3.5 P25 L14: "*These findings do not imply that isotopic signatures currently used to identify N_2O produced by denitrification should be replaced by a single, much broader range of delta values. Rather, they demonstrate that physiological state of the microbial community should be considered explicitly when defining and applying isotopic signatures for environmental interpretation.*"

2. Conclusion P26 L11: *"Instead of defining and interpreting isotopic signatures as fixed constants, our results dermonstrate that they should be interpreted in the context of the physiological and environmental conditions under which they were established.*"

Specific comments:

No 3): Some publications cited in the manuscript are missing in the bibliography, eg: Denman et al., 2007, Lewicka-Szczebak et al., 2016, Ostrom et al., 2011, Ye et al., 1991,

Added.

No 4): P2, L 25-56 „and upstream processes" - unclear and a bit confusing, what these processes are.

We agree that the term "upstream processes" was too vague. We have revised the sentence to explicitly refer to the transformations preceding NO reduction and N–N bond formation. The revised text now reads (L2 P28):

"In contrast, the oxygen isotopic composition of N_2O ($\delta^{18}O$–N_2O) reflects both NO reduction by NorB and the preceding transformations of nitrate and nitrite, and depends on the balance between nitrate-derived oxygen, nitrite–water exchange, and branching isotope effects."

No 5) P3, L3 I would add a problem on comparability of the experiments of pure culture studies and whole-soil or water communities. This seems to have a critical impact on the extent of O-exchange. While pure-culture studies show significant variations (Rohe et al., 2017, Haslun et al., 2018), whole-soil incubations favoring denitrification conditions typically show almost complete exchange (Lewicka-Szczebak et al., 2016; Kool et al., 2007). I suppose that the process

rates, inter-microbial relations and low substrate concentrations in comparison to pure-culture studies play here a crucial role.

We agree that a variety of mechanisms could play a role in controlling O atom exchange between nitrite and water. The exact mechanism underlying why and how O atom exchange varies with physiological state is beyond the scope of this study and will require future targeted investigations. We also note that the whole-soil studies cited by the reviewer represent one class of environmental system but should not be considered representative of all natural denitrifying environments. Environmental N_2O isotopic signatures exhibit substantial variability across ecosystems, reflecting differences in microbial communities, substrate availability, hydrology, and environmental conditions (Li et al., 2026, https://doi.org/10.1029/2025GL116045).

In contrast to the reviewer statement, differences in O atom exchange between nitrite and water have actually been interpreted as an indication of NirS versus NirK activity (e.g., Gruber et al., 2022, https://doi.org/10.1016/j.wroa.2022.100130). Importantly, our results demonstrate that this interpretation is not justified, as O atom exchange also varies substantially with physiological state.

No 6): P4, L6 I think that the "resuspension assay" needs a bit more explanation. I suppose that you meant to resemble the bacterial denitrification method, but it may not be clear to all readers how it is performed, that the bacteria are grown, washed and placed in known nitrate solution, right? Please add some more details on this. And I would also add the information that this treatment is done as in usual bacterial denitrification approach (if I am right on that)

We agree that the term "resuspension assay" would benefit from a brief explanation. We have therefore added a short description of the workflow, indicating that cultures were first grown, then pelleted by centrifugation, and subsequently resuspended in a defined nitrate-containing medium prior to isotope analysis. We also explicitly state that this approach is adapted from the denitrifier method. The text has been modified to (L4 P12):

"CB-R denotes a resuspension assay in which actively grown cultures were harvested by centrifugation and resuspended in a defined nitrate-containing medium, following an approach adapted from the bacterial denitrifier method used to analyse the isotopic composition of nitrate (Sigman et al., 2001; Casciotti et al., 2002; Weigand et al., 2015)."

No 7): P5, L5 "pelleted cultures" – not clear to me, what this means, just lyophilized bacteria?

We agree that the term "pelleted cultures" may not be clear to all readers. We have therefore replaced it with a more explicit description indicating that the cultures were harvested by centrifugation before being resuspended in the defined nitrate medium. We modified the text accordingly (P5 L11):

"Second, for stationary-phase resuspension incubations (CB-R_aur_nat, CB-R_chlor_nat), cultures were harvested by centrifugation (4700 rpm, 10 min) and resuspended in a defined nitrate medium prepared in natural-isotope-abundance water."

No 8): P5, L7 "replaced KNO_3 with USGS32, USGS34, IAEA-N3" – this is not clear, which KNO3? In above sentence you say " defined nitrate medium", is it KNO3? What are USGS32, USGS34, IAEA-N3? Not every reader has to know, that you refer to isotope standards abbreviations. Better say sth like: "… was prepared in the same way as stationary-phase resuspension incubations but replacing the defined nitrate solution with international nitrate isotope standards usually applied for bacterial-denitrification analytical procedure (USGS32, USGS34, IAEA-N3)"

We agree that this description could be clearer for readers unfamiliar with the denitrifier method and nitrate isotope standards. We have revised the text to explicitly state that the nitrate substrate was replaced by international nitrate isotope reference standards and clarified the role of these standards. We modified the text to (L5 P35):

"*For CB-RS_aur and CB-RS_chlor (closed-batch resuspension assays converting nitrate isotope reference standards with* P. aureofaciens *and* P. chlororaphis, *respectively; experiment codes defined in Sect. 2.1.3 and Table 1), the same resuspension procedure was used, except that the nitrate substrate (20 nmol* KNO_3*) was replaced with 20 nmol of an international nitrate isotope reference standard (USGS32, USGS34, IAEA-N3, or* ^{18}O*-enriched IAEA-N3-spike).*"

No 9): P5, L10 I feel that integration of this sub-chapter with the previous one would increase the clarity and eliminate some of above questions and unnecessary repetitions – in this chapter 2.1.3 you do not only say about media but also explain how the experimental approaches were conducted. This chapter explains some above questions, integrating into one would save some repetitions.

We agree that the original subsection title no longer accurately reflected the content, as the section describes not only the media formulations but also key aspects of the resuspension procedure. Rather than merging the sections, we have revised the title of subsection 2.1.3 to "*Media composition and resuspension procedure*" to better reflect its content and improve readability.

No 10): P5, L17 "pelleted" – how the cultures were pelleted, with centrifugation?

We agree that the term "pelleted" may not be sufficiently clear to all readers. We have therefore replaced it with a more explicit description indicating that the cultures were harvested by centrifugation before resuspension. We now write (L5 P25):

"For stationary-phase resuspension incubations, actively grown cultures were harvested by centrifugation and resuspended in a simplified medium (Weigand et al., 2015) containing 7.5 mM NH_4^+ *supplied as* NH_4Cl*, 30 g* L^{-1} *TSB, and 5 g* L^{-1} K_2HPO_4 *in the final solution.*"

No 11): P5, L22 "rather than to reproduce the exact conditions of the bacterial denitrifier method" – really? Why not? It is important comparison, and very needed, and your procedure is indeed the same as usually applied in the bacterial denitrification method, so I do not understand why you say this.

We agree that the previous wording could be misinterpreted. Our intention was not to imply that the resuspension assay differs fundamentally from the denitrifier method. Rather, we wished to distinguish between reproducing the biological resuspension approach and reproducing every analytical aspect of the denitrifier method. We have therefore revised the text to clarify that the resuspension protocol was adapted from the denitrifier method, while the headspace preparation was intended to standardize incubation conditions for our experimental objectives rather than to reproduce the complete analytical protocol. We modified the text as (P5 L31):

"The resuspension protocol was adapted from the bacterial denitrifier method,."

No 12): P6, L 13 I wonder what are your standards and how they were calibrated, I guess it must be self-produced standards, but the calibration of SP value of -93 permil is very challenging because normally the calibration scale is not

so wide. How sure are you about this value? This is important because if this value is not perfectly right your isotope calibration may be significantly biased, since this point is far away from your samples. I wonder why you apply this strange standard? The commercially available medical N2O has typically the SP value near 0 per mil, and would be here a perfect addition to your STD1 and STD3. Please check how much your SP values will differ if you omit the STD2 from your normalization.

We appreciate the reviewer's concern regarding the use of STD2 and the assigned SP value. For clarification, the three N_2O reference gases (STD1, STD2 and STD3) used in this study are produced at Empa by mixing of isotopically pure N_2O gases with commercial N_2O. Their preparation and value assignment by Sakae Toyoda (Science Tokyo) follow the procedures described by Mohn et al. (2014, http://doi.org/10.1002/rcm.6982). Furthermore, the IRMS analytical framework established at Science Tokyo has been shown to be linear over a wide range of isotope values (Mohn et al., 2022, https://doi.org/10.1002/rcm.9296). We therefore anticipate low uncertainty in the assigned values of the applied N_2O isotope reference gases.

We agree with Reviewer #1 that the ^{15}N site-preference of STD2 is very artificial, i.e., not observed in nature. For GC-IRMS site-preference (SP) measurements, calibration is not carried out on SP itself. Instead, the assigned $\delta^{15}N^{\alpha}$ and $\delta^{15}N^{\beta}$ values of the reference gases are used to calibrate individual isotope ratios after applying the ion-source scrambling correction and scale normalization. SP is subsequently calculated as $\delta^{15}N^{\alpha} - \delta^{15}N^{\beta}$ (Kelly et al., 2023, https://doi.org/10.5194/bg-21-3215-2024). Thus, the calibration is fundamentally based on the $\delta^{15}N^{\alpha}$ and $\delta^{15}N^{\beta}$ values of the reference gases, not on their SP values.

With respect to $\delta^{15}N^{\alpha}$ and $\delta^{15}N^{\beta}$, the use of the three reference gases spanning a wide range of isotopic compositions was intentional, and represents an advantage of the multi-point calibration approach, as it provides sufficient coverage to derive robust calibration factors. In addition, $\delta^{15}N^{\alpha}$ and $\delta^{15}N^{\beta}$ values of reference gases STD1-3 encompass the isotopic composition of most samples, which is considered good analytical practice. This would not be achievable using only STD1 and STD3, as suggested by Reviewer #1. Although such comprehensive calibration ranges are often unattainable in isotope studies because of the limited availability of reference materials, they are feasible here owing to the availability of STD1 – 3. Replacing STD2 with medical N_2O would narrow the calibrated $\delta^{15}N^{\alpha}$ and $\delta^{15}N^{\beta}$ range, thereby weakening the calibration and increasing the uncertainty of the resulting calibration factors.

As an additional quality check, STD1 was routinely analyzed as an unknown sample in several analytical runs. The recovered isotope values agreed well with the assigned values (typically within ~1–2 ‰ for $\delta^{15}N^{\alpha}$, <1 ‰ for $\delta^{15}N^{\beta}$ and $\delta^{15}N^{bulk}$, ~1–3 ‰ for SP, and ~1 ‰ for $\delta^{18}O$), providing an independent verification of the scrambling correction, scale normalization, and overall calobration procedure.

No 13): P7, L 12 – "N2O concentrations dropped below 50 ppm" – are you sure – 50 ppm is you limit for isotope analysis? Such huge concentration? (And later line 20)

The N_2O concentration chosen for isotopic analysis by QCLAS (50 ppm) does not represent a lower analytical threshold or detection limit but was selected to ensure optimal operation of both the experimental setup and the QCLAS analysis. Although lower concentrations (e.g. 5 ppm) could in principle be measured, doing so when reactor output concentrations are substantially higher (e.g. 300 ppm) would require extremely low sample gas flow rates during automated dilution. Furthermore, the direct absorption spectroscopy, as applied here, does not achieve the sensitivity of other spectroscopic approaches, such as ring-down spectroscopy (CRDS, Havsteen et al. 2026, https://doi.org/10.5194/amt-

19-3557-2026), although those techniques saturate at higher concentrations (e.g. >1.5 ppm for CRDS). We have clarified this point in the revised manuscript to avoid potential misinterpretation by adding this statement (P8 L3):

"Choosing 50 ppm as target concentration for N_2O isotope analysis was a compromise between data coverage and response time of the experimental setup but does not represent a lower threshold of the analytics."

No 14): P10, L16 Regression line method to quantify O-exchange was applied in Snider et al., 2009 and Lewicka-Szczebak et al., 2016

Added.

No 15): P12, Fig.3 I think it would be clearer if the X-Axis was the same for all 3 panels, it can be simplified or complete Raileigh equation for all, the trends will be the same and equally visible.

We appreciate the reviewer's suggestion. However, the use of different x-axes is intentional because the three panels address different scientific questions. Panel A uses the Rayleigh coordinate to estimate the apparent ^{15}N enrichment factor applying a Rayleigh model. Panels B and C are not interpreted within a Rayleigh framework but instead show the evolution of SP and $\delta^{18}O–N_2O$ with reaction progress ($1 - f$). While using the same x-axis for all panels might improve visual consistency, it would obscure the intended interpretation, by suggesting the use of a Rayleigh model for both SP and $\delta^{18}O–N_2O$, which is not intended.

No 16): P13, L 9-11 This very large range of enrichment factors is very surprising, I would double check all the values and calculations, because on the graph the fractionation seems quite stable. Have you calculated with initial substrate isotope signature or have you taken into account the increasing delta value of nitrate? Note that for both approaches different equations should be applied (see eg. Denk et al, 2017 – Eq 9 and 10). Since in your experimental approach you are dealing with instantaneous product (delta pi) you need to calculate the instantaneous substrate isotopic signature (delta s) for each (delta pi). The last point with very low nitrate residual fraction may provide biased results, due to complete reaction, and should be disregarded from calculating mean values. It would be interesting to see the graph, like Fig.3 with calculated epsilon values depending on the residual fraction. Your unique continuous data are here an excellent chance to investigate this dynamic. If the epsilon value is really so variable with residual fraction, the time-series of this change would be very interesting. Unfortunately, there are no original data given so that I could check the values myself.

We appreciate the reviewer's comments on this section. The enrichment factors and calculations have been checked and are correct. However, we believe there is a misunderstanding regarding how the enrichment factors were derived. In our study, the Rayleigh model analysis is applied to the cumulative isotopic composition of the accumulated N_2O product, following the cumulative-product approach described by Mariotti et al. (1981, https://doi.org/10.4141/cjss82-027) and Sutka et al. (2006, https://doi.org/10.1128/AEM.72.1.638-644.200). We do not estimate instantaneous isotope effects from the evolving substrate isotopic composition, as described by Denk et al. (2017, https://doi.org/10.1016/j.soilbio.2016.11.015). As we do not have temporally resolved $\delta^{15}N\text{-}NO_3^-$ data, we are unable to apply the mathematical framework suggested by the reviewer to the presented dataset.

We agree, however, that this distinction could be explained more clearly in the manuscript. We have therefore revised the beginning of Sect. 3.2 and the legend of Fig. 3 to explicitly state that the reported ε values are apparent enrichment factors derived from the cumulative N_2O product and to clarify the rationale for using this Rayleigh framework.

Changes made:

1. Beginning of Sect. 3.2 (P14 L3): "*... using the Rayleigh equation as presented in Mariotti et al. (1981) and Sutka et al. (2006) (Fig. 3A). The isotopic composition of the accumulated reaction product N_2O was calculated from the amount and isotopic composition of instantaneously formed and analysed N_2Ot.*"
2. Applicability pharagraph (P14 L25): *"Rather, the Rayleigh analysis is used as a first-order framework to derive apparent $\varepsilon^{15}N$ values from the cumulative N_2O product pool [...]*"
3. Literature comparison (P14 L30): "*This dataset, largely derived from Denk et al. (2017), includes enrichment factors determined under diverse experimental conditions and using different formulations of the Rayleigh model applied to either the instantaneous or accumulated product, or to the substrate. Nonetheless these enrichment factors provide a context for comparing the magnitude of isotope fractionation observed in the present study.*"
4. Fig. 4 (P15 L6): "*[...] which integrates enrichment factors derived from different experimental systems and using different mathematical formulations (e.g., soils, aqueous environments, and pure-culture incubations) [...]*"

No 17): P18, L 10-13 What value do you assume for branching? For equilibrated you used 10-15 permil (equilibrated nitrite – N2O) and for no-equilibration it makes 38 permil (nitrate-N2O). Is this difference due to additional effect by the nitrate-nitrite reduction step? I think you should provide values of branching effect that you assume here and any citation for these.

We thank the reviewer for this helpful suggestion. We agree that the fractionation factors underlying our conceptual framework were not stated explicitly, which may have caused confusion. We have therefore revised the manuscript to specify that the O-isotope fractionation factors assumed in our interpretation follow Casciotti et al. (2002, https://doi.org/10.1021/ac020113w) and Casciotti et al. (2007, https://doi.org/10.1021/ac061598h). This includes the approximate 25–30 ‰ difference in $\delta^{18}O$ between nitrate and nitrite and the approximately 10–15 ‰ branching isotope effect associated with the reduction of NO_2^- to N_2O. We have also revised the figure legend to specify that the expected $\delta^{18}O$–N_2O range under complete nitrite–water equilibration assumes this branching isotope effects and have added the corresponding references.

Modifications made:

5. Fig 6 (P19 L2): "*The shaded red band (+14.5 to +19.5 ‰) represents the expected $\delta^{18}O$–N_2O range assuming complete oxygen atom equilibration between nitrite and water and a branching isotope effect of approximately 10–15 ‰ (Casciotti et al., 2002; 2007)*"
6. Scenario introduction (P19 L14):" *To interpret the observed variability in $\delta^{18}O$–N_2O, three reference scenarios can be defined. Following the framework of Casciotti et al. (2002; 2007)), nitrate reduction to nitrite is associated with an oxygen isotope fractionation of approximately 25–30 ‰, while NO_2^- reduction to N_2O is associated with a branching isotope effect of approximately 10–15 ‰. In the absence of oxygen atom exchange between nitrite and water, $\delta^{18}O$–N_2O is therefore expected to be approximately 50 ‰, reflecting the nitrate-source signature ($\delta^{18}O$–NO_3^- = +12.4 ± 0.3 ‰) shifted by the combined isotope effects described above.*"

No 18): P21, L37-38 This statement actually only refers to *P. aureofaciens*. *P. chlororaphis* shows absolutely stable O-exchange across all experimental conditions. From your results I would say that rather high exchange is typical for these both strains but for *P.aureofaciens* the resuspension experiments create specific conditions that massively limit the O-exchange.

We agree that the marked influence of incubation conditions on O atom exchange between nitrite and water was observed primarily for *P. aureofaciens*, whereas *P. chlororaphis* exhibited consistently high O atom exchange across the experimental conditions investigated. We have revised the text accordingly to better reflect the distinct responses of the two strains and avoid overgeneralizing this observation.

We added in P. 22 an additional sentence: "I*n contrast, P. chlororaphis maintained consistently high oxygen atom exchange across the experimental conditions investigated.*"

No 19): P22, L20 Any conclusions why such differences are possible? For P.aureofaciens – from complete exchange in closed batch incubation to almost no exchange in resuspension experiments. These approaches are not so much different… was it the incubation time that differs? Is it the period of no nitrate available for bacteria in resuspension method for 24 hours – after this "hungry" period nitrate is rapidly consumed and therefore exchange is so low? Any conclusion here what the governing difference between these approaches could be ?

We thank the reviewer for this insightful comment. We agree that the mechanistic basis for the contrasting oxygen isotope exchange between nitrite and water observed under active-growth and resuspension conditions deserves further discussion. We have expanded the Discussion to include a mechanistic interpretation based on nitrite intermediate dynamics. During active growth, nitrate reduction, nitrite reduction, biomass synthesis, and enzyme expression occur simultaneously, which may prolong the residence time of nitrite (or downstream oxygen-bearing intermediates) in the aqueous phase and increase the opportunity for oxygen atom exchange with water. In contrast, resuspension assays employ fully induced cells that rapidly reduce the supplied nitrate, likely shortening the residence time of nitrite and thereby limiting oxygen atom exchange. We further note that additional factors, such as pH-dependent exchange kinetics or other physiological differences between growth states, may also contribute to the observed patterns. However, these effects cannotr not be resolved with the present dataset and therefore represent an important topic for future investigation.

We have added these aspects to the paragraph in question (P24 L16): *"A mechanistic explanation for this variability likely lies in the dynamics of the nitrite intermediate pool (Casciotti et al., 2007). Oxygen exchange between nitrite and water requires sufficient residence time of NO_2^- to approach isotopic equilibration. During active growth, differences in nitrate reduction rates, nitrite accumulation, and consumption can alter both the size and lifetime of this intermediate pool, thereby modulating the extent of exchange. In addition, because actively growing cultures simultaneously undergo biomass synthesis and enzyme expression, nitrate reduction and downstream nitrite reduction may be less tightly coupled than in resuspension assays, allowing nitrite to persist longer before reduction. In contrast, under resuspension conditions, fully induced cells rapidly reduce the supplied nitrate, and tighter coupling between nitrate and nitrite reduction likely limits nitrite accumulation and, consequently, oxyygen isotope exchange. Additional physiological factors, such as pH-dependent exchange kinetics (Buchwald and Casciotti, 2013) or changes in the cellular microenvironment, might also contribute but could not be resolved in the present study and therefore warrant future investigation. These observations indicate that oxygen exchange reflects system-level metabolic dynamics rather than fixed enzymatic behavior.*"

No 20): P22, L22 Also in Rohe et al., 2017

Added.

No 21): P23, Conclusion section

In this section it is very important to reasonably asses the significance of your findings into general field application of N2O isotopes in pathways partitioning. Should we increase the typical SP-denitrification values with all possibly SP values demonstrated here? These are probably very rare cases in natural conditions (if ever applicable) and would actually make the isotope N2O source -partitioning useless, because SP-ranges of nitrification and denitrification would overlap. It is reasonable to take these results into account? Which conditions in natural field studies could possibly enhance the untypical isotope values for N2O originating from denitrification.

We agree that the implications of our findings for environmental isotope applications deserve careful discussion. However, we do not think the available evidence supports the assumption that the elevated SP values observed here are necessarily rare or negligible under natural conditions. Our experiments demonstrate that denitrification itself can produce a substantially broader isotopic range than is generally assumed under specific physiological conditions. Together with the recent observations of Wang et al. (2024, https://doi.org/10.1073/pnas.2319960121), these results indicate that elevated SP values are an intrinsic property of denitrification under certain physiological states rather than an analytical artifact.

Our study was not designed to determine how frequently such physiological states occur in environmental systems. Consequently, we consider both the assumption that they are common and the assumption that they are rare to be premature. Instead, our results identify physiological state as an additional source of variability that should be considered when interpreting N_2O isotopocule data.

We therefore do not propose indiscriminately broadening existing denitrification endmember ranges indiscriminately. Rather, our findings suggest that these endmembers should be interpreted within the physiological and environmental context in which they were established. Our results expand the known isotopic space that denitrification can occupy under specific physiological conditions and demonstrate that applying fixed endmember values across fundamentally different physiological states may represent an oversimplification. The recent work of Strubbe et al. (2026, https://doi.org/10.1016/j.watres.2026.125580) illustrates how process endmembers can instead be constrained for the environmental system under investigation.

We have added this sentence in the conclusion (P26, L11): "*Rather than defining and interpreting isotopic signatures as fixed constants, our results support consideration of the physiological and environmental conditions under which they were established.*"

No 22): Are the elevated SP values at the beginning of the experiment not just an experimental artefact with no real impact on the real condition isotope characteristics? The initial experimental condition - before an equilibrium is established - show very distinct values – for all isotope values. Are these not very temporary values which do not occur when we deal with constant dynamic equilibrium conditions in natural environments?

The elevated SP observed at the beginning of the experiments were reproducibly observed across independent experiments, making an analytical or experimental artefact unlikely. Conventional closed-batch incubations measure the

isotopic composition of the cumulative N_2O pool present in the headspace. Even when sampled shortly after the onset of N_2O production, the measured isotope signature represents the integrated contribution of all N_2O produced up to that point rather than the instantaneous isotopic composition of newly formed N_2O. In contrast, our continuous flow-through approach resolves the isotopic composition of N_2O as it is produced, allowing transient early-stage isotopic dynamics to be observed directly.

The reviewer's comment further assumes that environmental denitrification generally occurs under steady-state conditions, such that these transient signatures would not be relevant. However, environmental systems are inherently dynamic, with fluctuating substrate availability, redox conditions, and microbial activity, such that steady-state conditions often cannot be assumed. There is currently no evidence that transient high-SP denitrification signatures do not occur in nature. In fact, elevated SP values observed in environmental studies are frequently attributed to nitrification or other pathways because they fall outside the canonical denitrification endmember range. Our results demonstrate that denitrification itself can produce substantially higher SP values, consistent with the findings of Wang et al. (2024, https://doi.org/10.1073/pnas.2319960121). Whether, and under which environmental conditions, these transient? signatures contribute to observed N_2O isotopic compositions remains an important question for future research.

No 23): P24, L2-4 Please publish your original data in accessible form in public repository. These are very important and complex measurements and possibly other researchers may need to perform further calculations with the data, eg. to add your findings into the common literature database on isotope fractionation factors.

From the dataset that you present in your current manuscript, the check of your calculated isotope effects is not possible – e.g. I tried to roughly check the epsilon15N values that you referred, and this is not possible with the available data. Please make sure that all the needed values for the calculation are provided in the repository files.

The dataset will be made available, as described in our updated Data Availability Statement.