Please see below the point-by-point responses to Referee 2, and the actions taken regarding their concerns.

Major changes

The revised manuscript includes relevant changes with respect to the submitted manuscript in order to address the reviewer's concerns. Please note that, due to the reorganization of certain sections, subsection numbering and figure references have been updated accordingly in the revised manuscript. In addition, we have replaced "predominant" with "dominant" in the title for grammatical correctness. In the text that follows, the suggestions and comments of the Referees are in black and plain font, and our responses are in ***italics and blue font.***

Referee 2

General comment:

This study aims to investigate N_2O production in two reservoirs and to distinguish its origin between nitrifying and denitrifying pathways. To achieve this, the authors combine natural-abundance isotopic analyses with rate measurements of N_2O production associated with partial and complete nitrification as well as denitrification, together with molecular tools to quantify and trace the relevant metabolic pathways at the genetic level. The authors find that denitrification appears to be the main source of N_2O, with consistently higher N_2O production rates and gene abundances than those associated with nitrification. The results highlight the value of combining isotopic and molecular approaches to understand nitrogen cycling in aquatic systems. The methodologies applied are well established. Overall, the manuscript addresses a timely and important topic in the context of climate change and contributes new insights into the origin of N_2O production in lakes. The text is generally well written, although I suggest a minor reorganization of some sections to improve the flow (see specific comments below).

We thank the reviewer for their thoughtful comment.

Specific Comments

Materials and Methods

Reorganization of isotopic abundance section: I suggest moving the section on natural isotopic abundances so that it follows immediately after the "Vertical profiles and biogeochemical characterization" section and precedes the "Functional genes" section. Because isotopic abundances are part of the chemical characterization of the water column, presenting them earlier would improve the logical flow of the manuscript. If this restructuring is adopted, the corresponding results section should be reorganized accordingly, presenting the natural isotopic abundance results right after the physicochemical characterization and before the genetic characterization. While not essential, I believe this change would strengthen the overall structure.

We thank the reviewer for their comments. We have reorganized the manuscript subsections to present the natural isotopic abundance earlier in the text. In the ***Materials and Methods*** *section, the subsection* ***Natural abundance of stable isotopes (δ15N and δ18O)*** *is now subsection 2.3 (previously 2.9). It appears after* ***Vertical profiles and Biogeochemical characterization*** *and before* ***Functional genes.*** *Similarly, the subsection* ***Changes in concentration and isotopic composition of N_2O and inorganic nitrogen*** *is now 3.2 (previously 3.5) and appears after the general description of vertical profiles. The*

rearrangement required substantial rewording of several other sections, which we have done in order to be consistent and provide necessary context for the interpretation of the natural abundance data.

Following the reorganization of the figures, we considered it necessary to include the Chl-a profile in Figure 1, as it is discussed in the Results section (3.1).

Subsection "Statistical tests": I recommend renaming this subsection to "Data analysis", which would allow the authors to describe more clearly the analytical criteria and tools used (e.g., the numbering system in the figures, the $\delta^{18}O$:$\delta^{15}N$ ratio, etc). Please also provide additional detail regarding the statistical procedures applied to linear and non-linear regressions. I assume that assumptions of normality, homoscedasticity, and independence were evaluated. Additionally, please specify the significance threshold used (e.g., $p < 0.05$).

The subsection Statistical tests have been renamed as Data Analysis (2.10). We have included more details on the statistical procedures applied. Please see the revised text below:

"Statistical analyses were conducted in R (R Core Team, 2014) version 4.4.0. Data visualization was also performed in R, with final figure adjustments made using Inkscape (Inkscape Project, 2017). We assessed normality using the Shapiro-Wilk test of normality analysis and homogeneity of variances across groups using Levene's test. For normally distributed data with equal variances, we applied one-way ANOVA (F). When normality was met but variances were unequal, we used Welch's t-test; otherwise, the standard t-test was applied. For data that violated normality assumptions, we employed the Kruskal–Wallis rank-sum test (K–W) or the Wilcoxon test (W). Outliers were identified using the Grubbs test (G). Statistical significance was set at $p < 0.05$. Linear regressions were used throughout the study to evaluate the rates and drivers of N_2O concentration and production. Model assumptions were assessed, and the model performance evaluated using adjusted R^2 values and predictor significance was determined using p-values ($\alpha = 0.05$). Each sample was assigned a unique identifier (#1-12), which is shown in Table 1 and in the figures to facilitate data interpretation and highlight observed trends." Lines 229 – 238 in the revised manuscript.

Technical Comments

Line 90: Please could you provide more detail on where and how the vertical profiles were measured? How many profiles were obtained per reservoir and sampling date? Was the same sampling site used in July and September?

We have provided additional details in the revised version of the manuscript. Please see the new text (underlined) below:

"We sampled the water column near the dam, in the open water of the reservoir, at the same location during both the July and September campaigns. First, we conducted a vertical profile of the water column using a Sea-Bird 19plus CTD profiler, obtaining continuous measurements of temperature (°C), dissolved oxygen (DO, µmol L^{-1}), and conductivity (µS cm^{-1}) in the reservoirs. Based on the temperature and DO profiles, we sampled three depths representing the epilimnion, oxycline, and hypolimnion or bottom waters. Water was collected at these three depths using a 5-L UWITEC bottle for further analyses and incubation experiments." Lines 95 – 100 in the revised manuscript.

Line 118: Please specify which nosZ clade (I or II) was quantified.

We have specified that we quantified clade I. Please see the text below:

"The nirS gene abundance was used as a proxy for denitrifiers, while nosZ gene (Clade I) abundance, was assessed only at the deepest layer, assayed only bacteria reducing N_2O to N_2" Lines 156 and 157 in the revised manuscript.

Line 124: Which was the headspace volume used for the oxic samples? Please, indicate it.

The headspace during the oxic incubation was similar to the anoxic one (=3 mL). This detail has been included in the revised manuscript, as follows:

"Once in the lab, samples from oxic water depths (refer to Table 1) were purged uncapped for 2 min to remove excess N_2O, and a 3-mL headspace with ambient air was maintained after being exposed to ambient air for 30 min." Lines 161 – 163 in the revised manuscript.

Line 212: A concentration >800 µmol O_2 L^{-1} is unusually high (>25 mg O_2 L^{-1}) … Considering the DO profiles shown, it may be worth double-checking the calculation. For instance, if 16 mg O were used instead of 32 mg O_2 for the conversion, this could partly explain the discrepancy. I kindly suggest verifying this value to ensure consistency.

We thank the reviewer for catching that error, the value should say 400 µmol O_2 L^{-1}. We have corrected the revised manuscript in the text (line 242) and in Figure 1.

Figure 1a: All N_2O concentration points for the Cubillas reservoir are the same color (orange). Additionally, the negative sign is missing from "-25" on the x-axis of the $^{15}N–NO_2^-$ panel.

We thank the reviewer for the comment. Yes, all the points for Cubillas, also for Iznájar, are shown in orange for July, versus purple for September. We have clarified that in the figure caption as follows "The color scheme for all data is the same for both reservoirs: July (orange) and September (purple)."

We have added the missing sign to (-)25. We thank the reviewer for catching that error.

Line 244: Please review spacing between symbols and values here and throughout the manuscript.

We thank the reviewer for this observation. We have carefully reviewed the manuscript and corrected the spacing between symbols and values throughout to ensure consistency and proper formatting.

Line 248: Please could you clarify more explicitly that these samples were excluded from the analysis?

Please see the text below in the revised manuscript:

"These two samples, which were excluded from this analysis, contained the highest NH_4^+ concentrations (>6 µmol L^{-1}). The N_2O production from NH_4^+ was an exponential function of the nitrification rates (Fig. 4b, adj $R^{2=}$0.60, value < 0.01)" Lines 295 – 297 in the revised manuscript.

Line 256: Since there is no statistically significant relationship between the two variables, please reconsider the use of the word "coupled." "Accompanied by" would more accurately describe the pattern.

The text has been modified following the reviewer's comment as follows:

"This decrease in the NO_3^- reduction rates was accompanied by a decrease in the NO_3^- concentration from July to September in both reservoirs" Lines 303 and 304 in the revised manuscript.

Lines 257–258: The formula and interpretation of NO_2^- turnover time (and N_2O turnover time) would be more appropriately placed in the Methods section rather than in the Results. Including this information earlier would help readers better follow the analyses and their interpretation.

We included this equation in the Methods section, in the subsection dedicated to ^{15}N-NO_2^- production (Lines 203 – 208 in the revised manuscript):

"Additionally, we also calculated the turnover time of NO_2^- ($\tau_{NO_2^-}$, days), which represents the average time required to replace the nitrite pool given the measured production rate following equation (3):

$$\tau_{NO_2^-} = \frac{[NO_2^-]}{R_{NO_2^- \text{ from } NO_3^-}} \quad (3)$$

where $[NO_2^-]$ represents the concentration of NO_2^- (nmol-N L^{-1}), and $R_{NO_2^- \text{ from } NO_3^-}$ represents the production rates of NO_2^- from NO_3^- (nmol-N L^{-1} d^{-1})".

Line 261: Consider using $p > 0.05$ for non-significant results, and report exact p-values only when results are marginally significant. (Same comment for lines 269, 270, and 275.)

We thank the reviewer for this suggestion. We have revised the manuscript to follow this recommendation: non-significant results are now reported as $p > 0.05$, and exact p-values are provided only for results that are marginally significant. This change has been applied consistently to those lines.

Figures 2 and 3: What do the numbers displayed next to N_2O concentrations represent? Please clarify this in the figure caption. The colour coding is also confusing: orange is used both for July samples and for N_2O production, regardless of sampling date. Please consider selecting a different colour for N_2O production.

The numbers displayed next to N_2O concentrations correspond to the sample IDs, which are initially reported in Table 1. We have clarified this in the figure captions for Figures 2 and 3 (3 and 4 in the revised manuscript).

Additionally, we have revised the colour scheme to avoid confusion in these figures. In the revised figures, the N_2O production is shown in magenta instead of orange.

Line 290: The dark-gray sediment colour referenced in the caption is not visible in any panel of Figure 3. Please remove this part of the caption.

We have removed that part of the caption.

Figure 4: Please consider using a lighter colour (or open symbols) for the excluded data points. As currently displayed, they are somewhat difficult to distinguish.

We thank the reviewer for this suggestion. To improve clarity, we have used open symbols for the excluded data points, which contrasts with the colors used for the included data.

Figure 5: Please explain what the numbers represent, ideally in the caption. Additionally, clarifying in the Methods how these numbered points relate to those in Figures 2 and 3 would help guide the reader through the Results and Discussion.

The numbering scheme for the samples has now been introduced in the Methods, which should clarify the figures. Please see the following text in the subsection "2.10. Data Analysis": "Each sample was assigned a unique identifier (#1-12), which is shown in Table 1 and in the figures to facilitate data interpretation and highlight observed trends." Lines 237 and 238 in the revised manuscript.

Additionally, we included the explanation in the caption as follows: "Correspondence between numbers and samples is shown in Table 1 and Figs. 2 and 3".

Figure 5a: Why is the segment connecting points 11 and 12 shown in red? I could not find an explanation in the text.

We thank the reviewer for noticing this. The red segment connecting points 11 and 12 in Figure 5a indicates a trend associated with N_2O consumption, as opposed to the black segments that represent trends associated with N_2O production. We have now clarified this in the figure caption, and in the text to avoid confusion. Besides, we added an extra legend in panel (a).

Figure 5c: There appear to be two red dotted lines. Which one is valid? Could you please specify and clarify this in the caption? I additionally suggest explaining the use of this ratio more clearly in the "Data analysis" section.

Both dotted lines are correct, and represent the ratio $\delta^{18}O$:$\delta^{15}N$ (2.5). To avoid confusion with the red lines in (a), we changed the color to green. The use of this ratio, which is indicative of active N_2O reduction (Ostrom et al., 2007), is explained in the discussion.

Line 314–315: Please could you provide a reference for the threshold defining suboxic conditions (DO < 10 µmol L^{-1}).

The threshold of 10 µM O_2 was chosen following the operational definition provided in the Springer Nature Encyclopedia of Astrobiology *entry "Suboxic," which states that the boundary between hypoxic and suboxic conditions is widely taken as 10 µM O_2. We have also used this threshold in previous works (e.g., León-Palmero et al., 2023).*

The references have been provided in the main text as follows:

"In both reservoirs, the higher N_2O concentrations were found in the deepest layers under suboxic conditions (i.e., DO < 10 µmol L^{-1}) (León-Palmero et al., 2023; Pinti, 2014)" Lines 345 and 246 in the revised manuscript

Line 317: The manuscript uses the term "relationship" for *nirS* vs. DO concentration, but "correlation" for nirS vs. cumulative Chl-a. Please use consistent terminology throughout.

We thank the reviewer for this comment. We have revised the manuscript to ensure consistency and replaced "relationship" with "correlation" where appropriate. For example, see lines 349 - 350 in the revised manuscript:

"It is thus consistent that nirS abundance showed a negative correlation with DO concentration (Fig. 6d) and a positive correlation with cumulative Chl a concentration (Fig. 6e)."

Line 367–368: Given the lack of detection issues for AOA in the Cubillas reservoir in September, I am not fully convinced that the presence of AOB and Comammox can be dismissed as easily.

Thank you for your comment. We agree that AOB and Comammox cannot be entirely ruled out. Our interpretation was based on previous evidence showing AOA dominance in these reservoirs (León-Palmero et al., 2023) and the absence of bacterial amoA measurements in this study. However, we acknowledge that pre-filtration and the lack of targeted analysis for AOB and Comammox may have limited our ability to detect these groups. We have revised the manuscript to clarify this point and avoid overgeneralization. Please see the revised text below:

"Previous work in San Francisco Bay revealed that dominant AOA clades were not amplified by commonly used primers, including those employed in this study (Rasmussen and Francis, 2022). It is therefore possible that important AOA lineages present in these reservoirs were missed, leading to an underestimation of amoA abundance. We did not measure the bacterial amoA gene abundance, because AOA had previously been identified as the dominant ammonia-oxidizers in the study reservoirs (León-Palmero et al., 2023). Therefore, we cannot assess the potential contribution of AOB. We tested for Comammox using specific primers and did not detect them in any sample. Additionally, sample water was pre-filtered before DNA extraction (pore size = 3 µm), which may have excluded microbes attached to particles or suspended sediment, potentially including AOA or Comammox groups." Lines 407 – 415 in the revised manuscript.

Line 375–376: Because no positive control for Comammox was available, the absence of amplification does not allow the rejection of the hypothesis that high nitrification rates without ammonium oxidation could be due to complete ammonia oxidation. I encourage the authors to consider this possibility.

We agree that the absence of amplification without a positive control does not allow us to conclusively reject the presence of Comammox. We have revised the text to acknowledge this limitation and to consider the possibility that complete ammonia oxidation could explain high nitrification rates without detectable ammonium oxidation intermediates. Please see the revised text below (Lines 418 – 423) :

"The detection of high nitrification rates, but no significant ammonia oxidation, might suggest that comammox is occurring at these depths. However, our PCR analysis showed no evidence of the presence of comammox bacteria (Fig. S2), although, because no positive control was available, we cannot completely exclude their presence. Therefore, we consider the possibility that complete ammonia oxidation could contribute to the observed nitrification rates. Alternatively, we hypothesize that the NO_2^- production by ammonia oxidation was tightly coupled to NO_2^- consumption by NO_2^- oxidizers, such that it could not be detected in the NO_2^- pool."

Line 411–412: If the earlier suggestion is incorporated, this description should be moved to the "Data analysis" section.

Thank you for your suggestion. We have incorporated the description into the "Data Analysis" subsection as recommended. However, we have also kept a brief mention in the Discussion to maintain clarity for readers who may not refer back to the methods while interpreting the results. We believe this helps contextualize the interpretation without redundancy.

Line 415: To support the interpretation, "which indicates net N_2O production" should specify by which process (i.e., denitrification, AOB, or Comammox).

We modified the text as follows (Lines 462 – 464):

"In general, the increase in the N_2O concentration with depth was coupled to the $\delta^{15}N$-N_2O decrease (e.g., #1-3, #5-6 or #7-9 in Figs. 1 and black trend lines in 2a), which indicates net production of N_2O by water column denitrification, nitrifier denitrification and/or bacterial nitrification"

Lines 417–418: The term "coupling" implies a relationship between variables that is not statistically supported. Please consider using "accompanied by" instead.

We have applied the reviewer's suggestion and replaced "coupling" with "accompanied by" in the text. The revised sentence now reads:

"There was also an increase in the $\delta^{18}O$-N_2O with depth in each profile, accompanied by an increase in N_2O concentration, which also suggests a parallel production and consumption of N_2O at the deeper layers" Lines 466 – 468 in the revised manuscript.

Lines 432–440: These results are very interesting, and the discussion provided here is excellent!

We thank the reviewer for the comment.

References:

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León-Palmero, E., Morales-Baquero, R., and Reche, I.: P inputs determine denitrifier abundance explaining dissolved nitrous oxide in reservoirs, Limnology and Oceanography, 68, 1734–1749, https://doi.org/10.1002/lno.12381, 2023.

Ostrom, N. E., Pitt, A., Sutka, R., Ostrom, P. H., Grandy, A. S., Huizinga, K. M., and Robertson, G. P.: Isotopologue effects during N2O reduction in soils and in pure cultures of denitrifiers, Journal of Geophysical Research: Biogeosciences, 112, https://doi.org/10.1029/2006JG000287, 2007.

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R Core Team: R: A Language and Environment for Statistical Computing, R Foundation for Statistical Computing, Vienna, Austria, 2014.

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