3 June 2026

We would like to thank the reviewers for the time and effort invested in carefully re-evaluating our manuscript. We have addressed all remaining issues in full. Please find below our point-by-point responses to the reviewer's comments (shown in blue).

We hope that the reviewer will now find the revised manuscript suitable for publication.

All the best,

Eyal Rahav and behalf of all co-authors.

Reviewer 1

The authors have adequately addressed my major concerns. I would suggest only a few technical corrections.

Reply: Thank you for the time and effort you invested in reviewing our work.

Line 104 and 107 - OPA method is still ammonium concentration. Just the oxidation is ammonia oxidation.

Reply: Sentence revised as suggested.

Line 132 - keep the McIlvin Altabet citation.

Reply: The whole paragraph was revised following Reviewer 2 comments. The relevant text, which now includes the McIlvin Altabet citation, now reads: "...The azide method (McIlvin and Altabet, 2005) and the denitrifier method (Casciotti et al., 2002; Sigman et al., 2001)..." (e.g., Lines 125-126).

Line 178 – caveat

Reply: Corrected.

Line 393 – occurring

Reply: Corrected.

Line 394 - "Additonally, the uncertainty in low nutrient concentration measurements in the GoA (most notably within the upper mixed layer depth) that may propagate into rate calculations…

Reply: We believe the original sentence reads better (same meaning).

Reviewer 2

The authors' response is timely and address lots of my concerns. However, I keep my original justify that this work possesses scientific merit but remains requires major clarifications in methods especially for isotopic measurement.

Reply: We thank the reviewer for acknowledging the scientific merit of the work and for emphasizing the need for clearer methodological details regarding the isotope-based rate measurements (i.e., Section 2.2). We have now substantially revised the Methods section and addressed all remaining concerns in detail.

Let us begin with the biggest concern:

Section 2.2 I found this section is still unclear for me even after revision.

Reply: Please see our detailed reply below to each of the concerns raised.

1. Look into the literature authors cited. The classic Sigman et al, 2001 marks the original protocol of the bacterial method. In the Experimental section, Sigman et al, 2001 noted "The concentrated [denitrifiers] cells are then aliquotted into 20-mL headspace vials (2 mL per vial), with each vial representing one analysis." The volume of sample is adjusted to achieve a final sample size of 10-20 nmoles N (5-10 nmol N2O), which is optimal for our system." "the volume of sample should not be more than 5-fold greater than the volume of cell concentrate." These standards didn't come from nowhere. The reaction time, isotopic blank from the bateria (0.5 nmoles of N blank associated with the 2 mL bacteria), conversion efficient will change with sample injection size. And the sampler of IRMS need to be changed dramatically with such a large sample volume up to hundreds of mL.

Reply: We thank the reviewer for this important clarification and for pointing us to the specific constraints outlined in Sigman et al. (2001). We agree that the original text was ambiguous and may have implied that the full incubation volume was introduced into individual denitrifier vials. This was not the case. To clarify the workflow: the 1-L incubation volume was required to ensure sufficient total nitrogen mass for IRMS analysis (as well as for the other variables measured), given the ultra-oligotrophic conditions of the GoA, where ambient NO_3 concentrations in surface waters were as low as 20 nmol/L. The denitrifier assay itself was conducted within the established analytical constraints of Sigman et al., (2001). Specifically, aliquots of 2-10 ml were introduced per vial, consistent with the 5-fold volume limit relative to the 2 ml bacterial concentrate. For surface samples with the lowest NO_3, sequential injections of multiple aliquots from the same filtrate were used to accumulate the 10-20 nmol N required for a reliable IRMS peak. For deeper samples single injections of 2-5 ml were sufficient to meet the optimal N-mass target. The isotope blank was accounted for in all cases, and standards and $HgCl_2$-killed controls were run alongside all samples.

We have revised the text to state explicitly:

"...Aliquots of 2-10 ml were introduced per denitrifier vial depending on ambient NO_3^- concentration, consistent with the volume constraints of Sigman et al., (2001) and McIlvin and Casciotti, (2011). For surface samples with the lowest NO_3^- concentrations sequential injections of multiple aliquots from the same filtrate were used to accumulate sufficient N mass per vial, while for deeper samples with higher NO_3^- single injections of 2-5 ml were sufficient... " (Lines 134-135).

"...Given that ambient NO_3^- and NO_2^- concentrations in GoA surface waters approached, or were below, the validated concentration ranges of these methods, the analyses were performed with careful attention to blank correction..." (Lines 135-138).

2. Another literature authors cited Buchwald et al., 2018 isn't a method paper.

The isotope method section in 2.2 is very short with citing McIlvin & Casciotti, 2011, Casciotti et al., 2002; Sigman et al., 2001, McIlvin & Altabet, 2005, Granger & Sigman, 2009. The authors of this manuscript under review didn't cite the DIRECT reference of the methods they used, and citing Buchwald et al., 2018 can not provide enough information for reproducibility. Even though when we look into Buchwald et al., 2018, the main system is for PORE WATER not OLIGOTROPHIC SYSTEM. When the concentration lower than a µM, Buchwald et al., 2018 also didn't report isotopic values (See Fig 3 of Buchwald et al., 2018).

Reply: We thank the reviewer for this observation. We have revised the text to cite the direct methodological references for each analytical approach used. For the denitrifier method applied to $^{15}NO_3$ analysis, we now cite the foundational protocol (Sigman et al., 2001) and the technical updates that specifically enable reliable analysis at low concentrations, including sub-micromolar nitrate (McIlvin & Casciotti, 2011). For the azide method applied to $^{15}NO_2$ analysis, we cite McIlvin & Altabet (2005). For nitrite removal prior to denitrifier analysis, we cite Granger & Sigman (2009). We acknowledge that the McIlvin & Casciotti (2011) technical updates paper is particularly relevant to our study: it specifically describes procedural modifications, including the use of low-volume sequential injections and a custom IRMS inlet, that extend the denitrifier method to samples with nitrate concentrations below 1 µM, which is the operational regime of the GoA surface waters in this study.

We have revised the text to reflect this directly: "...The azide method (McIlvin and Altabet, 2005) and the denitrifier method (Casciotti et al., 2002; Sigman et al., 2001), with technical updates for low-concentration analysis (McIlvin and Casciotti, 2011), are well established for isotopic analysis of nitrite and nitrate in oligotrophic seawater. Prior to denitrifier analysis, nitrite was removed from nitrate samples using the sulfamic acid procedure (Granger and Sigman, 2009) to ensure that the ^{15}N signal reflected only the nitrate pool..." (Lines 125-131).

3. For the indirect reference in Buchwald et al., 2018:

McIlvin & Casciotti, 2011: they noted : "Fresh or seawater NO3 samples are typically injected in volumes equivalent to 20, 10, or 5 nmol of NO3-, although measurements down to 1 nmol are possible (see below). A 6 mL headspace is recommended to prevent media from entering the purge and trap system. Therefore, the maximum volume of a sample is 11 mL (which when added to the bacteria culture in the vial totals 14 mL), and the minimum sample nitrate concentration is 0.1-0.2 µM." Also in this ref's Table 3, 1 nmol N group has been shown affecting by bacteria blank significantly. This ref also used an updated system that differed (and may have better effect) from the original system authors cited.

McIlvin & Altabet, 2005: This is the paper with chemical method for nitrite isotope (the authors should directly cite this, if they use the chemical method). The concentration range used in that study is 0.5-40 µM. And a blank of of 2 nmol is also found. Adding the volume can add to the blank problem.

Granger & Sigman, 2009. This paper related with nitrite removal, and again the authors should directly cite this. (PS. There are two or more protocols existing for nitrite removal, the authors should cite the one they actually used)

Reply: We thank the reviewer for this detailed and important methodological point. We comprehensively revised paragraph to accommodate the concerns raised:

"...For nitrite oxidation, we quantified the $^{15}NO_3^-$ in the dissolved nitrate pool after conversion to nitrous oxide with subsequent IRMS analysis. The azide method (McIlvin and Altabet, 2005) and the denitrifier method (Casciotti et al., 2002; Sigman et al., 2001), with technical updates for low-concentration analysis (McIlvin and Casciotti, 2011), are well established for isotopic analysis of nitrite and nitrate in oligotrophic seawater. Prior to denitrifier analysis, nitrite was removed from nitrate samples using the sulfamic acid procedure (Granger and Sigman, 2009) to ensure that the ^{15}N signal reflected only the nitrate pool. Aliquots of 2-10 ml were introduced per denitrifier vial depending on ambient NO_3^- concentration, consistent with the volume constraints of Sigman et al., (2001) and McIlvin and Casciotti, (2011). For surface samples with the lowest NO_3^- concentrations sequential injections of multiple aliquots from the same filtrate were used to accumulate sufficient N mass per vial, while for deeper samples with higher NO_3^- single injections of 2-5 ml were sufficient. Given that ambient NO_3^- and NO_2^- concentrations in GoA surface waters approached, or were below, the validated concentration ranges of these methods, the analyses were performed with careful attention to blank correction. Accordingly, rates derived from near-surface, low-concentration samples were interpreted conservatively. For sequential-injection analyses of low-concentration surface samples, the cumulative bacterial blank was estimated based on injection number and subtracted accordingly; samples where the ^{15}N signal could not be distinguished from the cumulative blank were excluded and treated as below the detection limit. In the most oligotrophic surface samples these established approaches were applied near their practical detection limits, and ^{15}N enrichments should therefore be regarded as conservative minimum estimates rather than evidence that the bacterial and azide methods are routinely robust at concentrations of only a few tens of nmol L^{-1}..." (Lines 124-146).

4. For The authors's response and the revised text:

For The authors's response "We would like to clarify that seawater incubations were conducted in 1 L bottles (not 0.5 L as mistakenly stated in the original manuscript), which allowed us to collect sufficient sample volume to ensured sufficient N mass for reliable IRMS detection, even at the low ambient summertime concentrations in the GoA.". "The larger sample size" is significantly beyond the ranges in papers authors cited (even beyond the paper indirectly cited.). And author's information here didn't suggest they overcome the limitations of reaction time, isotopic blank from the bacteria/reagents, conversion efficient etc.

Reply: Analyses were performed using the updated inlet system described by McIlvin & Casciotti (2011), which reduces the bacterial blank per injection to 0.04-0.08 nmol N compared to the 0.5 nmol N blank of the original Sigman et al. (2001) system. For the lowest-concentration surface samples, sequential injections of 2-5 mL were used, and the cumulative blank contribution was estimated and subtracted based on the number of injections performed. Conversion efficiency was monitored using co-injected isotopic reference standards run alongside each sample batch, and samples with anomalous peak areas or poor standard reproducibility were flagged and excluded. Samples where the ^{15}N signal could not be reliably distinguished from the cumulative blank were treated as below detection, consistent with the $HgCl_2$-killed control threshold.

The following text was added to clarify this point: "...For sequential-injection analyses of low-concentration surface samples, the cumulative bacterial blank was estimated based on injection number and subtracted accordingly; samples where the ^{15}N signal could not be distinguished from the cumulative blank were excluded and treated as below the detection limit..." (Lines 139-142).

For the revised text: "however, the established approaches used here are widely applied in oligotrophic systems and were sufficient for robust detection of 15N enrichment in the present study..." (Lines 132-136). This is true that bacteria methods has been used in oligotrophic systems BUT with samples higher than 0.1 or usually higher than 0.5 uM. The isotope methods can applied for a few dozens nM of nitrite/nitrate itself will be important news.

Reply: We agree with the reviewer that our previous wording was 'too strong'. The methods are now explicitly described as being applied near their practical detection limits in the most oligotrophic surface samples, and we state that ^{15}N enrichments at these concentrations should be interpreted as conservative, minimum estimates rather than evidence that the technique is routinely robust at tens of nmol/L.

The text was revised accordingly: "…In the most oligotrophic surface samples these established approaches were applied near their practical detection limits, and ^{15}N enrichments should therefore be regarded as conservative minimum estimates rather than evidence that the bacterial and azide methods are routinely robust at concentrations of only a few tens of nmol L^{-1}..." (Lines 142-146).

5. Jiang et al., 2026 Line 132-136 Jiang et al., 2026, the few lines here are unrelevant for methods here (they may be cited in limitation section but not method here). In original comment: I suggest "Of course, now there are some more advanced mass spectra or protocols for lower N amount, but authors didn't mention any of these in the methods". I intend to mean authors' citing method is unlikely to derive isotope measurement down to a few dozens of nM. So, if they adopt a different method scheme for low concentration N isotope, they should cite or describe for more information for reproducibility (see my reasons above).

Reply: We agree that the citation of Jiang et al. (2026) in the Methods may confuse readers. Our intention was not to imply that we employed the anion-exchange resin enrichment protocol described by Jiang et al. (2026) or that our analytical scheme enables routine nitrate and nitrite isotope measurements at a few tens of nmol/L. Rather, we used the established azide and denitrifier methods with the McIlvin & Casciotti (2011) updates, as now explicitly cited in Section 2.2, and we interpret surface measurements near the detection limit as conservative minimum estimates. To avoid confusion, we have removed Jiang et al. (2026) from the original paragraph and, instead, briefly mention it in the Discussion as an example of emerging approaches that may enable more robust isotope measurements at sub micromolar concentrations in future work.

The following statement was added: "...Future work in similar ultra-oligotrophic settings could benefit from newer low-concentration nitrate and nitrite isotope protocols (e.g., Jiang et al., 2026), which explicitly target sub nanomolar N species...." (Lines 401-404).

6. If the authors confirms with their detection limits, they should provide with enough original information of precision test in similar concentration ranges (~0 to 50 nM for nitrite or 10-90 nM for nitrate). The author even precent rates at 40 m (ammonia oxidation) and 60 m (nitrite oxidation) in July 23 (blue). The nitrite concentration and nitrate concentration are near 0 in that level July 23 (See fig 1), that is FAR lower than any method can detect, especially in the case no carriers are added.

Reply: Our operational detection limit for ammonia and nitrite oxidation is derived from the variability of $HgCl_2$-killed controls and background signals, rather than from dedicated low-concentration precision tests. This information is brought in detail (please see above text). We therefore treat rate estimates at the lowest ambient concentrations as conservative lower bounds. At the same time, we note that ambient NO_2 and NO_3 concentrations represent the instantaneous standing stock, not the integrated substrate supply over the 24 h incubation. In oligotrophic surface waters, rapid regeneration and uptake can sustain measurable nitrification fluxes even when bulk concentrations remain near or below detection, so near-zero concentrations at specific depths (e.g., 40-60 m in July) do not necessarily imply a complete absence of substrate availability over the incubation period.

The following text was added:

"...This operational detection limit is based on the variability of killed controls and background signals and does not represent a full validation of isotope analysis at ambient nitrite or nitrate concentrations of only a few tens of nmol L^{-1}..." (Lines 153-155).

"...At the same time, near-zero ambient NO_2^- or NO_3^- at specific depths and months (e.g., 40 m for ammonia oxidation and 60 m for nitrite oxidation in July; Figure 1) should not be interpreted as the absence of substrate availability, but as evidence of tight coupling between substrate supply and demand over the incubation period..." (Lines 342-345).

"...Nevertheless, isotopic measurements at ambient concentrations of only a few tens of nmol L^{-1} carry greater analytical uncertainty than measurements at higher concentrations, and the corresponding rate estimates are best viewed as conservative lower bounds on nitrification activity...” (Lines 345-348).

Minor notes

Line 38 Ammonium is fine when representing nutrient

Reply: Corrected as suggested.

Line 107-108 This statement is vague, no numbers here.

Reply: This statement is meant to show that our concentrations are consistent with those reported for other oligotrophic regions, providing environmental context for our measurements.

Supplementary information: missing author affiliation

Reply: Corrected.