Mdutyana et al. Controls on nitrite oxidation in the upper Southern Ocean: insights from winter kinetics experiments in the Indian sector

Response to reviewer's comments:

We thank the reviewers for their thoughtful comments and suggestions, which have definitely improved our paper. Both reviewers are positive about the manuscript and stress the significance of our unique dataset. Although reviewer 1 supports the ultimate publication of this work, they have raised a few larger concerns that we address in detail below. Reviewer 2 mainly had minor suggestions, to which we also respond below. Our responses to the reviewers are in blue text (with changes to the manuscript text indicated in bold) and their original comments are in black.

Comments from reviewer 1

Mdutyana et al. conducted a series of NO2- oxidation kinetics experiments on the surface waters along one section across the western Indian sector, as well as depth-profile NO2- oxidation rates determination along another section during a winter cruise in July 2017. This work provides reliable data/evidence that nitrite oxidizing bacteria require a minimum (threshold) nitrite concentration to produce nitrate. This result is a highlight of the paper. Yet, I have a few concerns that the authors need to deal with before I can recommend publication.

1. L16–17: This sentence is not easy to understand lacking explanations. Normally, "fuel productivity" means more CO2 fixation, which is logically incoherent with the second half-sentence "weakening the ….CO2 sink". It seems that the authors need to explain CO2 sink meaning export production or new production, which can be overestimated by nitrification. I agree nitrification complicates new production estimates but does not weaken new production (or carbon sink) itself.

Response: In a mass balance sense, nitrification in the surface layer *does* weaken the CO_2 sink. However, we take the reviewer's point and have modified the sentence as follows: "Across the Southern Ocean in winter, nitrification is the dominant mixed-layer nitrogen cycle process, with some of the nitrate produced therefrom persisting to fuel productivity during the subsequent growing season. **Because this nitrate constitutes a regenerated rather than a new nutrient source to phytoplankton, it will not support net removal of atmospheric CO_2**" (Ln 17-18).

2. L31–33: I do not agree with the authors about the understanding of "nitrite undersaturation of the … enzymes" in this paper. Please see below my comments on the relevant issues. In addition, the speculative conclusion should not be included in the abstract without the support of research data.

Response: We have removed the concluding sentence from the abstract and replaced it with the following: "Our findings have implications for understanding the controls on nitrification and ammonium and nitrite distributions across the global ocean" (Ln 33-34). We have responded to the reviewer's other comments regarding nitrite undersaturation of the enzyme in detail below (please see comments 30, 31, and 33, along with our responses).

3. L39–42: Carbon dioxide has no superscript "-"

Response: Apologies for this typo. The superscript on carbon dioxide has been removed, here and in other relevant places (Ln 40 and 43).

4. L51 and L59: Clarify the removal of CO2 from the atmosphere (not from the ocean) throughout the paper for a smooth understand.

Response: Perhaps we do not fully understand the reviewer's concern here – we thought that idea was clear and consistent in the text. We repeatedly refer to the removal of **atmospheric CO_2** in the introduction (and now also in the abstract). For instance, "The cycling of nitrogen (N) in the upper ocean is central to the role that phytoplankton and bacteria play in **atmospheric** carbon dioxide (CO_2) consumption and production" (Ln 38-39 of the original submission; Ln 39-40 of the amended submission); "Over appropriate timescales, new production is equivalent to "export production", the latter referring to the organic matter produced by phytoplankton that escapes recycling in surface waters and sinks into the ocean interior, thereby sequestering **atmospheric CO_2** at depth" (Ln 46-49 of the original submission; Ln 47-49 of the amended submission); and also in the discussion "Since phytoplankton consumption of regenerated NO_3^- yields no net removal of **atmospheric CO_2**" (Ln 601-602 of the original submission; Ln 506-507 of the amended submission).

5. L63–66: Again, iron-deplete conditions may restrict nitrification and thus weaken the overestimation of new productivity but not weaken the biological CO2 sink itself. It is not recommended to use such an ambiguous term "biological CO2 sink" unless it has already been defined/explained in the preceding part of the text. The use of more specific terms such as new production, export production, etc. helps readers easier to understand.
Response: We have amended the sentence as follows: "If this limitation is verified and proves widespread in the environment, one implication is that the iron-deplete conditions of the surface Southern Ocean may restrict mixed-layer nitrification and by extension, **may decrease the extent to which phytoplankton growth is fueled by regenerated nitrate**" Ln 64-67.

6. L97: Lomas and Lipschultz (2006) reported that PNM appeared at the base of the euphotic zone rather than the bottom of the mixed layer, which is different from this study. This study showed that the mixed layer of the Southern Ocean was much deeper than the euphotic zone. The authors should clarify these differences.
Response: The sentence has been modified as follows: "Generally, NO_2^- concentrations in the low-latitude oxygenated ocean reach a maximum near the base of the **euphotic zone**". Ln 97-98.
It should also be noted that nitrite concentrations in the winter Southern Ocean are high not only because the mixed layer is deeper than the euphotic zone; the Southern Ocean also hosts high mixed-layer nitrite concentrations in summer when the mixed layer can be shallower than the euphotic zone (e.g., Fripiat et al., 2019; Mdutyana et al., 2020).

7. L175, L183, and L198: The seawater in these incubation experiments was prefiltered through a 200 µm nylon mesh to remove zooplankton grazer. This operation may result in an overestimation of the phytoplankton uptake rate relative to the in situ rate and thus an underestimation of nitrification rates due to substrate competition with phytoplankton.
Response: We cannot rule out the possibility of nitrification rate underestimation due to phytoplankton outcompeting nitrifiers for substrate in the absence of zooplankton grazers, although we think this is highly unlikely in the case of our experiments. Our reasoning is as follows: 1) the South Ocean's winter mixed layer hosts elevated concentrations of NH_4^+ and NO_2^-, suggesting that neither phytoplankton nor nitrifiers are substrate limited, and 2) the nitrite oxidation experimental design was such (i.e., bottles were opaque) that phytoplankton activity should have been minimal. In addition, recent studies from our group that investigated the potential drivers of elevated NH_4^+ in the winter mixed layer of the Southern Ocean found that i) NH_4^+ production outpaces NH_4^+ removal in the mixed layer, which is inconsistent with substrate limitation of NH_4^+ oxidation (or phytoplankton NH_4^+ uptake) (Smith et al., 2022) and ii) that NH_4^+ oxidation is limited by substrate availability only at NH_4^+ concentrations <90 nM (typically far below those encountered in the Southern Ocean mixed layer in winter) (Mdutyana et al., 2022). This latter study further showed a positive relationship between the rates of NH_4^+ uptake by phytoplankton and NH_4^+oxidation by nitrifiers, which is the opposite of the relationship expected if the two groups were actively competing for NH_4^+ substrate. Therefore, we have not revised the text to include this caveat.

8. L226: The nitrification rate calculation based on the difference between two time-points values may be biased, especially when the added $^{15}NO_2^-$ tracer concentration (final concentration 200 nM) is higher than the in situ NO2- concentration (average 168±48 nM), the incubation time is long (23-30 h), and the inferred nitrification rate is relatively high. A linear fitting of at least 3 to 4 time-point values showing the variation of $^{15}NO_3^-$ content with incubation time helps to assess the stability of nitrite removal and the potential influence of $^{15}NO_3^-$ uptake by phytoplankton on the nitrite oxidation rate in the incubation system.
Response: We agree that fitting a linear regression to 3 or 4 timepoints would be preferable to using only 2 timepoints, and we will endeavour to do that in future. However, given the number of experiments conducted during this study, such an approach was unfeasible. Moreover, the main purpose of this research was to investigate whether NO_2^- oxidation in the wintertime Southern Ocean can be described by a Michaelis-Menten function. In the case of our Michaelis-Menten experiments, the goal was to stimulate the oxidation rates via progressively higher additions of NO_2^- substrate, so for these

results, the two-time-point approach is reasonable. For the vertical profiles (to which we believe the reviewer is largely referring in their comment above), we go to great lengths in the manuscript to address the issue of potential overestimation of the NO_2^- oxidation rates due to the high concentration of ^{15}N-NO_2^- added, demonstrating that NO_2^- oxidation rates can indeed be quite strongly affected by the addition of ^{15}N-NO_2^- concentrations in excess of the K_m for NO_2^- oxidation (Ln 276-291). This allowed us to calculate corrected NO_2^- oxidation rates (i.e., equation 4).

As to phytoplankton assimilation of ^{15}N-NO_3^- derived from ^{15}N-NO_2^- oxidation, this is unlikely to be an issue in the Southern Ocean at any time of year, and particularly not in winter. The ambient concentration of the mixed-layer NO_3^- pool is high year-round (4-28 µM) meaning that its assimilation by phytoplankton will have a negligible effect on the small fraction of the NO_3^- pool that is ^{15}N-labelled due to NO_2^- oxidation. Moreover, the rate of phytoplankton NO_3^- uptake in winter is extremely low (<13 nM d^{-1}; Mdutyana et al., 2020; Philibert et al., 2015; Smith et al., 2022), rendering phytoplankton removal of ^{15}N-NO_3^- even less likely. Finally, the NO_2^- oxidation experiments were conducted in opaque bottles in order to minimize phytoplankton activity (see Methods, Ln 168).

9. L300–302, 375–383, 529–531, 537–539, 661–662: Ammonia oxidation rates and kinetic parameters were mentioned and shown throughout the paper, including the results, Figure 3g-j, Figure 6, and a lot of discussions, but there was no description of the methodology. Similarly, the dissolved iron concentrations (L595-597) were shown in Figure 5, but the corresponding measurement methods were not given. The cited literature is a graduation thesis and cannot be retrieved. Please include these necessary contents in the paper so that the readers can fully understand the entire story.
Response: The information mentioned by the reviewer is now cited properly; we have changed the citation from the graduation thesis to our recently published paper on NH_4^+ uptake and oxidation kinetics in the Southern Ocean (Mdutyana et al. 2022), where the methods are fully described.

10. L347: delete a "from".
Response: The word "from" has been removed.

11. L351–353: This is a very important conclusion. Please give the correlation coefficient and statistical significance (r and p values).
Response: Correlation coefficient and statistical significance have been included in the text ($r^2 = 0.59$ and $p = 0.045$). Ln 332.

12. L357: Fig. 2e showed 56°S for St 05.
Response: This error has been corrected in the caption of figure 2, which now reads "e) St 05: 56°S (OAZ)".

13. L440–444: This is a discussion and should be moved to the discussion section.
Response: We have moved this sentence to the first paragraph of the concluding remarks section: "From depth-profile measurements, we deduce that the rate-limiting step for mixed-layer nitrification in the winter Southern Ocean is NO_2^- oxidation. Despite this, NO_3^- production from NO_2^- oxidation accounted for 63-237% of the NO_3^- consumed by phytoplankton, consistent with previous wintertime observations from the Atlantic sector (Mdutyana et al., 2020). **The implication of this finding is that most of the mixed-layer NO_3^- consumed by phytoplankton in winter, and likely also a significant fraction assimilated in spring, supports regenerated rather than new production (Yool et al., 2007; Mdutyana et al., 2020)"** (Ln. 703-706).

14. L447–450: These statements seem repeated with the content in the Introduction section.
Response: We have removed the repetition by deleting the first two sentences of the discussion so that the section now begins with a statement of our findings: "Across all the major zones of the wintertime Southern Ocean, the addition of NO_2^- to samples of surface seawater stimulated NO_2^- oxidation following a Michaelis-Menten relationship…" Ln. 379-389.

15. L481–482: Redundantly cited "(27-506 nM; Zhang et al., 2020)". It can be revised as "oxygenated coastal or open oceans (27-506 nM; Olson, 1981; Zhang et al., 2020)".
Response: The sentence has been amended as follows: "For the Southern Ocean, we report high substrate affinities of NOB, with K_m values ranging from 134 to 403 nM, which is largely within the range documented for **oxygenated** coastal **and** open ocean **waters** (27-506 nM; Olson, 1981a; Zhang et al., 2020) (Table 2)." (Ln. 409).

16. L482–484: This sentence reads confusing and needs to be reorganized. The Km values were high in Sun et al. (2021) (5-11μM), which is not similar to the low Km values mentioned earlier (Olson, 1981; Sun et al., 2017; Zhang et al.,2020).
Response: We have amended the sentence as follows: "In the low- to zero-oxygen waters of the ETNP ODZ, similarly low K_m values have been reported (254 ± 161 nM; Sun et al., 2017), **although values >5 μM have also been observed (Sun et al., 2021), with these** latter estimates associated with ambient NO_2^- concentrations >1 μM" (Ln. 410-412).

17. L505–506: Table 2 did not show Vmax values. Please add them.
Response: V_{max} values have been added to Table 2.

18. L513–515: There were several descriptive sentences in the Discussion section, e.g. focusing on the values distribution patterns. It is better to add some in-depth discussion about the causes of these phenomenon in order for a discussion to be effective.
Response: We do not completely understand the reviewer's comment here given that the line numbers to which they refer detail a finding of other studies (Sun et al. 2017; Zhang et al. 2020). However, we have tried to remove the very descriptive sentences from the text.

19. L524 and 686: The authors frequently used latitude as an indicator of light throughout the paper. I suggest directly using light intensity data (such as PAR) for analysis.
Response: We agree that it would be better to use directly measured PAR in our discussion of light availability; however, surface PAR was not continually measured during the cruise, which is why we use latitude as a (qualitative) proxy for light. We have added a sentence to the methods clarifying this: "Surface photosynthetically active radiation (PAR) was not measured continuously during the cruise; we thus use latitude as a qualitative proxy for light availability during Leg 1." (Ln 136-138). Given the large distance between our stations, we think that using latitude as a qualitative proxy for surface light availability is reasonable.

20. L602–604: The logical process of the sentence is unclear. I cannot understand nitrification weakens the biological pump. Nitrification supports primary production (carbon fixation), but indeed it can cause an overestimation of new productivity. The authors should accurately state the point.
Response: In a mass balance sense, surface layer nitrification **does** weaken the biological pump. This is particularly true in the Southern Ocean where the ambient mixed-layer nitrate pool remains high throughout the year. The fact that phytoplankton do not draw down more of the shallow nitrate reservoir amounts to a "leak" in the biological pump (i.e., the CO_2 released to the atmosphere is not compensated for by photosynthetic CO_2 removal; Sarmiento & Toggweiler, 1984; Sigman & Boyle, 2000). If nitrate is additionally added to the mixed-layer pool by recycling (which in net, results in CO_2 production even though we recognize that nitrification is an autotrophic pathway), then the biological pump will be further weakened because some amount of the nitrate consumed by phytoplankton is associated with CO_2 production (despite its consumption by phytoplankton causing the removal of CO_2). We have nonetheless edited the sentence as follows: "Since phytoplankton consumption of regenerated NO_3^- yields no net removal of atmospheric CO_2 **in a mass balance sense (Dugdale & Goering, 1967; Yool et al., 2007)**, an iron-related control…" (Ln 506-507).

21. L604: What does "It" mean here? Iron-limiting nitrification? or iron-limiting condition? Clarify it.
Response: "It" means iron-limiting nitrification, which has been clarified in the text: "… an iron-related control on mixed-layer nitrification would help to limit the extent to which this process can weaken the

Southern Ocean's biological pump **and** would lead to enhanced competition between phytoplankton and nitrifiers **for iron**" Ln 507-509

22. L628-632: Deep mixing events cannot explain the results of this study. The discussion does not make sense.
Response: We agree that deep mixing cannot explain our results; however, mixing has been suggested by others (e.g., Zakem et al., 2018) as the reason that nitrite accumulates throughout the mixed layer in the high latitudes (versus at the base of the euphotic zone in the subtropical ocean). We thus feel it necessary to address whether such an explanation applies to the observed near-invariant nitrite in the Southern Ocean mixed layer (and rapidly conclude that it is not). We have amended the paragraph as follows: "The persistence of elevated NO_2^- concentrations in the mixed layer at high latitudes has **previously** been attributed to the inability of iron- and/or light-limited phytoplankton to fully consume NO_2^- transported to the surface with NO_3^- during deep mixing events (Zakem et al., 2018). However, subsurface NO_2^- concentrations in the Southern Ocean are typically below detection (Figure 1b and 3a; Olsen et al., 2016), so it is unclear how deep mixing could supply measurable NO_2^- to the euphotic zone. **We thus discount subsurface mixing as a primary explanation for the elevated Southern Ocean mixed-layer NO_2^- concentrations, as were observed during our study and in other seasons (e.g., Fripiat et al., 2019)**". Ln 531-533

23. L655–657: "while in other cases, NH4+ oxidation is dominant …" seems redundant. This sentence needs to be reorganized.
Response: We don't understand the reviewer's comment. The sentence is evaluating past suggestions of which of the two nitrification steps is rate-limiting: "However, rate measurements from numerous ocean regions show contrasting results, with NO_2^- oxidation sometimes outpacing NH_4^+ oxidation while in other cases, NH_4^+ oxidation is dominant".

24. L606, 659, 665: The rates in Figures 5 and 6 were the corrected rates of ammonia and nitrite oxidation, right? Please accurately express them on the figure axes and Legends.
Response: Thank you for the suggestion. The figures and their captions have been amended to ensure that they are labelled as "corrected rates".

25. Figure 6: There were no error bars at all in Figure 6b. In addition, SE cannot be given based on two parallel measurements (n=2). Please use unified symbols for the same station in Figures 3, 5, and 6.
Response: Error bars have been added to Figure 6b. The station symbols in Figure 3 have been changed to match the symbols in Figures 5 and 6. We will change all references to SE where n = 2 to instead refer to the range of values.

26. L660: derived from?
Response: The sentence has been amended as follows "Additionally, the maximum rates of NO_2^- oxidation (V_{max}) **that we measure in this study** for the surface NOB community (~5 to 13 nM d^{-1}; Figure 2)…". Ln 560-561.

27. L693-694: Why dilute NOB particularly? not dilute AOA? The authors should give an explanation in order for the logic to be understood clearly.
Response: We apologize for the lack of clarify in the original text. The effect appears not be the direct result of dilution but rather the variable response of AOO versus NOB following dilution due to mixing. We have amended the sentence as follows: "In coastal waters, deep winter mixing has been shown to dilute the nitrifier community, **with AOO subsequently observed to recover more rapidly than NOB. This differential rate of recovery has been hypothesized to result in a period of low rates of NO_2^- oxidation during which the co-occurring NH_4^+ oxidation rates remain elevated, ultimately causing NO_2^- to accumulate in the surface** layer (Haas et al., 2021)" Ln 585-589.

28. L737-744: The discussion does not make sense. The consumption of N producing the same biomass of NOB and AOA and their growth rates cannot explain the results of this study. In another word, the

differences in the yield and growth rates (life strategies) of AOA and NOB cannot explain the coupling or decoupling of two steps of nitrification, which only depends on the rates of two steps of nitrification.
Response: We agree with the reviewer's concern and have removed this section of text from the manuscript.

29. L771: Nitrite concentration or oxidation rate?
Response: We are referring to nitrite concentration. The sentence has been amended as follows: "Practically, our findings suggest that Southern Ocean NOB require a minimum (i.e., "threshold") NO_2^- concentration below which, **the ambient NO_2^- concentration** becomes severely limiting." Ln 641

30. L776-787: Normally, the undersaturation by substrate of enzyme means the first order reaction is occurring. The reaction rate reaches the maximum with substrate saturation. However, the authors used substrate undersaturation to explain the substrate (NO2-) concentration threshold of the reaction below which no reaction occurred. The opposite meanings are confusing to readers.
Please see our response below to comment 31; we have addressed comments 30 and 31 at the same time.

31. L787-794: The logic is confusing too. Nitrospira and Nitrospina with a periplasmic NXR have a higher NO2- affinity than Nitrococcus and Nitrobacter with a cytoplasmic NXR. That means Km should be lower for Nitrospira and Nitrospina, and thus there should be no or lower threshold. But the authors explained the substrate threshold phenomena in the Southern Ocean with the high substrate affinity/low Km of Nitrospira and Nitrospina NXR. This is incomprehensible. The discussions about the substrate undersaturation of the enzyme and the response kinetics of the enzymes of different NOB to the substrate are too speculative and some discussions do not make sense.
We thank the reviewer for their insights in comments 30 and 31 above. In the amended version of the manuscript, we have significantly shortened and altered the text to which the reviewer refers (it is now just one paragraph), removing most of the speculation. We no longer invoke substrate undersaturation of the NXR enzyme to explain the apparent nitrite concentration threshold, nor do we discuss possible implications of the NXR location (periplasm/cytoplasm). Instead, we hypothesize that iron limitation may make it difficult for NOB to synthesize NXR in the Southern Ocean, as has been suggested for the tropical Pacific (Saito et al., 2020). In other words, we suggest that under conditions of low iron and low nitrite, nitrite oxidation is less likely to occur than under similarly low nitrite but higher iron conditions (i.e., when more NXR can be synthesized). At higher nitrite concentrations (i.e., following greater substrate amendment in our kinetics experiments), NOB will be less diffusion limited with regards to nitrite substrate than they were when the ambient nitrite concentrations were lower, rendering nitrite oxidation more favourable even as the iron concentration remains low. We have amended the text as follows (Ln 649-677):

"The existence of a NO_2^- concentration threshold may indicate limitation of the membrane-bound NXR enzyme, either by NO_2^- or by another essential nutrient. Recently, using NXR concentrations, estimates of NXR specific activity, and direct measurements of *in situ* NO_2^- oxidation rates, Saito et al., (2020) deduced that *Nitrospina* NXR is undersaturated with NO_2^- in the tropical Pacific, possibly due to iron limitation. The authors suggest that under iron-scarce conditions, it becomes increasingly difficult for NOB to synthesize NXR and thus to oxidize NO_2^-. A similar dynamic may be at play in the Southern Ocean, with limited synthesis of NXR at low iron concentrations resulting in a decrease in the efficiency of the NO_2^- oxidation pathway that manifests most strongly when the ambient NO_2^- concentration is also low. This inefficiency could be alleviated at higher NO_2^- concentrations since NOB (even with a paucity of NXR) are less likely to experience diffusion limitation with respect to NO_2^- when there is more of this substrate available (Pasciak & Gavis, 1974). Regardless of its mechanistic basis, limitation of NOB NXR would help to explain the perennially high concentrations of NO_2^- in the Southern Ocean mixed layer. Moreover, environmental factors unique to the Southern Ocean, such as limited iron availability, may be instrumental in setting the NO_2^- threshold and associated elevated mixed-layer NO_2^- concentrations.

Our observations raise the question of why a similar NO_2^- concentration threshold has not been reported for other ocean regions, particularly those characterized by similar conditions to the Southern Ocean. This may partly be due to the very limited number of NO_2^- oxidation kinetics experiments that have been conducted in the open ocean and/or to the fact that a classic Michaelis-Menten function is usually imposed upon kinetics data, with V assumed to increase as soon as S > 0. Additionally, depending on the maximum substrate concentration added during kinetics experiments (i.e., the maximum concentration on the x-axis of the V versus S plot), it can be difficult to discern a possible threshold NO_2^- concentration by simply examining the plots. Inspection of published Michaelis-Menten curves does reveal the possibility of a non-zero C value in some cases, including in the ETNP ODZ (Sun et al., 2021) and associated with the PNM in the South China Sea (Zhang et al., 2020). However, there are also published curves that clearly intercept the origin in V versus S space (Olson, 1981a; Sun et al., 2017), underscoring the need for further investigation of the conditions that lead to a threshold NO_2^- concentration requirement of NOB."

32. L801: What does "depending on the maximum substrate concentration added during kinetics experiments" mean? Normally a series of concentrations of substrate (not only the maximum substrate concentration) were added during kinetics experiments.
Response: We are referring to the x-axis scale of the MM plots presented in some previous studies. If the x-axis scale is linear between 0 and 10 μM nitrite, for instance, it is easy to ignore a <0.5 μM threshold because it appears very close to x = 0. For clarity, we have amended the sentence as follows: "Additionally, depending on the maximum substrate concentration added during kinetics experiments **(i.e., the maximum concentration shown on the x-axis of a Michaelis-Menten V versus S plot)**, it can be difficult to discern a possible threshold NO_2^- concentration on the order of 0.2 μM by simply examining the resultant plots." (Ln. 669-672)

33. L811-815: The findings from Saito et al. (2020) cannot explain/support the nitrite concentration threshold (C value) for nitrite oxidization here. Nitrospira and Nitrospina dominance does not necessarily cause the existence of a threshold. Nitrospira and Nitrospina usually distribute in the oligotrophic ocean with low concentrations of nutrients. According to the positive correlation between C and nitrite concentration (L351), the C value of Nitrospira and Nitrospina should be very low. This is not consistent with the high values of C observed in this study.
Response: This entire section has been re-written as outlined above (please see our response to comment 30 and 31), and much of the text to which the reviewer refers has been removed.

Comments from reviewer 2

In the manuscript Controls on nitrite oxidation in the upper Southern Ocean: insights from winter kinetics experiments in the Indian sector, Mdutyana and colleagues present strong evidence for nitrite oxidizing bacteria requiring a threshold nitrite concentration to produce nitrate in the mixed layer of the Southern Ocean in winter. Overall, the manuscript is well written, with a great set of figures, and the key findings and any associated limitations/caveats are clearly presented and thought through. Prior to publication I just have a few comments to enhance the clarity of the presentation in places.

Line 31 to 33: ending the abstract on a hypothesis / speculation seems out of place, I would suggest the authors consider instead a sentence focusing on the broader perspective of their work.
Response: We have removed the speculative concluding sentence from the abstract and replaced it with the following: "The decoupling of ammonium and nitrite oxidation, combined with a possible nitrite concentration threshold for NOB, may explain the non-zero nitrite that persists throughout the Southern Ocean's mixed layer year-round. **Additionally, nitrite oxidation may be limited by dissolved iron, the availability of which is low across the upper Southern Ocean. Our findings have implications for understanding the controls on nitrification and ammonium and nitrite distributions, both in the Southern Ocean and elsewhere.**" (Ln. 32-34)

Line 39 to 41: the superscript on CO2 needs to be deleted.
Response: This has been fixed, here and elsewhere in the manuscript.

Line 96 to 98: for clarity I think it would be important to clearly distinguish between the base of the euphotic zone and the mixed layer here, it is my understanding from Lomas and Lipschultz, 2006 (and other studies) that they have found the PNM at the base of the euphotic zone which sets it apart from your work.
Response: The sentence has been modified to specify that the PNM occurs at the base of the euphotic zone; "Generally, NO_2^- concentrations in the low-latitude oxygenated ocean reach a maximum near the base of the **euphotic zone**" (Ln. 97-99)

Line 150: Nutrient collection is not discussed in this section, so suggest you update the subheading
Response: We thank the reviewer for spotting this inconsistency; the subheading has been updated to "**Hydrography**".

Line 188: the custom built on-deck incubator use for the nitrite oxidation experiments, were these fitted with the neutral density screens mentioned for the nitrate update experiments, or were they carried out in the dark – this has important implications for your findings.
Response: The nitrite oxidation experiments were conducted in the dark in separate bottles from the nitrate uptake experiments. We have made sure that this detail is clear in the methods section (Ln 190-216). Conducting nitrite oxidation experiments in the dark (near-)eliminates phytoplankton activity, which one might be concerned could result in an overestimation of the nitrite oxidation rates. However, we recently published a paper (Mdutyana et al., 2020) detailing the results of nitrification and nitrate uptake experiments conducted in the same bottles under simulated light conditions; these rates from the winter Southern Ocean were very similar to the rates measured in the present study, suggesting that we are not significantly overestimating nitrite oxidation by incubating in the dark.

Line 180 to 205: it would be beneficial for the authors to comment on (here or in the discussion) the potential limitation of only having Tzero and Tfinal samplings for their rate experiments, and thereby assuming a linear relationship over the incubation period (potentially missing any time lags, flattening off, or exponential activity). Also, it would be worth mentioning the reasoning behind running the NO2- oxidation and NO3- uptake experiments for very different incubation periods?
Response: We agree that fitting a linear regression to 3 or 4 timepoints would be preferable to using only two timepoints, and we will endeavour to do that in future. However, given the number of experiments conducted during this study, such an approach was unfeasible. Moreover, the main purpose of this research was to investigate whether NO_2^- oxidation in the wintertime Southern Ocean can be described by a Michaelis-Menten function. In the case of our Michaelis-Menten experiments, the goal was to stimulate the oxidation rates via progressively higher additions of NO_2^- substrate, so for these results, the two-time-point approach is reasonable. For the vertical profiles (to which we believe the reviewer is largely referring in their comment above), we go to great lengths in the manuscript to address the issue of potential overestimation of the NO_2^- oxidation rates due to the high concentration of ^{15}N-NO_2^- added, demonstrating that NO_2^- oxidation rates can indeed be quite strongly affected by the addition of ^{15}N-NO_2^- concentrations in excess of the K_m for NO_2^- oxidation (Ln 276-291). This is why we calculate corrected NO_2^- oxidation rates (i.e., equation 4). Finally, numerous earlier studies (e.g., Olson, 1981a; Peng et al., 2016; Zhang et al., 2020), some of which we compare our results to in this manuscript, used the same T_0 and T_f sampling approach as we did, usually based on prior experiments to determine the timescale of the rates to maximize sensitivity/accuracy and minimize the number of bottles required.

As to the different incubation periods for NO_2^- oxidation and NO_3^- uptake – to some extent, this is experimental convention (e.g., Lipschultz, 2008). Experiments involving phytoplankton nutrient uptake can be compromised by the regeneration of the substrate pool if the incubation takes longer than a few hours – this is particularly problematic for NH_4^+, and potentially also for CO_2 (especially at night when regeneration processes are dominant). So, keeping the incubation time short helps to reduce isotope dilution and by extension, prevents underestimation of the rates (see Mdutyana et al., 2022 for a detailed discussion of this issue). In some of our previous work we have incubated phytoplankton for 24 hours and been subject to extensive criticism from reviewers (e.g., Mdutyana et al., 2020 – notice the

numerous caveats regarding potential isotope dilution due to regeneration). In the case of nitrification, the rates are typically so low that 24 hours are required for enough tracer to be transferred to the product pool that we can make a reliable measurement. Additionally, there was no light/dark cycle to worry about since the bottles were incubated in the dark.

Line 237: did you directly determine the fraction of the nitrite pool labelled with 15N i.e. concentration measurements before/after addition – this is not clear in the methods as currently written.
Response: The nitrite concentration were determined ashore, therefore the fraction of the nitrite pool labelled with ^{15}N was not known before the ^{15}N tracer was added. However, it was known (and used) at the time of rate calculation. The wording in the manuscript has been amended for clarify as follows: "$f^{15}_{NO_2^-}$ is the fraction of the NO_2^- substrate pool labelled with ^{15}N at the start of the incubation, **calculated following the direct measurement of the ambient NO_2^- concentrations**". Ln 217.

Line 362: directly related to my comment above, were these nitrite concentrations measured or assumed? This needs to be clearly documented in the methods section.
Response: Please see the response above, which addresses this issue.

Line 440 to 444: this is a nice point, but it belongs in the discussion.
Response: As per the reviewer's suggestion, we have moved this sentence out of the results section and into the first paragraph of our concluding remarks: ""From depth-profile measurements, we deduce that the rate-limiting step for mixed-layer nitrification in the winter Southern Ocean is NO_2^- oxidation. Despite this, NO_3^- production from NO_2^- oxidation accounted for 63-237% of the NO_3^- consumed by phytoplankton, consistent with previous wintertime observations from the Atlantic sector (Mdutyana et al., 2020). **The implication of this finding is that most of the mixed-layer NO_3^- consumed by phytoplankton in winter, and likely also a significant fraction assimilated in spring, supports regenerated rather than new production (Yool et al., 2007; Mdutyana et al., 2020)"** (Ln. 700-706).

Line 486: 'low ambient NO2-' can you be quantitative here? Also, how applicable are these 'low' concentrations to the rest of the ocean.
Response: We have amended the text as follows: "Our focus is on the K_m values derived under conditions of low ambient NO_2^- **(i.e., <250 nM)** given that (some of) the environmental factors affecting NO_2^- oxidation at high ambient NO_2^- concentrations appear to be unique. For example, oxygen has been shown to decrease the rate of NO_2^- oxidation in the ODZs (Sun et al., 2017, 2021) where novel clades of NOB have been detected (Sun et al., 2021). **Additionally, NO_2^- concentrations in the oxygenated open ocean seldom exceed 250 nM (Zakem et al., 2018).**" Ln 413-419.

Line 598: I would add in here as you have in the figure caption that this relationship is only for the euphotic zone. With the kinetics experiments only be conducted with surface waters the question remains, how applicable are these numbers/thresholds to deeper waters in the euphotic zone (where the community might be different, different light conditions etc), and while the authors do point this out in the manuscript, I think it also needs be articulated in this section as well and the potential impacts on the conclusions discussed.
Response: We have clarified in the text that this relationship is only for the euphotic zone: "While we have no iron data with which to compare our kinetic parameters, dissolved iron concentrations ([DFe]) were measured **throughout the euphotic zone** at the depth-profile stations…" Ln 495-497.

To address the reviewer's point about the broader applicability of our surface kinetics experiments, we have added the following text to the concluding remarks section: "Our kinetics experiments were conducted in surface waters only, which raises the question of the relevance of our findings for deeper euphotic zone waters. For instance, it is possible that surface nitrifier communities may be more iron limited than those nearer the base of the euphotic zone. However, in the winter Southern Ocean, the euphotic zone is always considerably shallower than the mixed layer such that both layers are typically very well-mixed, as is apparent from the near-invariant mixed-layer (and thus euphotic-zone) distributions of nutrients (Fig 1b-c), including trace metals (Cloete et al., 2019). One might therefore

expect the nitrifiers to also be evenly distributed over the euphotic zone and mixed layer. The light flux will not be homogenous over these layers, however. Indeed, light availability is frequently invoked to explain the vertical distribution of nitrification rates because nitrifier activity is impeded at high light (Horrigan et al., 1981; Olson, 1981b; Peng et al., 2018; Qin et al., 2014). Our nitrification depth profiles do not show a vertical trend, instead remaining similar throughout the euphotic zone (upper ~50-75 m) and only rising near the base of the mixed layer (Figure 3b-e). We thus consider the results of our surface kinetics experiments to be broadly applicable to the euphotic zone in winter" (Ln. 328-337).

Line 612 / Section 4.3: I largely enjoy this section, there are some really nice discussion points, but in a few places this section becomes a little like a literature review and could benefit from some streamlining to focus on your findings.
Response: We thank the reviewer for the suggestion. In responding to both reviewers' comments we have removed ~700-1000 words of text from this section in order to limit speculation and clarify our arguments. As such, the section has become significantly more streamlined. Please see our response to reviewer 1 above for specifics.

Line 666 (Figure 6): is it the revised rates that are shown? In panel b are the error bars smaller than the symbols?
Response: Yes, these figures are produced from the revised rates, as now made clear on the figure and in the caption. Error bars have been added to panel b.

Line 694: why particularly NOB?
Response: The effect appears not be the direct result of dilution but rather the variable response of AOO versus NOB following dilution due to mixing. We have amended the sentence as follows: "In coastal waters, deep winter mixing has been shown to dilute the nitrifier community, **with AOO subsequently recovering more rapidly than NOB**. **This differential rate of recovery has been hypothesized to result in a period of low** NO_2^- oxidation rates while NH_4^+ oxidation rates remain elevated, ultimately causing NO_2^- accumulation in the upper layer (Haas et al., 2021)". Ln 585-589.

Line 744: It is not clear how this line of discussion on life strategies links to a potential explanation for the decoupling observed.
Response: We agree with the reviewer that this text is overly speculative; we have removed it from the amended version of the manuscript.

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