Author responses to comments of Referee #1 for manuscript bgd-10-5803-2013, Santoro et al. "Measurements of nitrite production and nitrite-producing organisms in and around the primary nitrite maximum in the central California Current"

Referee 1 (R1): This study by Santoro and colleagues examined the dynamics of nitrite in/around the primary nitrite maximum (PNM) in the central California Current through a multidisciplinary approach. With a combination of nutrient profiling and 15-tracer experiment, the authors determined the relative importance of ammonia oxidation, nitrification and assimilatory nitrate reduction to nitrite at the PNM. They measured much higher rates of ammonia oxidation/nitrification, while nitrate reduction rates were low or undetectable. In parallel, with quantitative PCR they found increasing abundance of ammonia-oxidizing organisms towards the PNM and immediately below, as opposed to the shrinking populations of potentially nitrate-reducing primary producers, quantified by flow cytometry. In addition, rate measurements were compared against net production and consumption of nitrite estimated by 1-D modeling. The authors concluded that, contrary to previous conception, assimilatory nitrate reduction was found to be a minor contributor to nitrite dynamics at the PNM, whereas ammonia oxidation is the dominant source of nitrite.

Response: We thank the reviewer for these supportive comments and a thorough review of the manuscript.

R1: My major concerns for this manuscript lie in the rate determinations. While the described method for rate calculation was the same as the authors used before for nitrification rates (Santoro et al., 2010), it is not clear how the nitrate reduction rates to nitrite were calculated exactly. I presume it was the 15N /14N content of the nitrite pool being modeled. In that case, the isotopic compositions of nitrite and nitrate would have to be treated separately, but I didn't see these data presented (either as measured or calculated values).

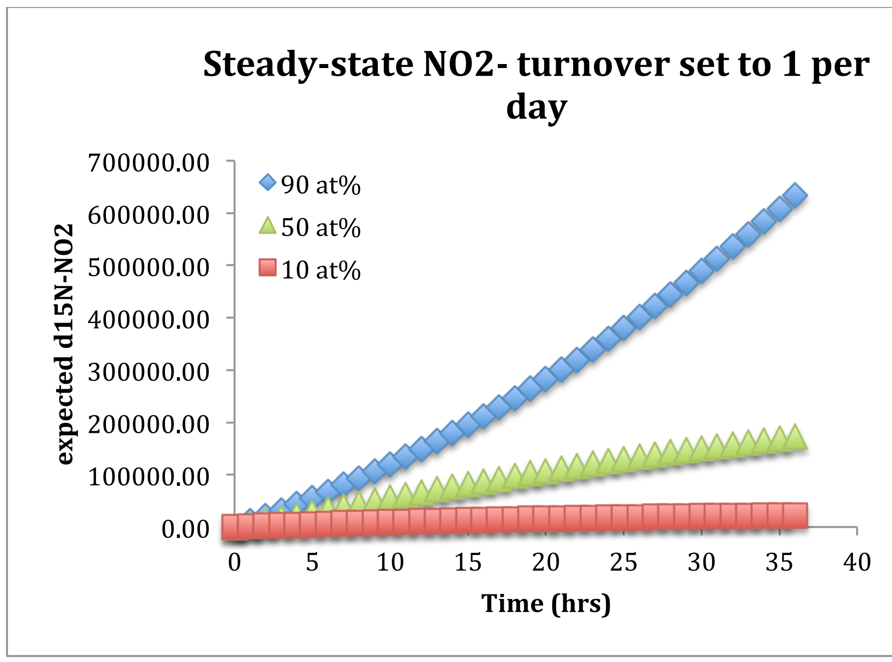
Response: We apologize for the confusion. Yes, in the case of NO_3^- reduction experiments, the $^{15}N/^{14}N$ ratio of NO_2^- was modeled with ^{15}N coming from NO_3^-, and fluxes leaving the NO_2^- pool either by NO_2^- assimilation and/or NO_2^- oxidation. As the reviewer points out, NO_2^- needs to be analyzed separately for this calculation, and indeed it was. The product pools being modeled for each rate calculation were indicated in Table 1, and the description of the sample preparation for NO_2^- analysis (the azide method) and for NOx (the denitrifier method) were described in Section 2.5 "Stable isotope analyses." If invited to submit a revised manuscript, we will add additional text to Section 2.6 "Rate calculations" to reiterate and clarify this point. As the rate determinations presented in this manuscript involved over 400 individual isotopic measurements, the data for each individual measurement are not presented, but rather only the calculated rate.

R1: In addition, nitrite assimilation would not be the only output term, but also nitrite oxidation to nitrite. Not only this process ought to be taken into account in the rate calculation, it is also very likely that the ^{15}N-nitrite production was considerably underestimated in the measurements. Given the fairly high nitrification rates measured in/around the PNM, it is possible that a substantial portion of the ^{15}N-nitrite produced from ^{15}N-nitrate reduction has been reoxidized back to nitrate. In theory, the contribution of nitrite oxidation may be corrected by the ammonia oxidation and nitrification rate measurements. However, how, or whether or not, the above-mentioned have been taken into account in the rate calculations, are unclear in the manuscript. Any complications or contribution from nitrite oxidizing organisms would also need to be discussed.

Response: We assume here that the reviewer meant to indicate nitrite oxidation to nitrate. Nitrite oxidation is certainly an important process in the California Current as in other pelagic ecosystems. The rates of NO_2^- production and consumption are simulated in the model to best fit the changes in $\delta^{15}N$ over time. The mechanism of NO_2^- consumption could come into play in the isotope effect chosen for the removal process (here chosen as 0.975 for nitrite oxidation) and, as the reviewer suggests, in the dilution of the $^{15}NO_3^-$ pool if NO_2^- is re-oxidized to NO_3^-. However, at the levels of enrichment we are working with, the model solution is not sensitive to the assumed isotope effect for NO_2^- removal, and with the size of the NO_3^- pool relative to a wide range of possible nitrite oxidation fluxes, we do not expect significant dilution of the $^{15}NO_3^-$ pool over the course of our experiments. Therefore, the model is not sensitive to the mechanism of NO_2^- consumption, which we agree could be through either nitrite oxidation or assimilation. We will revise the manuscript to reflect this.

We do not believe that we are underestimating the rates of $^{15}NO_2^-$ production for the following reasons. With a pool of NO_2^- that is held constant at steady state (inputs and outputs balanced), the rates of NO_2^- turnover that are required to saturate our ability to accurately model the input over 36 hours are on the order of 1/day. For example, a pool of NO_2^- that is 100 nM would saturate at rates of production and consumption of 100 nM d^{-1}. This finding is relevant to any size NO_2^- pool that is turning over once per day and is not sensitive to the substrate atom% ^{15}N in the range used here for $^{15}NH_4^+$ oxidation (35-93%) or $^{15}NO_3^-$ reduction (2-19%) (see figure below). Rates this high were not observed in our experiments, as we never observed plateauing of $\delta^{15}N$-NO_2^- during our experiments, which would be expected if we were saturating our ability to detect NO_2^- production, nor did we measure $\delta^{15}N$-NO_2^- values higher than 3 atm%. This is the beauty of using isotope tracers—one can detect a gross rate from the change in $\delta^{15}N$, regardless of whether the concentration is increasing, decreasing, or staying the same.

The reviewer may be correct that gross NO_2^- production could be underestimated in the euphotic zone and at 500 m, where the lack of measureable NO_2^- does not provide a trap for labeled NO_2^-. Our interest, however, in this manuscript was in the formation and maintenance of the PNM, for which the above arguments hold.



R1: The incubation experiments were conducted over the course 36 hours, but the authors also mentioned that some sampling stations were occupied at night (page 5822 line 17). Have all the incubations experienced the same number of hours in the dark/light? And were the cycles more like 12h light:12h dark:12h light, or 12h dark:12h light:12h dark? Obviously, it is more likely for the photosynethetic organisms to make any noticeable contribution in the former rather than the latter, so the light regime used could potentially introduce biases for/against these photosynthetic organisms.

Response: This is an excellent point that we neglected to address in the original submission. Due to the short duration of the cruise, the timing for occupying the two stations differed such that the incubation at station 67.70 received an additional ~8 hours of sunlight. Because most of our comparisons are within station rather than between stations, we do not feel this influences the conclusions of the manuscript. Furthermore, by analyzing intermediate time points (12 and 24 h) in addition to the final time point (36 h), we can show that the rates were linear over the 36 hour time course and did not appear to be linked to the day/night cycle. We will clarify this point in the Methods section if invited for a full submission.

R1: The amounts of 15N-amendments resulted in quite a large range of substrate enhancement: 0.5-13 x for ammonium and 0.02-0.23 x for nitrate. Because of the higher degree for ammonium amendment, I wonder if ammonia oxidation were more likely stimulated compared to nitrate reduction, and thus have contributed to the much higher rates measured?

Response: This is certainly a possibility, and if invited to submit a revised manuscript we will address this in the Discussion. Previous work by Newell and colleagues (2013) and by Martens-Habbena and coworkers (2009) indicate that the K_m for ammonia oxidation in the ocean and in ammonia-oxidizing archaea is very, very low (~100 nM) such that these organisms are likely not stimulated by additional NH_4^+ in excess of the K_m so we do not feel this significantly affected our rate measurements. We feel the bigger issue with the differing degree of initial labeling is the difference in sensitivity of the two rates being measured, such that a higher degree of initial

labeling of the substrate pool results in a lower detection limit in terms of the net rate. We addressed this somewhat in the first submission but will attempt to emphasize this point further in a resubmitted manuscript.

R1: While it is recommendable to employ alternative and independent method to estimate net consumption/production rates of nitrite, due to the high likelihood of horizontal advection in the California Current, I am not convinced that the use of a 1-D reaction diffusion model was appropriate. Besides, though the authors claimed to have "high'-resolution nitrite profiles, 12 depth spanning 200 m range, meaning every 16+ m on average is not exactly high-resolution in my opinion. There have been quite a few other studies where every 10 m (or down every 1-2 m in the extreme cases) were sampled. More importantly, it is rather apparent from e.g. Fig 5 that the sampling resolution was too coarse for the data to be used to model 1-m resolution (especially panel b which showed only 6 sampling depths over 200 m).

Response: We agree with the reviewer that the nutrient data presented here are not high resolution relative to some other studies. We will change this emphasis in the revised manuscript. However, we believe that the approach using 1 m resolution to model the distribution of physical and biological processes in the model is justified. One would not wish to represent these processes at a coarser scale than the measurements. It is preferable to have the model run at a physically realistic resolution, then subsample the results at the resolution of the observations to perform the model/data comparison.

R1: In an attempt to compare between modeled and measured net rates of nitrite, the authors simply added the measured ammonia oxidation and nitrate reduction rates together and then subtracted the nitrification rates as the "measured" net rates. Nevertheless, such mass balance calculation would require gross rates for individual processes, while the ones used are only net rates. Subsequently, the calculated net change became erroneously small, thus giving rise to a misleadingly long residence time. On the other hand, not all nitrite consumption and production processes have been taken into account – e.g. nitrite/nitrate assimilation.

Response: We appreciate this comment from the reviewer, which has caused us to rethink the measurement/model comparison as originally described. As discussed above, the rates calculated for $NH_4^+ \rightarrow NO_2^-$ and $NO_3^- \rightarrow NO_2^-$ using ^{15}N are best described as gross rates. This is due to the fact that regardless of NO_2^- consumption, the rate is derived from the change in $^{15}N/^{14}N$ of the NO_2^- pool. Given this, the gross rates of NO_2^- production from NH_4^+ and NO_3^- have been measured here. Less certain are the gross rates of NO_2^- consumption, but these were also derived in the model fits. Therefore, we believe that by combining the gross rates of NO_2^- production from NH_4^+ and NO_2^- with the estimated rates of NO_2^- consumption (from the $^{15}NH_4^+$ experiments, since they gave the most robust signals) we can compute a rate of net NO_2^- production that is comparable to the model. This is not what was done in the original manuscript, but would be included in a revised manuscript.

R1: In any case, why were there only three "measured net rates" shown in Fig. 5, when there were four incubation depths shown in Fig 3?

Response: Incubations were conducted at 5 depths at each station: the euphotic zone, above the PNM, at the PNM, just below the PNM, and 500 m (well below the euphotic zone). The purpose of the 1-D model was to estimate net NO_2^- production around the PNM, thus only the rate

measurements around the PNM are shown for comparison. As the reviewer points out above, NO_2^- assimilation could be a significant term in the NO_2^- budget in the euphotic zone. Because we did not measure NO_2^- uptake, we did not calculate net NO_2^- production at this depth. At 500 m, both NO_2^- and NH_4^+ are below detection limits, thus the 1-D model cannot be applied at this depth.

R1: While it was written in text that ammonia oxidation usually exceeded nitrification rates except for one occasion (St. 67.00, 55 m), Fig. 3 showed that rates of the former was always greater than nitrification. Please clarify.

Response: We agree that the conceptualization of "ammonia oxidation" and "nitrification" rates was confusing as originally described. In response to the reviewer's comments, we would clarify that the two rate measurements conducted should be expected to yield similar results. It is difficult to see both rate measurements when all depths are plotted on the same scale, but the "ammonia oxidation" rate was only 1-2 nM d^{-1} greater than "nitrification" at most depths above and below the PNM. In the case that they do not (St. 67.70, 55 m), where ammonia oxidation inferred from ^{15}NOx production is less than ammonia oxidation inferred from $^{15}NO_2^-$ production, some explanation is required. We will clarify this in the figure caption and throughout the text.

R1: With respect to molecular analyses, they seem a little unbalanced with greater effort spent on quantifying ammonia-oxidizing organisms, relative to the assimilatory nitrate reducing organisms. While, on one hand, I am not certain whether the qPCR and flow cytometry data are truly comparable due to the pros and cons of respective method, there was a lack of data for photosynthetic organisms below the PNM at station 67.155.

Response: As the reviewer correctly points out, one method (flow cytometry) we are counting cells based on pigments/morphology while in the other (qPCR) we are quantifying nucleic acids. Organisms catalyzing the two processes we are interested in (ammonia oxidation and nitrate reduction) have correspondingly very different patterns in their phylogenetic distribution. In the case of ammonia oxidizers, we can capture the majority of ammonia-oxidizing organisms with just two sets of qPCR primers. Nitrate reduction, however, has a much broader phylogenetic distribution and it would be impossible to capture the majority of potentially nitrate-reducing taxa with two or perhaps even ten sets of qPCR primers. Capturing just the cyanobacterial *narB* diversity requires a nested PCR assay not suitable for qPCR (Paerl et al. 2008), and 7 sets of qPCR primers to cover different *Synechococcus* ecotypes of *narB* (Paerl et al. 2011). Thus we feel that the two different approaches are justified for the two different groups. In theory qPCR is more sensitive, so if we were comparing samples at or below the detection limit of the flow cytometer it would be difficult to compare abundances. Paerl et al. 2011, however, found that their qPCR assay for *narB* gave about 40% of the abundance estimate of flow cytometric counts. We will include these discussion points in a revised manuscript. As seen in Fig. 1d, photosynthetic organisms were not present below the depth of the PNM at station 67.155.

R1: Furthermore, as mentioned in the introduction, assimilatory nitrate reduction may also be conducted by heterotrophic bacteria (and likely archaea too). There was, however, no attempt and no discussion to address their potential importance. Perhaps assimilatory nitrate reductase genes, e.g. nas, may be used as a biomarker gene to cover more organisms in this functional group?

Response: This is certainly an intriguing area of future research. We are not aware of a PCR assay that covers the full diversity of *nar* genes in marine bacteria and archaea. If invited for a full submission, we will include references to relevant studies that have attempted to quantify N uptake by heterotrophic bacteria. Previous studies have shown variable but significant contributions of bacterioplankton to total NO_3^- uptake in the upper ocean (4-14%, Kirchman et al. 1994; 5-60%, Kirchman and Wheeler 1998). The extent to which these organisms contribute to NO_2^- production would have been captured by our NO_3^- reduction rate measurements even though we did not attempt to quantify the organisms.

R1: Overall, I think this study has identified a very interesting topic in marine microbial ecology and biogeochemistry; yet the work did not appear sufficiently well planned and executed. Because of the number of critical issues associated with the rate measurements and calculations, as well as biases in molecular analyses, the conclusion that ammonia oxidation being a more dominant source of nitrite relative to assimilatory nitrate reduction, does not seem to be well supported. More clarification, discussion and probably more data would be necessary to help justify such a claim.

Response: The reviewer has provided insightful comments that we believe will help us to refine our interpretations and clarify the presentation. As highlighted above, we feel that we can address the majority of the reviewer's comments and concerns, and that the issues are more in the presentation of the results in the manuscript, rather than fundamental shortcomings of experimental design.

Specific Referee comments:

R1: Page 5808 line 8-12: sampling for nucleic acid extraction vs sampling for DNA extraction – aren't they supposed to be the same? Did you use 2-4L or 1-2L of seawater samples then?

Response: We apologize for the confusion. Water was collected in 2-4 L bottles, but only 1-2 L was filtered for each DNA sample. We will clarify this in the revised text.

R1: Page 5808 line 17 (also in abstract and throughout the manuscript): please avoid the hype of call it high-resolution, as it is not convincingly so.

Response: We will remove this language in the revised text.

R1: Page 5810 line 26: how were the sub-sampling at each time-interval performed? Through rubber septa, or?

Response: Samples were removed using a syringe from the mouth of the polycarbonate bottle. No attempt was made to perform the incubations under gas-tight conditions.

R1: Page 5818 lines 17-19: I'm a little confused with the numbers here. In Fig. 3, the rates for ammonia oxidation were always higher than nitrification rates, but here you mentioned there should be one exception at 55 m. Meanwhile, the number '23 nmol/L/d'

became ‘26’ on page 5823 line 8.

Response: Please see our response above for the ammonia oxidation vs. nitrification issue. We apologize for the typographic error on page 5823; the correct value is 23 nmol L^{-1} d^{-1}.

R1: Page 5820 line 9-10: if the contribution from nitrate reduction is minimal, at the PNM at station 67.155, for example, the measured ammonia oxidation rates were also very low and you modeled a substantial net production at that depth. How would you explain?

Response: We believe the reviewer is referring to the calculation for 150 m depth at 67.155. From Fig. 3: ammonia oxidation at this depth was 46 nmol L^{-1} d^{-1}, nitrate reduction was 4.5 nmol L^{-1} d^{-1}, and nitrification was 7.5 nmol L^{-1} d^{-1}. $46 + 4.5 - 7.5 = 43$ nmol L^{-1} d^{-1}, which is the value plotted in Fig. 5.

R1: Fig. 3: ammonium profile is missing for station 67.155

Response: Ammonium was below detection at all depths at 67.155. This was indicated in the caption for Fig. 3. We will also indicate this in the text of the revised manuscript.