Author responses to comments of Referee #2 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 2 (R2): Rate determinations: p. 5810: Although the authors argue that added nutrient concentrations were more or less around the ambient concetrations, an addition of nutrient one order of magnitude higher than present before always arises the problem that you measure potential rates instead of real ones. Moreover, the treatments for nitrate and ammonia supplementations are not really comparable, and it might happen, that ammonia oxidation is triggered stronger compared to nitrate reduction. How would you argue, here? Can you really exclude that you trigger the one or the other process with this?

Response: The induction of bottle effects is always a possibility in incubation type experiments. If invited to submit a revised manuscript we will address this more directly in the Discussion. Previous work by Newell and colleagues (2013) and by Martens-Habbena and coworkers (2009) indicate that the Km 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.

R2: Could you also add some statement on how the nitrate reduction rates are calculated, this is a bit unclear.

Response: Referee 1 also had a question about this point and the calculations for the nitrate reduction rates will be more explicitly described in a revised manuscript.

R2: The incubations were performed over 36 h. Is there any bottle effect to expect, here? Did you follow up the oxygen consumption in your incubations, which would be highly interesting anyway, and if so, how does oxygen behave compare to ammonia oxidation? Is there any influence of daylight on the rates (which would be expected)?

Response: We understand the concern of the reviewer about bottle effects. This is one reason that we collect multiple time points over the 36 hours (12, 24, and 36), which allows us to test for apparent changes (increases or decreases) in the rate over the full time course. In the experiments presented here, the increases in $\delta^{15}N$ were linear over the time course, which argues against changes in the processes during the period of containment in the bottle due to containment or day/light cycles. We agree that tracking O_2 dynamics in the experiments would be interesting, yet difficult to separate oxygen dynamics between the competing processes of respiration and photosynthesis. We did not conduct the experiments in air-tight containers, as we did not intend to follow O_2 dynamics.

R2: What about further nitrite oxidation to nitrate? Of course, there is always the problem, that you can't screen for this process using molecular tools, however, is this nitrite-loss term neglectable?

Response: Nitrite oxidation to nitrate is undoubtedly occurring. Our previous work (Santoro et al. 2010) quantified nitrite-oxidizing bacteria (NOB) of the genus *Nitrospina*, thought to be the most abundant and active NOB in the marine water column, and found that abundance of these organisms was linearly correlated with the abundance of ammonia-oxidizing archaea. This suggests that throughout most of the water column ammonia oxidation and nitrite oxidation are tightly coupled. Unless NO_2^- is accumulating in the water column, we assume that these two processes are in balance. In our original submission, we attempted to quantify the imbalance between NH_4^+ oxidation and NO_2^- oxidation by measuring complete nitrification (NH_4^+ to NO_3^-) and comparing it to the rate of ammonia oxidation (NH_4^+ to NO_2^-). In hindsight, we believe that this should be revised as both effectively measure the rate of ammonia oxidation. Nitrite oxidation rates would require $\delta^{15}N$ measurements of the NO_3^- pool alone, which we did not perform. In calculating the rates of ammonia oxidation from $^{15}NH_4^+$ experiments, we allow for NO_2^- consumption, and estimate the rate of NO_2^- consumption that is required to explain both the NO_2^- concentration and $\delta^{15}N$ over time. One of the great benefits of using ^{15}N tracer experiments is that one can measure the gross rate of NO_2^- production from changes in $\delta^{15}N$, whether the concentration is increasing, decreasing, or staying the same. Based on some additional calculations done in response to Reviewer #1, we believe that our ability to detect NH_4^+ oxidation in and around the PNM was not saturated, and thus was not underestimated.

R2: Fig.5: Why do you only present three out of four incubation experiments?

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. 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.

R2: Molecular methods: p. 5813 ff.: My major concern on this study is, that you might be missing one or more groups of important organisms, as you screened only for selected ones. How do you know about the diversity of the ammonia oxidizers and the nitrate reducers.? Did you perform any molecular screening based on sequences, e.g. a clone library? If so, it woud strengthen your study enormously to present that data, as it is completely unclear, whether you identified all important organisms or not. Particularly with regard to the ammonia oxidizers, you only quantified the beta proteobacteria and the archaea. Are there no gamma proteobacteria around, that might oxidize ammonia? The way you describe your screening for ammonia oxidizers gives me sort of a feeling that you want the AOA to be the important group, here. It would be good for the argumentation of your study to present this a bit more balanced.

Response: A previous study by our group in this region (Santoro et al. 2010) presented extensive cloning and sequencing data of both AOA and AOB across a 1000 km transect spanning the California Current. Sequences recovered in that study were used to design the qPCR primers used in the present study (Mosier and Francis 2010). Gamma proteobacterial ammonia oxidizing bacteria (γ-AOB) were detected in a few coastal samples within Monterey Bay using targeted primers specific for that group (O'Mullan and Ward 2004), but these organisms but do not appear to be abundant in pelagic systems (Mincer et al. 2007) and our own data from recent Illumina iTag sequencing (Smith and Francis, unpublished). Further, γ-AOB are rarely detected in

metagenomic and metatranscriptomic surveys of coastal systems (Hollibaugh et al. 2010), thus we feel justified in excluding them from the present study. The Santoro et al. 2010 paper also included a scaling argument based on rates of ammonia oxidation that AOA must be the dominant ammonia oxidizers in the central California Current, otherwise unreasonably high per cell ammonia oxidation rates would have to be invoked to explain the observed rates. At the editor's request when can include some of these previous results in the Introduction of the present manuscript.

R2: p.5817: The other thing, I have concerns, is on the comparability of flow cytometry to qPCR, how can you assure that both methods are quantitatively compareable?

Response: As the reviewer correctly points out, and was also noted by Referee #1, using 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. However, we note that in our manuscript we did not make any direct comparisons between the abundance of potentially NO_3^--reducing organisms and the abundance of ammonia-oxidizing organisms, but rather compared their relative depth distributions

Specific comments:
R2: p. 5808: The paragraph on DNA filtration and extraction is a bit confusing, please clarify, if there is any difference concerning nulceic acid filtration and DNA sampling, which filters, filtration times and voumes were used.

Response: We apologize for the differing terminology. 'Nucleic acid filtration' and 'DNA sampling' were meant to indicate the same thing. We will clarify this in the revised manuscript to indicate that 1-2L of water was filtered for DNA extraction.

R2: Same page: Which voumes did you sample for flow cytometry?

Response: Flow cytometery samples were collected from the rosette in 60 mL bottles and transferred to triplicate 1 mL cryotubes for fixation. We will include these details in the revised manuscript.

R2: p. 5816: The first datapoint you present is Chloropyll, but no method is provided for this, please add one sentence.

Response: We apologize for this omission. Chl *a* was measured by fluorescence determination on a Turner 10-005R fluorometer. Samples were filtered onto 25 mm glass fiber filters (GF/F) and extracted for 24 h in 90% acetone (Venrick and Hayward 1994). These methods will be added to the revised manuscript.

R2: Figures: fig. 3: Rates in the text and in the figue do not compare at 55m fig.5: What happened to the other incubation experiment?

Response: Figure 5 presents a calculated net rate of NO_2^- production, derived from the individual gross rate measurements at each depth (shown in Fig. 3). This calculation will be revised based on comments from the reviewers and presented in more detail in the revised manuscript.