Interactive comment on “Nitrogen fixation in the Southern Ocean: a case of study of the Fe-fertilized Kerguelen region (KEOPS II cruise)” By M. L. González et al.

Anonymous Referee #2

General comments In this study, the authors report for the first time N2 fixation activity as well as diazotrophic diversity along a natural Fe gradient in the Southern Ocean during the KEOPS II cruise and from this point of view, this study could be of interest for the scientific community. They compared their measured rates and the community composition to environmental conditions and try to depict the functioning of this under-studied ecosystem. Unfortunately I have a lot of concerns about the veracity of the results for both activity and diversity and I think these issues need to be addressed before a possible publication in BG (see my specific comments below). In general, this manuscript needs to be clarified (misspellings, station names and position, methods. . .), the result section is very difficult to follow, the conclusions should be supported by the results and not so much on speculations based on other studies and the figures are often too small and unclear.
A: We acknowledge all concerns raised by referee #2. We have checked our isotopic substrate, our calculations, figures and tables. We have extensively worked on the revised version of the manuscript and believe this version may be suitable for publication in BGC. We have ruled out a possible contamination in our isotopic gas stock. We also have clarified methodological issues in the text.

Abstract - slide 17153, line 8. For the calculation of the N*, why didnot you use a constant? (+2.9, Gruber and Sarmiento, 1997, GBC)
A: Actually we used the constant but did not write it down in the text. That was corrected in the revised version of the text.

- slide 17153, lines 12 and 15.
Redundancy: “Molecular analysis showed a diazotrophic community dominated by heterotrophic bacterioplankton.” and “diazotrophic community dominated by heterotrophic bacterioplankton”
A: The paragraph was rephrased

- slide 17153, lines 17 to 20. The last sentence is speculative and based on unrelated studies. In the present state of the study, this sentence should be removed. However, if you can provide statistical analysis showing the possible role of DFe for N2 fixation and regenerated primary production, it could support the first statement (lines 17-19) and, if they are available, dissolved organic matter concentrations (DOC, DON or DOP) and bacterial production should also be included in your statistical analysis to support your second statement (lines 19-20).
A: Because dFe data is not available for all sampled stations, we cannot fully test statistically the effect of iron on nitrogen fixation. The sentence was removed.

- Keywords: replace“dissolve” with “dissolved”.
A: Replaced

Introduction - slide 17154, lines 1-4. “the N cycle in the current ocean seems to be out of balance.” You forgot to say that most of the budgets and models have used stable isotopes incubations, which are for N losses “potential rates” (so, often overestimated rates) and for N2 fixation, underestimated rates as most of the incubations for the last decades have been done with “the bubble method”, which can underestimate the rates from 2- to 6-fold (Mohr et al., 2010, PlosONE; Grosskopf et al., 2012, Nature; Wilson et al., 2012, Applied and Environmental Microbiology).
A: We are aware of the possibility of underestimating our rates by using the bubble method. However, given the length of our cruise (4 months including shipment of reagents and materials) and

therefore the impossibility to verify the stability of our solution (in terms of 15N enrichment), we chose not to use an isotopic solution. A comment was added to the paragraph.

- slide 17154, line 14. Rephrase
“the diversity of diazotrophs is increasingly important and it currently includes groups within alpha and gamma-proteobacteria and archaea”, I would suggest “the diversity ofdiazotrophs is important and includes different groups within Cyanobacteria, alpha and gamma-proteobacteria and archaea, and the discovery of new diazotrophs is still increasing”.
A: The text was rephrased.

- slide 17154, line 21. Replace “Cianobacteria” with “Cyanobacteria”.
A: Replaced

slide 17154, line 25. After “oxygenic photosynthesis during the day”, add “such as Crocosphaerawatsonii” or “UCYN-B and UCYN-C” and spell what UCYN means.
A: The paragraph was rephrased

-C9140slide 17155, line 4. Add references.
A: Citation added

- slide 17155, lines 19-22. I donot understand thissentence, please rephrase.
A: The sentence was rephrased

Materials and Methods Section 2.1, - slide 17156. Here all the stations/transects mentioned later in the manuscript are not described and I would suggest to add all of them on the map (Figure 1) to make it clearer. I would also suggest to precisely say in the legend of this figure where the plateau is and add a line to represent the polar front.
A: Details on stations and transects were included in the text and also in Figure 1.

Section 2.2 - slide 17156, line 16. “Experiments were done at all process stations and at the pseudo-Lagrangian stations “E”.” is repeated again on slide 17157, line 6.
A: Repetition was deleted

- slide 17157, line 8.“Water samples (prefiltered by 25 µm)”. Why did you pre-filter your seawater on 25 µm? Especially you knew that diatoms are very abundant in this area (you mentioned a bloom in your Results section, slide 17160, line 22), and if there were present, you have possibly missed part of the activity of the Diazotroph-Diatom associations, which can be very important. Could you please comment about it.
A: We agree with the referee. However being in bloom conditions we wanted to avoid filter clothing that could prevent the molecular detection of diazotrophic groups. Also, we were not sure on our capacity to identify simbionts in this area and chose to leave the diatom-symbiosis out of the experimental setup.

- slide17157, line 9. In addition to the pre-filtration, you used the bubble method to do your measurements, which can again underestimate your rates. Your cruise took place 1.5 years after the Mohros paper, why did not you use the 15N2 dissolved method? Did you at least check your 15N2 enrichment at the different time points of your incubations? You donot even mention the possible underestimation of your rates until your conclusion and I think you should mention it before. Finally, you donot say anything about the company which have produced your 15N2. As you might know, some batches of 15N2 are not “clean” and are contaminated with 15N-NH4+ and 15N-NO3- (Dabundo et al., 2014, PlosONE) and could explain (1) why you have such high rates and (2) why you did detect “N2 fixation” (which could actually be NH4+ or NO3- uptakes) at every depths/stations. Could you please provide the name of the company and the batch number of your 15N2 bottles, and if they are part of the possible contaminated batches, could you please check the contamination, because at the moment, I am very suspicious about your results.
A: We agree with the referee. However, we purchased our $^{15}N2$ to ICON ISOTOPES which is not mentioned in the Dabundo paper. But even in the case of 15N2O contamination, this substrate can also be fixed by diazotrophs[*Farias et al.*, 2013] which would not alter the main conclusion of the occurrence of diazotrophy in our study area.

- slide 17157, line 16. “precombusted glass fiber filters(GF/F) of 0.7 µm pore (450_C for 12 h)” already said line 4.
A: The repetition was deleted

- slide 17157, line18. “filters were kept at 60_C until laboratory analysis”. Usually filters are kept dry but not at 60_C for so long. How did you ship/send them back to Europe? Do you have any references for this protocol?
A: The paragraph was rephrased for clarity. Filters were dried at 60_C for 72h and then kept at 40_C during the rest of the cruise. Filters were send back to Europe by plane (the same return plane as the scientific crew).

Section 2.3 - slide 17157, line 27. “150 µL of 1 % gluteraldehyde”. Usually 1% is the final concentration for the fixation, do you have any references for the fixation you used at _0.1% gluteraldehyde?
A: We included references in the text

- slide 17158. First, if your rates are real, you should have been able to amplify DNA at more stations, even with only 1L. In oligotrophic area, where the organisms are also rare and often less active, 1-2L are enough. Do you have any ideas of why it failed? It is usual to detect diazotrophs even when there is no activity, but the opposite is very surprising for me. Why the size fractionation is not the same as for the rates? At least you would have something comparable. From which size fraction your positive results are coming? The paper you cited line 12 (Beier et al., 2014) is now published in Environmental Microbiology Reports (2015). It is not very easy to access and the details about the DNA extraction are in the Supplementary informations, I would suggest to add few sentences about the protocol in the present manuscript.
A: We modified the entire section in the revised version of the ms.

- slide 17159, line8. What kind of technique have been used for the sequencing? (454, Illumina, other?), add a sentence.
A: The sequencing was done using the Sanger method (Magrogen, Korea).This information was added to the text.

- slide 17159, line 10. How did the software determine low quality sequences? Please add a threshold value.
A: The software carry out an end-trimming based on averaged quality scores (Q/N) identifying poor quality data usually found at the 5`end of the sequences. The quality (Q) is based on the tracer chromatograms of the sequences. However, the tracer data was also visually inspected before and after trimming.

If you only have one positive sample where you have been able to detect diazotrophs (at one depth of one station) and knowing that this region is highly variable, how can you state that this unique sample can be representative of the whole area?
A: We were careful in our discussion and results description not to exceedingly extrapolate our results.

Results Section 3.1 - slides 17159 and 17160. Some stations are new and not mentioned before. Please add them on the map (Figure 1) and describe then in the materials and methods section.
A: All stations were added to the map

- slide 17160. Define Ze as the depth of the euphotic zone
A: Ze was defined

line 9. Section 3.2 - slide 17160, line 19. TNS is not define and add this transect on the map.
A: Transect was represented on the map. Transect was defined in the text.

- slide 17160, line 24. Define precisely N*, the values you used and what it means in the materials and methods section.
A: Details were included in the Materials and Methodes section.

- slide 17161, lines 21-22.“In general,
a phytoplankton bloom with fluctuating biomass in time and space was developed at
all stations”. This sentence is very vague, please rephrase more specifically.
A: Rephrased

- slide 17161, line 24. “surface fluorescence values >2 µg L-1”. Do the authors mean fluorescence of chlorophyll a ? If yes, then the fluorescence is a proxy of the biomass
and it should be mentioned in the manuscript and precisions should be done on the
title of this section. Furthermore the bacterioplanktion should not be in a section called
“nutrients and fluorescencedistribution”.
A: Fluorescence used was obtained from the CTD cast and corrected for calibrated chlorophyll.

- slide 17162, line 6. Replace “picoeukariotes
andnanoeukariotes” by picoeukaryotes and nanoeukaryotes.
A: Replaced

Section 3.3 Why do the author talk again about bacterial abundances and nutrients in this section about N2 fixation? Please rewrite this section. As mentioned before, the statistical tools should be described and the authors should add the results here. I would rather suggest a RDA
instead of a PCA. It would help you to determine the possible relative “contribution” of
each variables (DFe, DOM, primary production, bacterial production) on your N2 fixation
rates and support the hypothesis in your discussion.
A: The section was rewritten.

-slide 17164, line 8. “totalcommunity” is not true if you have pre-filtered on 25 µm.
A: Replaced for “below 25 um”

-slide 17165, line 8. “heterotrophic
bacteria”. Are they all heterotrophs or do you mean non-cyanobacterials?
A: Rephrased

Discussion
- slide 17168, line 16.“Moreover the Fe limitation for diazotrophy is known to control diazotrophic cyanobacteria such as Trichodesmium (Bonnet et al., 2009), which were not detected in this study”. I would remove this sentence, which is not appropriate here for this discussion.
A: Deleted

- slide 17169, line 10. “Ammonium by diazotrophy could support 20 % of ammonium
oxidation”. Did you measure the NH4+ production from N2 fixation? These results
would be interested to show.
A: We could not measure ammonium released via N2 fixation but we did ammonium and nitrate assimilation measurements reported in a companion paper of the KEOPS2 special issue (Cavagna et al., in review). We used this data for evaluating the potential of N2 fixation to support ammonium phytoplankton demand.

Tables and figures

-Table 1. Defined MLD and PZ in the legend.Ze have been used
along the manuscript instead of PZ, please check and be consistent. It is not clear
what those rates mean. Are they areal rates? Then the units are wrong. Or are they
averages? Then it needs to be mentioned in the legend, with the number of depths,
the deepest depth and the number of measurements per depth.
A: MLD was defined. PZ was rephrased by Ze and defined in the Table legend. We also specified that values of N2 fixation are averaged of rates within the MLD.

-Table 2. Please add your integrative areal rates here.
A: Rates were added to the table.

-Figure 1. Please add dots for all the stations mentioned in the manuscript, lines for
the transects and a line for the Polar Front.
A: Figure 1 was modified as suggested.

-Figure 2 and 3. The subfigures are very small and difficult to read. You should add over a) and c) TNS and TWE respectively.
A: Added

For Figure 3, check fluorescence and itsunit, the titles on the graphs are not the same as in the legend.
A: There was a mistake with the legends. We corrected all imprecisions
.
-Figure 4. Too small again and check fluorescence.
A: The figure was corrected

-Figure 6. Add explanation about the day and the night time points.
A: Although we did incubations during a 24 h period, the time of day is not the same in all cases. Sampling time and therefore the 12 and 24h sampling points were determined by the hour of arrival to each station. Adding day and night to the figure would make it unclear to the reader. As we do not use the data for comparing daylight and nighttime fixation we did not think it necessary to add that information to the figure.

-Figure 7. Your sequences in the tree are not clear. Change OUT to OTU and check misspellings.
A: Figure 7 was modified

References

Farias, L., J. Faundez, C. Fernandez, M. Cornejo, S. Sanhueza, and C. Carrasco (2013), Biological N2O Fixation in the Eastern South Pacific Ocean and Marine Cyanobacterial Cultures, *PloS one*, *8*(5), e63956.