

Note: Since we only had a pdf of the reviewer suggestions we address the 3 paragraphs from the general comments without typing all text from the reviewer then address the specific comments below.

General Comments.

Surprise about the picoeukaryote.

This is a valid suggestion however at the time of the study the sequence data for the respective diatom hosts was unknown. A subsequent and current study in Foster lab is work developing new molecular methods (e.g. in situ hybridization, qPCR) for identifying on the diatom hosts by single cell PCRs, however this information was not concurrent with the work presented here so it is not included. Unfortunately we also have no more DNA template left to process as we used it all up on the 9 targets which we report on.

Discrepancies between ‘at sea’ and lab-based.

The comments and suggestions (see below) are also well noted, however we cannot address the extraction efficiencies given the nature of the samples (field based mixed communities. Although we attempted to be as uniform as possible in the sampling and archiving, it is likely that some heterogeneity in sampling resulted from that samples were take from different levels of the niskin. Perhaps all water could have been drained, homogenized prior to the subsampling of the volumes for the nucleic acids, however we were pressed to get the samples extracted and run on the qPCR in a timely manner to direct other points of interest for the cruise at large. We address some of the sources for the discrepancies in more detail in the revised version (Pgs 22-23, lines 13-4).

Restructuring and refining of introduction, results, etc.

We agree, and have made some reorganizing of the presented work.

It should be noted that this paper was submitted for a special issue of Biogeosciences which is entirely focused on the research cruise (OUTPACE) and so it is part of a collective body of work by all members of the expedition.

Specific Comments

Abstract:

In general, the Abstract could be improved by including quantitative nifH copy number rather than the percentage of nifH copies detected.

Agreed, and have provided ranges in the nifH copies enumerated for the various targets.

Line 9. What does the >47% refer to when you say the UCYN-A lineages were poorly detected?

Originally this was referring to the % of samples below detection, we have rewritten the sentence and this no longer applies (Pg 2, Lines 10-12).

Line 9-12. This is inconsistent-the hosts mirrored their respective symbionts yet were below detection?

We recognize that this was not very clear as we were referring to when the hosts were present they were only in samples where their respective symbionts were enumerated. We have re-written the result (Pg 2, Lines 13-15).

Lines 14-15. Perhaps re-phrase to state that they correlated with the surface group. Include the parameters that were significantly correlated with the deep UCYN-A group too.

Agreed and have followed the suggestions (Pg 2, Lines 19-20).

Lines 15-16. Could you expand on this briefly?

Added in a short comment on the most important environmental parameter in the meta-analysis: temperature (Pg 2, Line 18).

Line 18. ‘free-living cyanobacterial diazotrophs’

Agreed and rephrased (Line 2, Line 20)

Introduction:

Pg 4 lines 18-19. There is no clear link between these 2 paragraphs. Suggest moving.

Agreed and have restructured the introduction following these helpful suggestion.

Pg 5 lines 18-23. Perhaps expand on this slightly to indicate why understanding these differences are important for our understanding of marine N₂ fixation, especially within a predicted hotspot of N₂ fixation.

Added a sentence to emphasize the importance and benefits of a better understanding (Pg 5, Lines 3-5).

Pg 5 lines 24-25. Could you briefly outline why performing ‘at sea’ quantitation is/would be a preferential application for qPCR studies.

We have added a brief explanation as to why it was necessary and useful for the expedition. For example, the results from the qPCR was used to make informed decisions about regions high for target diazotrophs. Subsequently these stations were selected for long duration stations so intensive sampling could be implemented to better characterize the community, the microbial activities and hydrographic conditions (e.g. rate measures, drifter arrays, sediment traps, etc.) (Pg 6, Lines 3-8).

Materials and Methods:

Pg 6 21-24. Could you indicate here briefly which conditions you were aiming for with these LD stations.

A brief explanation is provided and also two articles in the special issue are referenced (Pgs 6-7, Lines 25, 1-4).

Pg 7 line 3. Which make and model of CTD was used.

The Seabird 911 was added (Pg 7 Line 15).

Pg 7, lines 9-11. Were these samples also stored with the glass bead mixture. Was the same amount of seawater filtered?

Details have been provided. The 4 samples taken for the ‘at sea’ processing were not stored, these were immediately extracted and the beads were necessary for the

agitation step. The same volume was always filtered (2.5L), except for a few cases with a lot of material clogging the filters (Pg 7 Lines 19-20; Pg 8, Lines 1-2).

Pg 7 line 16. How long were they stored?

The nutrient analysis samples were stored for a few months as all samples were re-analyzed in the land-based laboratory (Pg 9, Lines 7-8).

Pg 7 Line 22. Why are these methods included under the ‘Nutrient analyses’ sub-heading?

This was a mistake, the text has been replaced in an appropriate subheading (Pg 7, Line 11).

Pg 8 line 14. The *T. pelagicum* in Suppl. Table 5?

This was also a mistake, both should be K. pelagicum and have now been corrected.

Pg 8. Line 16. Were the host diatoms quantified too? If not why not?

The host diatoms were not quantified as their 18S rRNA sequences were unknown at the time of the study and hence qPCR primers and probes could not be designed. Subsequent and current work in Foster’s Lab is working on these types of assays. Unfortunately all DNA templates were used up on the 9 targets (Trichodesmium, UCYNA-1, UCYNA-2, UCYNA-1 host, UCYNA-2 host, UCYN-B, het-1, het-2, het-3).

Pg 9 Lines 2-4. Have you tested the two DNA extraction methods on identical samples to determine any potential differences between the two methods? It would be good to get a clear sense of how different these methods are here.

We wholly agree with this shortcoming in our approach, but unfortunately we do not have replicate samples to test. It should also be noted that replicate field samples are challenging to collect as one cannot be certain of uniform distribution in a niskin bottle. We have added a short summary of the latter in the discussion (Pg 22-23 Lines 13-4).

Pg 9 Line 14. What is the percent identity between the UCYN-A1 and UCYN-A2 host 18S rRNA sequences?

We have added this detail (97.95%) by determining a distance matrix on the 635bp fragment of the 18S rRNA for the following sequences: accession number JX291893 (UCYN-A1 Host) and accession numbers KF771248-KF771254 (UCYN-A2 host) (Pg 10 Lines 16-17).

Pg 9 Lines 13-21. This information might be better summarized in a table.

Agreed and have added an additional suppl. table 1 (1c).

Pg 10 lines 10-12. It’s great to see this information here but why wasn’t het-3 included in the het cross reactivity tests?

Earlier cross-reactivity tests reported in Foster et al. 2007 found no cross-reaction between the het-3 and the other het groups, so we felt it was redundant to repeat. This detail is added into the text. (Pg 11, Lines 6-8).

Pg 11 lines 8-10. Is there a particular reason why assays weren’t performed for these stations?

Overall qPCR assays 'at sea' were limited by time. The main reason for not performing qPCR at these stations was that they were not possible LD station candidates due to geographical and/or hydrological reasons. A short sentence has been added in the text (Pg 12 Line 5).

Pg 11 lines 22-24. What were the efficiencies of the other assays?

The efficiencies were only tested on the het groups as we were trying to be conservative with the limited template (Pg 12 Line 19)

Results:

I think the results of the cross-reactivity tests should be moved to become section 3.2 as this is important for the interpretation of the qPCR assays.

Agreed and have moved the text. Although it should be noted that the intention of this paper was not to be a 'methods' paper, and we are subsequently working on developing better qPCR assays which will be summarized in a separate body of work at a later date.

Pg 13 Line 7. Table 1 contains values for DIN-should they be bq?

We acknowledge the inconsistency and have amended the table where the bq is defined.

Pg 13 Lines 18-19. A comparison of the two DNA extraction methods is required to determine if they could have affected the qPCR results.

We agree with the statement that a comparison of the extraction method is necessary if one was to truly compare the results. However here our intention is to report the differences, which we attained. The differences in abundance could be derived from the difference in extraction method, the variation of extraction efficiency per target, the patchiness of plankton, etc. It is not clear how the reviewer suggests we correct this section.

Pg 14 Lines 1-5. Explanation for the differences in the at sea and the lab based. Can you explain these results? This needs to be discussed further on pages 20-21.

There are a few possibilities here, and are explained in the text (pg 22-23, Lines 20-25; Lines 1-11).

Pg 14 Lines 17-20. Here and in other places throughout the results where you report depths of maximum abundances, please include the *nifH* copy numbers in the text.

Agreed and have modified the text throughout section 3.4. Note that the depth of maximum abundances is the average from the two regions (MA and SG).

Pg 15. Lines 4-6. Please revise this sentence for clarity.

Agreed and have made it several sentences. (Pg 16, Lines 16-19).

Pg 15 Lines 7-11. The confusion with the LD and SD stations within the MA and SG.

Agreed and have followed the suggestion of the reviewer to indicate on Figures and also in the suppl. table 3.

Pg. 15 line 14. Sometimes you refer to number of stations and other times the number of samples when talking about prevalence of the different groups, please be consistent.

Noted and rephrased where appropriate. Although, we include the detail on samples when referring to the UCYN-A as it was striking that these were absent and/or patchy in distribution and we lose this context if we only refer to station.

Pg. 16 Section 3.4 indicate number of observations included when presenting the significant correlations.

Noted and fixed in the text.

Pg. 17 line 18. Perhaps indicate the significant clustering for group 1 and 2 on Figure 3 for clarity.

Agreed and amended the Figure with this detail.

Pg 18 lines 1-11. The RDA is explained very nicely, perhaps you should color code the dots in figure 4a to reflect the different response variables.

Figure 4a, the dots in the RDA represent samples and cannot be color coded according to diazotrophs, since they are represented by a vector (red labeled dot).

Pg 18 Section 3.5. I think the results of the meta-analysis would be more compelling if represented as a figure in the main text, perhaps as a heatmap/correlogram like Figure 3.

Agreed, we have added bar graphs (one for each diazotroph) as an additional figure (Figure 5).

Pg 19 Lines 14-24. I suggest moving this section to 3.2 of the results. Do you have the data for the “viceversa” e.g. UCYN-A2 host assay with the UCYN-A1 host target, and the het-2 assay with the het-1 target? This is not obvious from Supplementary Figure 1.

We did run the similar assays in the reverse (‘vice versa’) and have added the additional graphs in supplementary figure 1. Originally we limited the presentation since the results were similar and we were trying to keep things simplified. (Pg 14, section 3.2)

Discussion:

Pg 21 lines 2-8. Please discuss these results more thoroughly.

Agreed and have added text (Pg 22-23, Lines 13-4).

Specifically, can you comment on potential differences in DNA extraction efficiency between the two methods?

Agreed that this would be a valuable piece of information however we did not determine the DNA extraction efficiency, and it would also be quite difficult since these are mixed community field samples. One would need a known abundance of a particular target e.g. lab based culture work could address this for a particular target that has been isolated e.g. UCYN-B and Trichodesmium.

Are you comparing the same diazotroph community (e.g. from the same Niskin bottle/homogenized samples).

Samples were always taken from the same niskin bottle, but their entire volume was not homogenized.

Were there any inhibitors?

No inhibitors were added.

There doesn't appear to be a clear pattern in over/under estimated of the at sea versus lab assays based on Table S2, so perhaps you can't explain the differences, but possibilities should be at least discussed.

Agreed and further details have been added to the text (Pg 22 Lines 19-23).

Pg 21. Lines 23-25. Do you have a hypothesis as to why you observed these surprising results?

Assuming that the reviewer is referring to the restriction of the UCYN-A to one depth of LD C, perhaps one could hypothesize that it was a small-scale bloom or entrainment along an isocline. However we prefer to be conservative since it was only one observation and leave the text as is without drawing a larger conclusion.

Pg 22. Could you also compare the actual abundances throughout these paragraphs to give more context-perhaps also the seasonal timing of the different studies for comparisons?

*We are not sure how to address this suggestion. Is it that we should provide the exact *nifH* copy numbers for all the 11 datasets?*

Pg 24 lines 1-8. Indicating that DDAs are important for export production in this region, like the NPSG.

Agreed, added a sentence (Pg 26 Lines 13-14).

Pg 24. Lines 9-18. Could this also be due to a limited understanding/representation of UCYN-C diversity; how specific is the qPCR assay.

*The cited studies for UCYN-C have used qPCR (Turk-Kubo et al., 2015) and isolation, then subsequent 16S rRNA and *nifH* sequencing (Taniuchi et al., 2012). The qPCR study, which was conducted within a lagoon of the Melanesian archipelago, found UCYN-C to partly dominate diazotroph abundances, and they used the same qPCR assay as we did (Foster et al., 2007). We have added a short summary statement about the UCYN-C assay, which was evaluated in Turk-Kubo et al. 2015 that reported the UCYN-C assay to quantify the majority (up to 85% of the sequences in their study) of UCYN-C phylotypes (Pg 26, Lines 23-24).*

Pg 28 lines 14-22. Why do you think this was the case? What other factors (perhaps beyond what you measured) could have influenced the depth distributions of these groups.

UCYN-A has been shown to have a colder temperature optimum (and range) than the other cyanobacterial diazotrophs in this study, which could also drive a subsurface maximum. Moreover the distributions observed could be linked to viral infections and grazing by zooplankton, none of which was actually measured.

Pg 28-29 lines 23-19. It would be nice to see further discussion around these results of the meta-analysis: the similarities and differences to other regions and the local/environmental factors driving these patterns could be discussed.

Agreed. Added text (Pg 30-32 Lines 23-3).

Pg 30 Line 19. Could you provide the same context for the UCYN-A assay.

The cross reactivity for the two UCYN-A assays had a near perfect match when run as A2 assay with A1 standard, meaning that no matter the abundance of A1 (high or low), there would always be a risk of significant cross-reactivity and overestimation of A2 in the presence of A1.

Figures:

Figure 1. Does the white dashed line indicate the separation of the MA and SG? Please clarify. Would it be possible to overlay SST on the station map, as this was an important explanatory variable?

Yes, the dotted line separates the MA and SG and has been clarified in the caption. It is possible to overlay the map with SST but it will not be very informative in our relatively narrow region where the SST was mostly uniform (everything would be the same color). The large impact of temperature was mediated vertically rather than horizontally.

Figure 2. You mention specific depths in the text-perhaps indicate average depth on 2a or include % surface irradiance in the text. Can you make 2b slightly larger as the station numbers are difficult to distinguish (Perhaps also indicate the MA to SG transition).

Agreed

Figure 3. Indicate group 1 and group 2 on the hierarchical clustering for clarity.

Agreed. The two hierarchical clusters of group 1 (surface) and group 2 (subsurface) has been marked.

Figure 4. Color coordinated dots might help to support the text. Please include a y-axis label in 4b.

Agreed, however see previous reply in the results section regarding coloring the dots. Y-axis label (Variance) added.

I would also like to see the meta-analysis presented as a figure if possible.

Agreed and have added a new figure 5 which summarizes the meta-analysis.

A T-S plot as a supplementary figure would also help to distinguish the different water masses of the MA and SG.

This is a valid suggestion included a supplementary Figure 2 which has the upper 500m T-S plots for stations LD A and SD 15 (MA and SG, respectively). More details on the water masses will likely be addressed by the physical oceanographers whom have submitted work on the hydrography in the special issue.