

Interactive comment on “16S rRNA gene metabarcoding reveals a potential metabolic role for intracellular bacteria in a major marine planktonic calcifier (Foraminifera)” by Clare Bird et al.

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The authors sincerely thank the referee for their time and constructive comments regarding this manuscript. We discuss those comments further below.

General comments: 1) Title: The title is too general and promises something that cannot be shown yet by the data. Metabarcoding only reveals the presence of the bacteria not their metabolic role. The title further seems to refer to all planktonic foraminifera in general, while the study only analyzes one genotype of one morphospecies. The authors fully accept that the metabolic role of *Synechococcus* has not been estab-

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lished and will remove this from the title, and will be more specific in referring to the foraminifera. [Changes to manuscript] Title changed to: 16S rRNA gene metabarcoding reveals the presence of intracellular cyanobacteria in a major marine calcifier, *G. bulloides* (planktonic foraminifera).

2) Methodology: In part 4.1.4 of the discussion you mention the possibility of a primer bias against a certain group of bacteria introduced by the PCR based approach for the detection and identification of bacteria. I wonder if this may not be a more general problem in the study, making certain groups of bacteria appear more abundant than they actually are, since they are amplified more easily with the chosen primers than other groups. This issue needs to be discussed in the MS. We agree entirely with the referee that primer bias in this method needs to be addressed in the manuscript. So as not to repeat our comments on this point, we refer readers to both the “reply to SC1” document and to part (1) of “response to referee #2” on the discussion forum with regard to the bias in the primer set used in this study. [Changes to manuscript] In Section 2.6 we have added a fuller description of the primer set and the known biases, and discuss a lack of amplification bias towards *Synechococcus* in Section 4.1.4.

3) Discussion: Showing a metabolic role for intracellular bacteria in a eukaryote host sure is a difficult task. So for now this part should remain rather speculative and not appear for example in the title, as mentioned above. Yet, I agree that referring to the bacteria as endobionts is legitimate, as this purely describes their presence within another organism. As mentioned in part 4.1.2, *Synechococcus* are present in deep living benthic foraminifera as well as diatoms. In both cases photosynthesis would not play any role in the association between the bacteria and the hosts. I wonder if *Synechococcus* just uses the hosts as some kind of protection, more or less “infesting” them. In this regard, I am not sure how the authors conclude that *G. bulloides* actively and species-specifically takes up the bacteria from the water column. I think there are no data yet to show how the bacteria really end up in the foraminiferal cell.

The authors acknowledge that any metabolic interactions between the two parties have

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not yet been demonstrated and we have altered the title accordingly. We consider Section 4.2 to be a speculative discussion on the potential benefits to either party in agreement with the request of the referee. We agree with the referee that *Synechococcus* are endobionts, but that there is currently no data to show how large numbers of the cyanobacteria accumulate in the foraminiferal cell. For example does the *Synechococcus* population exist purely via cell division of a small number of cyanobacteria entering the cell, or does the foraminifera phagocytose many *Synechococcus* cells and actively maintain the population? Or are the *Synechococcus* cells able to avoid digestion unlike other phagocytosed bacteria, and how? So it is yet to be determined whether host or endobiont instigates this relationship.

However what is known is that there is selectivity in this process, as to date *G. bulloides* Type IId is the only planktonic foraminifera known to house *Synechococcus*, and we have also shown that *G. bulloides* Type IId cells retain only *Synechococcus* rather than all ingested cells, so rendering it a highly specific relationship. Never-the-less we will modify our conclusions and use more passive terms in describing the relationship.

[Changes to manuscript] In the introductory paragraph of the discussion (section 4.1) the sentence “However, we do have strong evidence that *G. bulloides* Type IId contains large numbers of the photoautotrophic picocyanobacterium, *Synechococcus*, and that they are preferentially taken up from the water column by *G. bulloides* and concentrated within the host cytoplasm” has been removed in favour of “However, we do have strong evidence that *G. bulloides* Type IId contains large numbers of the photoautotrophic picocyanobacterium, *Synechococcus*. *Synechococcus* accumulates within the host cytoplasm in abundances far greater than those found in bloom conditions in the California Bight or in other foraminifera species investigated. How this association occurs is unclear,”

This still stresses the unparalleled numbers of *Synechococcus* observed within the cell but avoids talking of the “active selectivity” objected to by the referee.

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The title of 4.1.3 is changed to “*Synechococcus* cells accumulate in the *G. bulloides* cytoplasm” to remove reference to *Synechococcus* being specifically taken up from the water column. The term “selectively accumulated” is replaced by “accumulate” in the first sentence and the word “selective” is removed from line 28.

Detailed comments: (1) Abstract: Line 2: Maybe edit to: “This marine protist is commonly used in micropaleontological investigations. . .”

[Changes to manuscript] Line 2 is amended as suggested above

(2) Abstract: Lines 4-5: The reasoning why the authors chose *G. bulloides* to search for bacteria symbiosis is not completely clear. What does “atypical geochemical shell signature” and “divergent ecology” mean?

[Changes to manuscript] In order to bring further clarity to the use of *G. bulloides* for this study we have altered lines 4-5, removing the words atypical and divergent accordingly, and instead briefly describing the atypical geochemistry and ecology.

(3) Introduction: Page 4: Lines 18-20: This sentence needs clarification: “. . .by more than any other extant, surface-dwelling species. . .Such large deviations. . .” Written like this, the statement needs quantification on how large the deviations actually are.

A single quantification is not appropriate here. The deviations from predicted values will vary by study and oceanographic location since the oceanographic setting likely affects the offsets from predicted values (e.g. if the carbonate ion concentration is different in two ocean basins, the carbon isotope offsets from predicted equilibrium will be different even at the same depth, temperature, etc.). For quantification, the reader should look to the cited studies.

[Changes to manuscript] Change in lines 18-20: The authors have re-worded these sentences to emphasise the point that *G. bulloides* precipitates its shell out of equilibrium with respect to both carbon and oxygen isotopes rather than focusing on the magnitude of that offset.

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(4) Introduction: Page 4: Line 31: Is there only this one genotype in the sampling area? If yes then paleontological analysis on that morphospecies from that area should not contain any noise due to genotypes as mentioned in the paragraph before.

There is only one extant genotype identified so far in this region (with the exception of a single individual considered to be potential contamination), and hence noise due to genotype variation is unlikely to occur here, rendering the geochemical calibrations from this area robust. The *G. bulloides* Type IId genotype has not, so far, been found elsewhere and as such the calibration for this genotype may not be appropriate for other genotypes. Understanding the ecology of each genotype and genotype-specific calibrations will be necessary across the morphospecies since mixed populations do exist that harbour different geochemical signatures.

[Changes to manuscript] Introduction: Line 31: Addition of phrase to confirm the presence of only one known genotype here to date. In addition a reference has been added to support the statement of different genotypes harbouring different geochemical signatures.

(5) Introduction: Page 5: Line 1: *Globigerina bulloides*: In general the genus name should be written out only once at the beginning of each chapter and then afterwards abbreviated.

[Changes to manuscript] *Globigerina bulloides* has been changed to *G. bulloides*

(6) Introduction: Page 5: Line 3-4: “We demonstrate. . .” I don’t think it is really demonstrated here that the bacteria are actively taken up by the foraminifera and I also don’t think it can be said yet if the association is really SPECIES-specific.

The authors acknowledge this point.

[Changes to manuscript] The sentence has been changed accordingly to “We demonstrate that intact viable cells of the picocyanobacteria *Synechococcus* accumulate in the cytoplasm of *G. bulloides* Type IId, that these cells are likely to be taken up from

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the water column and that *Synechococcus* should be considered an endobiont of *G. bulloides* Type IId.”

(7) Material and Methods: Page 5: Line 18: I think it would be helpful to put the sampling point for the bacteria analysis in the water column in Figure 1. Also Figure 1: The zoomed-out map is very small. I suggest making it larger and enhancing the contrast of the colors to make it more useful.

[Changes to manuscript] Figure 1 is amended as suggested.

(8) Materials and Methods: Page 5: Line 22: How do the dates chosen for sampling relate to the oceanography and the changes in foraminifera abundances?

The authors wish to thank the referee for this valid point.

[Changes to manuscript] Additional information is added to Section 2.2 highlighting the specific oceanographic conditions at the time of collection.

(9) Materials and Methods: Page 5: Line 26: “species level”: I assume this refers to morphospecies as the genotypes (which seem to be the actual species) cannot be differentiated morphologically as you mentioned before.

[Changes to manuscript] species level altered to morphospecies level.

(10) Materials and Methods: Page 6: Line 2: I wonder how it is possible to make sure that all external contaminants are removed. By putting the shell in RNALater I assume that also contaminant DNA gets preserved. How is it possible to separate the foraminifera cell from the contaminants?

The RNALater preserves all cells in stasis such that they do not lyse. Therefore any bacterial cells associated with the shell will remain intact and as the shell dissolves, those bacteria will be suspended in the RNALater. The washing steps are designed to remove as many of these bacteria as possible before the foram cell is transferred to DOC buffer for cell lysis, but we acknowledge that some contamination will remain.

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To test our method we used a benthic foraminifera known to feed on diatoms and perform kleptoplastidy, an Elphidium species (genetic Type S4; Darling et al., 2016, DOI: 10.1016/j.marmicro.2016.09.001). Because this foraminifera contains chloroplasts these should be abundantly present in the 16S rRNA gene profile in metabarcoding, and their abundance should be proportional to the numbers of bacteria associated with the foraminifera. We performed metabarcoding on four individuals. Three of these had their shells dissolved via RNALater and were washed according to the method described in this manuscript. A final individual was placed directly into DOC buffer for DNA extraction for comparison. The metabarcoding results were conclusive. The individuals that had been through the dissolution and washing steps contained average abundances of 85%:15% chloroplast:bacteria 16S sequences and the individual crushed directly in DOC buffer contained 8%:92% chloroplast:bacteria 16S sequences. The washing method used in this study therefore removes considerable amounts of bacterial contaminants from the shell.

[Change to manuscript] A sentence has been added to describe preliminary washing experiments with the benthic kleptoplastic foraminifera, Elphidium species, and to refer readers to an in preparation manuscript.

(11) Materials and Methods: Page 6: Line 9: Maybe mention here which genes were amplified.

[Changes to manuscript] "PCR amplification "of the foraminiferal 18S rRNA gene"" has been added.

(12) Materials and Methods: Page 7: Line 7: I am not sure I understand which samples were pooled for the sequencing. The different individuals? How are they told apart again later on?

[Changes to manuscript] Section 2.6 has been reworded where necessary to make more clear which samples were pooled and to emphasize the use of a barcoded primer series which gives each sample a unique and identifiable tag enabling identification

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(demultiplexing) after the sequencing process.

(13) Results: Page 9: Line 29: In Table S1 the *N. dutertrei* individual is called DUT59. We apologise for this error.

[Changes to manuscript] This sample has been relabelled DUT55 in accordance with the specimen used and referred to throughout this study.

(14) Results: Page 10: Line 4: I think it could be helpful to show the unstained *G. bulloides* in the supplementary files to have a comparison between stained and unstained images. Of course, even better would be a comparison to a stained species without (or with less) bacteria (e.g. *N. dutertrei*) to see the difference.

The authors thank the referee for this good suggestion. We are able to add an unstained *G. bulloides* fluorescence micrograph to the supplementary figures. However DAPI stained *N. dutertrei* images will be available in a separate manuscript.

[Changes to manuscript] Addition of supplementary image showing an unstained (no DAPI) *G. bulloides* cell under the DAPI filter set, labelled supplementary figure 1 and subsequent supplementary figure numbers changed sequentially.

(15) Results: Page 12: Line 3: I wonder how reliable the comparison between the bacteria in the foraminifera and the water column really is as the water column data were not taken together with the foraminifera sampling. I think it is necessary to further comment on these bacteria data to show how stable they are over time and how reliable it is to assume they were still valid at the time of sampling.

The concentration ranges and fluxes of *Synechococcus* across the world's oceans have been very well established over many years, and certainly it is particularly well monitored in the California Bight. Therefore a comparison between the bacteria in the foraminifera and the water column is entirely reliable. In this area maximum bloom concentrations (not always reached annually) have been measured at 6×10^5 cells per ml and hence our data which suggests concentrations of more than 1×10^9 cells per ml

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within the foraminifera are of huge significance.

[Changes to manuscript] In section 3.4 the well-established concentration range of *Synechococcus*, including bloom concentrations in the local area is highlighted more intensely.

(16) Discussion: Page 15: Line 30: “. . .with the majority of OTUs (>97%). . .”

[Changes to manuscript] Missing bracket has been inserted.

(17) References: In general species names must be in italics.

[Changes to manuscript] All species names have been changed to Italics where necessary.

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