

## 1. Response to Short Comment 1.

1. *16S rRNA gene metabarcoding and fluorescence microscopy can reveal the presence of bacteria in the cell (possibly digesting foods or endobionts), but cannot suggest ecological interaction between host and bacteria. How could you say the bacteria as endobionts?*

5 Future work will elucidate the nature of the relationship between *Synechococcus* and *G. bulloides* and until a benefit is demonstrated to either party we have refrained from using the term symbiont. We feel that the term endobiont is wholly appropriate in this instance.

The definition of an endobiont is of an organism that lives either below a surface (such as a sea bed) or inside another organism. It does not imply (beneficial) ecological interactions (although interactions must occur at a molecular level). We  
10 have used the term endobiont as our evidence suggests that *Synechococcus* are alive inside the *G. bulloides* cell. *Synechococcus* cell counts in TEM images demonstrate large numbers of *Synechococcus* cells inside *G. bulloides*, and in addition, that 5% of these cells are going through cellular division, i.e. they are reproducing.

- 15 2. *The 16S rRNA gene metabarcodings were coming from amplicon sequences. Amplicon sequences are biased by primer, thus ratio of amplicon sequences did not mean the ratio of the bacteria community inside the cell.*

The primer set used in this study is that designed and used by the Earth Microbiome Project (Gilbert et al., 2010). The biases in this primer set are well known and have recently been corrected for (Apprill et al., 2015; Walters et al., 2016; Parada et al., 2016). The primer set has been tested with mock communities (Parada et al., 2016) and compares well with FISH results (Apprill et al. 2015) giving a good representation of the bacterial assemblages targeted. The bias in this primer set does not  
20 include an over amplification of *Synechococcus*. Therefore we believe that the proportions of *Synechococcus* demonstrated by this method are accurate and taken with the TEM cell counts, do reflect the true proportions of *Synechococcus* within the *G. bulloides* cell.

- 25 3. *Also, the TEM image of possible Synechococcus is difficult to observe thylakoid membrane. It is unclear for me to distinguish them as Synechococcus.*

The thylakoid membranes can be observed circling the periphery of the cell, but we acknowledge that the clarity of these is not perfect. However, the carboxysomes, only found in cyanobacteria, are very clear, and the cell division in a single plain is also obvious. Both are characteristic of *Synechococcus*. TRITC excitation of *G. bulloides* cells under fluorescence microscopy (see new uploaded image), also demonstrates the presence of phycoerythrin-containing bacteria throughout the  
30 cell. Phycoerythrin is a pigment characteristic of *Synechococcus* and therefore all the evidence points to these cells being *Synechococcus*.

## 2. Response to Short Comment 2

The authors wish to thank T. Grebert for taking the time to read this manuscript and for his useful and constructive comments, which the authors have enjoyed discussing here.

1. *The title is misleading as it is not supported by any result presented in this study, and should be changed.*

The authors accept this in full. Future work will determine the metabolic role of the cyanobacteria identified within the foraminiferal cell in this study. Therefore the authors acknowledge that the title should be amended to “16S rRNA gene metabarcoding reveals the presence of intracellular cyanobacteria in a major marine planktonic calcifier (Foraminifera)”

2. *Synechococcus clade I also contains green-light specialists (e.g. strains ROS8604 or SYN20, see Pittera et al. 2014, ISME J, doi 10.1038/ismej.2013.228). Similarly, only two strains of clade IV have been characterized for pigmentation. The claim that all clade I and IV Synechococcus are chromatic acclimators (discussion 4.1.5 and 4.2) is thus false for clade I and should be moderated for clade IV.*

Thank you for this valid point. The text in discussion sections 4.1.5 and 4.2 will be modified accordingly to more accurately state that some strains within clade I and those so far characterized for pigments in clade IV are chromatic adapters. The rest of the discussion regarding the potential benefit of chromatic adaption to the host, remains valid.

3. *In paragraph 4.1.1, you claim that PCR amplification of 16S RNA and rbcL "provides additional evidence that Synechococcus DNA [is] not grossly degraded by nucleases". However, you do amplify 16S marker gene for metabarcoding of bacteria that you claim are digested by the host. You thus use the same result to draw diametrically opposed conclusions.*

This is an excellent point that does need clarification. DNA degradation in “dietary samples” limits the size of DNA fragments that can be successfully amplified. For investigation of prey items target fragments should be limited to ~100-250 bp. Whilst longer fragments can be utilised, they will limit the success of amplification, so that sequences will not always be obtained (see Pompanon et al., 2012 doi: 10.1111/j.1365-294X.2011.05403.x).

In our study using 16S rRNA gene metabarcoding, we have amplified a fragment of 253 bp which will therefore give us information not only on intact bacteria but also on those bacteria that have been phagocytosed for food. Since the diet of foraminifera is not wholly known this information is also of value. TEM imaging has then enabled us to further discriminate between food and endobiont.

We also PCR amplified partial *Synechococcus* 16S rRNA genes of 422 bp from *G. bulloides* total DNA for cloning and Sanger sequencing. This is a longer fragment than ideal for identifying prey (i.e. >250 bp) suggesting that the *Synechococcus* DNA is more intact than might be expected if it were the DNA of prey bacteria. Therefore the authors consider this to be supporting evidence (not stand alone evidence) that *Synechococcus* are living cells, endobionts, and not prey bacteria. The term “not grossly degraded by nucleases” is used in the manuscript to avoid over exaggeration of the significance of this data.

The authors consider that a further sentence in section 4.1.1 regarding the sizes of target DNA in helping to discriminate between prey and endobiont bacteria will be a helpful addition to the discussion.

4. *In paragraph 4.1.3, you observe 100% identity between 16S RNA and rbcL markers between “endobionts” and clade IV Synechococcus CC9902. However, strains CC9902 and BL107 share 100% and 98.9% nucleotide identity for these markers, yet share an average nucleotide identity of 91.3% for the core proteins (see Dufresne et al 2008, Genome Biology, doi 10.1186/gb-2008-9-5-r90), lower than the threshold value commonly used for bacterial species definition (94%). The conclusion “This strongly supports a strategy of horizontal rather than vertical transmission” should thus be moderated, as two strains exhibiting high degrees of similarity for these markers can be quite divergent for the rest of their genome: these markers thus provide evidence but are not sufficient to totally exclude genetic drift between internal and free-living Synechococcus.*

Unlike in the example given above, both the 16S rRNA gene and the *rbcL* gene amplified and cloned from total *G. bulloides* DNA gave 100% identity to *Synechococcus* CC9902. However, we accept this point and will moderate our conclusion in section 4.1.3 accordingly with the caveat that there is a high degree of diversity among strains seemingly closely related through analysis of 16S and *rbcL* phylogenies.

### 3. Response to Referee #1

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 established 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 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.

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 focussing on the magnitude of that offset.

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 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.

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](https://doi.org/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 (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  $6 \times 10^5$  cells per ml and hence our data which suggests concentrations of more than  $1 \times 10^9$  cells per ml 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.

#### 4. Response to Referee #2

The authors wish to thank referee #2 for their time and contribution to this enjoyable discussion, and address her/his points below.

#### **General comments:**

1. *Amplicon sequencing does not exactly show the quantity of DNA in the cell. The authors used amplicons, which were amplified by PCR, for 16S rDNA sequencing. In this process, the primers never randomly attach to DNA molecules. Even though 16S amplicon shows high abundance of DNA sequences of the cyanobacteria (Result 3.3), this does not mean high abundance of cyanobacteria in the foraminiferal cell. A large variation (37–87%) of the abundances of the cyanobacterial DNA sequences possibly shows a bias of PCR amplification. The authors removed some contaminated sequences from the samples based on the three negative controls (Method 2.6.1). However, we still find the sequences of the class Alphaproteobacteria in Fig. 3. What are they? Are they contaminants, preys, or symbionts?*

The authors agree with referee #2 that amplicon sequencing is not a quantitative method and hence we have presented our data as proportionality bar charts and not absolute numbers. However, the next-generation sequencing methods employed in



this study are currently routinely used to assess bacterial populations in a variety of environments. We accept that primer bias is a general and acknowledged weakness in any amplification procedure but it is not a weakness specific to our study *per se*.

As discussed with Yoshiyuki Ishitani, author of SC1 (many thanks to this contributor), the primer set employed in this study is that designed and used by the Earth Microbiome Project (Gilbert et al., 2010). The biases in this primer set are well known and have recently been corrected for (Apprill et al., 2015; Walters et al., 2016; Parada et al., 2016). The primer set has been tested with communities of known species composition (Parada et al., 2016) and compares well with FISH results (Apprill et al. 2015) giving a good representation of the bacterial assemblages targeted. The bias in this primer set does not include an over-amplification of *Synechococcus*. This is further demonstrated in the second foraminifera species presented in this study, *N. dutertrei*, where *Synechococcus* sequences were present but in a very low proportion. There is, therefore, no evidence to support the implication by referee #2 that this primer set is biased in favour of *Synechococcus*.

Instead, we suggest that the variation in the proportion of *Synechococcus* OTUs (37%-87%) in the dataset is exactly what we would expect given the sporadic nature of predation. Depending on whether a *G. bulloides* individual has recently phagocytosed bacteria or not at the point of sampling, will shift the proportions of “other” bacteria compared to the *Synechococcus* endobiont. See section 3.3 in the manuscript where this is stated, and also referee #2 comments in part 2 below where she/he agrees with this hypothesis.

The Alphaproteobacteria are a class of bacteria made up of a number of orders and families which encompass hundreds of species. One of the families of the Alphaproteobacteria, family *Bradyrhizobiaceae* is represented in our dataset by a large number of OTUs (OTUs are separated by a >3% difference in the DNA sequence). One of these OTUs was removed because it was the major contaminating OTU, as were a further 8 OTUs from the class Alphaproteobacteria (see section 2.6.1). The other OTUs within the class Alphaproteobacteria (including OTUs of the *Bradyrhizobiaceae* family) were not contaminants, i.e. they were not significantly amplified within the three controls and therefore they remained in the dataset and are represented in Figure 3. These Alphaproteobacteria along with all other bacteria are considered to be food because we were unable to observe any intact bacteria other than *Synechococcus* in TEM imaging.

[Changes in 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. We also make clear that we interpret the remaining Alphaproteobacteria OTUs as food items for the reasons laid out above via small additions to Section 4.3.

2. *The data of 16S rDNA sequences does not show “living” bacteria in the foraminiferal cell. The DNA fragments are highly remained in the cell, if the cell (foraminifer) takes bacteria through endocytosis. As mentioned in the method section 2.2, the samples were immediately put in the buffer after collection. In this case, foods were not digested in a planktonic foraminifer. If G. bulloides is a bacteria-feeder, the DNA fragments of foods could be detected in 16S rDNA sequencing. This hypothesis (bacteria-feeder) is also reasonable to explain why the components of 16S rDNA sequences without cyanobacteria were different among the specimens (Fig. 3).*

We agree with referee #2 that 16S rRNA sequences are not conclusive of “living” bacteria, and that our method of transferring samples to RNALater after collection would indeed mean that recently phagocytosed bacteria would not be completely digested. We refer back to comment 3 in our response to SC2 on this discussion forum: It has been demonstrated that DNA degradation in “dietary samples” limits the size of DNA fragments that can be successfully amplified. For investigation of prey items target fragments should be limited to ~100-250 bp. Whilst longer fragments can be utilised, they will limit the success of amplification, so that sequences will not always be obtained (see Pompanon et al., 2012 doi: 10.1111/j.1365-294X.2011.05403.x).

In our study using 16S rRNA gene metabarcoding, we have amplified a fragment of 253 bp which will therefore give us information, as referee #2 rightly points out, not only on intact bacteria but also on those bacteria that have been phagocytosed for food. Since the diet of foraminifera is not wholly known this information is also of value. TEM imaging has then enabled us to further discriminate between food and endobiont. Observations of intact and dividing *Synechococcus* cells, and of no other intact bacteria, demonstrates that the 16S rRNA genes amplified from other groups of bacteria cannot belong to an endobiont, and are most likely therefore, to be ingested prey.

We also PCR amplified partial *Synechococcus* 16S rRNA genes of 422 bp from *G. bulloides* total DNA for cloning and Sanger sequencing. This is a longer fragment than ideal for identifying prey (i.e. >250 bp) suggesting that the *Synechococcus* DNA is more intact than might be expected if it were the DNA of prey bacteria. Therefore the authors consider this to be supporting evidence (not stand alone evidence) that *Synechococcus* are living cells, endobionts, and not prey bacteria. The term “not grossly degraded by nucleases” is used in the manuscript to avoid over exaggeration of the significance of this data.

[Changes in manuscript] The authors consider that additional sentences in section 4.1.1 regarding the sizes of target DNA in helping to discriminate between prey and endobiont bacteria will be a helpful addition to the discussion.

3. *The images of DAPI and TEM are not enough to support the presences of “living” bacteria in the foraminiferal cell. As the image of DAPI (Fig. 2) was unclear, it’s difficult for me to follow the description in the result section 3.2. Although the TEM image was clear, there is no explanation about cytoplasm. Which are vacuoles? Are there phagosomes? The TEM images indicated the cell structures, which have carboxysomes and thylakoid membranes, as a character of the cyanobacteria. However, these images were not enough to certify that Synechococcus are living in the foraminiferal cell. As the body size of bacteria is very small rather than foraminifera, they can be remained in the foraminiferal cell. At least, the authors will need to count the number of bacteria-like cells in a planktonic foraminifer and show how these bacterial cells are universal. Moreover, I recommend the authors to use the FISH (Fluorescence in situ hybridization) method for detecting the “living” bacterial cells.*

DAPI: The authors understand that the unclear nature of the DAPI images was as a result of their failure to upload properly in the first instance, an issue which has now been rectified. The DAPI images demonstrate the presence of thousands of bacteria within the host *G. bulloides* cell. The authors acknowledge that a comparison with an unstained (no DAPI) *G. bulloides* cell (suggested by referee #1) would be of benefit and will add such an image to the supplementary material. In addition, we will add an additional figure to the main manuscript of a *G. bulloides* cell observed with a TRITC filter set

which excites phycoerythrin, a highly labile pigment characteristic of *Synechococcus*. It can be observed confined in bacteria-sized bright spots right across the *G. bulloides* cell. (Such an image was uploaded in reply to SC1 and can therefore be assessed by the editor and interested parties). This confirms that these multitude of bacteria are indeed *Synechococcus* and that their cell membranes are intact. Water soluble phycoerythrin would very rapidly diffuse into the surrounding aqueous milieu if the cell membrane was compromised (see section 4.1.1).

TEM: The authors acknowledge a need for more labelling on Figure 4. TEM images. We feel justified however in labelling the cyanobacteria in these images as *Synechococcus*. The genus *Synechococcus* includes all unicellular cyanobacteria of the order *Chroococcales* that lack a laminated sheath but possess thylakoids, carboxysomes and divide by binary fission in a single plane (Rippka et al., 1979) as demonstrated in the TEM image. Other names have been used for individual species but not all (e.g. *Microcystis*) have the authority of the Bacteriological Code.

The authors disagree with referee #2 that the evidence provided does not indicate living *Synechococcus*. The authors assert that since 5% of all cyanobacterial cells counted within the *G. bulloides* cell were dividing, that this is strong support for a living endobiont, particularly given that cyanobacterial cells are within the cytoplasm and not within vacuoles. We believe that the use of the term endobiont as opposed to symbiont is wholly appropriate given the evidence. We refrain from using the term symbiont (or endosymbiont), since, as referee #2 quite rightly points out, we have not yet demonstrated a benefit to either party.

FISH: As suggested by referee #2, the authors did investigate the possibility of performing FISH, as an excellent method for determining living endobionts and their metabolic activity. However, in our hands the unparalleled foraminiferal cell autofluorescence observed under fluorescence microscopy, would have drowned out the signal generated by FISH, or indeed CARD-FISH. Under such unusual circumstances, therefore, FISH was considered an unsuitable method at this time for this organism.

As referee #2 suggests, we did in fact perform cell counts, but rather than using FISH and a fluorescence micrograph, we used TEM imaging. We calculated an average *Synechococcus* cell concentration up to 4 orders of magnitude greater in *G. bulloides* compared to concentrations known in the water column in this area (section 3.4). This high count is borne out by Fluorescence microscopy, particularly using TRITC (new figure to be added).

[Changes in manuscript] Addition of supplementary image (suggested by referee #1) showing an unstained (no DAPI) *G. bulloides* cell under the DAPI filter set to be labelled supplementary figure 1 and subsequent supplementary figure numbers to be changed. Addition of an extra figure to be labelled Fig. 3 (subsequent figure numbers to be changed) of a *G. bulloides* cell under the TRITC filter set demonstrating the fluorescence of the pigment Phycoerythrin which is characteristic of *Synechococcus*. Additional labelling on Figure 4 TEM images (which will be relabelled Figure 5).

4. *General characters of Synechococcus did not prove their metabolic functions in foraminiferal cell. Synechococcus generally take or use nitrogen, carbon, and phosphorus. Their uptakes are different depending on species or clades. The current data and discussion (4.2) have no evidence which Synechococcus clade (I to IV) take these elements.*

Moreover, the authors should investigate intracellular distributions and assimilations of these elements. Please see one good example: Nomaki et al. (2016) published in *Frontiers in Microbiology*. Because of these reasons, the title of this MS is not supported by any results. Although this study may show the presence of bacteria in a planktonic foraminifer, it is open to question whether those bacteria are endosymbionts or not. If the authors won't show any additional information to answer the questions as mentioned above, the title, abstract, and main text should be modified to report finding bacterial cells in a planktonic foraminifer. In particular, please delete the descriptions concerning the carbon isotope of the foraminiferal shells, because no data is suggesting this topic.

The authors acknowledge that the current study does not present data on the metabolic interactions between *G. bulloides* and *Synechococcus*. Future work will elucidate the benefits to either party. Therefore we are happy to modify the title and moderate aspects of the manuscript accordingly. However, the data strongly support our conclusions that *Synechococcus* is an endobiont of *G. bulloides* Type IIId. As a living endobiont, the respiration of *Synechococcus* is absolutely pertinent to the carbon isotopic ratio of the *G. bulloides* shell and demands discussion. Geochemical proxies based on *G. bulloides* are particularly important for palaeoceanographic reconstructions because they provide a link between subtropical and high-latitude species. Bacterial respiration could contribute isotopically depleted respiratory carbon to the calcite shell, explaining part of the offset between measured and predicted  $\delta^{13}\text{C}$ .

[Changes in manuscript] Title changed to: 16S rRNA gene metabarcoding reveals the presence of intracellular cyanobacteria in a major marine calcifier, *G. bulloides* (planktonic foraminifera).

### **Minor comments:**

1. Abstract (lines 3-4): Not “unusual”. *G. bulloides* is one of spinose species without algal symbionts.

We used the term “unusual” to refer to two qualities of *G. bulloides*. Firstly to its lack of algal symbionts. Unusual does not imply unique, but it does mean distinct: the distinction being that many other spp. have algal symbionts. Out of the 11 spinose species living in the photic zone whose symbiont status is reported by Hemleben et al. 1989, 3 lack symbionts, but one of these (*Globigerinella calida*) has since been reported to have symbionts. Only 5 of the 11 are paleoceanographically important and out of those, *G. bulloides* is the only one without symbionts, and hence is unusual. Secondly we used the term “unusual” in reference to *G. bulloides* shell geochemistry which is universally accepted as being so, and hence we are fully justified in using the term “unusual”.

2. Abstract (line 8): There is no direct and own data about the bacterial populations in the water column.

This is correct, and the authors acknowledge that having our own data for comparison would be of value. Unfortunately it was not possible to collect such data during this work. However, the SPOT site has been studied extensively over 10 annual cycles. The microbial composition and seasonal variability are well established therefore giving a clear overview of the water column assemblage (compared to a handful of snap shot samples) with which to compare the *G. bulloides* assemblage.

[Changes in manuscript] Line 8 is modified to read “To investigate the ecological interactions between *G. bulloides* and marine bacteria,.....”

3. *Abstract (line 16): This study does not show the presence of bacterial endobiont.*

We have refrained from using the term symbiont as we would agree that since no mutual benefit, or indeed a benefit to one or the other partners has been demonstrated in this study the term symbiont should not be used. However all the data presented in this study points to *Synechococcus* living inside the *G. bulloides* cell, so the term endobiont is absolutely appropriate.

4. *Abstract (lines 17-19): Please delete.*

The authors consider that, given that we have presented many convincing lines of argument to suggest that *Synechococcus* is an endobiont of *G. bulloides*, lines 17-19 are valid statements pertaining to the manuscript discussion.

Keywords: “symbiosis”, “endobiont”, and “carbon isotope” are deleted.

We accept that symbiosis should be deleted, but assert that “endobiont” and “carbon isotope” should remain, for reasons discussed above.

[Changes in manuscript] keyword “symbiosis” deleted

5. *Introduction: The first two paragraphs should be omitted. Instead of them, the authors need to describe how the other studies demonstrated the presence of symbionts in Foraminifera and/or other organisms.*

Referee #2 makes an excellent suggestion to describe how other studies have demonstrated the presence of symbionts/endobionts and we thank them for it. However the authors feel that the first two paragraphs on the foraminiferal contribution to the carbonate flux and their importance to palaeoceanography is of significance and therefore needs to be introduced, giving weight to the need for investigation of these organisms.

[Changes in manuscript] The first two paragraphs are revised and shortened rather than omitted. A short new paragraph is inserted, as suggested, to describe how the presence of protist algal symbionts has been demonstrated.

6. *Section 2.2 Sample collection: Samples were collected from the vertical net-towing or surface water. In this case, it is very difficult to compare the hosts with bacterial components in different depths.*

Off Santa Catalina Island, near the SPOT site, where the bacterial population in the water column is well documented (see point (b) above), the foraminifera were collected by scuba or net tows in the surface waters (<25m) and therefore these specimens can be compared with the well-documented bacterial composition of the water column.

At Bodega Head samples were collected by vertical net tows, but these are not close to the SPOT site and therefore the two *G. bulloides* specimens collected from here and used in metabarcoding were not directly compared with the water column assemblage. However, statistical analysis (Bray-Curtis and LEfSe analysis) showed no significant difference between the bacterial composition of *G. bulloides* individuals from Catalina and those from Bodega Head.

7. *Section 2.3 Decalcification: Have the authors decalcify and wash the cell for all specimens?*

Yes, see section 2.3.

8. Section 2.6. DNA extraction: Why did the authors use only one comparison (*N. dutertrei*)?

*N. dutertrei* was used as a comparison because it was the species collected at the same time and location as the Santa Catalina *G. bulloides* and therefore provides a direct comparison to both validate the methods used here and highlight that there are differences between species and also consistency across the *G. bulloides* individuals.

5 [Changes in manuscript] A small addition to section 3.3 clarifies why *N. dutertrei* was used as a comparison.

9. Discussion 4.1, 4.1.4: Based on amplicon sequencing, it is difficult to discuss the “abundance” of bacteria. Please see my comment (1).

In the title of Section 4.1 we use the term “abundant” to refer to the *Synechococcus* endobiont. It is a statement not based on amplicon sequencing, but on our microscopy data and cell counts which clearly demonstrate that *Synechococcus* is indeed  
10 abundant within the *G. bulloides* cell. We refer the editor particularly to the new figure (this will be figure 3. in the revised manuscript) highlighting phycoerythrin fluorescence. In Section 4.1.4 we would like to point out that the term “abundant” is preceded by the word “relative” when referring to OTUs (which are based on amplicon sequencing) thereby indicating the proportional, and not absolute, nature of the data.

10. Discussion 4.1.3: The authors used “selective uptake” in line 28. It’s wrong, because there is no evidence.

15 The authors suggest that the evidence for selective uptake is the 4 orders of magnitude greater numbers of *Synechococcus* inside the *G. bulloides* cell compared to the highest numbers of *Synechococcus* found in the region. However the authors agree that it is not yet known how the *Synechococcus* arrive in the foraminiferal cell, whether the cyanobacterial population exists purely via cell division of a small number of bacteria entering the cell, or whether *Synechococcus* are phagocytosed in large numbers, and hence will modify this section accordingly.

20 [Changes in manuscript] 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. An additional sentence is added to stress that it is not yet known how the *Synechococcus* cells accumulate in the cytoplasm in order to move away from the concept of “selective uptake”.

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11. Discussion 4.1.4: A visual coloration of planktonic foraminiferal cytoplasm does not demonstrate the characters of *Synechococcus* clades

We assume that this is in reference to Discussion section 4.1.5. The referee is correct. The colouration of the foraminiferal cytoplasm isn’t evidence of *Synechococcus* clades; the systematic evidence from both 16S and *rbcL* genes is: these are  
30 sequences predominantly from Clade IV *Synechococcus*

[Changes to manuscript] An additional few words have been added to clarify that not all Clade IV *Synechococcus* have yet been characterised for pigments, which is further clarified in the following Section 4.2.

12. Figure 2: Unfortunately, Fig. 2 was somehow incomplete. Especially, I cannot find black arrows in Fig. 2a

The authors understand that this was a technical issue regarding the initial upload of the manuscript. This has since been rectified and Figure 2 is now complete.

**5. List of Changes to manuscript. Please note page and line numbers referred to are relevant to this document only.**

1. **Title.** Changed to: Cyanobacterial endobionts within a major marine, planktonic, calcifier (*Globigerina bulloides*, Foraminifera) revealed by 16S rRNA metabarcoding. (requested by all)
2. **Abstract. Page 19. Line 2.** Addition of the word microplaeontological (requested by ref#1)
3. **Abstract. Page 19. Lines 5-7.** Addition of a sentence to describe the “atypical” shell geochemistry and “divergent” ecology (requested by ref#1)
4. **Abstract. Page 19. Line 10.** Replacement of “bacterial populations in the water column” with “marine bacteria” (requested by ref#2)
5. **Introduction. Page 20-21.** An initial paragraph has been added regarding symbiosis in planktonic foraminifera, the original first two paragraphs describing the importance of planktonic foraminifera have then been revised and shortened.
6. **Introduction. Page 22. Line 23-24.** 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 focussing on the magnitude of that offset. (requested by ref#1)
7. **Introduction. Page 23. Lines 2-10.** The term “species-specific” has been replaced with “genotype-specific” and sentences have been added to describe the genotypes in the region. In addition a reference has been added to support the statement of different genotypes harbouring different geochemical signatures (requested by ref#1).
8. **Introduction. Page 23. Line 11.** *Globigerina bulloides* has been changed to *G. bulloides* (ref#1)
9. **Introduction. Page 23. Lines 15-19.** These lines have been altered to avoid use of the term active and species-specific uptake (requested by ref#1 and #2).
10. **Materials and methods. Section 2.2. Page 24. Lines 4-8 and 14-15.** Addition of information regarding the specific oceanographic conditions at time of sample collection. (requested by ref#1)
11. **Materials and methods. Section 2.2. Page 24. Line 12.** Addition of the prefix “morpho” to “species” (requested by ref#1)
12. **Materials and methods. Section 2.3. Page 24. Lines 22-24.** Addition of s sentence describing the efficacy of the washing treatment. (requested by ref#1)
13. **Materials and methods. Section 2.4. Page 24. Line 27.** Addition of the gene amplified. (requested by ref#1)
14. **Materials and methods. Section 2.6. Pages 25-26.** This section has been reworded with a number of additions for further explanation of (i) the biases in the primer set used, (requested by refs#1 and 2 and SC1) and (ii) clarification on the pooling and demultiplexing of DNA samples (requested by ref#1).
15. **Materials and methods. Section 2.6.1. Page 26. Line 24.** Correction of “genus” Alphaproteobacteria to “class” Alphaproteobacteria.
16. **Results. Section 3.2. Page 29. Lines 2-3.** Insertion of reference to “Supplementary Figure S1” which is now an unstained *G. bulloides* cell observed under the DAPI filter set (requested by ref#1).
17. **Results. Section 3.2. Page 29. Line 11.** Insertion of reference to new figure 3, the *G. bulloides* cell under TRITC excitation.
18. **Results. Section 3.3. Page 29. Line 27.** Change Supplementary figure 1 to supplementary figure 2.
19. **Results. Section 3.3. Page 29. Lines 30-31.** Addition of phrases to describe the use of *N. dutertrei* as a comparison to *G. bulloides* (requested by ref#2).
20. **Results. Section 3.3. Page 29. Lines 31 and 32.** Change Fig. 3 to Fig. 4 due to additional figure.

21. **Results. Section 3.4. Page 30. Lines 29, 31 and 33.** Change to “Fig. 4a/b/c” to “Fig. 5a/b/c” due to additional figure.
22. **Results. Section 3.4. Page 31. Lines 6-13.** Additional phrases to stress that the concentrations of *Synechococcus* including bloom concentrations in the water column are well known and established (requested by ref#1).
23. **Results. Section 3.5. Page 31. Line 21.** Change Supplementary figure 2 to supplementary figure 3.
24. **Discussion. Section 4.1. Page 32. Line 11.** *Globigerina bulloides* is changed to *G. bulloides* as it has been referred to previously (requested by ref#1)
25. **Discussion. Section 4.1. Page 32.** Rephrasing of this paragraph to remove emphasis on selective/active uptake of *Synechococcus* by *G. bulloides* (requested by refs#1 and #2).
26. **Discussion. Section 4.1.1. Pages 32-33.** This section has been rephrased to discuss the size of target DNA in helping to discriminate between prey and endobiont bacteria (requested by SC2 and ref#2).
27. **Discussion. Section 4.1.1. Page 32. Lines 28, 29 and 30.** Figure numbers have been changed due to the new additional figure.
28. **Discussion. Section 4.1.1. Page 33. Line 9.** Reference to Figure 3 has been inserted.
29. **Discussion. Section 4.1.2. Page 33. Lines 18-19.** Addition of phrase “(identified as cyanobacteria by...) and a correction to the reference from “Seckbach” to Buck and Bernhard 2006. (Seckbach is the editor of the book containing the Buck and Bernhard paper now referenced).
30. **Discussion. Section 4.1.3. Pages 33-34.** The title of this section and aspects of this paragraph have been altered to remove emphasis on selective/active uptake (requested by refs #1 and #2)
31. **Discussion. Section 4.1.3. Page 34. Lines 23-24.** Addition of the sentence “Whilst there can be a high degree of diversity among strains seemingly closely related through *rbcL* and 16S rRNA gene phylogenies, this evidence supports.....” to acknowledge that although it is likely that the phylogenies accurately depict the cladal groupings, they are not absolute (requested by SC2).
32. **Discussion. Section 4.1.4. Page 34. Line 28.** Removal of “very specific uptake” again to remove emphasis on selective/active uptake of bacteria b the foraminifer (change in emphasis requested by ref#1 and ref#2).
33. **Discussion. Section 4.1.4. Page 35. Lines 6-8.** Addition of a sentence regarding the lack of bias towards *Synechococcus* in the primer set used (requested by SC1, ref#1 and ref#2).
34. **Discussion. Section 4.1.4. Page 35. Line 15.** The term species-specific is changed for genotype-specific.
35. **Discussion. Section 4.1.4. Page 35. Line 17.** Fig. 3 changed to Fig. 4
36. **Discussion. Section 4.1.4. Page 35. Line 18.** Missing bracket inserted (ref#1).
37. **Discussion. Section 4.1.4. Page 35. Line 20.** “species-specific” is replaced with “morphospecies/genotype-specific” as it is a comparison between the morphospecies of *N. dutertrei* and *G. bulloides* and also between the specific genotypes.
38. **Discussion. Section 4.1.5. Page 35, Line 23 –33.** This section has been reworded for clarity. A sentence has been added to confirm that the discussion pertains to *G. bulloides* collected in this area only. Also a sentence has been added to clarify that not all clade IV *Synechococcus* have yet been characterised for pigments (requested by SC2).
39. **Discussion. Section 4.2. Page 36. Lines 10-11.** Sentence re-phrased to clarify that not all clade IV *Synechococcus* have yet been characterised for pigments and that some clade I and those characterised from clade IV are chromatic adapters (requested by SC2).
40. **Discussion Section 4.3. Page 37. Lines 4-5 and 6-7.** Information regarding the oceanography at the time of sampling is added to give further reasons for the differences in the specimens from the different sampling locations (requested by ref#1)
41. **Discussion Section 4.3. Page 37. Lines 13 and 20.** Fig. 3 is changed to Fig. 4.
42. **Discussion Section 4.3. Page 37. Lines 17-18.** Addition of bracketed phrase confirming that some OTUs of the family Bradyrhizobiaceae were not contaminating OTUs and remained in the data set (requested by ref#2).
43. **References.** Species names have been put into italics where necessary (ref#2).



44. **References.** Deletion and addition of various references resulting from changes to the introduction, methods sections 2.2 and 2.6 and a correction in section 4.1.2 outlined above.
45. **Figure 1. Page 57.** This has been modified with corresponding figure legend amendment **Page 56** (requested by ref#1)
- 5 46. **New Figure 3. Page 59.** This is a new addition for further clarity of the fluorescence microscope work, with new figure legend inserted **Page 56**.
47. **Old Figure 3. Page 60.** Bar chart of 16S rRNA gene OTU abundances and corresponding legend changed to Figure 4. **Page 56**.
- 10 48. **Old Figure 4 Page 61.** TEM image changed to Figure 5 and an additional label added highlighting the unusual fibrillary bodies with corresponding additions to the figure legend. **Page 56** (requested by ref#2).
49. **Table 1 Page 62.** Correction, DUT59 corrected to DUT55 (ref#1)
50. **New Supplementary Figure 1. In supplementary material document.** Addition of extra supplementary figure (new S1) with additional figure legend showing an unstained *G. bulloides*. All subsequent supplementary figure numbers are changed in sequence.
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# **Cyanobacterial endobionts within a major marine, planktonic, calcifier (*Globigerina bulloides*, Foraminifera) revealed by 16S rRNA metabarcoding.**

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**Abstract.** We investigated the possibility of bacterial symbiosis in *Globigerina bulloides*, a palaeoceanographically important, planktonic foraminifer. This marine protist is commonly used in micropalaeontological investigations of climatically sensitive subpolar and temperate water masses as well as wind driven upwelling regions of the world's oceans. *G. bulloides* is unusual because it lacks the protist algal symbionts that are often found in other spinose species. In addition, it has a large offset in its stable carbon and oxygen isotopic compositions an atypical geochemical shell signature compared to other planktonic foraminifer species, and also that predicted from seawater equilibrium. This is suggestive of novel differences in *G. bulloides* ecology and life history, divergent ecology, making it a good candidate for investigating the potential for bacterial symbiosis as a contributory factor influencing shell calcification. Such information is essential to evaluate fully the potential response of *G. bulloides* to ocean acidification and climate change. To investigate possible ecological interactions between *G. bulloides* and marine bacterial populations in the water column, 18S rRNA gene sequencing, fluorescence microscopy, 16S rRNA gene metabarcoding and transmission electron microscopy (TEM) were performed on individual specimens of *G. bulloides* (Type IId) collected from two locations in the California Current. Intracellular DNA extracted from five *G. bulloides* specimens was subjected to 16S rRNA gene metabarcoding and, remarkably, 37–87 % of all 16S rRNA gene sequences recovered were assigned to operational taxonomic units (OTUs) from the picocyanobacterium, *Synechococcus*. This finding was supported by TEM observations of intact *Synechococcus* cells in both the cytoplasm and vacuoles of *G. bulloides*. Their concentrations were up to four orders of magnitude greater inside the foraminifera than those reported for the Californian Current water column and approximately 5 % of the intracellular *Synechococcus* cells observed were undergoing cell division. This suggests that *Synechococcus* is an endobiont of *G. bulloides* Type IId, which is the first report of a bacterial endobiont in the planktonic foraminifera. We consider the potential roles of *Synechococcus* and *G. bulloides* within the relationship and the need to determine how widespread the association is within the widely distributed *G. bulloides* morphospecies. The possible influence of *Synechococcus* respiration on *G. bulloides* shell geochemistry is also explored.

**Key words.** *Globigerina bulloides*; Planktonic foraminifera; 16S rRNA gene metabarcoding; *Synechococcus*; symbiosis; endobiont; carbon isotopes

## 1. Introduction

Mutualistic associations between organisms in marine ecosystems can provide the partners involved with the capacity to adapt to environmental stresses such as energy or nutrient limitation, and provide robustness under the challenges caused by climate change. For example, the close association between photosynthetic microalgae and planktonic foraminifera, supplies valuable fixed carbon and other benefits to the host and is a common feature of oligotrophic, surface waters (Decelle et al., 2015). Since Murray first proposed a symbiotic role for these intracellular phototrophs in 1897 (Murray, 1897), many cytological and ultrastructural studies using light, fluorescence, and transmission electron microscopy have confirmed the presence of intracellular photosynthetic dinoflagellates or chrysophyte algae in a wide range of planktonic foraminifera (see Gastrich, 1987; Hemleben et al., 1989). Symbiosis was demonstrated first by Bé et al. (1982) in *Orbulina universa* by the elimination of its dinoflagellate endobionts and their successful reinfection. Other experimental techniques focused on tracing radiolabelled C and N (Gastrich and Bartha, 1988), stable isotope analysis (Uhle et al., 1997; 1999), and microsensor studies of the chemical microenvironment (Jørgensen et al., 1985; Rink et al., 1998). However, direct microscopic observations remain an important first step in assessing potential symbiotic associations.

Since not all planktonic foraminifera harbour protist microalgae, there is obvious diversity in the requirement for this type of mutualistic relationship. Technological advances now allow the investigation of the potential diversity and physiological role of other organisms (e.g., bacteria) entering into relationships with these abundant, calcifying protists. Understanding and investigating the full range of their mutualistic relationships is of great importance in planktonic foraminifera because of their considerable importance in Earth's biogeochemical cycles. Their calcium carbonate shells play a significant role in marine carbon cycling within the water column and ocean sediments. They contribute up to 40% of the biogenic carbonate exported from the surface ocean (Schiebel, 2002; Schiebel et al., 2007) and shell dissolution at depth provides a significant buffering of ocean carbonate chemistry and atmospheric CO<sub>2</sub> (Holligan and Robertson, 1996; Iglesias-Rodríguez et al., 2002; Schiebel, 2002; Feely et al., 2004; Ridgwell and Zeebe, 2005; Sarmiento and Gruber, 2006). However, calcification rates of foraminifera are now known to be affected by the continued release of anthropogenic CO<sub>2</sub>, and are sensitive to the associated changes in surface seawater pH (Ridgwell and Zeebe, 2005; Manno et al., 2012). Assessment of the impact of climate change on planktonic foraminifera and the implications for future biogenic carbonate production is hampered currently by a lack of basic ecological information (Lombard et al., 2011; Roy et al., 2015), including information on the diversity of symbiont-foraminifera associations that might allow different species to adapt to future environmental conditions.

In addition to their role in marine carbon cycling, the geochemistry of foraminiferal shells buried within the sediments provides a long-term repository of information that reveals past changes in ocean conditions. Reconstructions of past seawater temperature, pH, dissolved inorganic carbon (DIC) concentrations and other environmental parameters based on these shell geochemical proxies provide essential constraints for refining climate change projections (Kucera 2007; Katz et al., 2010; Henderson, 2002). The underlying rationale in the use of foraminiferal shell chemistry for this purpose is that it reflects conditions in bulk seawater at the time of deposition. Yet this chemical signal can be altered by protist algal

symbionts within some foraminifer species, complicating the interpretation of proxy records (Spero et al., 1991; Bemis et al., 1998; 2002; Anand et al., 2003; Russell et al., 2004). Important geochemical proxies such as  $\delta^{18}\text{O}$ ,  $\delta^{13}\text{C}$ , and  $\delta^{11}\text{B}$  are influenced by the consumption or addition of  $\text{CO}_2$  to the calcifying microenvironment through photosynthesis by algal symbionts and symbiont-host respiration (Mashiotta et al., 1997; Rink et al., 1998; Wolf-Gladrow et al., 1999; Hönisch et al., 2003; Eggins et al., 2004). The presence of other symbiotic or endobiotic organisms like bacteria within a foraminiferal host could also potentially complicate the interpretation of the proxy record via cryptic isotope fractionation through respiration or other metabolic processes.

Planktonic foraminifera are marine protists that precipitate an external shell composed of calcium carbonate. They are found at densities averaging 20–50 individuals  $\text{m}^{-3}$  throughout the meso- and oligotrophic oceans, but can reach densities of  $>1,000$  individuals  $\text{m}^{-3}$  in polar ocean blooms (Schiebel and Hemleben, 2005; Kucera 2007; Lombard et al., 2009). Most planktonic foraminiferal species have rapid turnover rates with a generation time of a month or less (Hemleben et al., 1989; Bijma et al., 1990; Erez et al., 1991; Schiebel et al., 1997; Lončarić et al., 2005; Jonkers et al., 2015) and are the source of up to 40 % of the biogenic carbonate exported from the surface ocean (Schiebel et al., 2002; 2007). Dissolution of this carbonate within the water column and sediments provides a significant buffering of ocean carbon chemistry and atmospheric  $\text{CO}_2$  (Holligan and Robertson, 1996; Iglesias-Rodríguez et al., 2002; Schiebel, 2002; Freely et al., 2004; Ridgwell and Zeebe, 2005; Sarmiento and Gruber, 2006). Calcifying organisms like the planktonic foraminifera are under threat, however, from the continued release of anthropogenic  $\text{CO}_2$  and associated changes in surface seawater pH due to ocean acidification (Ridgwell and Zeebe, 2005). Efforts have been made, therefore, to model the global abundance and distribution of the major species of planktonic foraminifera in an attempt to determine the impact of climate change on these organisms and to understand the implications for biogenic carbonate production over the coming decades (Lombard et al., 2011; Roy et al., 2015). However, the sensitivity of such models is hampered by the lack of basic ecological information (food preferences, symbiotic associations, response to changing calcite saturation states, mortality rates, etc.) required for their successful implementation (Roy et al., 2015).

The deposition and burial of planktonic foraminiferal calcitic shells at the sea floor generates a fossil record that dates back 180 million years (Hart et al., 2002). The geochemical signatures of their shells represent the contemporary physical and chemical nature of the water column in which they were precipitated. Their shell geochemical concentrations are used, therefore, as proxies for the reconstruction of past water column and climate conditions (Kucera 2007; Katz et al., 2010), allowing sensitivity testing of climate models to refine climate change projections (Henderson, 2002). For accurate palaeoclimate reconstructions, it is important to understand the relationship between water column conditions and planktonic foraminiferal shell geochemical signatures. Individual species of extant planktonic foraminifera differ substantially in their environmental preferences and life histories (habitat, temperature, physiology, feeding, behaviour, reproduction, symbiotic associations; e.g. Hemleben et al., 1989) which directly impacts on their shell geochemistry, resulting in the need for species-specific geochemical calibrations for some environmental factors, such as temperature (e.g. Erez and Luz 1983; Spero and Williams 1988; Spero et al., 1991; Bemis et al., 1998; 2000; 2002; Bijma et al., 1999; Anand et al., 2003; Eggins 2004;

~~Russell et al., 2004). Many planktonic foraminifera also house protist algal symbionts (dinoflagellates or chrysophytes) within their cells (Bé et al., 1982; Spero, 1987; Gastrich, 1987; Hemleben et al., 1989; Lee and Anderson, 1991; Siano et al., 2010) that contribute photosynthetic products to the host (Gastrich and Bartha 1988; Caron et al., 1995; Uhle et al., 1997). Both symbiont photosynthesis and symbiont and host respiration alter the immediate chemical microenvironment, including C and O isotope ratios ( $\delta^{13}\text{C}$  and  $\delta^{18}\text{O}$ ) surrounding the host shell, which has been shown to influence shell geochemistry (Mashiotto et al., 1997; Rink et al., 1998; Wolf-Gladrow et al., 1999; Hönisch et al., 2003; Eggins et al., 2004).~~

Although the role and importance of the protist algal symbionts within planktonic foraminifera is widely recognized and relatively well understood, the association of planktonic foraminifera with bacteria has received very little scientific attention. Apart from a few studies reporting the presence of living bacteria inside benthic foraminifera from dysoxic sediments (Bernhard et al., 2000; Bernhard et al., 2006; Bernhard et al., 2012; Tsuchiya et al., 2015), there has been little consideration of specific endosymbioses between foraminifera and prokaryotes. Indeed, there are no reports of planktonic foraminiferal relationships with bacteria other than a single report observing the external association of *Globigerinella siphonifera* Type I with the marine nitrogen-fixing, filamentous, cyanobacterium, *Trichodesmium* (Huber et al., 1997). This oversight is surprising, since the occurrence of bacterial symbiosis within other protists is well established, as is their great potential for providing highly specialised metabolic processes to their hosts (e.g. van Hoek et al., 2000; Schweikert and Meyer, 2001; Beier et al., 2002; Ashton et al., 2003; Foikina et al., 2003; Gast, 2009; Nowack et al., 2010; Orsi et al., 2012; Gilbert et al., 2012).

*G. bulloides* is a spinose planktonic foraminifer lacking protist algal symbionts (Febvre-Chevalier, 1971; Spero and Lea, 1996) that is abundant in the subpolar and temperate regions and also in the lower latitude upwelling systems (Kleijne et al., 1989; Naidu and Malmgren, 1996). In these climatically sensitive areas it dominates the downward flux of foraminiferal shells to the sea floor and, as a consequence, is of considerable importance for palaeoclimate reconstructions (Sautter and Thunell, 1991; Spero and Lea, 1996). A complication in using *G. bulloides* for palaeoclimate reconstruction, however, is that despite its lack of protist algal symbionts, the shell secreted by *G. bulloides* is still out of isotopic equilibrium with respect to both carbon and oxygen isotopes, deviating the shell geochemical signatures (e.g.  $\delta^{18}\text{O}$  and  $\delta^{13}\text{C}$ ) of *G. bulloides* deviate from predicted values by more than any other extant, surface-dwelling species (Deuser et al., 1981; Kahn and Williams, 1981; Curry and Matthews, 1981; Kroon and Darling, 1995; Spero and Lea, 1996; Bijma et al., 1999). Such deviations are difficult to explain in the absence of protist algal symbionts, although some of the disequilibrium has been potentially linked to growth and ontogeny or even to *G. bulloides* respiration rates (Spero and Lea, 1996). The presence of intracellular bacteria may provide an additional or contributing explanation.

The study of *G. bulloides* is further complicated by our current inability to morphologically distinguish the numerous genotypes of *G. bulloides* identified within the morphospecies (Darling and Wade, 2008; Seears et al., 2012; Morard et al., 2013). The majority of the genotypes have been elevated to species level status (Andre et al., 2014) and all are potentially ecologically distinct, though they are commonly found in the same water column, where their adaptive ranges overlap (Darling and Wade, 2008; Morard et al., 2013). Where this occurs, aggregation of two ecologically distinct *G. bulloides*

species could introduce significant noise into palaeoclimate calibrations (Darling et al., 2000; Kucera and Darling, 2002), particularly if they exhibit ~~speciesgenotype~~-specific geochemical signatures as has recently been demonstrated in the Arabian Sea (Sadekov et al., 2016).

In this study we have focussed on the cool water lineage *G. bulloides* Type IId. With the exception of a single specimen of Type IId appearing off Santa Barbara in January (Darling et al., 2003), Type IId was the only genotype identified off the coast of California throughout the year in both upwelling and non-upwelling hydrographic regimes (Darling et al., 2003; Darling and Wade, 2008). ~~This is, which~~ also ~~corresponds to~~ the region where the majority of experimental geochemical studies on the *G. bulloides* morphospecies have been carried out, which means that current calibrations for this area should be robust (e.g. Spero and Lea, 1996; Bemis et al., 1998; 2000; 2002), but may not be globally applicable since this genotype has not been found elsewhere to date (Darling and Wade, 2008; Morard et al., 2013).

We examined the intracellular bacterial population of individual specimens of the planktonic foraminifer ~~*Globigerina*~~-*G. bulloides* using a multiphasic approach. We used 18S rRNA gene sequencing to identify the genotype of the host cells and fluorescence microscopy, 16S rRNA gene metabarcoding via next-generation sequencing, transmission electron microscopy (TEM), and genetic characterisation using the polymerase chain reaction (PCR) to investigate the distribution and taxonomic affiliations of the intracellular bacteria. We demonstrate that intact, viable cells of the picocyanobacterium *Synechococcus* spp. accumulate in large numbers within the cytoplasm of *G. bulloides* Type IId. We propose that these cells are likely to be taken up directly from the surrounding water column from the surrounding water column and that *Synechococcus* should be considered an endobiont of *G. bulloides* Type IId. ~~and enters into a species-specific association.~~ We ~~go on to discuss~~ consider the nature of this association and its potential metabolic and geochemical implications. We also discuss the power of the methodological approach adopted for improving ecological knowledge of planktonic foraminifera.

## 2. Materials and Methods

### 2.1 Oceanographic setting

Sampling was undertaken off the Californian coast in waters influenced by two opposing currents. The Californian Current flows equatorward from the North Pacific Current (~50° N) to Baja California (~15–25° N). Southerly along-shore winds drive upwelling of cold nutrient-rich waters in early spring and summer in central California, and weaker but more sustained upwelling further toward the south. The relatively warm, saline Davidson Current and California Undercurrent flow poleward over the continental shelf. During the Southern California summer, the California Current moves farther offshore, and the Davidson Current predominates near shore (Checkley and Barth, 2009). For this study, samples were collected along the narrow Central California shelf ~1 km off Bodega Head, California (38.3° N, 123.0° W) and in the Southern California Bight off Santa Catalina Island (33.4° N, 118.4° W; Fig. 1). At both sites, local variation in foraminiferal abundances and species composition is well understood (Thunell and Sautter, 1992; Field, 2004, Davis et al., 2016), driven by periods of upwelling, relaxation or downwelling, and/or seasonal predominance of the Davidson Current. The Santa Catalina Island site

is close to the San Pedro Ocean Time-Series (SPOT; 33.55°, 118.4° W) station where the composition of the bacterial assemblage in the water column has been monitored for over a decade (Chow et al., 2013; Cram et al., 2015).

## 2.2 Sample collection

~~Samples were collected during July/August 2013, November 2014, and April 2015 (Table 1).~~ The Bodega Head samples were collected during November 2014 when there is a relaxation in the upwelling before the winter storms, and also in April 2015 (Table 1). Normally the Southerly along-shore winds start to drive renewed upwelling in early spring (Garcia-Reyes and Largier, 2012) but the samples for this study were collected prior to the delayed upwelling season of 2015, an unusual phenomenon possibly due to the strong ENSO (e.g. Jacox et al., 2015). Bodega Head samples were obtained from vertically integrated 150µm mesh-size net tows, deployed to a maximum depth of 160 m, or to 10 m above the seafloor at shallower sites. Tow material was transferred to ambient surface seawater and kept chilled during transit to shore at the Bodega Marine Laboratory where live foraminifera were wet picked. *G. bulloides* were then identified morphologically to the morphospecies level, rinsed in 0.6 µm filtered surface seawater and preserved in RNALater® (Ambion™). This reagent conserves cell integrity, inhibits nucleases at ambient temperatures, and dissolves the calcite shell. The Santa Catalina Island samples were collected by scuba diving or net tows during July/Aug 2013 towards the end of the weaker upwelling season off Southern California. Collected foraminifera were treated as at the Bodega Marine Laboratory and transferred to RNALater® at the Wrigley Marine Science Center.

## 2.3 Decalcification and washing of samples

To remove the shell and shell-associated, external contaminants, each individual specimen was decalcified by exposure to RNALater® (Ambion™). The cell was then washed in filter-sterilised, salt-adjusted phosphate buffered saline (PBS) or sterile artificial seawater, transferred to a new sterile 1.5 ml tube and washed a further three times before being transferred to DOC DNA extraction buffer (Sect. 2.4; Holzman and Pawlowski, 1996) for DNA analysis, or 4 % (w/v) paraformaldehyde in salt-adjusted PBS for microscopy. Preliminary experiments with the kleptoplastic foram, *Elphidium* species confirmed the efficacy of this treatment. The majority of prokaryote shell contaminants (>80%) were removed in the washing steps following dissolution in RNALater® (Bird et al., in preparation).

## 2.4 Foram genotyping and Sanger DNA sequencing

DNA was extracted from individual foraminifer specimens using the DOC extraction method to identify the specific genotype (Holzman and Pawlowski, 1996). PCR amplification of the foraminiferal 18S rRNA gene was performed according to Sears et al., (2012). DNA sequencing was carried out using the BigDye® Terminator v3.1 Cycle Sequencing Kit and an ABI 3730 DNA sequencer (both Applied Biosystems).



## 2.5 DAPI staining and fluorescence microscopy

Foraminifer cells were stained with 4',6-diamidino-2-phenylindole (DAPI) which forms a highly fluorescent DAPI-DNA complex that allows the visualisation of bacterial cells and eukaryotic cell nuclei under fluorescence microscopy. Individual decalcified and washed foraminifera were fixed in 4 % (w/v) paraformaldehyde in salt-adjusted PBS for four hours at 4 °C. Fixed cells were transferred to a polylysine-coated microscope slide and dehydrated through an ethanol series of 70 %, 90 % and 100 % ethanol. Cells were stained in 1 µg ml<sup>-1</sup> DAPI (dilactate, Sigma-Aldrich) in PBS for three minutes and then rinsed with sterile deionised water. The stained preparations were mounted in AF1 mountant solution (Citifluor) and bacteria and eukaryotic nuclei visualised using a Zeiss Axio Imager Fluorescence microscope equipped with a DAPI filter set. An unstained specimen of *G. bulloides* was also examined by fluorescence microscopy to observe the background levels of autofluorescence under the DAPI filter set to compare with the appearance of DAPI stained individuals. A TRITC filter set (excitation wavelength 540 nm, emission wavelength 580 nm) was used also on unstained individuals to investigate for the presence of autofluorescent, phycoerythrin-containing, cyanobacterial cells.

## 2.6 DNA extraction, amplification and 16S rRNA gene metabarcoding

DNA for 16S rRNA gene metabarcoding of the bacterial population within the foraminifera was extracted from decalcified and washed planktonic foraminiferal cells by the DOC extraction method (Holzman and Pawlowski, 1996). The DNA from the six samples: three independent *G. bulloides* isolates (BUL34, BUL36, BUL37) and a single non-spinose *Neogloboquadrina dutertrei* (DUT55) collected in July/Aug off Santa Catalina Island and two additional *G. bulloides* cells collected in November off Bodega Head (BUL22, BUL23; Table 1), was amplified alongside three reagent controls containing (i) no DNA template (two replicates) and (ii) DOC buffer only. The V4 region of the 16S rRNA gene was amplified-chosen for amplification using the 515F forward primer and a barcoded 806R reverse primer series (Caporaso et al., 2012). This primer set is widely used by the Earth Microbiome Project (Gilbert et al., 2010), and therefore the amplification biases are known and well documented. For example, there is a bias against amplification of the SAR11 group of marine Alphaproteobacteria, and a bias towards over amplification of Gammaproteobacteria (Apprill et al., 2015, Walters et al., 2016, Parada et al., 2016). Each DNA sample and control was PCR amplified with a unique barcode tag that enabled demultiplexing of the samples after being pooled for sequencing. The thermal cycling conditions are detailed by Caporaso et al., (2012). PCR reactions contained 1 x *Taq* buffer plus additional MgCl<sub>2</sub> (final concentration 2.5 mM), 0.2 mM of each dNTP, 0.25 µM of each primer, 1 µl of template DNA and 1.25 U of *Taq* DNA polymerase (Roche Applied Science), with the volume made up to 25 µl with PCR grade water (Sigma). All PCR reactions were set up in a PCR6 vertical Laminar Airflow Cabinet with UV sterilization (Labcaire Systems, Bristol, UK) as described by Pagaling et al., (2014). Reaction tubes and PCR mixtures were treated for 15 minutes with 15 W UV light (wavelength = 254 nm) to destroy contaminating DNA, prior to addition of dNTPs, *Taq* polymerase and template DNA (Padua et al., 1999). ~~Three negative controls containing (i) no DNA template (two replicates) and (ii) DOC buffer only were cycled alongside functional PCR reactions.~~

The ~~six functional, and three control~~ PCR reactions were run on a 1 % agarose gel and the products were purified with the Wizard® SV Gel and PCR Clean-Up System (Promega). The purified amplicons were quantified using a Quant-iT PicoGreen ds-DNA Assay Kit (Life Technologies) prior to pooling ~~samples~~ at equimolar concentrations for DNA sequencing. ~~The samples analysed were the PCR products from three independent *G. bulloides* isolates (BUL34, BUL36, BUL37) and a single non-spinose *Neogloboquadrina dutertrei* (DUT55) collected in July/Aug off Santa Catalina Island and the products from two additional *G. bulloides* cells collected in November off Bodega Head (BUL22, BUL23; Table 1).~~ The total number of quality-filtered sequencing reads including controls was 862,954. DNA sequencing was performed at Edinburgh Genomics using an Illumina MiSeq v3 to generate 250 base pair (bp) paired-end reads.

### 2.6.1 Quality filtering and contaminant removal

The Quantitative Insights in Microbial Ecology (QIIME, v1.8.0, Caporaso et al., 2010) pipeline was used to assemble paired-end reads and quality filter the sequences. Raw reads were paired with an overlap of 200 bp and quality filtered with a minimum Phred score of 20 for maximum accuracy (Kozich et al., 2013). Reads of less than 245 bp (i.e. short reads) were removed from the dataset with the python script filter\_short\_reads.py from <http://gist.github.com/walterst/7602058>. Chimeras were detected using Usearch v6.1.544 default settings (Edgar et al., 2011) and version 13\_8 of Greengenes 16S rRNA gene reference database (DeSantis et al., 2006). Given the low yield of endogenous bacterial DNA in these small-sized samples, we anticipated that amplicon contamination from PCR amplification reagents, DNA extraction reagents, and the ultra-pure water system would contribute a significant number of DNA sequences and OTUs from contaminant genera to the sample set (Salter et al., 2014; Laurence et al., 2014). Operational taxonomic units with greater than 1000 sequences in any of the three control samples were considered to be potential contaminants and were removed from the sample set. Two OTUs were removed due to contamination in the two PCR controls; a *Bradyrhizobiaceae* of the class Alphaproteobacteria and an *Acinetobacter* of the class Gammaproteobacteria. Twelve contaminating OTUs were removed due to contamination via the DOC buffer, seven of these were also of the class Alphaproteobacteria, order *Rhizobiales*: two additional *Bradyrhizobiaceae* OTUs; *Methylobacterium*; *Mesorhizobium*; *Pedomicrobium* and two further *Rhizobiales* OTUs; and one final OTU from the ~~genus-class~~ Alphaproteobacteria, *Sphingomonas* of the order *Sphingomonadales*. The final four OTUs were *Burkholdaria bryophila* of the class Betaproteobacteria; two *Sediminibacterium* OTUs of the phylum *Bacterioidetes*; and lastly an OTU identified as *Streptophyta* chloroplast. A single *Bradyrhizobiaceae* OTU was by far the largest contaminant, with a total of 284,636 sequences from all samples and controls, and it is known to be a common contaminant of next-generation sequencing data, along with other Alphaproteobacteria (Laurence et al., 2014).

### 2.6.2 Operational taxonomic unit (OTU) picking and taxonomic assignment

The default QIIME pipeline was used for data analysis: OTU picking and taxonomic assignment. De novo picking (pick\_de\_novo\_otus.py) clusters DNA sequences into OTUs with 97 % similarity with no external reference and selects a representative sequence of each OTU for alignment and subsequent assignment of taxonomy. This script keeps all diversity,

including unknowns in the sample set. Closed reference picking was also performed which removes OTUs that are not closely matched (< 97 %) with OTUs in the Greengenes database (pick\_closed\_reference\_otus.py). This output is required for normalisation by copy number (NBCN) using the online Galaxy tool (<http://huttenhower.sph.harvard.edu/galaxy/>). This corrects the abundance of each OTU to better reflect the true organism abundance by normalising predicted 16S rRNA gene copy number for each OTU. In both OTU picking methods, OTUs with fewer than 10 sequences across all samples were removed from the sample set (filter\_otus\_from\_otu\_table.py).

### 2.6.3 Alpha-rarefaction and sequencing depth

In QIIME, the script alpha\_rarefaction.py was used to assess whether the sequencing depth was adequate to detect foraminiferal bacterial diversity. Samples were rarefied to the lowest sequencing depth observed across all samples (10,551 in closed reference picking in sample BUL22) and OTU richness curves were generated, using the Observed Species metric which counts the number of unique OTUs found in a sample.

### 2.7 TEM

TEM was used to observe and document the structural relationships between the endobiotic bacteria and foraminiferal cells. Decalcified *G. bulloides* were fixed in 3 % glutaraldehyde in 0.1 M Sodium Cacodylate buffer, pH 7.3, for 2 hours followed by three 10 minute washes in 0.1 M Sodium Cacodylate. Specimens were then post-fixed in 1 % Osmium Tetroxide in 0.1 M Sodium Cacodylate for 45 minutes, followed by a further three 10 minute washes in 0.1 M Sodium Cacodylate buffer. Specimens were then dehydrated in 50 %, 70 %, 90 % and 100 % ethanol (X3) for 15 minutes each, then in two 10-minute changes in Propylene Oxide prior to being embedded in TAAB 812 resin. Sections, 1 µm thick were cut on a Leica Ultracut ultramicrotome, stained with Toluidine Blue, and then viewed under a light microscope to select suitable specimen areas for investigation. Ultrathin sections, 60 nm thick were cut from selected areas, stained in Uranyl Acetate and Lead Citrate and then viewed with a JEOL JEM-1400 Plus TEM.

### 2.8 Genetic identification of *Synechococcus* cells identified in *G. bulloides*

*Synechococcus* cells were found in large numbers inside *G. bulloides* and were genetically characterised. A 422 bp fragment of the *Synechococcus* 16S rRNA gene was amplified from total DNA extracted via the DOC method from individual specimen BUL34 (Table 1). This provided a larger, more informative fragment for phylogenetic analysis compared with the 253 bp generated by 16S rRNA metabarcoding. Cyanobacterial specific primers were used (CYA359f 5'-GGGGAATCYTTCCGCAATGGG-3' and CYA781R a and b 5'-GACTACWGGGGTATCTAATCCCWTT-3', Nübel et al., 1997) and thermocycler conditions were as follows: 94 °C for 2 minutes followed by thirty cycles at 94 °C for 15 seconds, 55 °C for 15 seconds and 72 °C for 30 seconds followed by a final extension at 72 °C for 5 minutes. PCR reactions were performed with MyTaq REDDY mix (Bioline) and 0.25 µM of each primer, 1 µl of template DNA with the volume

made up to 25 µl with PCR grade water (Sigma). The PCR product obtained was cloned (TOPO®–TA cloning kit, Invitrogen) and Sanger sequenced.

Clone sequences were aligned with reference *Synechococcus* 16S rRNA gene sequences retrieved from the GenBank database (NCBI) using ClustalW software within the package MEGA6 (Tamura et al., 2013). Phylogenetic trees (maximum likelihood; neighbour-joining; minimum evolution; UPGMA; maximum parsimony; Sect. 3.5) were generated using the default settings of MEGA6 with 500 bootstrap resamplings to determine the closest taxonomic affiliations (i.e., clade designation *sensu* Fuller et al., 2003) of the *G. bulloides*–associated *Synechococcus*. Informed by this analysis, further primers were designed that target other signature genes harboured by the *Synechococcus* clades identified including that for *rbcL*. This phylogenetically informative gene encodes the large subunit of RubisCO (ribulose–1,5–bisphosphate carboxylase/oxygenase), the primary CO<sub>2</sub>–fixing enzyme found in cyanobacteria. Primers (SynrbcL\_For 5′–CGGCAACTTCTTCGATCAGG–3′; SynrbcL\_Rev1 5′–ATGTCGCGGCTTTCTTTCTC–3′; SynrbcL\_Rev2 5′–CCGGCTTCCATAAGGATGTC–3′) were designed with Primer 3 (<http://primer3.ut.ee/>) that target a 252 bp fragment of *rbcL* from the most closely related *Synechococcus* spp. (i.e., strains CC9902, CC9311 and WH8120, see below).

Purified DNA from fourteen *G. bulloides* specimens (Table 1) generated products of the correct size on PCR amplification with the *rbcL* primers. The product obtained from isolate BUL34 was selected and TA cloned and DNA sequenced as described above. PCR reactions were performed in a Biometra Personal Thermocycler using MyTaq REDDY mix (Bioline) and 0.25 µM of each primer, 1 µl of template DNA with the volume made up to 25 µl with PCR grade water (Sigma). Thermocycling conditions were 94 °C for 2 minutes followed by 30 cycles at 94 °C for 15 seconds, 56 °C for 15 seconds and 72 °C for 30 seconds followed by a final extension at 72 °C for 5 minutes.

### 3. Results

In total, 29 individual specimens of *G. bulloides* collected from waters off Santa Catalina Island and Bodega Head (Fig. 1) were investigated during this study. The sampling information and analyses performed on each specimen is detailed in Table 1, and the sampling strategy and genetic characterisation are described in the methods.

#### 3.1 Genotyping of foraminifera

Partial 18S rRNA (SSU) gene sequences amplified from BUL34 and DUT55 have been submitted to Genbank (NCBI, accession numbers KX816046 and KX816048 respectively). *G. bulloides* specimen BUL34 is Type IId and *N. dutertrei* specimen DUT55 is Type Ic and it is the first time a ~1000 bp fragment has been amplified for this genotype. Both genotypes have been found routinely in the Southern California Bight (Darling et al., 2003).

### 3.2 Fluorescence microscopy and DAPI staining

Fluorescence microscopy examination of an unstained, fixed *G. bulloides* specimen (BUL21; Table 1; [supplementary Fig. S1](#)) under the DAPI filter set demonstrated high levels of diffuse autofluorescence across the entire cell. *G. bulloides* cells that were first stained with DAPI (n=6; Table 1) also showed background autofluorescence but in addition some more highly fluorescent regions 3–10  $\mu\text{m}$  in size were also observed (Fig. 2a). These stained structures are probably DNA from the foraminiferal nucleus and also DAPI–DNA complexes in organisms sequestered within food vacuoles. Of greater present significance, however, were the very many fluorescent, globular structures of approximately 1  $\mu\text{m}$  diameter observed consistently within all of the *G. bulloides* cells analysed. Their small, regular size is consistent with the presence of intact intracellular (coccoid) bacteria residing within the foraminiferal cell (Fig. 2b). Further microscopic examination of *G. bulloides* using the TRITC filter set also revealed high autofluorescence across the cell but, in addition, brighter fluorescence from many of these approximately 1  $\mu\text{m}$  diameter DNA-containing structures ([Fig. 3](#)). This observation is entirely consistent with the presence of the photosynthetic pigment, phycoerythrin (excitation maxima ~495, 545 nm, emission maximum 565–580 nm), within many of these intracellular (cyano)bacteria (see below).

### 3.3 16S rRNA gene metabarcoding

16S rRNA gene metabarcoding was carried out on five specimens of *G. bulloides* and a single specimen of the non-spinose species *Neogloboquadrina dutertrei* for comparison (Table 1). The raw dataset has been submitted to the sequencing read archive (SRA, NCBI), Bioproject accession number SRP090165, SRA accession numbers: SRR4271458, SRR4271479, SRR4271493, SRR4271505, SRR4271506, SRR4271507). A total of 862,954 sequences was generated from the six samples and three controls after quality filtering, and removal of short reads (<245 bp) and chimeric sequences (Sect. 2.6.1). In closed reference picking, after removal of pynast failures, all control sequences (288,985) contaminant OTUs (including 161,282 sequences as result of the single contaminating *Bradyrhizobiaceae* OTU across all six samples) and OTUs with an abundance of less than 10 sequences across all samples, a total of 214,087 sequences were clustered into OTUs and assigned taxonomy (Sect. 2.6.1 and 2.6.2). The numbers of sequences and OTUs generated in individual specimens for both closed reference picking and de novo picking are listed in supplementary Table S1. The OTU profiles within a specimen were highly similar between de novo picking and closed reference picking with normalisation by copy number (NBCN, Sect. 2.6.2). Therefore, we present results for closed reference picking with NBCN, and indicate when de novo picking OTUs are being represented. Rarefaction curves ([supplementary Fig. S12](#)) for OTU richness confirmed that sequencing depth was sufficient to capture the full bacterial assemblage diversity.

All replicates of *G. bulloides* contained a highly distinctive assemblage of OTUs. The OTU assemblage of an individual *N. dutertrei* (Table 1) sampled at the same time [and from the same water mass](#) is shown for [direct comparison of the bacterial assemblages of two morphospecies of foraminifera](#) (Fig. [34](#)). Within the five BUL specimens investigated (Table 1), 37–87 % of all sequences belonged to five OTUs assigned to the unicellular, cyanobacterial genus, *Synechococcus* (Fig. [34](#)). This

was by far the greatest abundance of a single genus of bacteria; no other genera, or, indeed, family or order of bacteria was found across all five BUL specimens at relative abundances consistently more than 2 %. The next highest relative abundance group, therefore, must be described at the class level. Across four (BUL22, BUL23, BUL34, BUL36) of the five BUL specimens 15–31 % of sequences belonged to the class Alphaproteobacteria and were dispersed amongst 81 OTUs. The fifth and outlying specimen (BUL37, containing 87 % *Synechococcus* sequences), contained only 2.6 % Alphaproteobacteria across 37 OTUs. It contained marginally more phylum Actinobacteria (1.8 %) and class Betaproteobacteria (1.4 %) sequences, but the sequence abundances of these classes were more similar to the other BUL specimens. The high relative abundance of *Synechococcus* within this specimen might be due to a lack of feeding on other bacteria or algae immediately prior to sampling, indicative of a high turnover rate for prey bacteria and algal cells.

There were no chloroplast-affiliated OTUs in specimens BUL22 and BUL23. However, 6.4 %, 27 % and 3 % of sequences in specimens BUL34, BUL36 and BUL37 respectively were allocated to chloroplast 16S rRNA gene OTUs from a variety of sources. These were two OTUs from a mixotrophic protist belonging to the diverse, protozoan phylum, Cercozoa (Cavalier-Smith and Chao 2003); three OTUs from the phylum of Haptophyte algae that includes coccolithophores; eight OTUs from the phylum Stramenopile that includes both diatoms and chrysophyte algae; and, finally 13 OTUs from the group Streptophyta (an unranked clade of plants that includes green algae).

In de novo picking, *G. bulloides* specimens BUL34, BUL36 and BUL37 contained varying percentages of sequences (25.4 %, 0.3 % and 1.6 % and respectively) in three taxonomically unassigned OTUs whereas BUL22 and BUL23 did not contain any unassigned sequences. 94 % of all the unassigned sequences belonged to a single OTU (e.g. 5,878 sequences in sample BUL34) that was 99 % identical (100 % coverage of 253 bp) to an unidentified marine bacterial clone (accession number HQ673258) retrieved from the northeast subarctic Pacific Ocean (Allers et al., 2013). The nearest match (87–89 % similarity over 100 % coverage) to an identified phylum was to a large number of uncultured Verrucomicrobiae bacteria of the phylum Verrucomicrobia from a wide range of habitats, including marine environments. Whilst this was not a particularly close match, it is within the defined limit of >85 % DNA identity that delineates a phylum (Hugenholtz et al., 1998; Rappé and Giovannoni, 2003) albeit based only on a 253 bp fragment. The variation in abundances of this OTU found in each *G. bulloides* individual analysed (25 % in BUL34; 0.3 % in BUL36 and 1.6 % in BUL37) might indicate this bacterium has a patchy distribution and is an opportunistic food source.

### 3.4 Transmission electron microscopy

Transmission electron microscopy (TEM) imaging was carried out on four *G. bulloides* specimens (BUL32, BUL39, BUL69, BUL71; Table 1, Fig. 4~~5~~) to observe whether any endobionts were present within the cell. No intracellular eukaryotic cells were observed, confirming a lack of algal symbionts. However, numerous intact coccoid cells containing carboxysomes (Fig. 4~~a~~5a) within the central cytoplasm surrounded by thylakoid membranes, characteristic of the cyanobacterium, *Synechococcus*, were observed throughout the cytoplasm and also in vacuoles of all individual *G. bulloides* observed (Fig. 4~~b~~5b). Approximately 5 % of the observed *Synechococcus* cells were undergoing cell division (Fig. 4~~e~~5c).



To compare foraminiferal cellular *Synechococcus* concentrations with those of the water column, the concentration of *Synechococcus* cells ml<sup>-1</sup> of foraminiferal cytoplasm was calculated by assuming a conservative average host cell diameter of 200 µm (Spero and Lea, 1996; Aldridge et al., 2012), a spheroid morphology (Geslin et al., 2011) and that the cytoplasm was equivalent to 75 % of the shell volume (Hannah et al., 1994). Based on averaged cell counts from the TEM images, the total number of *Synechococcus* cells within *G. bulloides* occupied less than 2 % of the foraminiferal cell volume but was equivalent to 3.8 x10<sup>9</sup> *Synechococcus* cells ml<sup>-1</sup>; This is far higher than the well established range of concentrations of *Synechococcus* found throughout the global ocean that range from compared with between 1x10<sup>2</sup> – 1.5x10<sup>6</sup> *Synechococcus* cells ml<sup>-1</sup> of seawater throughout the global ocean (Partensky et al., 1999; Paerl et al., 2011). In the Southern California Bight, *Synechococcus* cell counts are generally fewer than 1.5x10<sup>5</sup> cells ml<sup>-1</sup> but can reach 6x10<sup>5</sup> cells ml<sup>-1</sup> during the blooms that are generally observed in late spring to early summer (Tai and Palenik, 2009; Tai et al., 2011). The concentration of *Synechococcus* in the *G. bulloides* cell, therefore, was up to 4 orders of magnitude greater than peak bloom concentrations measured in the California Bight. This that in the surrounding water column, indicating suggests that *Synechococcus* cells accumulate within the cytoplasm of *G. bulloides* Type IId. selectively concentrate *Synechococcus* in their cytoplasm.

### 3.5 Genetic characterisation of intracellular *Synechococcus*

Five *Synechococcus* OTUs were assigned in 16S rRNA gene metabarcoding with closed reference picking. However, more than 99 % of the BUL *Synechococcus* sequences were assigned to just one of these OTUs. The representative nucleotide sequence (253 bp) of this OTU is a 100 % match to the coastal, clade IV *Synechococcus* sp. strain CC9902, originally isolated from the California Current. Two further OTUs were highly similar to this abundant OTU and were 99 % identical to *Synechococcus* sp. strain CC9902. The remaining two OTUs both had a nucleotide match of 99 % with *Synechococcus* sp. Strain WH8020, a Clade I strain also found typically in coastal waters. In order to confirm these clade assignments, phylogenetic analysis (supplementary Fig. S23) of a larger (422 bp) fragment of the *Synechococcus* 16S rRNA gene generated from BUL34 total DNA was performed. Ten clones (GenBank accession numbers KX815969–KX815978) clustered with the Clade IV *Synechococcus* strain CC9902 and two clones (GenBank accession numbers KX815979 and KX815980) clustered with Clade I strains CC9311 (another California Current isolate) and WH8120, in agreement with the 16S rRNA gene metabarcoding data. The topologies of the phylogenetic trees produced were all in overall agreement with well-established analyses of *Synechococcus* 16S rRNA genes (Scanlan et al., 2009) confirming the phylogenetic resolution of the sequence data included in the present study.

In addition, a 252 bp fragment of the *Synechococcus rbcL* gene was cloned and 230 bp of this clone was DNA sequenced (GenBank accession number KX816048) from BUL34 (Table 1). A GenBank BLAST search (NCBI) found 100 % nucleotide sequence identity with the RuBisCo large subunit coding region of *Synechococcus* CC9902 and 92 % identity with *Synechococcus* WH8020, confirming the presence of *Synechococcus* CC9902, or a very closely related Clade IV strain. The DNA of thirteen further *G. bulloides* specimens (Table 1) also yielded products of ~252 bp on amplification with the

*Synechococcus rbcL* primers confirming the consistency of the association between *G. bulloides* Type IId and *Synechococcus* strains in the California Current year round.

#### 4. Discussion

Our results highlight a novel endobiotic association between the usually free-living, photoautotrophic picocyanobacterium, *Synechococcus* spp., and its host, *G. bulloides* Type IId, a genotype of a spinose planktonic foraminiferal morphospecies, barren of protist algal symbionts. Below, we discuss the evidence for this endobiosis, and possible roles of *Synechococcus* in *G. bulloides* host metabolism and its characteristic cytoplasm colouration. A better understanding of *G. bulloides* genotype ecology will ultimately provide ecological information for modelling foraminiferal distribution, abundance and seasonality under different climate regimes, and improve the accuracy of the palaeoceanographic proxy records.

##### 4.1 Evidence for *Synechococcus* as an abundant endobiont of *Globigerina bulloides* Type IId

~~*Globigerina-G. bulloides* has consistently been reported to be barren of protist algal symbionts (Febvre–Chevalier, 1971; Gastrich, 1987; Hemleben et al., 1989; Spero and Lea, 1996). The current study supports this conclusion, since no intact algal cells were found in any of the *G. bulloides* cell sections examined using TEM. However, we do have strong evidence that *G. bulloides* Type IId contains large numbers of the photoautotrophic picocyanobacterium, *Synechococcus*. Intact *Synechococcus* cells accumulate and that they are preferentially taken up from the water column by *G. bulloides* and concentrated within the host cytoplasm in abundances far greater than those found under bloom conditions in the California Bight or in any other foraminiferal species investigated. How this association occurs is unclear, but *G. bulloides* Type IId is the only foraminiferal species currently known to associate with *Synechococcus*, an observation that implies a specific potentially mutualistic benefit, particularly since *G. bulloides* does not exploit protist algal symbionts as in other spinose species. Based on the observations discussed below, we propose that these picocyanobacteria are abundant, metabolically active endobionts living within the *G. bulloides* cell, rather than prey.~~

##### 4.1.1 *Synechococcus* cells are intact and viable

~~DNA degradation in prey items limits the success of amplification of DNA sequences greater than ~250 bp (Pompanon et al., 2012). In this study we targeted a 253 bp fragment of the 16S rRNA gene via metabarcoding thus providing information not only on intact, undigested bacteria, but also on those bacteria phagocytosed for food. Subsequent TEM imaging has enabled us to distinguish between prey and endobiont. Indeed, TEM images have demonstrated that, of the diversity of bacteria identified in 16S rRNA gene metabarcoding, only *Synechococcus* cells were observable in the *G. bulloides* cell. showed that~~ ~~‡~~~~The *Synechococcus* cell membranes were physically intact (Fig. 4a5a) and, whilst some *Synechococcus* cells were observed within vacuoles, many were distributed throughout the cytoplasm of *G. bulloides* (Fig. 4b5b) where digestion does not occur.~~  
As many as 5 % of the intracellular *Synechococcus* population were observed to be in the process of cell division (Fig. 4e5c)



indicative of actively growing, viable individuals (Campbell and Carpenter, 1986). Significantly, Bernhard et al., (2000) considered as few as 3 % dividing cells a substantial enough proportion of the population to suggest a symbiotic role for the intracellular bacteria they observed in the benthic foraminifer *Buliminella tenuata*. Further, successful PCR amplification of a longer, 422 bp fragment, of the *Synechococcus* 16S rRNA gene suggests that the *Synechococcus* DNA was more intact than might be expected if it were the DNA of a prey organism (i.e. >250 bp, Pompanon et al., 2012). This and the amplification of a second short fragment of the *rbcL* gene provides additional evidence that *Synechococcus* DNA was not grossly degraded by nucleases. In further confirmation of the intact nature of the intracellular *Synechococcus* population, autofluorescence in the orange/red spectral region arising from the photosynthetic pigment phycoerythrin, was readily detected within these DNA-containing endobionts within *G. bulloides* (Fig. 3). Phycoerythrin, a water-soluble biliprotein found routinely in marine *Synechococcus* spp. rapidly diffuses into the aqueous surroundings if the cell membranes are compromised (Stewart and Farmer, 1984; Wyman, 1992). ~~In further confirmation of the intact nature of the intracellular population — two partial *Synechococcus* genes (the 16S rRNA gene and *rbcL*) were successfully amplified by the PCR, providing additional evidence that *Synechococcus* DNA was not grossly degraded by nucleases.~~

#### 4.1.2 *Synechococcus* are endobionts in marine protists

Whilst *Synechococcus* spp. are known primarily as free-living organisms (Waterbury et al., 1979; Richardson and Jackson, 2007), an endobiotic lifestyle has also been observed in association with a number of different marine protist groups. *Synechococcus* has been identified in the benthic foraminifer *Fursenkoina rotundata*, sampled from the benthos at 600 m using both fluorescence microscopy (identified as cyanobacteria by Bernhard et al., 2000) and in TEM imaging (identified as *Synechococcus*. ~~Seekbaeh,~~Buck and Bernhard, 2006). At these depths, however, the *Synechococcus* endobionts would be unable to photosynthesise, which rules out the most obvious functional metabolic role for this potential symbiont. *Synechococcus* also have been found living embedded within the extracellular matrix surrounding a marine diatom (Buck and Bentham, 1998), and within a polycystine radiolarian (Yuasa et al., 2012). This study now confirms that they are also to be found within the living cells of at least one type of planktonic foraminifer.

#### 4.1.3 *Synechococcus* cells ~~are specifically taken up from the water column and~~ accumulate in the *G. bulloides* cytoplasm

~~Not only are the intracellular~~Intact *Synechococcus* cells ~~intact, but they are also selectively~~ accumulated within the *G. bulloides* cytoplasm at densities ( $\sim 3.8 \times 10^9$  cells ml<sup>-1</sup>) that are four orders of magnitude more concentrated than those reported in the surrounding seawater (Tai and Palenik 2009; Tai et al., 2011). Whilst DNA sequences from other bacteria were identified by 16S rRNA gene metabarcoding (Fig. 34), no bacterial cells lacking carboxysomes were observed by TEM, indicating that, unlike *Synechococcus*, other bacteria were rapidly digested once taken up. Quite how *G. bulloides* *Synechococcus* accumulate in the foraminiferal cell is yet to be established. For example, does the host or endobiont instigate the association? Are the cyanobacteria passed on via parental gametes or is the association established via direct uptake of

~~*Synechococcus* from the water column? first selects and then accumulates *Synechococcus* cells, however, is uncertain.~~ In the case of planktonic foraminifera harbouring protist algae, ~~the a small number of~~ symbionts are taken up directly from the water column (horizontal transmission) rather than being inherited through vertical transmission via parental gametes (Hemleben et al., 1989; Bijma et al., 1990). Juveniles with only 2 to 3 chambers already have ~3 to 5 symbionts, and it is assumed that they are taken up from the water column exclusively since no protist symbionts (5–10 µm cell diameter) have been observed within the much smaller flagellated gametes (~2.5 µm; Hemleben et al., 1989). Although picocyanobacteria such as *Synechococcus* are much smaller in size (~1 µm diameter) than algal symbionts and could potentially be inherited via parental gametes, we favour the hypothesis that the *Synechococcus* population within *G. bulloides* is similarly taken up from the water column, despite evidence for both horizontal (Ashton et al., 2003) and vertical transmission (Schweikert and Meyer, 2001) of bacteria within protist hosts.

To investigate the potential mode of transmission of the *Synechococcus*, we compared the strain assemblages within *G. bulloides* to those of the surrounding water column. If the *Synechococcus* endobionts were horizontally transferred to *G. bulloides* via ~~selective~~ uptake from the water column, we would expect that the diversity of the internal strain assemblage would mirror that of the surrounding waters closely. Alternatively, if the endobionts were vertically transmitted, a degree of genetic drift would be expected between the internal and free-living strains of *Synechococcus* as the result of genetic isolation over time (Wernegreen, 2002; Bright and Bulgheresi, 2010). Off the coast of California, the most prevalent strains of *Synechococcus* are those belonging to Clades I and IV (see Fuller et al., 2003) that display seasonal population differences throughout the annual cycle (Tai and Palenik, 2009; Tai, et al. 2011). The *Synechococcus* 16S rRNA gene sequences cloned from a *G. bulloides* specimen collected in July/Aug (Table 1) show that the strain composition strongly reflects the seasonal cladal distribution patterns that are observed in the water column at that time of year (Tai and Palenik, 2009). Up to 100 % nucleotide identity was found for the 16S rRNA gene clones and the *rbcl* gene sequences of the internal endobionts and those of the free-living, clade IV *Synechococcus* strain CC9902, originally isolated from waters off the California coast. Whilst there can be a high degree of diversity among strains seemingly closely related through *rbcl* and 16S rRNA gene phylogenies, this evidence ~~strongly~~ supports a strategy of horizontal rather than vertical transmission for the *G. bulloides* endobionts.

#### 4.1.4 Intracellular OTU relative abundances do not reflect those of the water column

The intracellular 16S rRNA gene OTU profiles of *G. bulloides* were very different from those of the water column assemblages, indicating ~~very specific~~ uptake of specific bacteria from the general microbial population. The foraminifer collection site off Santa Catalina Island in the San Pedro Channel is adjacent to the SPOT sampling location, where seasonality and trophic interactions within the microbial assemblages in the water column have been studied routinely for over a decade (Chow et al., 2013; Cram et al., 2015). In both the surface waters and deep chlorophyll maximum layer, the microbial assemblage at the SPOT sampling site is dominated by OTUs from the ubiquitous SAR11 group (Giovannoni 1990; Morris et al., 2002) of marine Alphaproteobacteria, that represent over 30 % of the assemblage. In addition, members

of the Actinobacteria account for approximately 15 % of OTUs while the picocyanobacteria represent just 2–5 % of the total bacterioplankton. Of the latter, *Prochlorococcus* dominates the assemblage although *Synechococcus* is also present year round (Chow et al., 2013). The remaining 50 % of the microbial population comprises a series of OTUs from a variety of marine bacteria each representing less than 2 % of the assemblage (Chow et al., 2013). This water column assemblage contrasts strongly with the intracellular 16S rRNA gene OTUs of *G. bulloides*, where between 37 % and 87 % of the total number of sequences recovered belong to *Synechococcus* OTUs. It should be noted that no amplification bias towards *Synechococcus* has been reported for this primer set and data from a number of marine locations supports this (Apprill et al., 2016). Strikingly, *Prochlorococcus* sequences were not identified in the three *G. bulloides* specimens collected close to the SPOT sampling location (BUL34, BUL36 and BUL37), even though *Prochlorococcus* represents the majority of the picocyanobacteria in the water column in this region. Further, < 4.5 % of OTUs in the amplified *G. bulloides* specimens were assigned to the Actinobacteria (compared to ~15 % in the water column) and no OTUs of the ubiquitous SAR11 group of Alphaproteobacteria were identified in our sample set. However, in part this is likely to be a result of bias against SAR11 clades (Apprill et al., 2015; Walters et al., 2015) in the primer set used in this study (Caporaso et al., 2012).

The composition of the internal microbial population of the *G. bulloides* cells clearly does not mirror that of the surrounding water column, highlighting the species/genotype-specific nature of the OTU assemblages observed within the *G. bulloides* cell. This observation is reinforced by the fact that the intracellular OTU assemblage within *G. bulloides* also differs substantially from those identified within specimens of the non-spinose species *N. dutertrei* (for e.g. DUT55; Fig. 34), collected at the same time and location. *N. dutertrei* contains ~ 2 % bacterial OTUs with the majority of OTUs (> 97 %) being assigned to Stramenopiles (a group that includes diatoms and chrysophyte algae; 53 %) and Cercozoa (a diverse phylum of mixotrophic protists; 44.5 %). This highlights again the morphospecies/genotype-specific nature of the *G. bulloides* intracellular OTU assemblage.

#### 4.1.5 Unusual cytoplasm colouration of *G. bulloides*: a role for endobiotic *Synechococcus*

Living *G. bulloides* cells often exhibit a distinctive brown colouration in the specimens found off the coast of California (Spero and Lea, 1996) that is not a general feature of the other spinose species in the region. The discovery of phycoerythrin-containing *Synechococcus* spp. within the cytoplasm of the foraminifera reported here provides a plausible explanation for this unusual property. A number of *Synechococcus* spp. isolated from the California Current are brown in colour owing to the production of urobilin-rich phycoerythrins (Toledo and Palenik, 1997). Many of the clade I and IV strains with which the *G. bulloides* endobionts cluster in phylogenetic analysis are type IV chromatic adapters that exhibit elevated concentrations of this urobilin-rich phycoerythrin (Six et al., 2007) as well as the photoprotective carotenoid, zeaxanthin, (Bidigare et al., 1989) under blue light (i.e., under the illumination conditions typical of the oligotrophic waters off the California coast from which the samples were obtained during the present study). The presence of these pigments within the *Synechococcus* endobionts, therefore, probably contributes to the unusual cytoplasm colouration observed in *G. bulloides* from this location.

## 4.2 Potential metabolic roles for the *G. bulloides* endobionts

There are some obvious potential metabolic benefits to each organism in a *G. bulloides*–*Synechococcus* partnership. Firstly, the foraminifer might benefit from a supply of photosynthetically fixed carbon, as is the case with the foraminifera that harbour protist algal symbionts (Caron et al., 1995; Uhle et al., 1997; 1999). If this were the sole benefit, however, one would question why *G. bulloides* preferentially recruits *Synechococcus* for this purpose, rather than the more conventional algal symbionts found in other species. One possible explanation is that *G. bulloides* inhabits a wide range of depths that often extend below the photic zone and it is also common in unstable upwelling waters where potential algal symbionts may not thrive. *Synechococcus* has been found alive in aphotic waters at depths of 600m (Bernhard et al., 2000), and has been shown to assimilate carbon mixotrophically (Paoli et al., 2008). It could therefore augment phototrophy with carbon assimilated through (photo)heterotrophy, depending on the water column depth of the host. In addition, some *Synechococcus* strains within clade I and those so far characterised in clade IV ~~groups of *Synechococcus*~~, as found in *G. bulloides*, are chromatic adapters, able to modify their pigment composition and absorption properties depending on the underwater light field (Six et al., 2007). Such adaptability might make *Synechococcus* a more compatible symbiont for the *G. bulloides* lifestyle.

Alternatively, *Synechococcus* may have additional or quite separate functional roles in association with *G. bulloides* beside endobiotic photosynthetic activity within the photic zone. For example, approximately half of the nitrogen assimilated by the host cell in the *Orbulina universa* foraminifer–symbiont system is transferred via the algal symbionts; a contribution that increases further to ~90–100 % in nitrate–depleted waters (Uhle et al., 1999). *Synechococcus* spp. have a very high affinity for combined nitrogen (for e.g. nitrate, nitrite and ammonium) and accumulate expanded stores of this element within their light–harvesting phycobilisomes under N–replete conditions (Wyman et al., 1985). Likewise, *Synechococcus* sequester large stores of P within their cells as polyphosphate, even under low external concentrations (Martin et al., 2014). These nutrient reservoirs could be readily mobilised and exploited by the foraminiferal cell, particularly prior to gametogenesis, when planktonic foraminifera require extra elemental resources for DNA production (Hemleben et al., 1989). For *Synechococcus*, being housed within a foraminiferal cell could protect it from grazers and the multitude of cyanophages present in the water column (Suttle and Chan, 1994; Mühling et al., 2005). *Synechococcus* may also benefit presumably from a supply of host metabolic by–products or from specific nutrients as products of prey digestion.

## 4.3 Feeding preferences and life strategy of *G. bulloides* Type IIId

TEM in combination with 16S rRNA gene metabarcoding enables identification of both bacteria and eukaryotic chloroplasts within the foraminiferal cell. This methodology does not amplify eukaryotic, nuclear–encoded, (18S) rRNA genes and, as a result, does not provide any information about the non–chloroplast–bearing zooplankton prey of *G. bulloides*. Observations of large numbers of freshly collected specimens of *G. bulloides* confirm that they feed on small zooplankton prey as well as phytoplankton (Spero and Lea, 1996). Amongst the latter, a preference for some species of diatoms and chlorophytes over

dinoflagellates or chrysophytes has been reported (Lee et al., 1966). Interestingly, however, two of the five *G. bulloides* specimens in this metabarcoding study (BUL22, BUL23) did not contain any chloroplast DNA, indicating that they had not fed on phytoplankton prior to sampling. However, these specimens were sampled in November (Off Bodega Head) during the period of relaxation in upwelling, from vertically integrated net tows and may have been obtained from resident populations as deep as 150 m. In contrast, the three individuals in which chloroplast 16S rRNA sequences were present (BUL34, BUL36 and BUL37) were sampled from shallow water nets in July/Aug (off Santa Catalina Island) towards the end of the weak summer upwelling period. These differences in OTU composition, therefore, could be as a result of location, depth or seasonal differences in available diet. The three *G. bulloides* with chloroplast sequences (6.4 %, 27 % and 3 %, respectively) were clearly feeding on a range of photosynthesising eukaryotes (Sect. 3.3). OTUs indicate these to be Cercozoa (mixotrophic protists), Streptophyta (includes green algae), Haptophyta (includes coccolithophores) and Stramenopiles (includes both diatoms and chrysophyte algae).

Our data suggest that *G. bulloides* may also utilise bacteria as a significant food source. *G. bulloides* contained 33.7–62.5 % of non-*Synechococcus* bacterial sequences within the cell, (BUL37 was an outlier with only 10 %, Fig. 34) corresponding to a diverse assemblage of 200 OTUs. We assume that these sequences are derived from prey species because no intact bacteria lacking the carboxysomes and thylakoid membranes found in *Synechococcus* were observed in TEM images of the *G. bulloides* cytoplasm or non-digestive vacuoles. The most abundant group of sequences recovered (15–31 %, outlier BUL37 contained 2.6 %) comprise 81 OTUs belonging to the class Alphaproteobacteria (including those OTUs of the family Bradyrhizobiaceae that were not excluded as contaminants), perhaps indicating a preferential selection of specific members within this class. The remaining 17–47.5 % (outlier BUL37, 7.5 %) of sequences were made up of a diverse collection representing other major phyla of bacteria (Sect. 3.3; Fig. 34) that include the Acidobacteria, Actinobacteria, Bacteroidetes, Firmicutes and Planctomycetes and the classes Beta- and Gammaproteobacteria of the phylum Proteobacteria.

*G. bulloides* Type IId is found throughout the year in the Southern California Bight, where it is exposed to cool, upwelling periods of high productivity and also to warmer periods characterised by more stratified, less productive conditions (Darling et al., 2003; Darling and Wade, 2008). It has a relatively high growth rate, possibly reproducing within 2–3 weeks (Spero and Lea, 1996; Lombard et al., 2009). In combination with our data, this suggests that *G. bulloides* Type IId is a generalist predator with an opportunistic feeding strategy, utilizing bacterioplankton as well as phyto- and zooplankton, the proportions of which may be seasonal and depth dependent. Such opportunism may enable *G. bulloides* to grow and reproduce rapidly within its diverse habitat. We propose two hypotheses for the life strategy of *G. bulloides* Type IId to survive the challenges presented within the broad seasonal changes in the region. The first is that it is a mixotrophic feeder (Mitra et al., 2016) and that the *Synechococcus* endobionts are photosynthesising symbionts contributing fixed carbon to the foraminiferal host. In this scenario, they would be fulfilling a functional role similar to that of algal symbionts in other spinose species, and in particular could provide additional resources during the maximum growth phase of the shallow-dwelling juveniles. Alternatively, or concurrently, *Synechococcus* may be exploited by *G. bulloides* Type IId for its nutrient

assimilation and storage capacity and then digested as an extra energy, nitrogen and phosphate source for DNA replication at reproduction.

#### 4.4 The importance of genotype ecology

Since *G. bulloides* occurs in great abundance in cool, high latitudes and mid to lower latitude upwelling systems (Kleijne et al., 1989; Naidu and Malmgren, 1996), it is one of the most commonly used planktonic foraminifera for palaeoclimate reconstruction (Sautter and Thunell, 1991; Spero and Lea 1996). In order to reconstruct past changes in oceanic conditions using the shell geochemical data, it is important to obtain a thorough understanding of the relationship between foraminiferal ecology and the geochemistry of its shell. This relationship is based on the assumption that each foraminiferal morphospecies represents a genetically continuous species with a unique habitat preference. However, since *G. bulloides* inhabits such a wide range of different ecosystems, it is not surprising that several ecologically distinct genotypes have been recognised (Darling et al., 1999; Kucera and Darling, 2002; Darling and Wade, 2008; Seears et al., 2012; Morard et al., 2013). Indeed, recent species delineation studies support species status for several of the *G. bulloides* genotypes (André et al., 2014) including *G. bulloides* Type IId. Such diversity could result in genotype-specific geochemical signatures across the morphospecies (Healy-Williams, 1985; Bijma et al., 1998; de Vargas, 2001; Kucera and Kennet, 2002; Sadekov et al., 2016). Both Kucera and Darling, (2002) and Morard et al., (2013) have demonstrated that, based on ecological knowledge, integrating *G. bulloides* genotypes into assemblage-based SST reconstructions significantly improves resolution. This demonstrates the value in understanding the ecology of genotypes within a morphospecies and the necessity of establishing whether the association between *G. bulloides* Type IId and *Synechococcus* is universal across this foraminiferal morphospecies complex.

#### 4.5 Implications for palaeoceanography

The discovery of intracellular bacteria within a palaeoceanographically significant foraminiferal host may lead to a significant improvement in our current understanding of foraminiferal shell geochemistry. The carbon isotopic composition of planktonic foraminifera has the potential to help reconstruct changes in the chemocline of the surface ocean, providing insights into changes in ocean circulation (Spero et al., 2003). However, the interpretation of  $\delta^{13}\text{C}$  data is often complicated by poor understanding of the causes of offsets between shell  $\delta^{13}\text{C}$  and the  $\delta^{13}\text{C}$  of the dissolved inorganic carbon from which foraminifera build their shells. In particular, the  $\delta^{13}\text{C}$  of *G. bulloides* shells deviates from predicted values more than that of any other extant species (Deuser et al., 1981; Kahn and Williams, 1981; Curry and Matthews, 1981; Kroon and Darling, 1995; Spero and Lea, 1996; Bijma et al., 1999), implying consistent use of metabolic carbon during calcification by this morphospecies (Deuser et al., 1981).

Symbiont photosynthesis as well as symbiont and host respiration alter the chemical microenvironment surrounding the host shell, which in turn influences their shell geochemical signatures (Rink et al., 1998; Wolf-Gladrow et al., 1999; Eggins et al., 2004). In this *G. bulloides*/*Synechococcus* association, respiration of both endobiont and host would contribute  $^{13}\text{C}$ -depleted

CO<sub>2</sub> to the calcifying microenvironment (Spero and Lea, 1996), whilst *Synechococcus* photosynthesis would counteract this by preferentially removing <sup>12</sup>CO<sub>2</sub> and hence elevating <sup>13</sup>C/<sup>12</sup>C ratios in the remaining dissolved CO<sub>2</sub>, as occurs in protist algal symbiont bearing planktonic foraminifera (Spero et al., 1997). The large offset towards <sup>13</sup>C-depleted values measured in *G. bulloides* suggests that *Synechococcus* respiration dominates the shell geochemical signature, and implies that photosynthesis is not the primary role of *Synechococcus* in this association (Sect. 4.2). The presence of metabolically active *Synechococcus* in *G. bulloides* Type IId, therefore, may account for the unusual shell δ<sup>13</sup>C determined via culture-based studies conducted at Santa Catalina Island. Since *G. bulloides* Type IId is abundant here, it is unlikely that variation in genotype has contributed to uncertainties in these calibrations, but applying these culture-based calibrations to other regions in which different genotypes dominate may produce erroneous results (Darling et al., 2003). It is of particular importance therefore, to determine whether the *Synechococcus*/*G. bulloides* association exists in other *G. bulloides* genotypes in order to generate and apply genotype-specific palaeoclimate calibrations.

## 5. Conclusions

This is the first report of bacterial endobionts within a planktonic foraminiferal species. Our results show that the picocyanobacteria *Synechococcus* spp. are found in large numbers within the protist algal symbiont-barren foraminifer, *G. bulloides*. *Synechococcus* spp. are taken up from the water column by the host and live and divide within the host cytoplasm at substantially higher concentrations (~4 orders of magnitude) than those found in the surrounding seawater. Their role is not yet known, but their potential for both phototrophy and (photo)heterotrophy makes *Synechococcus* an ideal symbiont for *G. bulloides* as it occupies water depths both within and below the photic zone. Additionally, the ability of *Synechococcus* to store P as polyphosphate, and N within biliproteins under nitrogen replete conditions, would be beneficial for a foraminiferal host exhibiting fast reproductive turnover, with a high nutrient and energy demand at gametogenesis. Further experiments are required on the *G. bulloides* Type IId/*Synechococcus* association to elucidate the full relationship between the two organisms. More investigations are also needed of the *G. bulloides* morphospecies globally, to determine how widespread the association is to improve understanding and accuracy of this species as a palaeoclimate proxy.

In this study we have demonstrated that 16S rRNA gene metabarcoding of the intracellular DNA of planktonic foraminifera and TEM has the potential to provide new insights into the biological associations and seasonal feeding preferences of ecologically distinct genotypes of planktonic foraminifera. With the addition of 18S rRNA gene metabarcoding to target protist and multicellular zooplankton, next generation DNA sequencing technologies could transform the usefulness and accuracy of planktonic foraminiferal global distribution and seasonality models by providing the essential ecological information currently unavailable (Fraile et al., 2008; Lombard et al., 2011; Roy et al., 2015).

## Author contribution

CB conceived and devised the project and methods, carried out the lab work and prepared the manuscript. KFD and BTN advised on methodology. ADR, CVD and JF collected planktonic foraminifera. AF contributed to next-generation sequencing and analysis. MW contributed to analysis of *Synechococcus* DNA. CB and KFD wrote the manuscript with contributions from all co-authors.

## Competing interests

The authors declare that they have no conflict of interest.

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### Figure Legends

Figure 1. Map of the Californian coast and, at higher magnification, the region within the red box (insert) showing the Bodega Head and Santa Catalina Island sampling locations, (black circles) and the SPOT sampling site (white circle). The hydrography of the region is described in Section 2.1, whilst the directions of flow of the two major coastal currents, the California Current and the Davidson Current, are indicated by the arrows.

Figure 2. Fluorescence micrograph of a DAPI stained, decalcified *G. bulloides* cell. (a) Diffuse autofluorescence not associated with DNA-stained inclusions can be observed throughout the cytoplasm. The black arrow highlights an example of the bright regions, 3-10µm in size, that correspond to vacuoles containing condensed prey items. The many ~1 µm diameter brightly stained globular structures that occur throughout the cell are consistent with the presence of intracellular bacterial cells. The white rectangle denotes the area magnified in Figure 2(b) and shows examples (white arrows) of these DNA-containing, bacteria-sized structures and their cytoplasmic location.

Figure 3. Fluorescence micrograph of a decalcified *G. bulloides* cell examined under the TRITC filter set (excitation 540 nm, emission wavelength >580 nm). (a) The cytoplasm from two of the chambers is shown. The autofluorescence localized to the ~1 µm diameter globular structures found throughout the cell is consistent with the presence of phycoerythrin-containing *Synechococcus*. The white rectangle denotes the area magnified in Fig. 3(b) in which the autofluorescing structures can be seen as discrete entities demonstrating that the cyanobacterial cell membranes are intact (see text). The white arrowheads

highlight a group of bacteria that can also be identified by DAPI staining when examined under the DAPI filter set in Fig. 3(c).

Figure 34. Relative abundance of taxonomically assigned 16S rRNA gene sequences from bacteria and chloroplasts within the cytoplasm of six individual foraminifer specimens; five *G. bulloides* (BUL22, BUL23, BUL34, BUL36 and BUL37) and one *N. dutertrei* specimen (DUT55). Sequences are assigned to operational taxonomic units (OTUs) grouped at different levels of taxonomic classification (see key). 16S rRNA gene sequences assigned to OTUs of the genus *Synechococcus* are the most abundant within *G. bulloides* and are at the highest level of classification when compared to the other OTUs assigned to individual classes or phyla.

Figure 45. Transmission electron microscope images of *Synechococcus* cells inside *G. bulloides*. (a) A *Synechococcus* cell with characteristic polyhedral carboxysomes in the central cytoplasmic region (white arrow) surrounded by thylakoid membranes. (b) Numerous *Synechococcus* cells within a *G. bulloides* cell are observed in both the cytoplasm and vacuoles (black arrow). This is a region of cytoplasm rich in fibrillar bodies (white arrow) found only in planktonic foraminifera, and whose function is unknown. (c) *Synechococcus* cell within a *G. bulloides* cell undergoing cell division as indicated by the presence of a constriction at the cell midpoint (white arrow).



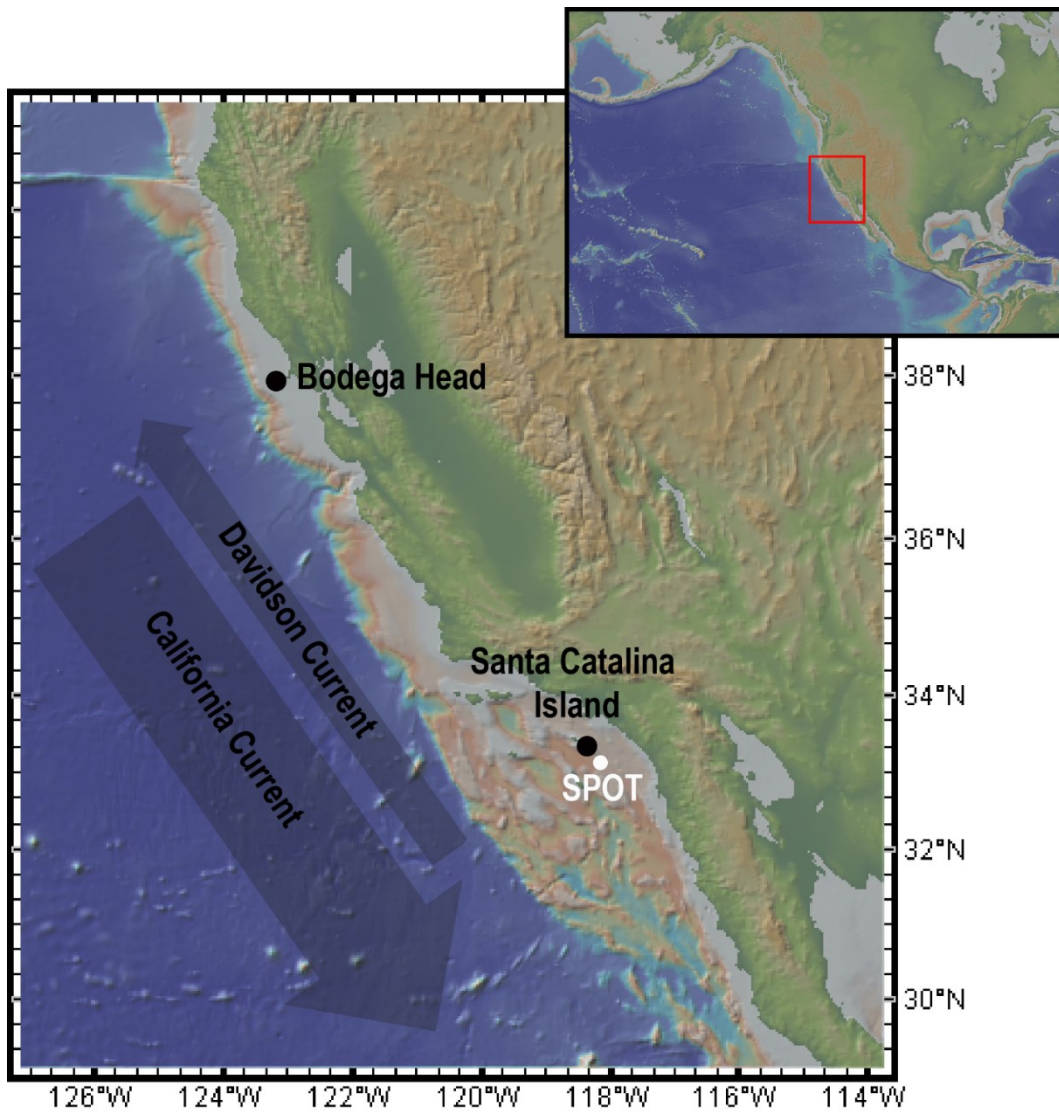


Fig01

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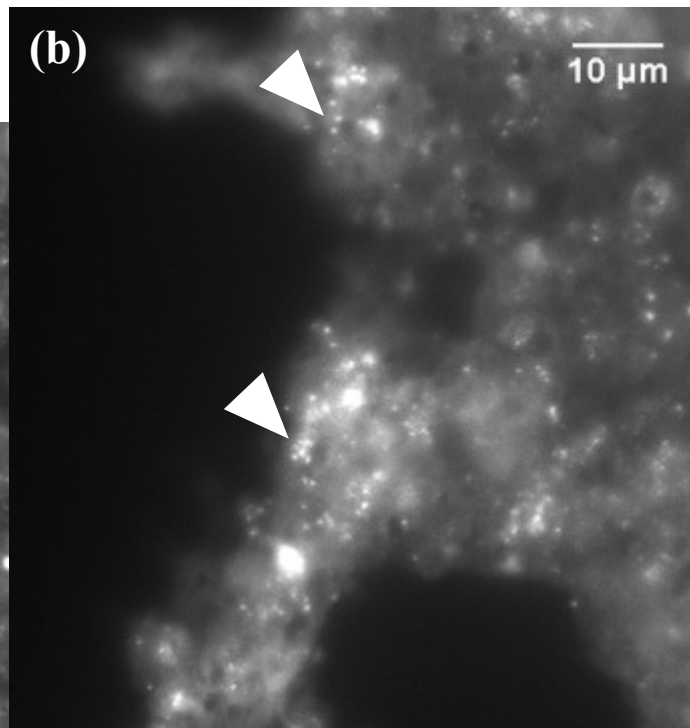
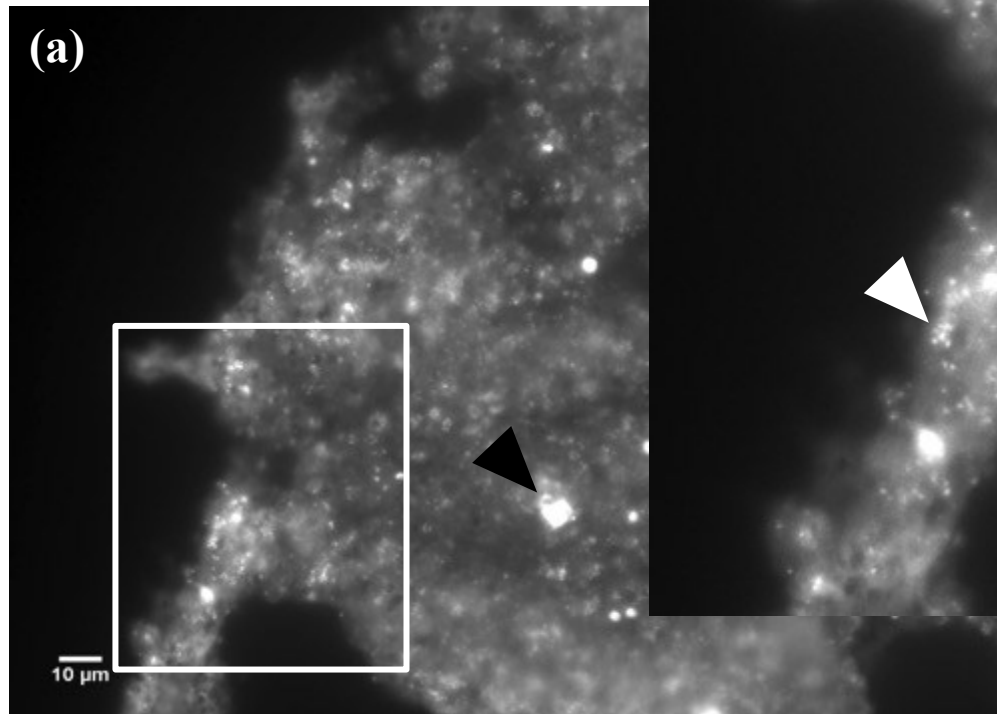


Fig02

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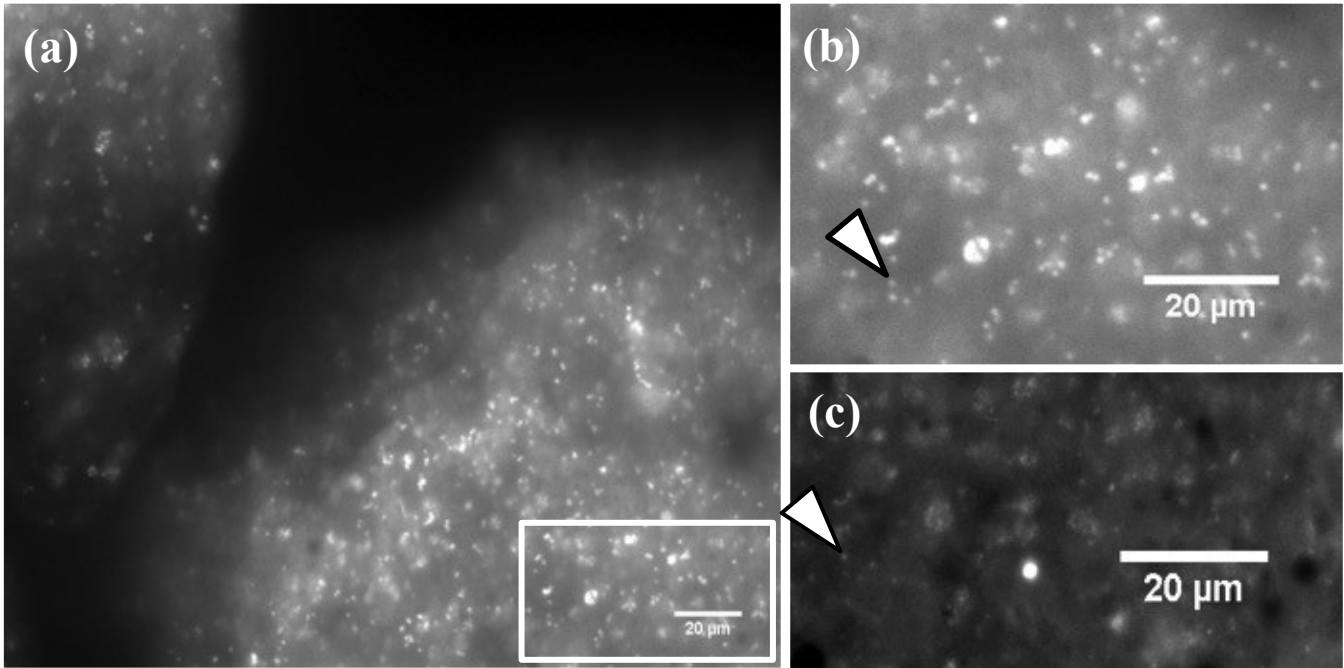
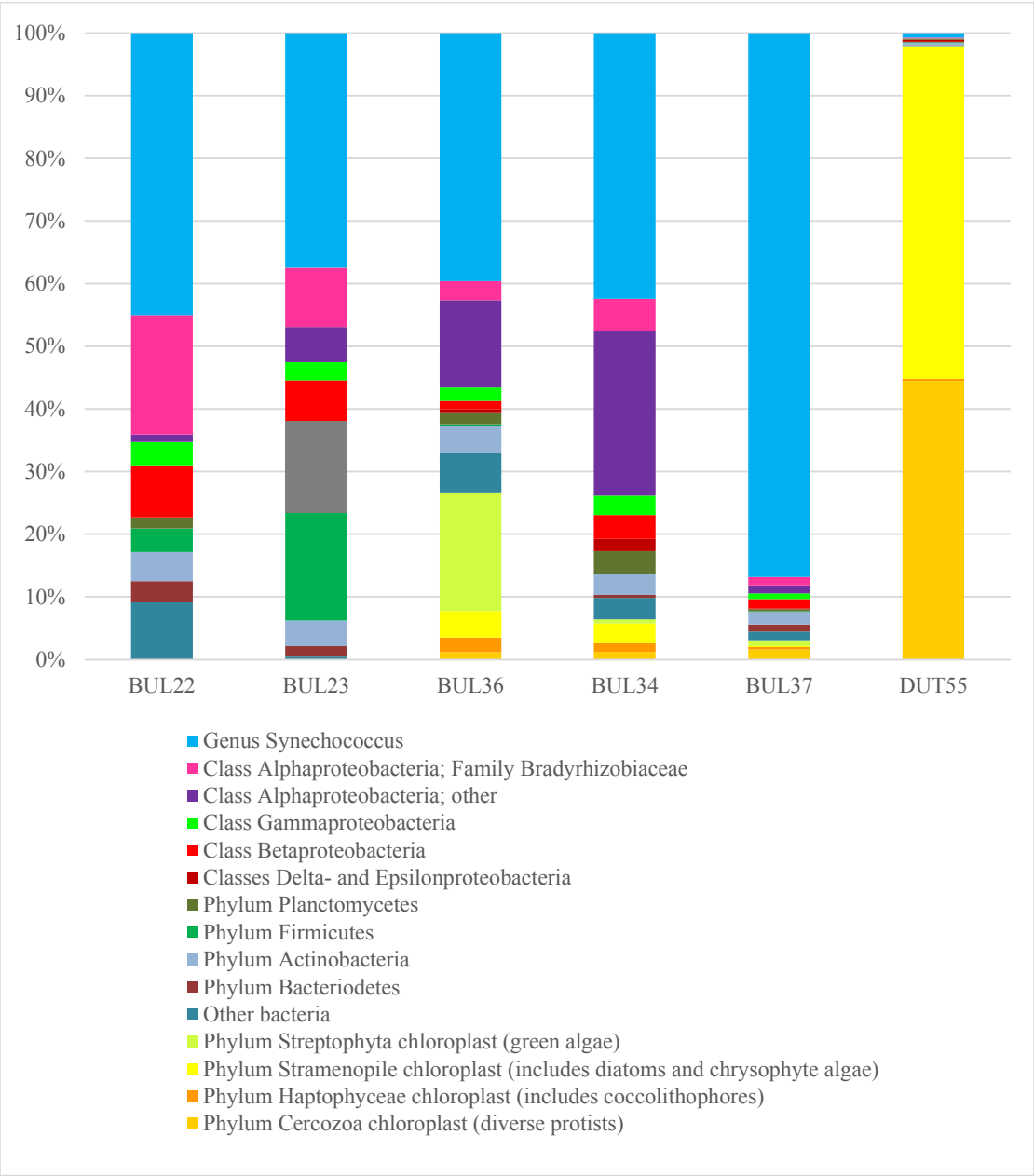


Fig03



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Fig03Fig04

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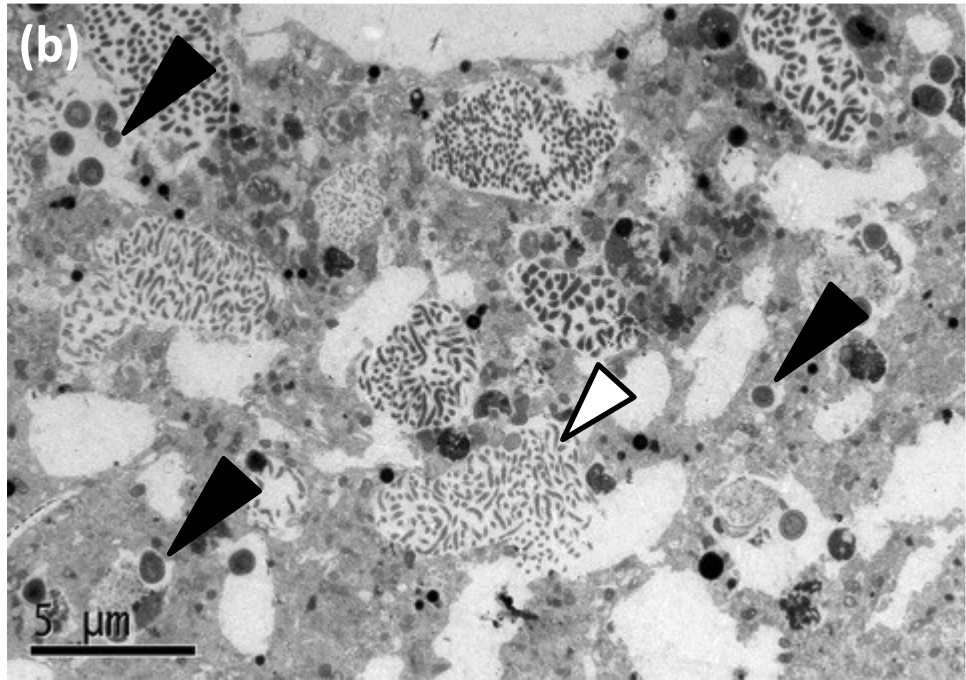
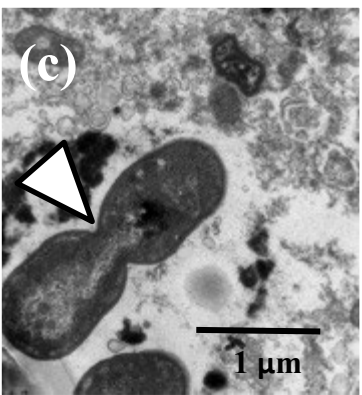
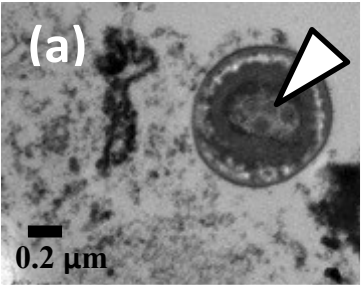


Fig04Fig05

Table 1. Sampling information and details of analyses performed for each planktonic foraminifer specimen collected

Morphospecies	Sample ID	Sampling site	Sampling date	Co-ordinates	Sea Surface Temperature	Analysis
<i>G. bulloides</i>	BUL21	Santa Catalina Island	July/Aug 2013	33.4°N, 118.4°W	18°C -21.5°C	Control for fluorescence microscopy
<i>G. bulloides</i>	BUL24	Santa Catalina Island	July/Aug 2013	33.4°N, 118.4°W	18°C -21.5°C	DAPI staining and fluorescence microscopy
<i>G. bulloides</i>	BUL25	Santa Catalina Island	July/Aug 2013	33.4°N, 118.4°W	18°C -21.5°C	DAPI staining and fluorescence microscopy
<i>G. bulloides</i>	BUL26	Santa Catalina Island	July/Aug 2013	33.4°N, 118.4°W	18°C -21.5°C	DAPI staining and fluorescence microscopy
<i>G. bulloides</i>	BUL28	Santa Catalina Island	July/Aug 2013	33.4°N, 118.4°W	18°C -21.5°C	DAPI staining and fluorescence microscopy
<i>G. bulloides</i>	BUL29	Santa Catalina Island	July/Aug 2013	33.4°N, 118.4°W	18°C -21.5°C	DAPI staining and fluorescence microscopy
<i>G. bulloides</i>	BUL30	Santa Catalina Island	July/Aug 2013	33.4°N, 118.4°W	18°C -21.5°C	DAPI staining and fluorescence microscopy
<i>G. bulloides</i>	BUL32	Santa Catalina Island	July/Aug 2013	33.4°N, 118.4°W	18°C -21.5°C	TEM
<i>G. bulloides</i>	BUL34	Santa Catalina Island	July/Aug 2013	33.4°N, 118.4°W	18°C -21.5°C	Metabarcoding, genotyping and <i>Synechococcus</i> 16S and <i>rbcL</i> cloning and sequencing
<i>G. bulloides</i>	BUL36	Santa Catalina Island	July/Aug 2013	33.4°N, 118.4°W	18°C -21.5°C	Metabarcoding
<i>G. bulloides</i>	BUL37	Santa Catalina Island	July/Aug 2013	33.4°N, 118.4°W	18°C -21.5°C	Metabarcoding
<i>G. bulloides</i>	BUL39	Santa Catalina Island	July/Aug 2013	33.4°N, 118.4°W	18°C -21.5°C	TEM
<i>G. bulloides</i>	BUL69	Santa Catalina Island	July/Aug 2013	33.4°N, 118.4°W	18°C -21.5°C	TEM
<i>N. dutertrei</i>	DUT5 <sup>59</sup>	Santa Catalina Island	July/Aug 2013	33.4°N, 118.4°W	18°C -21.5°C	Metabarcoding and genotyping
<i>G. bulloides</i>	BUL04	Bodega Head	Nov 2014	38.3°N, 123.0°W	14°C-15°C	<i>Synechococcus</i> 16S and <i>rbcL</i> **
<i>G. bulloides</i>	BUL05	Bodega Head	Nov 2014	38.3°N, 123.0°W	14°C-15°C	<i>Synechococcus</i> 16S and <i>rbcL</i> **
<i>G. bulloides</i>	BUL13	Bodega Head	Nov 2014	38.3°N, 123.0°W	14°C-15°C	<i>Synechococcus</i> 16S and <i>rbcL</i> **
<i>G. bulloides</i>	BUL14	Bodega Head	Nov 2014	38.3°N, 123.0°W	14°C-15°C	<i>Synechococcus</i> 16S and <i>rbcL</i> **
<i>G. bulloides</i>	BUL15	Bodega Head	Nov 2014	38.3°N, 123.0°W	14°C-15°C	<i>Synechococcus</i> 16S and <i>rbcL</i> **
<i>G. bulloides</i>	BUL22	Bodega Head	Nov 2014	38.3°N, 123.0°W	14°C-15°C	Metabarcoding
<i>G. bulloides</i>	BUL23	Bodega Head	Nov 2014	38.3°N, 123.0°W	14°C-15°C	Metabarcoding
<i>G. bulloides</i>	BUL71	Bodega Head	April 2015	38.3°N, 123.0°W	10.5°C	TEM
<i>G. bulloides</i>	BUL73	Bodega Head	April 2015	38.3°N, 123.0°W	10.5°C	<i>Synechococcus</i> 16S and <i>rbcL</i> **
<i>G. bulloides</i>	BUL74	Bodega Head	April 2015	38.3°N, 123.0°W	10.5°C	<i>Synechococcus</i> 16S and <i>rbcL</i> **
<i>G. bulloides</i>	BUL82	Bodega Head	April 2015	38.3°N, 123.0°W	10.5°C	<i>Synechococcus</i> 16S and <i>rbcL</i> **
<i>G. bulloides</i>	BUL83	Bodega Head	April 2015	38.3°N, 123.0°W	10.5°C	<i>Synechococcus</i> 16S and <i>rbcL</i> **
<i>G. bulloides</i>	BUL84	Bodega Head	April 2015	38.3°N, 123.0°W	10.5°C	<i>Synechococcus</i> 16S and <i>rbcL</i> **
<i>G. bulloides</i>	BUL85	Bodega Head	April 2015	38.3°N, 123.0°W	10.5°C	<i>Synechococcus</i> 16S and <i>rbcL</i> **
<i>G. bulloides</i>	BUL86	Bodega Head	April 2015	38.3°N, 123.0°W	10.5°C	<i>Synechococcus</i> 16S and <i>rbcL</i> **
<i>G. bulloides</i>	BUL88	Bodega Head	April 2015	38.3°N, 123.0°W	10.5°C	<i>Synechococcus</i> 16S and <i>rbcL</i> **

\*PCR amplification of *Synechococcus* 16S rRNA gene and *rbcL* (RuBisCo large subunit)