Author responses to review of Anonymous Referee #1 to "Carbonic anhydrase is involved in benthic foraminiferal calcification"

by Siham De Goeyse, Alice E. Webb, Gert-Jan Reichart, and Lennart J. de Nooijer

We are very grateful to the anonymous reviewer for the detailed comments on our manuscript. We reply below to the specific comments. Reviewer comments are given in italic font and our response in bold font.

OVERVIEW OF THE MANUSCRIPT AND BASIC ASSESMENT:

"This manuscript addresses an important question regarding calcification in foraminifera: "Does carbonic anhydrase play a role in inorganic carbon uptake?" The authors address this question using a pair of experiments using probably the second-most widely studied benthic foraminifer, Amphistegina lessonii. This is a warm-temperate to tropical species that is abundant throughout the Indo-Pacific and which grows abundantly in some large-scale reef aquaria, which was the source of the experimental specimens. The results of the experiment support previous experimental work showing that Amphistegina spp. can live and calcify at elevated pCO2 levels (e.g., Glas et al., 2012; McIntyre-Wressnig et al., 2013; Knorr et al., 2017).
Unfortunately, the manuscript itself, while reporting interesting data, is not suitable for publication as currently written. There are numerous deficiencies in statements and assumptions regarding foraminifera, methods descriptions, and referencing, that must be addressed to bring this manuscript to publication quality."

We are very happy with the constructive comments by this reviewer and changed our manuscript accordingly. Below, we reply point-by-point and indicate what we have changed in the text of the revised version of our manuscript.

SPECIFIC COMMENTS:

"Title: Because the paper is written with the assumption that this experiment represents "benthic foraminiferal calcification", an erroneous assumption that will be addressed next, please change the title to" Carbonic anhydrase is involved in calcification in Amphistegina lessonii, a benthic foraminifer that hosts diatom endosymbionts". Recommendation: Change the Title. "

We agree with the reviewer that the original title may have been a bit too general. Therefore, we changed the title into: "Carbonic anhydrase is involved in calcification of the benthic foraminifer *Amphistegina lessonii*"

"Introduction: A fundamental problem with the title and the paper overall is the inherent assumption that calcification in A. lessonii represents calcification in the benthic Foraminifera. While at least some of the co-authors know that is not a valid assumption (e.g., de Nooijer et al, 2009), the manuscript should at least make the distinction between calcification in Globothalmea (in this case, a hyaline, perforate foram) and Tubothalmea (imperforate, porcelaneous forams). This distinction is important because, as shown by Pawlowski et al. (2013) together with Mikhalevich (2014), these two groups evolved calcification independently. Moreover, since Amphistegina spp. host diatom endosymbionts, the carbonic anhydrase could be associated with the diatoms, in which case, the observations would not apply to hyaline taxa that do not host algal symbionts. Recommendation:

Revise the Abstract, Introduction and Discussion to note that this experimental study applies to hyaline forams hosting diatom symbionts."

We fully agree with the reviewer on this point. We often neglected calcification as it is done by imperforate species, as they are not often used in paleoceanography. We have carefully assessed our abstract, introduction and discussion to emphasize that our results apply in principal to perforate foraminifera. For example, we added 'perforate' to lines 20 and 26 (abstract) and 61 (Introduction). We also refer to the potential difference between Globo- and Tubothalamea and added the two suggested papers on foraminiferal phylogeny in lines 256.
Section 4.2 describes the potential effect of the symbionts and their CA on our results. The reviewer is right, that species without (diatom) symbionts may react differently to incubation with AZ than *A. lessonii*. Therefore, we added a cautionary sentence at lines 225-226.

"Methods: There are many studies in the literature that discuss culture of Amphistegina spp. and Heterostegina depressa, as well as other benthic forams that host algal symbionts. The authors do not mention two important culture parameters, illumination (i.e., light intensities) and salinity. The latter may not be as critical to experimental results, since alkalinity is reported. However, light is a widely established, extremely important environmental parameter (e.g., Muller, 1978; Hallock, 1981; Hallock et al., 1986; Talge and Hallock, 2003; Williams and Hallock 2004)."

We thank the reviewer for pointing out this omission. Now, we added the light intensity at line 75: "Illumination was approximatively 180 µmol photons m^{-2} s^{-1}, during the 12h of light"
The salinity was added to line 74: "They were fed with freeze-dried *Dunaliella salina* and incubated in North Atlantic sea water (salinity: 36)."

"In addition, the authors do not report the size range of individuals used in the experiments. If they had been aware of the earlier experimental studies, they would know that growth rates in Amphistegina are size dependent, which pertains to the comparability of their results to other studies (more on this in comments on the Discussion). Finally, in line 72, the authors mention that specimens were incubated in calcein prior to starting the experiment, with no mention of why and no further mention of calcien in the manuscript. Recommendations: Please report the light environment of the cultures, the salinity of the culture media, and the approximate starting size (or ending, since the experiments were very short) of the experimental specimens. Also, either elucidate on the use of calcein or delete mention of it."

The calcein was used to analyze whether the number of chambers formed matches the alkalinity decrease during the experiment. Results show that there is a consistent addition of new chambers with changes in total alkalinity: we now added a table with those results as supplementary information (Table S1).
We chose using individuals of the maximum possible size range in order to account for any size-specific responses. We agree with the reviewer and have added the range of starting diameters to the text (line 80).

"Results: The results are relatively straightforwardly presented. The only suggestion is that, in Table 1 and Table 2, reporting the decimal values for initial TA and initial DIC are not meaningful, given the standard deviations of the changes reflect whole numbers that represent ~10–40% of the changes in TA and DIC. "

We followed recommendation of the reviewer 1 and rounded all values in table 1 and Table 2.

"Discussion: See the comments and recommendations under "Introduction. That is, the Discussion should be focused on Amphistegina as a model for hyaline forams with algal endosymbionts, not all benthic forams."

We agree and have added a number of cautionary sentences to the discussion.

"Moreover, the authors state in lines 159–160, "The only previous study using Amphistegina spp."; that statement is inaccurate. Ter Kuile and Erez (1984, 1987); and Hallock et al. (1986), all reported rates of calcification in units equivalent to those reported. And indeed, the calcification rates reported in the submitted manuscript are lower than most of the previously reported rates for Amphistegina, which is why this reviewer questioned the light environment of the experiments. If the light levels inside the culture flasks were limiting photosynthesis and growth of the experimental specimens, the calcification rates would of course be relatively low. See, for example, Table 1 in Hallock et al. (1986), who reported growth rates in µg/day dry weights at five different light intensities for both A. lessonii and A. gibbosa. The growth rates at the lowest light levels are similar to those reported in the submitted study. Moreover, the authors should note the starting diameters of the specimens used in the Hallock et al. (1986) paper. The experiment reported in Table 1 in that paper used recently produced juveniles, while the experiments reported in Table 3 included one trial with intermediate-sized specimens (500–600 um diameter), while the other trials also used small juveniles. If the experiments reported in the submitted paper used specimens in the 1–1.5 mm size range, the biology of the forams indicates that only a few specimens would have added new chambers."

We agree with the reviewer and have re-phrased the end of the discussion's first paragraph accordingly. We summarize the previously published growth rates to compare them to ours and we added the suggested references.

"Lines 177–179: The authors suggest that calcification in Amphistegina might differ from that reported in G. sacculifer, which is interesting, because, elsewhere, they are equating calcification in Amphistegina with calcification in miliolids, which are far more distantly related, as noted above."

We see the reviewer's concern, which we now have hoped to have repaired by focussing the discussion of our results to perforate foraminifera.

"In the paragraph in lines 187–196, the authors appear to assume that photosynthate produced by the algal symbionts is primarily used for organic matrix. They do not consider the production of simple sugars that can be used in ATP production that drives the ion pumps. In the case of Amphistegina, TEM studies have shown the abundance of lipid storage bodies in the vicinity of the symbionts. Indeed, the authors' conclusion that more research is needed on the types of organic molecules produced is certainly true, but they overlooked pertinent information in papers by Lee, Stuhr, Talge, Toler, and probably others. They also overlooked pulse-chase studies by Muller (1978) and ter Kuile and Erez (1987)."

The suggestion that the symbionts are producing sugars for the foraminifer was added to line 197-199 including the suggested papers in this and following sentences. We extended the discussion by citing reports on the positive effects of photosynthesis on calcification, which is in line with our results.

"In lines 193–194, the authors mention "symbiotic dinoflagellates and zooxanthellae". Zooxanthellae are symbiotic dinoflagellates."

We removed "and zooxanthellae" from this line.

"Conclusions: Of course, it is photosynthesis that enhances growth and calcification in Amphistegina; and light is required for photosynthesis. The phylogenetic and physiological capability to calcify is inherent in the hyaline forams (that is why Amphistegina can exhibit some calcification in the dark). But because calcification is an energy-driven process, the substantial energy and organic matter provided by photosynthesis by algal symbionts substantially enhances growth, including calcification."

We fully agree that photosynthesis contributes to calcification by providing energy. However it was suggested that calcification and photosynthesis might be competing processes (Ter Kuile et al., (1989b, 1989a) which is contrary to what we observed. Therefore, we mentioned this in the conclusions.

"References: The references are typically "end-note" formatting-problematic and need extensive editing if Biogeoscience requires consistency in referencing."

We carefully edited the reference section.