Assessment of "Carbonic anhydrase is involved in benthic foraminiferal calcification"

1. Overview of the manuscript and basic assessment:

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 pCO_2 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.

2. Specific deficiencies.
   a. 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.
   b. 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.
   c. 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). 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.

d. 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.

e. 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.
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.
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.
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).
In lines 193–194, the authors mention "symbiotic dinoflagellates and zooxanthellae". Zooxanthellae are symbiotic dinoflagellates.
Recommendations: See below.

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

Lines 259, 272, 288, 291, 303, 305, 316, 332, 335, 337, 228: genus and species names are not italicized
Lines 264, 290–291, 315–316, 335, 337–338: in manuscript titles, the nouns and some other words start with capital letters, inconsistent with referencing format for other journal articles.
Line 329 and 332, use subscripting, superscripting and Greek notation, as appropriate.

Overall recommendations: The authors should become much more familiar with the rather extensive literature on culture, growth, calcification, photosynthesis, physiological and cytoplasmic studies of *Amphistegina* spp. Then rewrite the entire paper, correcting misunderstandings, being more rigorous regarding what other taxa these observations may apply to, and incorporating appropriate citations. The experimental protocol and basic results appear to be sound and can be an important contribution to understanding calcification in hyaline forams that host algal endosymbionts. But the manuscript, as currently written, contains errors and misleading interpretations that detract substantially from the experimental results.