

Author responses to review of Anonymous Referee #2 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 helpful comments on our manuscript. We reply below to the specific comments. Reviewer comments are given in italic font and our response in bold font.

GENERAL COMMENTS

“The authors present a set of experiments, performed on a high number of foraminifera specimen of a same common symbiotic species. In this experimental work, the effect of an extracellular inhibitor of the carbonic anhydrase (CA) enzyme is compared to the effect of a photosynthesis inhibitor, as well as the sole light deprivation. Biomineralisation change is evaluated through measurements of DIC concentration and alkalinity change, and using solely that approach to evaluate “biomineralisation yield” is also a main aspect of the article. These results evidence, in my opinion, the role of carbonic anhydrase, but I do believe that additional simple information should be given in order to confirm that no other phenomenon can explain, or interfere with, those results. If those information can be given (see below) and the role of CA is confirmed, then the scientific significance of these results is excellent. The scientific quality is good, the method and experimental aspects are good despite the few information lacking, as great effort were provided to replicate the experiments and perform them on a significant amount of specimens. The discussion however and the manuscript text in general is not as good as I believe necessary for publication in an international journal. There are not enough references backing information, several aspects of the results are not discussed, a part of the discussion is just a description of results, there are words missing in some sentences, one name on a figure and a table do not match, one figure permitting the comparison of all results is missing, and there are several typography mistakes. I am not able to properly judge the english, but I found the manuscript perfectly understandable. If the text of the manuscript can be improved by the authors, I would recommend publication of the article as the results constitute a major advance in the understanding of biomineralisation by foraminifera (and in my opinion, it gives insight on biomineralisation mechanism in general considering how widespread is CA). For that reason, I hope the authors will improve the text, scientific content and discussion of the article in order to provide these interesting results the context they deserve to become a well referred to article.”

We thank the reviewer for the kind words and helpful comments. We have critically assessed the text of our manuscript and listed our answers to all comments below. We hereby hope that our manuscript now is fit for publication in *Biogeosciences*.

SPECIFIC COMMENTS:

“I understand that solely using chemical solution parameters to describe the evolution of biomineralisation is one of the suggestion of the article, I however believe it is not enough as

some other parameters can affect DIC concentration and alkalinity: ex: microbial proliferation or open system phenomenon (improved gas /liquid phase exchanges in one experiment because of slightly different pH, or temperature differences due to the use of aluminum foil etc. . .). In my opinion the interest of a laboratory experiment on living organism cultured in vials is to be able to observe directly these organisms, which is not possible in other type of experiments. Are, in the end of the experiment, the vials clean enough with no particular microbial proliferation in one treatment? What proportion of foraminifera survived the experiment in all setups?"

We carefully monitored for signs of biofilm proliferation, of which there was no sign. In addition, there was no difference in appearance of the water, nor the vials between treatments, which is likely due to the relatively short incubation period and use of nutrient-poor seawater. We added the notion of the potential effect of biofilm proliferation to the end of the methods (lines 133-136).

"How are the new chambers? The author used calcein, they should thus be able to image the new chambers formed in each media. I believe any experiment of that type should present some kind of imaging, or at least a description of the visual aspects of the experiment, validating that new chambers formed, and evaluating that no microbial proliferation could have explained death of several microorganisms, that could explain less biomineralisation. For example, in the acetazolamide experiment, if a microbial proliferation occurred and a third of the foraminifera died, while the other survived and biomineralised regularly, wouldn't it give the impression, just by measuring DIC and alkalinity variation that only biomineralisation was affected by acetazolamide? These are simple information that would strengthen the results and the methods, that should be provided in the manuscript before publication. "

We agree that we cannot distinguish between 'normal chamber addition rates' by less specimens, and less chambers added by all specimens during the incubation after addition of AZ. We added this to the manuscript, although we would like to stress that both these options (less chambers by all specimens versus less specimens that added chambers) lead to largely the same conclusion. We now mention the number of chambers added to the supplementary information: these results also suggest that the same number of individuals added less chambers. By far most of the specimens added only one chamber after addition of AZ instead of two/ three in the control vials (now added as a supplementary table, S1). If half of the specimens would not have added chambers at all and the other half would have 'normal' chamber addition rates, more specimens would have been found with two/ three chambers added after addition of AZ. This implication has been added to the discussion (lines 190-192).

"In my opinion, authors should find a way to represent the results of all the different experiments together in one figure to ease comparison. As an example they could use the "corresponding g/L precipitated calcite" calculated for each experiment. "

We propose keep figures 3 and 4 separate, since there are (minor) experimental differences that may complicate a direct comparison. Length of the experiment and

initial size distribution of the foraminifera may have been slightly different and therefore may artificially increase differences between treatments.

“When discussing the effect of photosynthesis on calcification (line 192) the author do not mention the effect of lowering ATP production and rather suggest that photosynthesis promotes the production of molecules that are used in organic templates of calcification. The role of ATP in chamber formation is, in my opinion, impossible to ignore, the author must discuss it in the manuscript. On the other hand, organics produced by the symbionts may help biomineralisation (this indeed need further investigation), but it should be mentioned that there are (many?) benthic foraminifera with a hyaline test that do not bear symbionts. This should be discussed by the authors as well.”

We agree with the reviewer and, as replied to reviewer 1 too, we added this possibility to 4.2 (lines 216-220) in the revised version of our manuscript.

“Technical corrections Missing words or information: L29: “saturation state” the author should specify that it is towards calcium carbonate”

We changed this into “the saturation state of sea water with respect to calcite” and changed the sentence in L39 similarly: “Addition of CO₂ to sea water not only reduces saturation state with respect to calcite”.

“L35-36: The authors could specify the foraminifera species (benthic ? planktonic ? Amphistegina ?)”

We added “benthic” here.

“L44: “Since this uptake. . .” The sentence sounds odd, a word is probably missing, it should be rephrased. “

We removed ‘it’ after ‘and’ from this sentence.

“L48: There is a dot after the bracket “

We removed it.

“L48: The sentence states “It was recently suggested that CO₂ uptake by foraminifera is achieved through proton pumping” is that correct or did the authors used a shortcut to say that proton pumping (and thus ATP consumption) is used to modify pH and thus favor CO₂ uptake/or that a proton pump is used to actively cotransport CO₂? This imprecision should be corrected. “

It was indeed a shortcut to indicate that foraminifera use proton pumping to locally shift the pH around the site of calcification and thereby increase the CO₂ gradient and hence promotes inward CO₂ diffusion. We now extended this statement by explaining

the mechanism proposed by Toyofuku et al. (2017). This is added to lines 51-55 of the revised version of our manuscript.

“L50: In my understanding Bentov paper rather says that CO₂ gets concentrated in low pH vesicles, and that from there it diffuses to the high pH vesicles where it converts into charged DIC species and is thus trapped inside the vesicle. The authors should clarify that point if I am not mistaking. “

We agree with the reviewer, but essentially, Bentov et al. (2009) and Toyofuku et al. (2017) describe the same carbon concentrating mechanism. They only differ in the location where this happens (i.e. either within vesicles or between SOC/ outside medium), but both propose differences in pH and thereby $p\text{CO}_2$ as a way to promote diffusion of CO₂ towards the location where calcification proceeds. We have extended the description of this process in the revised version of our manuscript (lines 52-55) to clarify this.

“Line 112: I am not familiar with the “T” symbol signification next to each alkalinity species, could it be clarified? “

The T was only here for “total”. We have removed the ‘T’ from equations (1) and (2) as it was unclear.

“L115: Is that equation calculating “the alkalinity” or “the change in alkalinity”, this should be clarified. “

We changed the text from “the observed change in alkalinity” to “the observed alkalinity” (now line 123).

Figure 4 caption: there is a dot after “represents”

We removed this dot.

L193: the sentence is missing a word

We changed this sentence into: “Utilization of photosynthate as an organic template for calcification may explain this observation” (now line 123 of the revised version of our manuscript).

“L198 : “it has been shown that symbiotic dinoflagellates and zooxanthellae can trigger the activity of carbonic anhydrase (CA) in their host organisms [. . .] thereby explaining how photosynthesis enhances calcification”. The authors need to specify whether they mention the symbiont CA or if they refer to the host CA. Additionally the link between CA and photosynthesis must be explained. “

As we say in this sentence: ‘...of CA in their host organisms’, although the reviewer may be right when hinting to enhanced activity of CA in the symbiont and thereby only indirectly helping calcification. Therefore, we have added the following sentence:

‘Alternatively, increased activity of CA in the symbiont may also promote the flux of products to the host and thereby promote calcification indirectly.’

This section of the discussion has been altered according to other comments too, so that it now describes several possible interactions between CA, photosynthesis and calcification.

“Missing references: L27: “1/3rd of the carbon. . .” a reference should be provided”

We added a reference (Sabine and Tanhua, 2010).

“L40: Who suggested it? a reference is missing “

The reviewer is correct: there is no evidence for this, but the idea is sometimes brought up since other calcifiers are known to have such bicarbonate transporters. We therefore changed this sentence to avoid the suggestion that this uptake path has been shown to exist for foraminifera.

“L52: “many prokaryotes and virtually all eukaryotes” a reference should be provided.”

We added Lionetto et al., 2016 and Pastorekova 2004 to this sentence.

“ L60: “ a membrane impermeable inhibitor of this enzyme” a reference must be added. (A reference attesting that DCMU inhibits photosynthesis should be added as well if not provided in the manuscript). “

We added a reference to Moroney et al., 1985. Plant Physiol 79: 177-83. For the function of DCMU, we added a reference to Metz et al., 1986. FEBS Letters 205: 269.

“L207 : “Ca promotes [. . .] into the calcicoblastic space” this information should be supported by a reference”

We added a reference to Bertucci et al., 2013. Bioorg Med Chem 21: 1437-1450.

“L214: this reference and thus probably the whole sentence (except if another reference can be given) must be suppressed (as mentioned on Biogeosciences website “Works cited in a manuscript should be accepted for publication or published already”). “

We deleted this sentence.

OTHER COMMENTS:

“#1 Given that TA is measured with a 3µmol/kg precision and given the errors given in the tables, the decimals should be suppressed. “

We followed recommendation from reviewer 2 and reduced the number of decimals.

“#2 L78: What is the final concentration of dimethyl sulfoxide in the final flasks? The effect of dimethyl sulfoxide at that concentration on foraminifera should have been checked in a control experiment, if not, it should at least be discussed. “

The final concentration of DMSO was 0.05% (v/v) and the absence of impact had been tested on a preliminary experiment. We did not include these results since they would be merely a repetition of results shown by Moya et al. (2008), which we now added to line 85 of the revised version of our manuscript.

“#3 Figure 3 and 4, error bars should be represented or mentioned in the caption if smaller than symbols, or, even better, each 3 point replicates could be represented. “

We agree and have added values for ΔT_A and ΔDIC for the individual measurements (in light gray). In case of the added 4, 8 and 16 µM AZ, we did not include them since the variability was very low: this is now indicated in the caption of figure 3.

“#4 Can the author explain why on figure 4 and 3 the control point is not at the same position (is there an explanation for these two different control results?). Additonally, in table 1 and figure 3 there is two different names for one treatment, “No AZ” and “0 µM”, please choose one wording.”

The reviewer notes correctly that the averages for the two controls are not the same. This is due to the fact that results presented in figure 3 and 4 were obtained from experiments carried at two different time. Therefore, the initial size distribution of foraminifera was not the same. This explains why the ‘control’ vials gave different calcification rates. We also corrected the ‘No AZ’ from figure 3 to ‘control, 0 µM’.

“#5 From line 159 to 170 it is a summary of the results that should not be, in my opinion, in the discussion. “

Here we respectfully disagree with the reviewer. It is indeed a summary of the results, but with no interpretation of them. Therefore, we propose to keep it where it was.

“#6 Line 175: The “extracellular” specificity of CA is mentioned here but not discussed in part 4.3 and then comes back later in the manuscript. This should be restructured to clarify the message of the authors. “

We agree and have specified where necessary ‘extracellular’ in 4.3.

“#7 Line 183: “the discrepancy between results may be caused by differences in the process involved in calcification between these species” Can the author mention one or more process they are referring to?”

We have included the following (now line 205-208):

“For example, it has been suggested that calcification may involve seawater transport (Erez, 2003; Segev and Erez, 2006) as well as transmembrane transport (Nehrke et al., 2013; Toyofuku et al., 2017), of which the relative contribution may vary between groups of foraminifera.”