Dear Anonymous Referee #2,
We want to thank you very much for the very constructive, helpful and valuable comments and corrections, which helped us to ameliorate the manuscript.
Please find below my responses to all comments:

Title: Variation in brachiopod microstructure and isotope geochemistry under low pH–ocean acidification–conditions
Authors: Facheng Ye, Hana Jurikova, Lucia Angiolini, Uwe Brand, Gaia Crippa, Daniela Henkel, Jürgen Laudien, Claas Hiebenthal, and Danijela Šmajgl
MS No.: bg-2018-332
MS Type: Research article

The responses to referees are structured following this sequence using different colors:
(1) Comments from Referees;
(2) Author's response;
(3) Changes in the manuscript: original sentences/revised sentences

Comments from Anonymous Referee #2, Received and published: 19 September 2018
Dear Editor,
The manuscript 'Variation in brachiopod microstructure and isotope geochemistry under low pH–ocean acidification–conditions' examines the change to micro-structure and biogeochemistry of the shell of the brachiopod Magellania venosa in natural conditions versus experimentally cultured brachiopods under low pH environments. The authors apply scanning electron microscopy (SEM) to understand changes to the microstructure in the form of the distribution of endopunctae in the anterior margin and the thickness of the primary layer. In addition, the study applies stable carbon and oxygen isotopes (^{18}O, ^{13}C) to relate the shell growth to the surrounding seawater chemistry in which the brachiopods were grown. This is a thorough investigation of the changes to the brachiopod shell formation under low pH environments. The authors comment from a biogeochemistry point of view relating the changing microstructure and isotope geochemistry of the shell to environmental carbon isotopes therefore providing further evidence for brachiopods to be useful as an ocean acidification proxy in geological samples. A very good multi-disciplinary approach to determine the impacts on the brachiopods shell growth.

1) I do have a few concerns in the way the samples were prepared using 5% acid etching, and if this could possibly mask the impacts of the experimental acidification on the microstructure.
Answer:
The time of 5% acid etching was so rapid (3 seconds) that it did not affect the microstructure, as shown by a first screening and by previous published studies on preparation methods (e.g. Zaky et al., 2015; Crippa et al., 2016). In any case all the samples experienced the same treatment, with exactly the same possible effects; so for the comparative goals of the manuscript, this would have been negligible. Furthermore, if this treatment could have affected the microstructure- which was excluded by our screening and previous studies -, it could have only slightly affected the outline of the structural units (fibre), but not their size, and not the density of the endopunctae.

2) However, these are minor concerns outlined below for the authors. The use of calcein staining to mark the new growth of the shell distinguishes where natural and low pH environments impact the shell growth in the brachiopod. This should ensure that the authors can identify any similarities in the low pH treatment versus the acid etching. However, this should be discussed in the manuscript and perhaps guide the reader to the nice figures representing this. For example, can the authors provide figures 4 for each treatment for comparison? These are very nice visual representations of how the brachiopod microstructure is affected under low pH treatments versus the natural growth ahead of the calcein staining.
Answer:
There are: Figure 2 to show the growth lines marked with calcein, and Figure 11 to summarize the visible microstructure difference under different pH treatments. Also, the onset of culturing could be seen by a 'break' in the shell structure-quite visible on the surface. Calcein has been widely used for staining calcium carbonate structures. Calcein has been shown to be incorporated passively into growing calcium carbonate of various taxa (e.g. Moran and Marko 2005; Riascos et al., 2007; Herrmann et al., 2009), including brachiopods (Rowley and Mackinnon 1995). None of the authors reported enhanced mortality or other negative influences on life histories or

physiology.
Following the request of the reviewer, an additional plate was added in the supplementary material (supplementary figure 1) to show how the brachiopod microstructure is affected under different treatments.

The manuscript is appropriate and well-suited for publication in Biogeosciences Discussions, I would recommend for publication with minor edits as detailed below.
Minor comments to the authors.
3) Introduction Please re-phrase, 'pH has dropped by 0.1 pH units and will probably drop another 0.3-0.5', these are projections based on modelling of historic data, I would suggest predicted or projected instead of probably.
Answer:
We have corrected them in the revised manuscript
Page 2 Line 2:
pH has dropped by 0.1 pH units and will probably drop another 0.3-0.5
changed to
pH has dropped by 0.1 pH units and was predicted to drop another 0.3-0.5

4) Line 15 states 'calcifying organisms', the table 1 refers only to a few brachiopod studies, please change to brachiopods. I could not comment on the supplementary table here. I would instead suggest including a sentence referencing some of the key papers outlining the consequences of experimental acidification on biomineral formation in other calcifying organisms.
Answer:
Supplementary Table 1 contains all the information on calcifying organisms.
We are sorry that the reviewer could not find the supplementary table, which we report also here in the attached file.
The sentence referencing to some of the key-papers on experimental acidification on biominerals is already written in the manuscript, just below at Page 2 Line 18: Only a few studies deal with the effect of acidification on microstructure (Beniash et al., 2010; Hahn et al., 2012; Stemmer et al., 2013; Fitzer et al., 2014a, b; Milano et al., 2016), and all of them focused on bivalves and show that neither microstructure, nor shell hardness seem to be affected by seawater pH.

5) There are such studies examining acidification impact on the microstructure of the sea urchin spicules for example Bray et al., 2014 (Med. Mar. Sci.), PUPA Gilberts group including studies by Politi et al., the authors only list here studies applied to molluscs.
Answer:
We have added the suggested citations to the supplementary table 1: Bray et al., 2014; Wolfe et al., 2013.
However, the paper of Politi et al., 2008 is about the "Transformation mechanism of amorphous calcium carbonate into calcite in the sea urchin larval spicule" so it is not very relevant for this aim.

6) Materials and methods. Page 8, line 2, how long were the brachiopods acclimated for prior to calcein staining and CO_2 induced acidification?
Answer:
We have added the time of the acclimatisation and changed the sentence:
Brachiopods were first left to acclimatize,

changed to
Brachiopods were first left to acclimatize at control conditions for five weeks,

7) Low-pH culture of several brachiopods was done under two phases, what was the justification for this? Were these two phases comparable in their treatments?
Answer:
Regarding these two phases, basically we had a control and low pH aquarium. The two phases were comparable in their treatments. One reason for increasing the CO_2 treatment from 2000ppm to 4000ppm was that the brachiopods have been done obviously well under the 2000ppm treatment and we aimed to increase the chemical impact as much as possible but with considering the survival of the specimen. Before the 4000ppm treatment has been started individuals of *M. venosa* have been stained again with calcein in order to mark the new material grown under 4000ppm.

8) In general, the treatments appear clear in the table 2 and 3, however it is difficult to understand the experimental design without the details which are currently not obtainable from Jurikova et al., in review.
Answer:
Jurikova et al. manuscript (submitted to Geochimica et Cosmochimica Acta) was revised and sent to the Editor for final decision. We will update the information as soon as Hana Jurikova receive the notification about acceptance.

9) Microstructural analyses – this section is much easier to understand with sufficient detail for the reader to reproduce. The authors used 5% hydrochloric acid for etching the shell prior to SEM analyses. Although a standard protocol for SEM imaging, the authors should comment on how they can be sure that this has not affected the microstructure in comparison to the experimental acidification of the culture. How would the impact the microstructure be distinguishable compared to the acid etch of the microstructure of shells?
Answer:
As explained above, the time of 5% acid etching was so rapid (3 seconds) that it did not affect the microstructure, as shown by our screening as well as in previous published studies on preparation methods (e.g. Zaky et al., 2015; Crippa et al., 2016). In any case, all the samples experienced the same treatment, with exactly the same possible effects; so for the comparative goals of the manuscript, this would have been negligible. Furthermore, if this treatment could have affected the microstructure- which was excluded by our screening and previous studies -, it could have only affected the outline of the structural units (fibre), but not their size, and not the density of the endopuncta.
In any case, in the revised manuscript we have added a sentence stating that the effect of 5% acid etching was negligible.
Page 10, line 5
The sectioned surfaces were manually smoothed with 1200 grit 5 sandpaper, then quickly (3 seconds) cleaned with 5% hydrochloric acid (HCl), immediately washed with tap water and air–dried.
changed to
The sectioned surfaces were manually smoothed with 1200 grit 5 sandpaper, then quickly (3 seconds) cleaned with 5% hydrochloric acid (HCl), immediately washed with tap water and air–dried. The time of acid etching was so rapid that it did not affect the microstructure, as also shown in the experiments by Crippa et al. (2016b).

10) Figure 4 and 5, can the authors please provide a specimen reference to which sample and treatment these images relate to?

Answer:

We have added the information to the figures in the revised manuscript.

Figure 4A: 9006ddv; 4B: 8005dv

Figure 5: 43vv

11) The manuscript suggests just two specimens #8005 and #9006 were used for these analyses. Can the authors conclude that these are representable as a sample population? Are these images available in supplementary information for comparison?

Answer:

#43, #63, #158, #223, #8005 and #9006 were analysed for the microstructure of secondary layer, additionally, #8005 and #9006 were also analysed for the thickness of primary layer and the size/density of endopuncta. All the measurement data are available in the Appendix dataset.

12) Carbonate stable isotopes analyses Likewise, the authors use 5% hydrochloric acid to clean shell prior to sample preparation for stable isotopes. Please detail why this was used, for example to remove organic material? If so why was a bleach or plasma ash treatment not chosen for this purpose to avoid potential issues with comparing experimental acidification treatments with hydrochloric acid treated shells?

Answer:

As explained in the method description (paragraph 2.3), we used 10 % HCl to remove the primary shell layer and surface contaminants; then we immediately rinsed with distilled water and air–dried. This is an important and fundamental step before doing isotope analyses. As already proved by previous studies (e.g., Veizer, 1992; Carpenter and Lohmann, 1995; Brand et al., 2003, 2013), the primary layer is not secreted in isotope equilibrium with the seawater in which the brachiopod lives, so we have to remove it in order to avoid contamination when sampling the shell. In this way we analysed only the in-equilibrium secondary layer (Parkinson et al., 2005; Cusack et al., 2012; Brand et al., 2013, 2015). To remove the organic material in the shell, as written in paragraph 2.1 we used 36 volume hydrogen peroxide. This in another important step to get clear images of recent brachiopods at the SEM (see Crippa et al., 2016b).

We have added a sentence to explain wht we have removed the primary layer before isotope analyses.

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Page 14 Line 10:

For specimens #8005 and #9006, the primary layer and surface contaminants were manually and chemically removed by leaching with 10 % HCl, rinsed with distilled water and air–dried

changed to

For specimens #8005 and #9006, the primary layer and surface contaminants were manually and chemically removed by leaching with 10 % HCl, rinsed with distilled water and air–dried. As the primary layer is not secreted in isotope equilibrium with ambient seawater (e.g. Veizer, 1992; Carpenter and Lohmann, 1995; Brand et al., 2003, 2013) it is important to chemically remove it in order to avoid contamination

during the analyses.

13) Section 3.3.2 During culturing Page 23, the authors state that 'The results from specimens (#43 and #63) grown under low pH conditions (pH3 and pH4) for a short time interval of 214 days are difficult to interpret, as in this case, there is no direct control experiment sample to compare', can the authors confidently relate the changing microstructure and geochemistry here to acidification is the only comparison are those samples grown under natural conditions?

Answer:

As written in the manuscript, to assess the change in microstructure and geochemistry, we compared both the differences between parts produced before-culturing and during-culturing, as well as the differences between low-pH treated specimens and control specimens. For specimens (#43 and #63), a control specimen was not available so we were not confident in interpreting the results we obtained and we preferred to underline it in the manuscript

14) It appears that the experimental treatments here are similar despite the pH used (Figure 9). I would question the relevance of this section, perhaps omit or justify how this is comparable.

Answer:

We think that these data are very important to be represented as they show the results of the different methods used. Also this diagram answers to reviewer question 13 and it shows that specimen cultured under low–pH conditions had smaller fibres when compared to that of control. It also shows the differences in fibre size among different subzones of the same specimens. In conclusion, this section clearly syntheses and displays the numerous measurements taken in this this study, so it is very important for the reader.

15) Section 3.4 Stable isotopes Nice figure 10, it is clear to see trends between three specimens for significantly lighter stable carbon isotopes with experimental low-pH treatment compared to natural versus control treatments. Did the authors compare these data statistically?

Answer:

We thank the reviewer for this suggestion. We have done additional *t*-tests to compare the data from different pH treatments, and added them in the revised manuscript (Supplementary table 2).

16) Discussion Page 28, 'electron back scattering diffraction' should be electron backscatter diffraction.

Answer:

We have corrected it in the revised manuscript

Page 28 Line 9

Analyses of electron back scattering diffraction

changed to

Analyses of electron backscatter diffraction

17) Line 20, 'May this indicate a greater amount of organic components in this part of the shell?' is this what the authors suggest? Please rephrase not as a question but a statement with references or omit.

Answer:

We have deleted this sentence in the revised manuscript, because there is no conclusive evidence about it in the literatures.

18) Lines 28, 'living organism' this should be living organisms.
Answer:
We have corrected it in the revised manuscript
Page 28 Lines 28:
living organism
Changed to
living organisms

19) Page 34-35. The discussion of the depleted ^{18}O, ^{13}C is related to changes in percentage, can the authors present the statistical significance here of the changing isotope values?
Answer:
We thank the reviewer for this suggestion. We have done additional *t*-tests to compare the data from different pH treatments, and added them in the revised manuscript (Supplementary table 2).

20) The authors state that there is individual specimen variability, does this remove the significance of the low pH treatment over the isotope depleted values? Or are the authors suggesting here that there are insufficient specimen numbers to make significant statements relating to the isotope data? Page 35, line 11 'More measurements are however needed to fully answer this.'?
Answer:
We think that analyses on more specimens could help to understand in greater details the $d^{13}C$ variability, but this does not undermine that we have robust data to support the fact that brachiopod produce shells near brachiopod equilibrium even in changing external conditions. We have results from both dorsal and ventral valves, if there was uncertainty it might show up in one but hardly both, the matching and concurrent isotope results speak clearly to the robustness of the values and trends.

21) Page 35, line 16 'Thus, we think', perhaps the data suggest?
Answer:
We have changed it in the revised manuscript
Thus, we think that large part of the secondary layer isotope record
changed to
Thus, the data suggest that large part of the secondary layer isotope record

22) The authors end in the statement 'secondary layer isotope record may reflect the environmental conditions supporting the interpretation of brachiopod shells as good archives of geochemical proxies, even when stressed by ocean acidification.'. This is also stated in the abstract as one of the main implications of this study. Following the current discussion on page 34-35 I would question whether the authors can make this statement, and whether there is sufficient evidence to support this, although Figure 10 does suggest this is the case. Please directly refer to the data here; are there sufficient samples, what is the n-number? This will enable the reader to determine if the manuscripts data do support these conclusions. If this data is not available then the authors will need to remove this emphasis from the abstract and conclusion statements.

Answer:
We think that our data are robust because we have analysed 9 specimens, 6 specimens for microstructure analyses, 5 for isotope geochemistry. We measured the size and shape of 540 fibres plus 1392 fibre at the anterior margin, we selected and measured 388 sub-zones for boundary calculations; we took 170 measurements for primary layer thickness; we selected and measured 29 zones for endopunctae density, 227 for diameter of endopunctae; we analysed 79 samples for isotope geochemistry. Following the suggestions of the reviewer, we have added these numbers in the conclusions.
Page 35 lines 19-20:
This study combined the analysis of shell microstructure and stable isotope geochemistry on brachiopods cultured at low pH conditions for different time intervals, and suggests the following conclusions.
changed to
This study combined the analysis of shell microstructure (based on 6 specimens, 1932 fibre size measurements; 170 primary layer thickness measurements; 256 punctal density and diameter measurements) and stable isotope geochemistry (5 specimens, 79 sample analyses) on brachiopods cultured at low pH conditions for different time intervals, and suggests the following conclusions.

23) Page 35 conclusions 'This was related to the source of carbon dioxide gas used in the culture setup', could this not be due to a change in the carbonate compositions as a result of adding CO_2 impacting the DIC? Did you test the carbon isotopes of the gas?
Answer:
We did not test the $d^{13}C$ of the gas, but we did measure the $d^{13}C$ in water, as we written in the manuscript Page 34 Line 31: the $\delta^{13}C_{DIC}$ in the water during the cultivation process of our specimens was low ($\delta^{13}C$ VPDB: -23.63 ‰ for the low pH conditions and -2.03 ‰ for the control conditions, which corresponds to the pH_2 phase), This is essentially the $d^{13}C$ of the DIC which comes from the source so it is pretty much almost the same.

24) I have seen this drop in carbon isotopes in the natural seawater samples where increasing CO_2 from run-off caused a lighter carbon isotope value. The authors should expand this discussion to the previous paragraph.
Answer:
As the main goal of the paper is to understand the impact of acidification on cultured specimens, we did not expand the discussion on what happened before culturing in the natural environment. However, this was the object of a paper by Romanin et al. (2018). In fact, as we have already written in manuscript at Page 34 Line 14, Romanin et al. (2018), who also analysed specimens collected from Comau Fjord, attributed the negative isotope excursion to environmental perturbations, in particular, to changes in seawater productivity and temperature, and/or to anthropogenic activities. Here, we follow the interpretation of Romanin et al. (2018) to explain the mid–shell excursion observed in our specimens.