Response to Anonymous Referee #2

We are grateful to the Referee #2 for the insightful reviews which have raised several very helpful points that we have taken on board. We consider that the manuscript has been much improved after the revisions and hope it is suitable for publication in Biogeosciences.

Our responses follow the referee's comments below in bold text.

GENERAL COMMENT.

Review BG-2018-204 Basic reporting In this study, the authors placed oysters from the genus Crassostrea in a range of 4 pCO2 scenarios to establish the quantitative relationship between microstructural and mechanical properties of juvenile oyster shells under increased OA conditions. The authors investigated into structural and mechanical properties using the SAM, the ESBD and nanoindentation tests. It is a straight forward paper, relatively well written and critical in filling gaps of current knowledge on the hierarchical structural organization of oyster shells under elevated pCO2 conditons. However, I have a few concerns regarding especially the methods and discussion that the authors should consider and address prior to publication.

REPLY: We thank the Referee #2 for the appreciation of our work and the manuscript has been carefully checked accordingly and corrected throughout.

MAJOR COMMENTS:

The supplementary table should be placed within the main text, this is valuable information showing robustness of experiment.

REPLY: We agree with the referee's suggestion. The supplementary table, is now, moved into the main text.

A figure illustrating a schematic of the pCO2 system set up with tanks should be added to methods to improve reader's understanding.

REPLY: Agreed. A schematic of the experimental system has been added in the method.

Was growth monitored (and did it differ with pH)? This may be important to deepen the discussion: could it be that differences in crystallography are essentially due to (impaired) growth or does the process of calcification (e.g. calcification rates) appear not be hampered and are most of the differences imprinted after shell formation?

REPLY: Our research team had earlier experiment that focused on shell growth. Considering this comment, we have now added more information into the discussion. Briefly, previous studies within our lab found that the larval shell growth of the Portuguese oyster is not affected by ocean acidification (Thiyagarajan and Ko, 2012). We have not monitored the juvenile shell growth in this study due to the calcitic juvenile oyster shell being less soluble

than aragonite larval oyster shells under ocean acidification. However, we have added this reference accordingly into the revised discussion.

The reference:
Thiyagarajan, V., and Ko, G. W. K.: Larval growth response of the Portuguese oyster (*Crassostrea angulata*) to multiple climate change stressors, Aquaculture, 370-371, 90-95, 10.1016/j.aquaculture.2012.09.025, 2012.

It is a shame that not a few measurements were done on specimens collected from the field. This would have allowed the authors to check whether the shells formed in the experiment are representative (crystallographically) of those found in nature.

REPLY: Agree – more field samples would be great. However, in this study we have not focused on field specimens. This may be a nice future experiment, especially to correlate multiple environmental drivers and shell plasticity in nature. Nevertheless, now we have discussed this point in the discussion. Specifically, according to Checa *et al*., (2018), in field samples of the Portuguese oyster, the crystallographic orientation maps of foliated layers showed a preferred crystallographic orientation with a ~40 degree of variation which concurs with the results of this study. We have added this reference in the revised version.

The reference:
Checa, A. G., Harper, E. M., and González-Segura, A.: Structure and crystallography of foliated and chalk shell microstructures of the oyster *Magallana*: the same materials grown under different conditions, Sci. Rep., 8, 7507, 10.1038/s41598-018-25923-6, 2018

MINOR COMMENTS:

Title
I think 'reduction of a property' is a bit meaningless. Consider changing it into: '...reduces hardness and stiffness of the...' or something similar.

REPLY: Referee #2 suggests changing the title and so does Referee #1 although their suggested revised titles are different. We consider that it is more informative to state the way in which mechanical properties are altered so we have adopted the more specific suggestion of Referee #2 and propose the following title "Ocean acidification reduces hardness and stiffness of the Portuguese oyster shell with impaired microstructure: a hierarchical analysis".

ABSTRACT

line 19: have been very well documented (not has)
line 22: see comment to title
Line 23: shell takes an "s"
Line 31: she's defensive function

Line 31: “surfaces” not used correctly (shows?)

REPLY: We apologize for the typos and thank the referee for the detailed suggestions. We have revised the abstract accordingly.

In particular:

line 16: please remove ‘coastal areas’ or rephrase. Particularly near-coast, OA is hard to detect due to the relatively large fluctuations in inorganic carbon chemistry in such environments due to seasonality, river runoff, sedimentary geochemistry, etc.

REPLY: We have removed “coastal areas”.

line 22 and further: please mention here that your study deals with juvenile oysters (<35 days old). Previous studies have shown that juveniles may be affected differently (usually more severe) by OA than adults.

REPLY: We have stated here that our study focused on “juvenile oysters”.

INTRODUCTION

Line 35: change belong for belonging
line 40: please delete ‘fascinating’
line 45: protect takes an s
line 52: shells “developing” under: : : add word
Line 61: correct the word “demonstrate”
Line 62: Stating “elevated CO2 conditions” is self-explanatory to how it affect the carbonate system. Remove “and OA”
Line 62: correct “structural”

REPLY: We apologize for the typos and thank the referee for the detailed suggestions above. We have revised the introduction accordingly.

In particular:

line 38: I don’t see how calcite is relatively brittle. It is, for example, more resistant to dissolution.

REPLY: The calcite is brittle in relation to its mechanical properties. We have revised the sentence to improve clarity.

line 53: dissolution occurred in Ries et al. (2011) high CO2 scenario due to the saturation state being less than 1. Oceans’ Arg and Calc saturation states are quite far from being lower than 1 even with ongoing ocean acidification. I would remove this part or mention dissolution only occurred in very high CO2 scenario.

REPLY: We stated the dissolution "…only occurred in very high *p*CO_2 scenarios…" in the revised introduction.

Line 66 + 69: repetition specifically

REPLY: We have removed the second "specifically".

Line 67 and throughout the text: this phrasing "high CO2 induced decreased ph" is a not very elegant. Replace by something like high CO2 scenarios/treatments. The decreased pH is implied.

REPLY: We have replaced "high CO_2 induced decreased pH" throughout with "high CO_2 scenarios induced decreased pH".

Line 69: materials science techniques?? Correct sentence

REPLY: We have removed the "materials science". The revised sentence is "…by using a variety of characterization and imaging techniques…".

METHODS

Line 77-78: bad wording. They were left to acclimatize in flow-through: : :
line 78 and further in the manuscript: salinity is unitless, so please remove 'psu'.
Line 82: remove word process
Line 96: oyster takes an s
Line 129: remove word "of" before "greater"

REPLY: We apologize for the typos and thank the referee for the detailed suggestions above. We have revised the method accordingly.

In particular:

line 75 and on: how many specimens were incubated? How many survived/ grew into maturity? Did pH have any effect on the mortality?

REPLY: For this study we did not focus on the larval survival or mortality, so we are unable to provide this data. Our focus was on the effect of OA on the biomineralized structure of oyster shell and for this, we concentrated on surviving larvae. However, from our previous studies on the similar species and similar pH levels, OA had no significant effect on survivorship of the Portuguese oyster larvae (Thiyagarajan and Ko, 2012). Therefore, we assumed there was no significant selection of individuals under selected pH levels.

The reference:
Thiyagarajan, V., and Ko, G. W. K.: Larval growth response of the Portuguese oyster (*Crassostrea angulata*) to multiple climate change stressors, Aquaculture, 370-371, 90-95, 10.1016/j.aquaculture.2012.09.025, 2012.

line 102: was pH measured daily? Please include the 'n' in the (suppl) table.

REPLY: The pH was measured daily. Daily pH measurements were firstly averaged within and among days per each replicate tank. The pH level was calculated, by averages of the replicate culture tanks within each treatment (n = 4), which was included in the caption of Table S1.

line 105: TA was measured every four days, although the supplementary table indicates that TA was calculated.

REPLY: TA was measured instead of being calculated. We apologize for the typo in supplementary table. The wrong superscript character has been deleted.

line 107-110: the calculated inorganic carbon parameters are accompanied by error estimates. How were they calculated?

REPLY: All provided inorganic carbon parameters were calculated using CO2SYS software program (Pierrot et al., 2006) based on the measured pH, temperature, salinity and total alkalinity (TA) from each replicate tanks. The errors here are standard deviation of the 4 replicate tanks each treatment (n = 4). We have revised the sentence in method section to improve clarity.

The reference:
Pierrot, D., Lewis, E., and Wallace, D.: MS Excel program developed for CO_2 system calculations, ORNL/CDIAC-105a. Carbon Dioxide Information Analysis Center, Oak Ridge National Laboratory, US Department of Energy, Oak Ridge, Tennessee, 2006.

line 128-130: Is the 'thresholding' susceptible to settings (i.e. contrast) of the SEM?

REPLY: The porosity thresholding was calculated using the non-diffracted regions of images produced by backscattered electrons. Therefore, a change in contrast, or focus position of the SEM would not affect the ability to distinguish between brightly well diffracted calcite and the porous space in between. In addition, all specimens were examined with same settings of SEM. Therefore, the results would be comparative in this study.

Line 156: not clear. Average per specimen?

REPLY: There were 6-11 indentations made in each specimen. Afterwards, the measurement per specimen was obtained by averaging the data among the 6-11 indentations. We have revised the sentence in method section to improve clarity.

Line 157: not clear, why not compare all values?

REPLY: Five to six specimens per treatment were randomly selected. As these specimens were belonging to individuals cultured in different replicate tanks, comparing all the specimens' values in the analysis would have led to pseudo-replication. Therefore, firstly, the

values of specimens within each replicate tank were averaged. The tank averages (n = 3 ~ 4) were then used as replicate in the analysis of variance to compare the mechanical properties among treatments. We have revised the sentences in method section to improve clarity.

RESULTS

Line 180: correct Decreased pH in title

REPLY: We apologize for this typo. The word has been corrected.

line 183: 'erosion' or 'physical damage' sounds as if the formed prismatic layer was intact at first and later dissolved or damaged. Is there any evidence for this or could it also be that the calcification of the prismatic layer was hampered to begin with?

REPLY: We thank the referee for the detailed suggestions above on wording. The shells of juveniles raised at pH 7.8 and pH 7.5 showed signs of dissolution or physical damage when compared to the controls, which suggests that the prismatic layer was damaged after formation. However, there is evidence to suggest that this damage is there to begin with. We have discussed this in context to the shell microstructure in relation to the EBSD and porosity data. EBSD indicated that, although the aragonite was affected at the lower pH treatments, the overall crystallographic growth of the calcite fraction did not change between treatments. The porosity and mechanical properties did suggest a microstructure impairment, which is discussed in lines 245-260. We thank the referee for their wording suggestions here, which makes this clearer within the results (lines 195-200). We have added a sentence to clarify this into line 260 "… Although the juvenile shells show signs of physical dissolution, the EBSD and porosity data suggest that the microstructure growth is impaired initially".

DISCUSSION

Line 233: revealed
Line 245: remove word "is"
Line 264: bad wording, "reduces with" instead of has started reducing
Line 268: bad wording. "reduces with" instead of has started to reduce
Line 273: replace run by "occur throughout"
Line 309: wrong use of indeed, remove please.

REPLY: We apologize for the typos and thank the referee for the detailed suggestions above. We have revised in the revised version of discussion accordingly.

In Particular:

The fact that many oyster larvae were capable of producing new foliated calcite at undersaturation (at pH 7.2) is highly interesting and although the authors are not the first ones to show this, discussing this result may improve the manuscript.

REPLY: It is an interesting point, and consistent to early observations such as in Ries 2010. Marine invertebrate's calcification has highly controlled mechanisms and remained to be explored by further studies (Toyofuku et al., 2017). Animals are capable in actively increasing the site of calcification by pumping proton out of the calcification site, thereby enabling calcium carbonate precipitation (added in revised discussion Line 286). We have added a sentence into the discussion in the revised version line 256 "The presence of such porous foliated layers was an obvious impairment of decreased pH. However, the larvae were still able to produce a new foliated layer under these treatments whilst at undersaturation (at pH 7.2)."

Reference:

Ries, J. B., Cohen, A. L., and McCorkle, D. C.: A nonlinear calcification response to CO_2-induced ocean acidification by the coral *Oculina arbuscula*, Coral Reefs, 29, 661-674, 10.1007/s00338-010-0632-3, 2010.

Toyofuku, T., Matsuo, M. Y., de Nooijer, L. J., Nagai, Y., Kawada, S., Fujita, K., Reichart, G.-J., Nomaki, H., Tsuchiya, M., Sakaguchi, H., and Kitazato, H.: Proton pumping accompanies calcification in foraminifera, Nat. Commun., 8, 14145, 10.1038/ncomms14145, 2017.

I miss references to some papers dealing with the crystallography of bivalve shells (below), which may help to compare the overall patterns found here with those published previously (i.e. in addition to their between-treatment comparison).
Dauphin and Denis, 2000. Comp Biochem Phys A, 126: 367.
Krauseâ˘AR˘ Nehring, J., Klügel, A., Nehrke, G., Brellochs, B., & Brey, T. (2011). Impact of sample pretreatment on the measured element concentrations in the bivalve Arctica islandica. Geochemistry, Geophysics, Geosystems, 12(7).

REPLY: Thanks for these references. We have included these references in the comparison.

Line 237: they are many more recent papers

REPLY: Thanks for this suggestion. We have included more recent references papers in the revised version.

Line 309-312: You state that previous studies have shown that on C. gigas and blue mussel increase their shells strength and size under higher CO2 levels, this should be in main discussion and more explanation to why this may occur. You mention that the pCO2 level is 100uatm which is twice and 4 times lower than your higher treatments.

REPLY: We agree that this is an important point, which we have raised in the discussion lines 300 – 310. Specifically, we discussed the differences being species-specific and the larvae being vulnerable at near-future pH 7.8. We have considered this discussion sufficient to address the points you raise here.