Response to Anonymous Referee #1

The authors wish to acknowledge thank Referee #1 for the detailed suggestions. We have taken all of these comments into consideration and are confident that the improvements made will improve the manuscript and make it suitable for publication in Biogeosciences.

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

GENERAL COMMENT.
Meng et al. propose here to further our knowledge of the negative effects of Ocean Acidification on marine calcifiers, i.e. reduced calcification, by characterizing various properties of shells of oyster Crassostrea angulata. This study describes the effects of experimentally induced OA on the shell surface, structure, crystallographic composition, crystallographic orientation, mechanical strength and density of C. angulata exposed to four different pH treatments (including the control treatment). This multimodal characterization and imaging approach adds to the scientific understanding of the effects of OA of the shell structure of a commercially important species of oyster.

The science presented here is sound, as are the statistical analyses associated to the findings. The main issues here reside with the redaction of the manuscript itself, the wording and terminology. Parts of the Methods, Results and Discussion sections are confused, and I do have a couple of questions regarding the methods (e.g. control treatments, and testing under hydrated conditions) that could expand the discussion further.

This study warrants publications but the text needs to be reworked to avoid confusion and some references need to be added. It took some time to carefully annotate the pdf file to help with wording and English but the authors should be more careful in future. The confused English distracts from the data and information presented. I hope this helps.

REPLY: We thank Referee #1 for the appreciation of our work and the carefully annotated pdf file. The manuscript has been carefully checked accordingly for any imprecise wording, terminology and references, and corrected throughout.

SPECIFIC COMMENTS
1. Title: consider changing title to "Ocean acidification affects mechanical and structural properties of Portuguese oyster shells (Crassostrea angulata)".

REPLY: Referee #1 suggests changing the title and so does Referee #2 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".

2.Wording and vocabulary:
- "corrode/corrosion": this relates to metal not carbonates, "dissolution" is more adapted to carbonate calcification;

REPLY: We have replaced the word "corrode/corrosion" with "dissolve/dissolution".

- "loose": my understanding is that this work is used for structural studies in engineering, not in crystallography. If the authors insist on using this word, I think it should be defined clearly in the ms;

REPLY: We have replaced "loosened" by "porous" referring to the suggestion in the PDF file.

- "microstructure": this word refers to the structure of the crystals themselves, not the structure of the shell. So if you are talking about crystal orientation or shell porosity you are talking about structure not microstructure. Please review the ms and change the terminology accordingly;

REPLY: We appreciate this suggestion for the replacement of "microstructure". However, "microstructure" is a term commonly used in biomineralization for oyster shell structural characterization when referring to the shell structure at micrometer scale, for example MacDonald *et al*., (2010). We have added this reference in Line 114 for clarification.

The reference:
MacDonald, J., Freer, A., and Cusack, M.: Alignment of crystallographic *c*-axis throughout the four distinct microstructural layers of the oyster *Crassostrea gigas*, Cryst. Growth Des., 10, 1243-1246, 10.1021/cg901263p, 2010.

- "down-sifting": can't you just say decrease (?) why make it complicated;

REPLY: We have replaced the word "down-sifting" with "decrease".

- "bottom-up": this is more of an ecological (i.e. food chain interactions) or physical oceanography (i.e. seawater mixing) term. I would just delete this term from the ms totally

REPLY: We have deleted the word "bottom-up".

- "erode/erosion": this is a geological term, use "dissolution" instead.

REPLY: Noted, we have replaced the word "erode/erosion" throughout with "dissolution".

3. Methods and Discussion: I was wondering whether the authors considered the fact that certain carbonate materials produced by marine calcifiers have increased strength when hydrated. For example, pearl oysters are very solid underwater but very brittle once dried. Using ethanol to preserve the samples is the easiest way but could it have affected the shell strength by extreme dehydration?

REPLY: The authors agree that this is a very interesting point. However, our work focuses on the comparing the effect of ocean acidification on the biomineralized structures. Since all samples from control and treatments were preserved and examined with identical methods, this ensures that the results and conclusions are appropriate for this objective. Therefore, the hydration of the shell in this study would not be

considered as one of the compounding factors of this comparative study. We do not consider it necessary to discuss the use of ethanol as a preservation method here because it is commonly used in studies that investigate the effect of ocean acidification on biomineralized shells, for example Chan et al., (2012). We have added this reference in Line 109 accordingly to justify our choice of preservation method.

The reference:
Chan, V. B., Li, C., Lane, A. C., Wang, Y., Lu, X., Shih, K., Zhang, T., and Thiyagarajan, V.: CO_2-driven ocean acidification alters and weakens integrity of the calcareous tubes produced by the serpulid tubeworm, *Hydroides elegans*, PloS ONE, 7, e42718, 10.1371/journal.pone.0042718, 2012.

Is micro-CT and nano-indentation doable in a medium that would preserve the shell (i.e. neutral)?

REPLY: Both micro-CT and nanoindentation measurements were carried out in ambient air conditions so we can reassure the referee that the analytical conditions are neutral. This methodology was consistently used in the control and treated samples and allowed us to make conclusions about the impacts of ocean acidification.

Please discuss Technical corrections See pdf document

Please also note the supplement to this comment:
https://www.biogeosciences-discuss.net/bg-2018-204/bg-2018-204-RC1- supplement.pdf

REPLY: All suggestions on phrasing for clarity and corrections of typos in PDF documents have been carefully considered and revised.

In particular:

Line 47 technically speaking, seawater is not getting more acidic but less basic (i.e. less alkaline). The pH scale is such the seawater pH predicted for the near future are not acidic because acidic pHs are found below ~6. "Ocean acidification" is a generally accepted "layman's term" to describe the seawater getting less basic but you can't really say the seawater is getting acidic (yet). I suggest you reword this sentence.

REPLY: We have reworded the sentence in Line 45 "…Oceans currently absorb about a third of anthropogenic CO_2, which dissolves in seawater forming carbonic acid and increases the concentration of hydrogen ion, this chemical process is popularly known as ocean acidification (OA)."

Line 48: Define the term "pCO_2"

REPLY: We have defined "pCO_2" in Line 47. "… is highly vulnerable to high carbon dioxide partial pressure (pCO_2; μatm) …."

Line 84 you need to specify which one is considered the control.

REPLY: We have specified the control in the method section in Line 82-83 "Four environmentally and climatically relevant pH levels (the control: pH 8.1; the low pH

treatments: pH 7.8, 7.5, and 7.2) were selected as proxies to investigate the effect of CO$_2$-driven OA on oyster shells." In addition, we have added a schematic of the experimental system in the method for clarity.

Line 120 consider replacing with "lip"

REPLY: We have replaced "bill" by "edge" in Line 119, a term defined by Galtsoff (1964) for the description of edible oyster shells. We have added the reference in the revised version.

The reference:
Galtsoff, P. S.: The American oyster, *Crassostrea virginica* Gmelin, Fish. Bull., 64, 1-480, 1964.

Line 121 Is this due to dissolution? Other reasons??

REPLY: The referee asked why the edge of shell is fragile. It is because the edge region is newly formed and naturally thin which can refer to the scanning electron micrograph (SEM) of the full shell cross-sectional surface in Fig. 3a (previously Fig. 2a). We have also reworded the sentence in line 119 to improve the clarity.

Line 127-128 Please describe you standardization and the thresholds used for the images. Depending on exposure, thresholds can be very variable...

REPLY: The porosity thresholding was calculated using the non-diffracted regions of SEM 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, so we considered the results would be comparative in this study. To improve the clarity, we revised the sentence in the method Line 125 "…The cross-sectional porosity of foliated laminated structure was calculated using ImageJ software by standardizing and converting an SEM image to thresholding where the non-diffracted regions of SEM images were defined as pores."

Line 163 Which ones? If they are all in resine, which shells did you use?

REPLY: The referee asked about the specimens used in Micro-CT scanning. Those were the complete individuals directly scanned by Micro-CT without being embedded in resin. They were randomly selected from the treatment (n = 3) which we have raised in Line 168.

Line 178 It would be great to have a 3D video/file of the micro-CT density results for one shell of each pH level as supplement material

REPLY: The authors thanks the suggestion on the micro-CT density results. We presented the 3D shell density maps in Figure 6a-d (previously Fig. 5a-d) which showed the overall decrease of shell density with decreasing pH. We have considered this figure sufficient to support the points we concluded.

Line 194 What does this means in terms of orientation? Be clear. Why should we care about colour variations? Use the correct terminology.

REPLY: The "color variation" represented the changes of crystallographic orientation corresponding to the color key showed in Fig. 4 (previously Fig. 3), which is a term commonly used to describe the results obtained by EBSD when referring to the crystallographic orientation map or the pole figures, for example Fitzer et al., (2014). To improve the clarity, we have added the reference and sentences into line 194 "…The crystallographic orientation maps (Fig. 4.i) showed changes in crystallographic orientation from the control (pH 8.1) to low pH conditions (pH 7.8, 7.5 and 7.2) as represented by color change corresponding to the color key. The spread of data points in pole figures (Fig. 4.ii) highlighted the variation in crystallographic orientation between the juvenile oysters under the low pH and the control conditions. …"

The reference:
Fitzer, S. C., Cusack, M., Phoenix, V. R., and Kamenos, N. A.: Ocean acidification reduces the crystallographic control in juvenile mussel shells, J. Struct. Biol., 188, 39-45, 10.1016/j.jsb.2014.08.007, 2014.

Line 207 Do you not have more to say here? This section seems very short. The methods section talk about hardness (H) and stiffness (S), could you give some values for these variables maybe?

REPLY: We apologize for the inconsistency of using the terminology - "Young's modulus" which is the measurement of the stiffness. We have revised the result section in Line 207 accordingly.

Line 212 – 221
This entire section needs work. It is very unclear. You need explain better how you got the density/volume in order for us to understand what you are measuring: shell density/volume or mineral density volume?? I'm really confused right now.

REPLY: We measured "Volume ratio (%)" of the corresponding partial density range and utilized linear regressions to determine the relationships between "Volume ratio (%)" and "density (g/cm^3)" which we have raised in the method Line 173. For clarity, we have revised sentences in line 213. ".. A similar decrease is visible in the linear regressions (Volume ratio (%) = b × density (g/cm^3) +a) in Fig. 6. f (previously Fig. 5f).."

Line 244 Consider changing to: "Oyster shells mechanical properties under OA"

REPLY: In order to be consistent and informative with the later subheading in discussion, we have kept the subheading "4.1 Effect of ocean acidification on shell mechanical features: a hierarchical analysis".

Line 286-289 Unclear. What are you trying to say?

REPLY: We were discussing the potential explanation of the porous foliated layer based on the calcification mechanism of mollusk. This section in Line 289-299 has been revised and added new reference. "…Marine invertebrate's calcification has highly controlled mechanisms and remained to be explored by further studies. Animals are

capable in actively increasing the site of calcification by pumping proton out of the calcification site, thereby enabling calcium carbonate precipitation (Toyofuku et al., 2017). Supersaturated calcite conditions of oysters were found restricted to the shell edge including the outer mantle and the first intracellular nucleation site (Mount et al., 2004). Undersaturated calcite conditions may be maintained elsewhere in contact with the inner shell surface (Addadi et al., 2006; Thomsen et al., 2010). Therefore, in low pH conditions due to OA, these inner areas of newly formed minerals, which are precipitated as structural building blocks for the prismatic and foliated layers, may still be prone to dissolution. When the shell dissolution rate is faster than the mineralization rate, organisms tend to produce thinner and lighter (less dense) shells resulting in impaired shell microstructure. This may explain the multiple negative effects of reduced pH in our results, including porous and less dense foliated layers…."

The new reference:
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.

Line 320 Why are you not talking about the commercial implications like aquaculture and so on?

REPLY: The authors agreed with the referee. We have added the commercial implications in the revised discussion in Line 331. "….This biological effect of OA on shell structures and mechanical features should be incorporated to the coastal oceanographic biophysical models to accurately project the survival of oysters in near-future coastal oceans which is vital for commercial shellfisheries to plan for sustainable growth under climate change induced acidification."

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 in Line 312 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 in Line 302.

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 in Line 37 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 "… in very high pCO$_2$ scenarios…" in the revised introduction in Line 53.

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 in Line 105-108 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 Line 155-158 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 Line 155-158 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 195-210). We have added a sentence to clarify this into line 261 "… 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 289). We have added a sentence into the discussion in the revised version line 260 "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˘ARˇ 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 310 – 325. 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.

Ocean acidification reduces hardness and stiffness of the Portuguese oyster shell with impaired microstructure: a hierarchical analysis

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Abstract. The rapidly intensifying process of ocean acidification (OA) due to anthropogenic CO_2 is not only depleting carbonate ions necessary for calcification but also causing acidosis and disrupting internal pH homeostasis in several marine organisms. These negative consequences of OA on marine calcifiers, i.e. oyster species, have been very well documented in recent studies, however, the consequences of reduced or impaired calcification on the end-product, shells or skeletons, still remains one of the major research gaps. Shells produced by marine organisms under OA are expected to show signs of dissolution, disorganized microstructure and reduced mechanical properties. To bridge this knowledge gap and to test the above hypothesis, we investigated the effect of OA on juvenile shells of the commercially important oyster species, *Magallana angulata* at ecologically and climatically relevant OA levels (using pH 8.1, 7.8, 7.5, 7.2). In lower pH conditions, a drop of shell hardness and stiffness was revealed by nanoindentation tests, while an evident porous internal microstructure was detected by scanning electron microscopy. Crystallographic orientation, on the other hand, showed no significant difference with decreasing pH using Electron Back Scattered Diffraction (EBSD). These results indicate the porous internal microstructure may be the cause of the reduction in shell hardness and stiffness. Overall decrease of shell density observed from Micro-computed tomography analysis indicates the porous internal microstructure may run through the shell, thus inevitably limiting the effectiveness of the shell's defensive function. This study shows potential deterioration of oyster shell induced by OA, especially in their early life stage. This knowledge is critical to estimate the survival and production of edible oysters in future ocean.

1 Introduction

Edible oysters belonging to the genus *Magallana* have a complex life cycle, in which the free-swimming larvae attach onto a suitable hard substrate and then metamorphose into sessile juveniles within a few hours (Medaković et al., 1997; Salvi and Mariottini, 2017). The oyster larval shell is primarily made of aragonite, a denser and mechanically stronger form of calcium carbonate ($CaCO_3$) compared to calcite which is a stable but mechanically brittle polymorphous $CaCO_3$ (Lawn and Wilshaw, 1993; Han et al., 1991). Upon metamorphosis, the fraction of calcite rapidly increases and becomes the main component in the juvenile and adult oyster shell (Medaković et al., 1997; Weiner and Addadi, 1997). The composition of the mineral and

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its organic matrix define a wide array of composites that relate to the mechanical strengths of the shell of each of these life stages (Lee et al., 2011). Early-life stages of marine invertebrates, oysters included, are highly vulnerable to predators (Newell et al., 2007) and environmental stressors (Thomsen et al., 2015) when compared to the adult stages. Production of mechanically strong shells during larval and juvenile stages, is essential to the post-larval phase because shell integrity and strength acts as a protective barrier against shell-breaking and drilling predators.

Oceans currently absorb about a third of anthropogenic carbon dioxide (CO_2), which dissolves in seawater forming carbonic acid and increases the concentration of hydron ion, known as ocean acidification (OA). A study showed that the calcification rate of marine organisms, including oysters, is highly vulnerable to high carbon dioxide partial pressure (pCO2; μatm) driven decreases in seawater pH (Feely et al., 2009; De Bodt et al., 2010). If the early-life stages of edible oysters are vulnerable to near-future OA, then it could directly harm oyster survival and aquaculture production. Evidence of the negative effects of OA is, for example, the decline of spat production in oyster hatcheries on the west coast of the USA due to poorly calcified larval shells consequence of upwelled high-pCO$_2$ waters (Barton et al., 2012). Previous studies on calcifying organisms suggest that OA not only reduces calcification rates, but also increases dissolution of formed shells in very high pCO$_2$ scenarios (Ries, 2011; Bednarsek et al., 2012). The decreased pH depletes carbonate ions necessary for $CaCO_3$ mineralization, as well as weakens marine organisms physiologically by causing acidosis and impairing internal pH homeostasis needed for optimal calcification (Dupont and Portner, 2013) . Recently, an increasing number of studies have captured the importance of the mechanical properties of calcareous shell, the end-products of calcification, under OA scenarios (Dickinson et al., 2012; Ivanina et al., 2013; Li et al., 2014; Fitzer et al., 2015; Collard et al., 2016; Teniswood et al., 2016; Milano et al., 2016). For instance, it has been reported that the Pacific oyster, *Magallana gigas* (previously *Crassostrea gigas*), and the Eastern oyster, *Crassostrea virginica*, produced softer shells with reduced mechanical strength under OA conditions (Beniash et al., 2010;Dickinson et al., 2012). Despite these OA threats to oyster calcification process, studies are yet to demonstrate the structural organization of oyster shells under elevated CO_2 conditions. Importantly, modulating effects of OA on the inherent relationship between shell structural and mechanical features is yet to be studied in detail.

This study is designed specifically to fill this gap in knowledge using the ecologically and economically important edible oyster *Magallana angulata* (previously *Crassostrea angulata*) also as model species. Here, the quantitative relationship between microstructural and mechanical properties was examined using the newly formed juvenile oyster shells. Specifically, the effect of OA on this relationship was tested using three levels of environmentally and climatically relevant levels of high-CO_2 scenarios induced decreased pH. As the calcitic foliated layer is the major shell structure for mechanical support in oysters (Lee et al., 2008), we examined its structural and mechanical properties by using a variety of characterization and imaging techniques such as scanning electron microscopy (SEM), electron backscatter diffraction (EBSD) and nanoindentation tests. To further evaluate the overall shell integrity, we quantified shell density, shell density - volume ratio relationships using high-resolution micro-computed tomography scanning (Micro-CT).

2 Materials and methods

2.1 Experimental design

Sexually matured adult oysters of the Portuguese oyster, *Magallana angulata,* were collected from the coastal area in Fujian, China (26°05′53.36″N 119°47′45.81″W) in the South China Sea on July 29th, 2014. The adults were transported to the laboratory at the Swire Institute of Marine Science, The University of Hong Kong. They were left to acclimate in flow-through tanks in natural seawater at ambient conditions (31 salinity, 29 °C and pH(NBS) 8.1) for a week. They were fed with a

mixed algae diet (*Isochrysis galbana* and *Chaetoceros gracilis*). Sperm and eggs were obtained from more than 10 males and 10 females by the "strip spawning" method (Dineshram et al., 2013), and cultured under ambient conditions. Twenty-four hours postfertilization, embryos developed into D-shaped veliger larvae.

Four environmentally and climatically relevant pH levels (the control: pH 8.1; the low treatments: pH 7.8, 7.5, and 7.2) were selected as proxies to investigate the effect of CO_2-driven OA on oyster shells. According to IPCC projections, the average pH of oceans (currently pH 8.1) is expected to drop to pH 7.8 and 7.5 by the year 2100 and 2300, respectively (Feely et al., 2009). Lowest pH treatment was included in this study to understand the impact of extreme environmental conditions in the coastal habitats of *M. angulata* (pH 7.2), manifest naturally fluctuating pH levels up to -0.8 units due to river runoff and microbial respiration (Duarte et al., 2013; Thiyagarajan and Ko, 2012). Treatment levels of pH were maintained by bubbling filtered natural seawater with air enriched with CO_2 at the required concentrations using gas flow meters/controllers (Cole-Parmer, USA) (Fig. 1). Oyster larvae were raised from the D-shaped veliger stage to the juvenile stage under the four pH levels with four biologically independent replicates tanks for each treatment. D-shaped larvae (10 larvae/mL, 50L replicate tanks, 1 μm FSW, 31 salinity, at 29°C ± 2°C) were reared until the pediveliger stage following methods described by Dineshram et al. (Dineshram et al., 2013). After about 2 to 3 weeks, larvae attained competency for attachment and metamorphosis. Larvae were transferred from each 50 L replicate tanks to 1 L replicate tanks containing plastic substrates coated with 7-day-old natural biofilms. Attachment and metamorphosis took place within 24 hours. Attached oysters were reared in 1 L replicate tanks with the same pH level as before attachment for 35 days until collection. Larvae and juveniles were fed twice a day using a mixture of live *I. galbana* and *C. gracilis* (5-10×10^6 cells/mL, 1:1 ratio). Seawater pH (NBS scale) and the temperature were measured using a Metter-Toledo (SG2) probe, and salinity was measured using a refractometer (ATAGO, S/Mill0E; Japan). The probe was calibrated using NIST buffers (pH =4.01, 7.00, and 9.21; Mettler Toledo, Gmbh Analytical CH8603 Schwerzenbach, Switzerland). In each culture, tanks levels of pH, temperature and salinity were measured daily. Daily measurements were firstly averaged within and among days per each replicate tank. Samples of seawater (50 ml) from each culture tank were collected every 4 days and poisoned with 10 μl of 250 mM mercuric chloride for total alkalinity (TA) analysis using the Alkalinity Titrator (AC-A2, Apollo SciTech's Inc., U.S.). The TA measurement was standardized with a certified seawater reference material (Batch 106, A.G. Dickson, Scripps Institution of Oceanography, U.S.). The carbonate system parameters, i.e. carbon dioxide partial pressure (pCO_2; μatm), carbonate ion concentration (CO_3^{2-}; μmol kg^{-1}), calcite and aragonite saturation state (Ω_{Ca}, Ω_{Ar}), were calculated from pH, salinity, temperature and TA measured from each replicated tanks (n = 4), using the CO2SYS software program (Pierrot et al., 2006) with equilibrium constants K_1, K_2 and KSO_4 (Mehrbach et al., 1973; Dickson and Millero, 1987). The treatment level (Mean ± SD; Table 1) was calculated, by averages of the replicate culture tanks within each treatment (n = 4). On the 35th day post-metamorphosis, juveniles were collected and preserved in 75% ethanol for the following analyses (Chan et al., 2012).

2.2 Shell microstructure analysis

The sessile juvenile oyster permanently cements the left valve of its shell to substratum, whereas its right valve provides protection from predators and the environment. In this study, only the right valve was used in the shell analysis. The surface topography of the intact shell was examined under variable pressure at 30 kV using a scanning electron microscope (SEM; Hitachi S-3400N VP SEM, Hitachi, Japan). To examine sectional surface microstructures (MacDonald et al., 2010), shells were embedded in epoxy resin (EpoxyCure, Buehler) and sliced along the dorsal-ventral axis using a diamond trim saw blade. This allows for a more controlled comparison between the hinge region and the middle region of the shell. The hinge region (hereafter also referred to as "older shell") is the part of the shell that is deposited first by the juvenile oyster, whereas the middle region (hereafter also referred to as "younger shell") is the part of the shell that is deposited more recently. The

edge region (Galtsoff, 1964), formed most recently, was not included in this study because it is too thin and fragile to handle.

120 The sectioned surfaces were polished for 2 to 5 min using grit papers (P320, P800, P1200, P2500, and P4000) and etched for 20 seconds using 1% acetic acid, and then washed with distilled water and air dried. The sectioned resin blocks were mounted on aluminium stubs using carbon adhesive tape with the polished side up. The area surrounding the specimen was painted with silver to reduce charge build-up, and the sectioned surfaces were sputter-coated with 50-nm thick gold−palladium alloy. The shell microstructure was examined under SEM with an accelerating voltage of 5 kV using a LEO

125 1530 Gemini FSEM (Zeiss, Germany). The cross-sectional porosity of foliated laminated structure was calculated using ImageJ software by standardizing and converting an SEM image to thresholding where the non-diffracted regions of SEM images were defined as pores. The pore area was then calculated by using the ImageJ "Analyse Particles" feature due to the divergence in the size of pores. The pores area was sized with a confidence area greater than 0.001 µm^2. Three to four specimens from each treatment were randomly selected and examined (n = 3 ~ 4). All data was tested for normality of

130 residuals, normality, and homogeneity of variance before analysing by ANOVA. Student-Newman-Keuls test was used to compare the means following one-way ANOVA.

2.3 Shell crystallographic orientation analysis

Shell crystallographic orientation was analysed by Electron Backscatter Diffraction (EBSD). Shells were prepared according to the above method, minus etching. The shell surfaces were ultra-polished for 4 min using cloths with 1 µm and 0.3 µm

135 Alpha alumina powders and for 2 min using colloidal silica. In order to investigate both larva aragonite and juvenile calcite composition, an area throughout the sectional surface of the older hinge regions were selected. The EBSD analyses were carried out under low vacuum mode (~50 Pa) with a beam voltage of 20 kV using an FEI Quanta 200F with the stage tilted at 70° to examine backscatter Kikuchi patterns (Perez-Huerta and Cusack, 2009). Diffraction intensity, phase, and crystallographic orientation maps were produced using the OIM Analysis 6.2 software. Data was partitioned through two

140 clean-up procedures to display grains with a confidence index (CI) greater than 0.1. Pole figures were used to illustrate the spread of crystallographic orientation (Perez-Huerta and Cusack, 2009). The colours in the crystallographic orientation maps and pole figures were used to quantify the crystallographic orientation. Two randomly selected specimens were examined per treatment.

2.4 Shell mechanical properties analysis

145 After SEM and EBSD analysis, the resin blocks were re-polished for 5 min using grit papers (P2500 and P4000) and for another 5 min using cloth with colloidal silica to remove the gold-palladium coating and etched shell surface. The mechanical properties of the polished longitudinal cross sections were determined by measuring the hardness (H) and stiffness (E) using load and displacement sensing nanoindentation tests (Perez-Huerta et al., 2007). Hardness and stiffness of foliated layers were measured in the older hinge and younger middle regions of the specimens used in the SEM analysis. The

150 nanoindentation tests were carried out from the interior to the exterior shell in these regions at ambient temperature with a Hysitron TriboIndenter TI 900 (TI 900, Hysitron, MN, USA) equipped with a Berkovich indenter (with a half-angle of 63.5°). Indentations were made in each specimen using a 6−11 indent-per-row pattern and a maximum load of 2000 µN with valid contact depth of 16 to 184 nm. The hardness and stiffness from each indentation were obtained from the loading-unloading curve using the Oliver-Pharr model (Doerner and Nix, 1986; Oliver and Pharr, 1992). Five to six specimens of

155 each treatment were randomly selected for nanoindentation tests (1 to 2 specimens per replicate tanks). Measurements per replicate tanks were calculated by firstly averaging the values among indentations per specimen, and then among specimens per replicates. Afterwards, the effect of decreased pH on the hardness and stiffness of juvenile oyster shells was compared by

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three to four replicate measurements (n = 3 ~ 4). All data was tested for normality of residuals, normality, and homogeneity of variance before analysing by ANOVA. Student-Newman-Keuls test was used to compare the means following one-way ANOVA.

2.5 Shell density analysis

The three-dimensional shell density maps, the overall shell density and the density - volume ratio relationships were obtained using a high-resolution micro-CT scanning system (SkyScan 1076, Skyscan, Kontich, Belgium) with a spatial resolution of 9 μm. Individual shells were placed in a small plastic container held securely in the chamber of the micro-CT scanner. Shell densities and the corresponding volume ratios of partial density were calculated by relative comparison using standardized phantoms used for bone density measurement in the analytical software CT-Analyser v 1.14.4.1 (SkyScan) (Celenk and Celenk, 2012). The 3D digital data was converted from ~1000 2D layers using reconstruction software CT-Volume v 2.2.1.0 (SkyScan). Three randomly selected specimens were used per treatment (n = 3). The volume ratio with partial density ranges of 0 to 0.5 g/cm^3, 0.5 to 1 g/cm^3, and >1.5 g/cm^3, and density of the treatment groups were compared with the controls by one-way ANOVAs. For the datasets that did not meet the requirement of variance homogeneity, i.e., the volume ratio with a partial density range of 1 to 1.5 g/cm^3, Kruskal-Wallis tests were used to compare the effect of pH on these shell properties. For all other datasets, Student-Newman-Keuls test was used to compare the means following one-way ANOVA. Otherwise, Dunn's test was used after Kruskal-Wallis test. Linear regressions (Volume ratio (%) = b × density (g/cm^3) + a) was utilized to determine the relationships between shell density and the corresponding volume ratio, a is the y-intercept and b is the scaling exponent of consumption. To compare slopes of the resulting linear models, analysis of covariance (ANCOVA) was performed by using log_{10} transformed volume ratio as the dependent variable, pH levels as the independent variable, and shell density range as covariates. All data met the homogeneity of variance and normality assumptions of parametric tests. ANCOVA were implemented in R 3.3.2 using the statistical package Linear and Nonlinear Mixed Effects Models (Team, 2013).

3 Results

3.1 Shell surface and internal microstructure

As shown by the SEM, decreased pH altered both shell topography (Fig. 2) and internal microstructure (Fig. 3). Mineral dissolution was prominent on the outer surface layers of shells under decreased pH. The shells of juveniles raised at pH 7.8 (Fig. 2b, f) and pH 7.5 (Fig. 2c, g) showed signs of dissolution or physical damage when compared to the controls (pH 8.1) (Fig. 2a, e). At the lowest pH of 7.2 with undersaturated calcite conditions, the outer prismatic layer was completely absent at the older hinge and younger middle regions of the shell (Fig. 2d, h). Though the overall calcitic foliated laminas alignment were retained, those in the shells of controls (pH 8.1) were compactly arranged and well-ordered with minimal gaps between layers (Fig. 3c, e). In contrast, the foliated layers in shells under all three decreased pH treatments presented a more porous alignment that the foliated laminas were less tightly packed and irregularly arranged (Fig. 3g, i, k, m, o and q). The area porosity of foliated layers increased significantly with decreasing pH levels, regardless of older and younger shell (Older region: $F_{(3,11)} = 3.683$, $p = 0.045$; Younger region: $F_{(3,11)} = 7.480$, $p = 0.005$) (Fig. 3r, s).

3.2 Crystallographic orientation

Electron backscatter diffraction (EBSD) intensity mapping analysis showed diffraction patterns for both calcite and aragonite crystals of older hinge regions in the juvenile shells (Fig. 4). The crystallographic orientation maps (Fig. 4.i) showed changes

in crystallographic orientation from the control (pH 8.1) to low pH conditions (pH 7.8, 7.5 and 7.2) as represented by colour change corresponding to the colour key (Fitzer et al., 2014). The spread of data points in pole figures (Fig. 4.ii) highlighted the variation in crystallographic orientation between the juvenile oysters under the low pH and the control conditions. Though the foliated layers of shells under decreased pH showed colour variations within a limited area (~ 5-10 foliated laminas) close to the interior, the majority of calcite crystal units showed uniform orientation, the same as those in the control (Fig. 4.i and ii). It was confirmed by the pole figures that the preferred crystallographic orientation of foliated layers was identical, resulting in the extent of the variation in crystal orientation of 40 degree regardless of pH treatments, corresponding to the colours in the orientation maps (Fig. 4.i). But notably, there was an absence of aragonite in the shells formed under pH 7.2 (Fig. 4.iii). Although the aragonitic crystals are not present in the most extreme treatment (pH 7.2), the overall crystallographic orientation of the calcitic fraction did not change between treatments.

3.3 shell hardness and stiffness

Shell hardness was significantly reduced as treatment pH decreased, relative to control (Older region: $F_{(3,11)}$ = 21.987, $p <$ 0.001; Younger region: $F_{(3,11)}$ = 4.135, p = 0.034). Similarly, shells at pH 7.5 and 7.2 had reduced stiffness compared to the controls (Fig. 5c, d) (Older region: $F_{(3,11)}$ = 4.525, p = 0.027; Younger region: $F_{(3,11)}$ = 7.369, p = 0.006). The reduced mechanical features due to decreased pH were observed in both the older hinge regions and younger middle regions (Fig. 5).

3.4 Shell density

Three-dimensional (3D) shell density maps (Fig. 6a-d), the overall shell density and the relationship of shell density - volume ratio by micro-computed tomography (Micro-CT) showed an overall decrease of shell density with decreasing pH (Fig. 6e) ($F_{(3,8)}$ = 5.318, p = 0.026). A similar decrease is visible in the linear regressions (Volume ratio (%) = b × density (g/cm^3) + a) in Fig. 6. f. Volume ratios were decreased with the increased shell density in all pH treatments (ANCOVA; shell density, $F_{(1,263)}$ = 1253.14, $p <$ 0.001). There was an interaction between pH and shell density (ANCOVA; pH × density, $F_{(3,263)}$ = 4.994, p = 0.002), indicating that the effect of pH on the density - volume ratio relationship was different. The lower scaling of consumptions at pH 7.8 (mean exponent -0.063), pH 7.5 (mean exponent -0.065), pH 7.2 (mean exponent -0.062) versus the control pH level of 8.1 (mean exponent -0.052), indicating the volume ratio of denser shell was reduced with decreased pH while the volume ratio of less dense shell was increased correspondingly (Fig. 6f). 3D shell density map (Fig. 6 a-d) reinforced the effect of decreased pH on the density - volume ratio relationships. In the controls, shells were produced with denser minerals compared to shells in decreased pH (Fig. 6a). Shells in pH 7.8, pH.7.5 and pH 7.2 had larger proportions of lower shell density regions or "pores" (Fig. 6b-d). These pores were observed in the 3D density maps as density values below the detection threshold (Fig. 6a-d). With classifying the shell volumes into four density categories, i.e., < 0.5 g/cm^3, 0.5-1 g/cm^3, 1-1.5 g/cm^3 and > 1.5 g/cm^3, the proportions of high (>1.5 g/cm^3) and low (< 0.5 g/cm^3) shell density areas were significantly different between pH treatments (Fig. 6a-d). The volume ratios of high density areas were significantly reduced in each pH treatments (pH 7.8, pH 7.5 and pH 7.2) when compared to the controls (pH 8.1) ($F_{(3,8)}$ = 4.856, p = 0.033). Meanwhile, the volume ratios of low density areas (< 0.5 g/cm^3) significantly increased in each of the lower pH treatments (pH 7.8, pH 7.5 and pH 7.2) compared to the controls ($F_{(3,8)}$ = 6.945, p = 0.013). There were no significant differences in the volume ratios of the middle densities (0.5-1 g/cm^3: $\chi^2_{(2)}$ =5.615, p = 0.132; 1-1.5 g/cm^3: $F_{(3,8)}$ = 3.713, p = 0.061) among treatments (Fig. 6a-d).

4 Discussion

This study provided new compelling information of structure - property relationships in calcareous shells of commercially important oyster species at different spatial scales and under a variety of environmentally and climatically relevant OA scenarios (i.e. different levels of decreased pH via pCO$_2$ increase). The revealed structural information and subsequent analysis of mechanical features in this study provided an important experimental basis for developing models to forecast the impact of ocean acidification on marine calcifying organisms. The rate of calcification of many marine organisms is expected to be significantly reduced in near-future oceans with a reduced pH of 7.8 due to OA (Ries, 2011; Bednarsek et al., 2012; Duquette et al., 2017; Chatzinikolaou et al., 2017). This study observed the same calcification trend in *Magallana angulata* because OA is not only depleting carbonate ions necessary for CaCO$_3$ mineralization, but it is also metabolically weakening marine organisms through the altered physiological processes, i.e. acidosis (Dupont and Portner, 2013). Importantly, this study provides a strong evidence to support the argument that shells produced by oysters under OA suffer from dissolution with disorganized or impaired crystal orientation and microstructures, and reduced mechanical properties. The possible mechanisms and consequences underlying such negative effects of decreased pH on mechanics of shell structure are discussed in the following sections.

4.1 Effect of ocean acidification on shell mechanical features: a hierarchical analysis

In any given biologically formed materials, mechanical properties at macroscale generally depends on composition of material component and materials micro-structural features (Rodriguez-Navarro et al., 2002; Meng et al., 2018). In this study, oyster shell material is composed of two calcium carbonate polymorphs, calcite and aragonite. Oysters begin their life (larvae) with aragonite-based shell, but it is completely replaced by calcite in adult shells though juvenile shells may retain a tiny portion of aragonite (Weiss et al., 2002). Calcite is relatively less soluble in seawater compared to aragonite regardless of environmental pH. Calcite is a relatively less soluble form of CaCO$_3$ to decreased environmental pH when compared to aragonite (Lippmann, 1973). This chemical feature of calcite may have made feasible for the juvenile oysters to successfully mineralize and retain the laminated calcareous structure even under undersaturated CaCO$_3$ saturation levels, e.g. decreased pH 7.4 (Fig. 3).

Like previously described oyster shell microstructure (Dauphin and Denis, 2000; MacDonald et al., 2010), the materials used in this study are composed of structurally organized layers. The bulk of the microstructure is characterized by foliated layers of crystal units organized in lamellae. In order to understand the modulating effect of environmental pH on the relationship between the shell structural and mechanical features, we have quantified the "space or pore" size between laminated layers within the folia. The decreased pH significantly increased size and quantity of the pore in the folia layer. The presence of such laminated folia with pores or gaps was an obvious consequence of decreased pH. However, the larvae were still capable to produce a new foliated layer under these treatments whilst at undersaturation (at pH 7.2). Although the juvenile shells show signs of physical dissolution, the EBSD and porosity data suggest that the microstructure growth is impaired initially This micro-structural impairment was observed even under the near-future level of decreased pH 7.8, where the porosity was increased by 10 folds (Fig. 3r). On the other hand, the preferred orientation of crystal units within the folia layer showed no difference in all low pH treatments, with *c*-axis of calcite units approximately perpendicular to the outer and inner shell surface. Thus, the significantly reduced hardness and stiffness of the foliated layer under decreased pH might be due to the impaired microstructure with significantly higher pore size and numbers.

Furthermore, we measured the impacts of decreased pH on whole shell density and thus on "pores or gaps" in foliated layers using micro-CT analysis. Notably, higher density shell volume reduces with decreasing pH. This result supports our finding on the effect of decreased pH on microscale structure and mechanical features in the folia. Calcite shell

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materials are brittle in nature, like egg shells or ceramics, therefore their resistance to deformation (or breaking force) is largely depend on stiffness parameter of the shell (Lawn and Wilshaw, 1993). Here, we found that both hardness and stiffness of the folia layer reduces with decreasing pH, which may have triggered shell fracture under simulated external attack. A folia layer with lower stiffness and hardness resulting from a porous laminated microstructure is expected to be highly vulnerable to predatory attack even though the preferred orientation remains unaffected (Kemeny and Cook, 1986). In addition, the overall decrease of shell density detected by Micro-CT analysis indicates the porous internal microstructure may occur throughout the juvenile shell. In other words, the juvenile oyster shell with impaired microstructural features is more prone to predator attack under near-future level of decreased pH due to OA processes.

4.2 Effect of ocean acidification on shell microstructure and crystallography

The outermost prismatic layers of the older hinge and younger middle regions had completely disappeared when juvenile oysters were exposed to the extreme scenarios (pH of 7.2 and calcite undersaturation $\Omega_{cal} \approx 0.66$) (Fig. 2h and Fig. 3n, p). Undersaturated waters, with regards to calcite ($\Omega_{cal} < 1$), have for consequence the dissolution of calcitic materials (Bednarsek et al., 2012; Lippmann, 1973). Similar impacts were observed in *Argopecten irradians* (pH 7.8 and pH 7.5) (Talmage and Gobler, 2010), *Mercenaria mercenaria* (pH 7.7) (Dickinson et al., 2013) and , *Saccostrea glomerata* (pH 7.8 and pH 7.6) (Watson et al., 2009).

The juvenile oysters exposed to decreased pH exhibited porous microstructure in foliated layers (Fig. 3). Firstly, it may be due to the decreased calcification rate resulted from the metabolic depression and/or energy shortage in the decreased pH conditions (Gobler and Talmage, 2014; Lannig et al., 2010). Secondly, it could be due to the dissolution of newly formed minerals of the inner surface in the lower pH conditions (Melzner et al., 2011). Marine invertebrate's calcification has highly controlled mechanisms and remained to be explored by further studies (Krause-Nehring et al., 2011). Animals are capable in actively increasing the site of calcification by pumping proton out of the calcification site, thereby enabling calcium carbonate precipitation (Ries et al., 2010; Toyofuku et al., 2017). Supersaturated calcite conditions of oysters were found restricted to the shell edge including the outer mantle and the first intracellular nucleation site (Mount et al., 2004). Undersaturated calcite conditions may be maintained elsewhere in contact with the inner shell surface (Addadi et al., 2006; Thomsen et al., 2010). Therefore, in low pH conditions due to OA, these inner areas of newly formed minerals, which are precipitated as structural building blocks for the prismatic and foliated layers, may still be prone to dissolution. When the shell dissolution rate is faster than the mineralization rate, organisms tend to produce thinner and lighter (less dense) shells resulting in impaired shell microstructure. This may explain the multiple negative effects of reduced pH in our results, including porous and less dense foliated layers. Similarly, mussel shells grown in lower pH conditions (pH 7.65) showed inner shell surface dissolution (Melzner et al., 2011) and impaired shell microstructure (Hahn et al., 2012), which were consistent with the results in this study. The crystallography of marine shell is the other important proxy to environmental stressors (Milano et al., 2017). The crystallographic orientation maps of foliated layers showed a preferred crystallographic orientation with a ~40 degree of variation regardless of pH treatments which concurs with the results of field samples (Checa et al., 2018). Compared to calcite, aragonite represents a small fraction of the oyster shells and is more soluble under decreased pH conditions (Fitzer et al., 2014). which could explain the absence of aragonite in the older hinge regions at pH 7.2 (Fig. 4.iii) is observed in this study. A similar absence of aragonite was also reported in mussel shells in high pCO$_2$ (1000 µatm) conditions (Fitzer et al., 2014). Nevertheless, aragonite dissolution may be very relevant for oyster shells during early-life stages but not so much for the adult shell. Therefore, it plays insignificant role in shell mechanical properties of the calcite predominant adult shells and thus the adult oyster survival.

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4.3 Ecological implications and conclusion

Although previous studies showed that early larval life stages of several edible oyster species were relatively physiologically tolerant to the near-future OA conditions (Dineshram et al., 2013; Ko et al., 2013; Ko et al., 2014; Thiyagarajan and Ko, 2012), this study shows that they are still vulnerable due to the effects decreased pH has on shell characteristic like porosity, hardness, and stiffness.. Similar negative impact of OA on shell mechanical properties was reported in various marine calcifiers. For example, the pearl oyster, *Pinctada fucata*, produced a 25.9% weaker shell after exposure to seawater at pH 7.8 (Welladsen et al., 2010). Decreasing shell mechanical properties in decreased pH conditions was also observed in *Mytilus californianus* (Gaylord et al., 2011) which produced 20% weaker shells under pH 7.8, *Mercenaria mercenaria* (Dickinson et al., 2013; Ivanina et al., 2013) which produced approximately 60% softer shells under pH 7.77 (16 salinity), and *Hydroides elegans* (Li et al., 2014) which produced 80% softer tube under pH 7.8. However, the effects of increased carbon dioxide partial pressure (pCO_2) on shell mechanical properties are species-specific. Near-future conditions (pH 7.8) did not affect shell hardness in the sea urchin *Paracentrotus lividus* (Collard et al., 2016) or in the barnacle *Amphibalanus amphitrite* (McDonald et al., 2009). Furthermore, juvenile oysters of *C. gigas* significantly increased their shell strength and size as a compensatory adaptive response to low pH condition (i.e., pH 7.8) (Wright et al., 2014), and the blue mussel, *Mytilus edulis*, produced a stiffer and harder calcite layer under in increased pCO_2 condition (i.e., pH 7.3) (Fitzer et al., 2015).

The long-term survival strategy of oysters with mechanically softer shells as yet to be studied. However, as shown in a recent study (Sanford et al., 2014), it appears that weaker shell structures will result in compromised defence abilities. Moreover, results from a recent study suggest that oysters with reduced and impaired calcification mechanisms have lower repair capabilities (Coleman et al., 2014). This hierarchical study revealed that the OA conditions may cause a deterioration on oyster shells, thus pose a serious threat to oyster survival and the health of coastal oyster reef structures in the near-future ocean. This biological effect of OA on shell structures and mechanical features should be incorporated to the coastal oceanographic biophysical models to accurately project the survival of oysters in near-future coastal oceans which could be used for commercial shellfisheries to plan for sustainable growth under climate change induced acidification.

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Table 1: Seawater physicochemical parameters in the experimental system.

Treatments/parameter	Control	pH 7.8	pH 7.5	pH 7.2
pH	8.14 ± 0.04	7.88 ± 0.02	7.46 ± 0.01	7.23 ± 0.01
Temperature (°C)	27.04 ± 0.14	27.02 ± 0.08	24.35 ± 0.12	27.50 ± 0.08
Salinity (psu)	31 ± 0.5	31 ± 0.5	31 ± 0.5	31 ± 0.5
TA (μequiv kg^{-1})	2053.77 ± 46.51	2032.63 ± 25.60	2061.50 ± 4.56	2091.37 ± 39.37
pCO$_2$ (μatm) [a]	352.93 ± 11.04	861.37 ± 130.34	1997.23 ± 124.42	4091.73 ± 447.85
CO$_3$$^{2-}$ (μmol kg^{-1}) [a]	175.66 ± 24.96	97.92 ± 16.38	48.82 ± 6.07	26.59 ± 4.72
Ω_{Ca} [a]	4.59 ± 0.25	2.43 ± 0.41	1.21 ± 0.15	0.66 ± 0.12
Ω_{Ar} [a]	3.01 ± 0.18	1.59 ± 0.28	0.79 ± 0.10	0.43 ± 0.08

Data are mean ± s.d. of the replicate culture tanks (n = 4) for the seawater physicochemical parameters measured or calculated during the duration of the experiment: pH (National Bureau of Standards scale), temperature (°C), Salinity (psu), TA (μequiv kg^{-1}), carbon dioxide partial pressure (pCO$_2$; μatm), carbonate ion concentration (CO$_3$$^{2-}$; μmol kg^{-1}), calcite saturation state (Ω_{Ca}), and aragonite saturation state (Ω_{Ar}). Values were first averaged within and among days per each of the replicate culture tanks. Afterwards, the treatment mean was computed.

[a] Parameters were calculated using the CO2SYS software program (Pierrot et al., 2006) with equilibrium constants K$_1$, K$_2$ and KSO$_4$ (Mehrbach et al., 1973; Dickson and Millero, 1987).
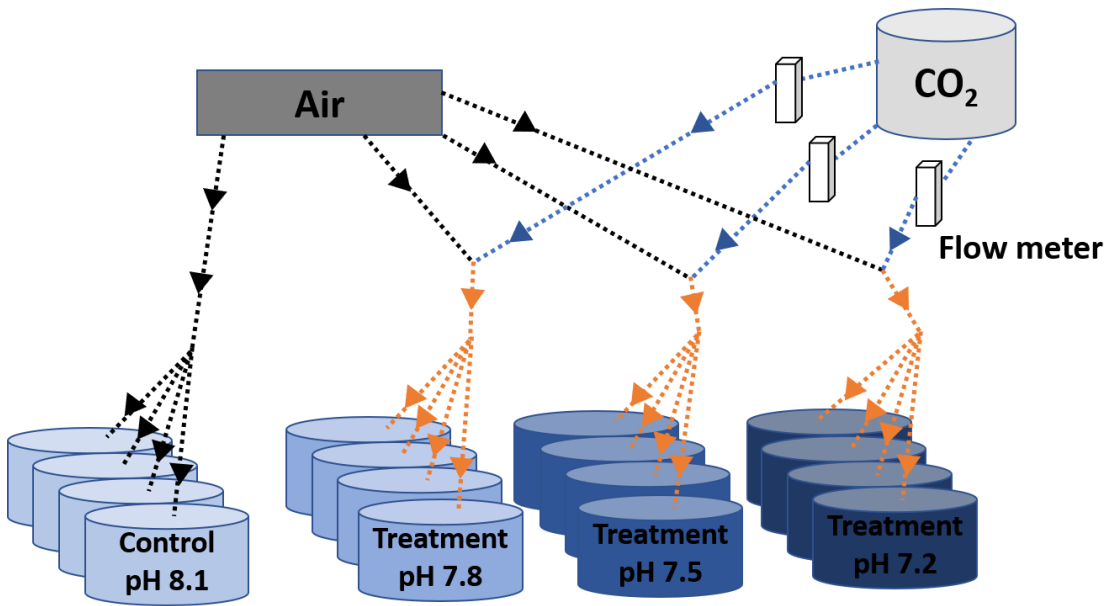
Figures

Figure 1: Scheme of the experimental system. Decreased pH conditions were obtained through bubbling CO₂-enriched air with the appropriate CO₂ concentration. Black and blue solid arrows indicate air and CO₂ flow respectively and orange arrows indicate CO₂-enriched air flow. The appropriate CO₂ concentrations in the injected CO₂-enriched air flow were controlled by adjusted using a dual variable area flow meter.
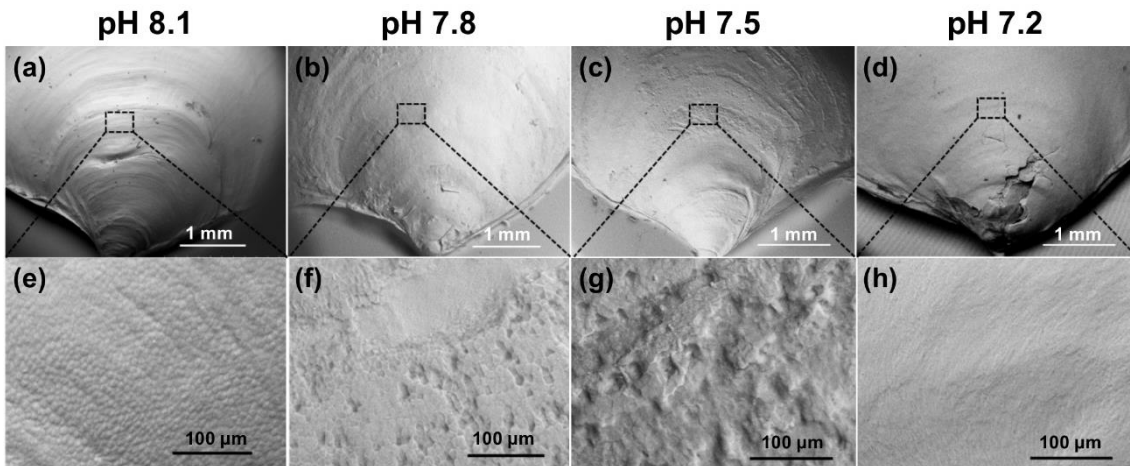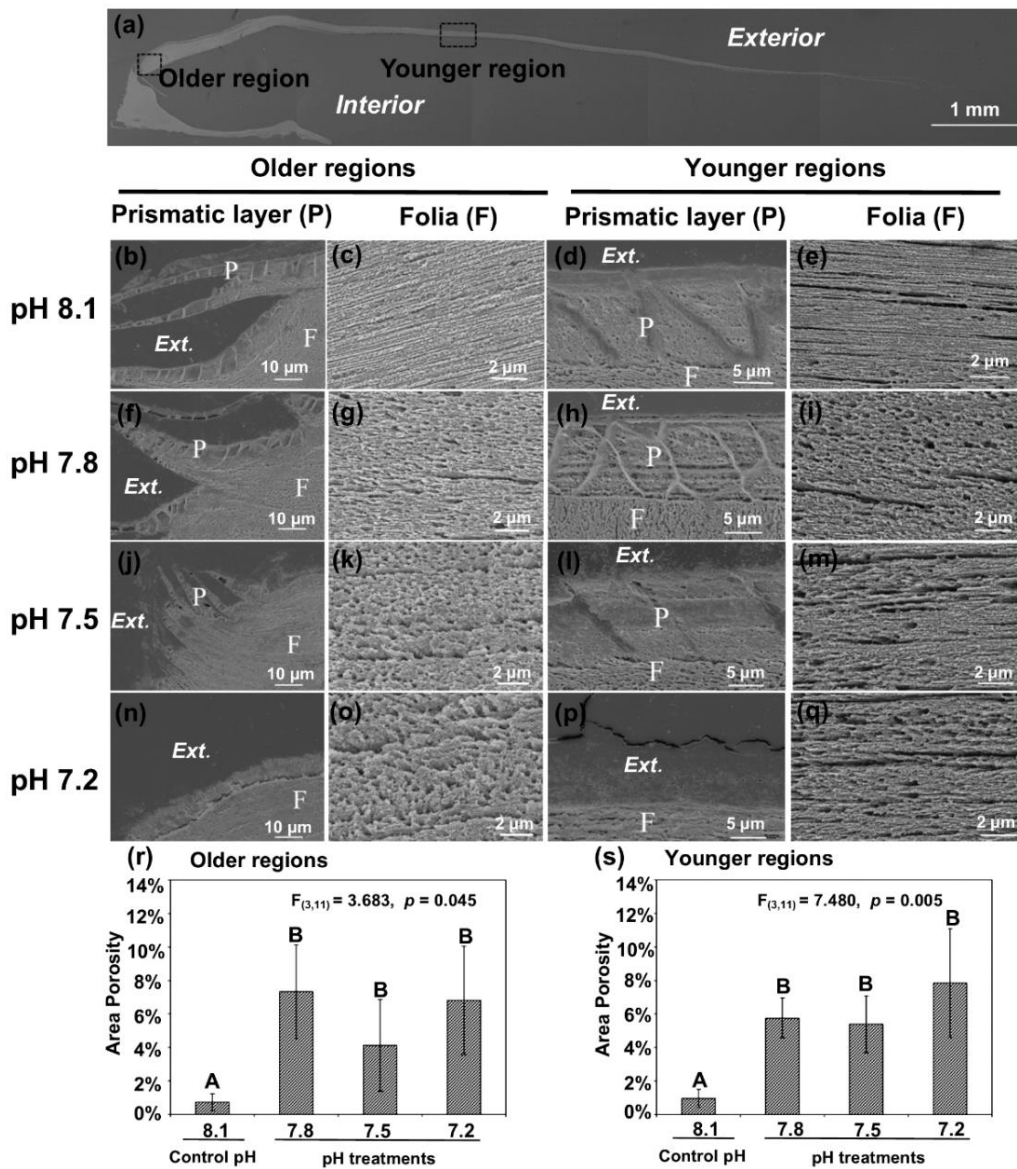

Figure 2: Scanning electron micrographs of 35-day-old juvenile *Magallana angulata* shells cultured at ambient or control pH 8.1 (a and e), treatment pH 7.8 (b and f), pH 7.5 (c and g) and pH 7.2 (d and h) were compared. Top row: low magnification tomography of the juvenile shells. Bottom row: enlarged view of the crystallite units (top view). (e) The prism units were arranged in compact prismatic structures at pH 8.1; (f) prismatic arrangement was partially lost at pH 7.8; (g) rough surface was observed demonstrating a much lower level of organization at pH 7.5; (h) a smooth surface was observed with no prismatic arrangement due to dissolution by environmental seawater.

Figure 3: Microstructures were observed in the cross-sectional shell surfaces of 35-day-old juvenile *Magallana angulata*. Scanning electron micrographs were taken near the older hinge region (b, c, f, g, j, k, n and o) and the younger middle region (d, e, h, i, l, m, p and q). First row: scanning electron micrograph of the full shell cross-sectional surface (a). Second row: the prismatic layer (b and d) and tightly packed foliated structure (c and e) at pH 8.1. Third row: the prismatic layer (f and h) and the foliated structure with more and bigger pores (g and i) at pH 7.8 compared with at pH 8.1. Fourth row: the incomplete prismatic layer (j and l) and more porous foliated structure (k and m) at pH 7.5 compared with at pH 8.1. Fifth row: the prismatic layer was not detectable (n and p) with porous foliated structure (o and q) at pH 7.2. The porosity of foliated layers at the older (r) and younger regions (s) of the shell reared under control and low pH treatments. The mean values are presented in the bar chart (mean ± SD, n = 3 - 4). Annotations: P-prismatic layer; F- foliated layer.
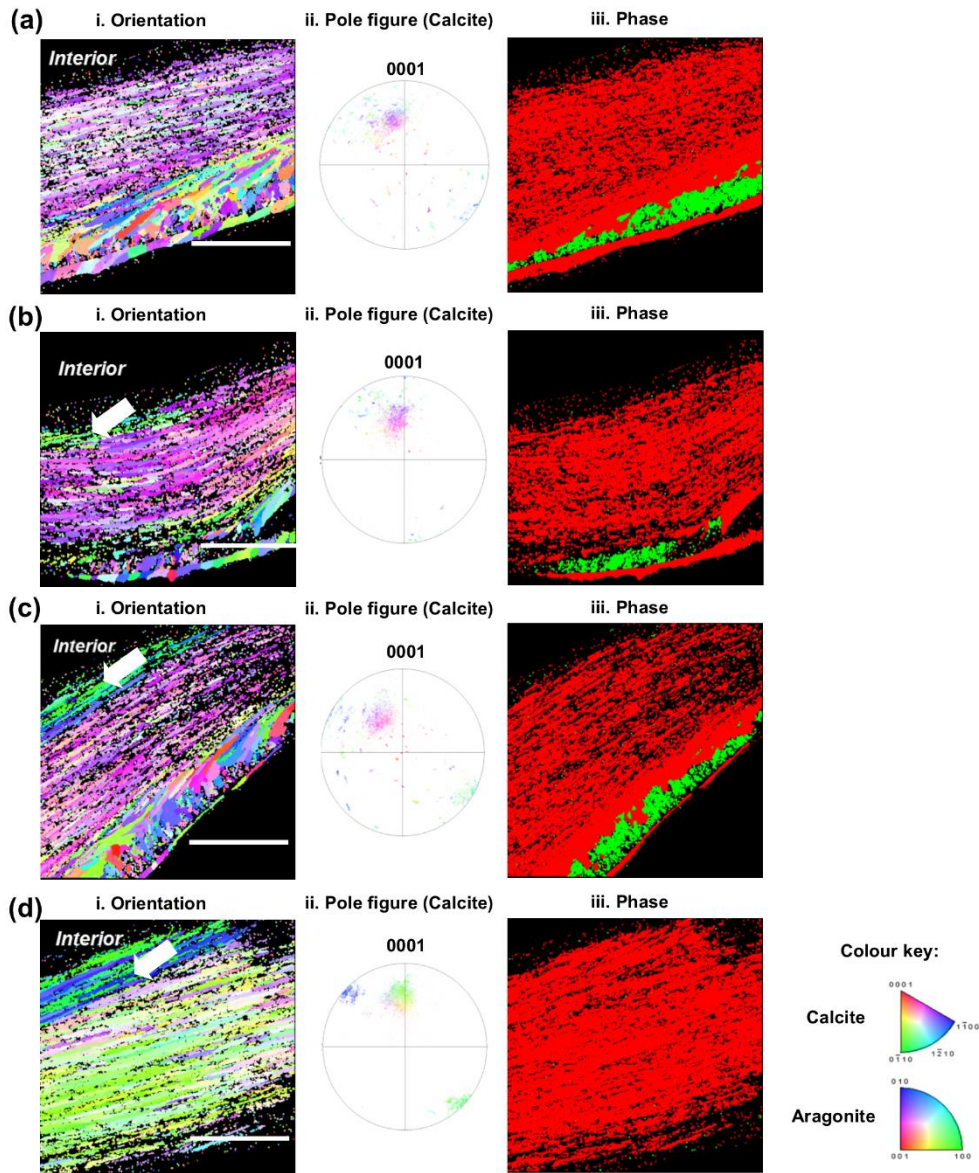
Figure 4: Electron Backscatter Diffraction analyses of shells grown for 35 days at control pH 8.1 (a), treatment pH 7.8 (b), pH 7.5 (c) and pH 7.2 (d). Crystallographic orientation maps (i) of calcite crystals in reference to the {0001} plane and aragonite crystals in reference to the {001} plane. Crystallographic planes of calcite are colour-coded according to the normal crystallographic direction shown in the colour key (Perez-Huerta and Cusack, 2009). Pole figures for calcite (ii) corresponding to the crystallographic orientation maps with the same colour key. (iii) Phase map of calcite exhibited in red and aragonite in green. White arrow: change in colour of 5 to 10 marginal foliated laminates. Scale bar = 45 µm.
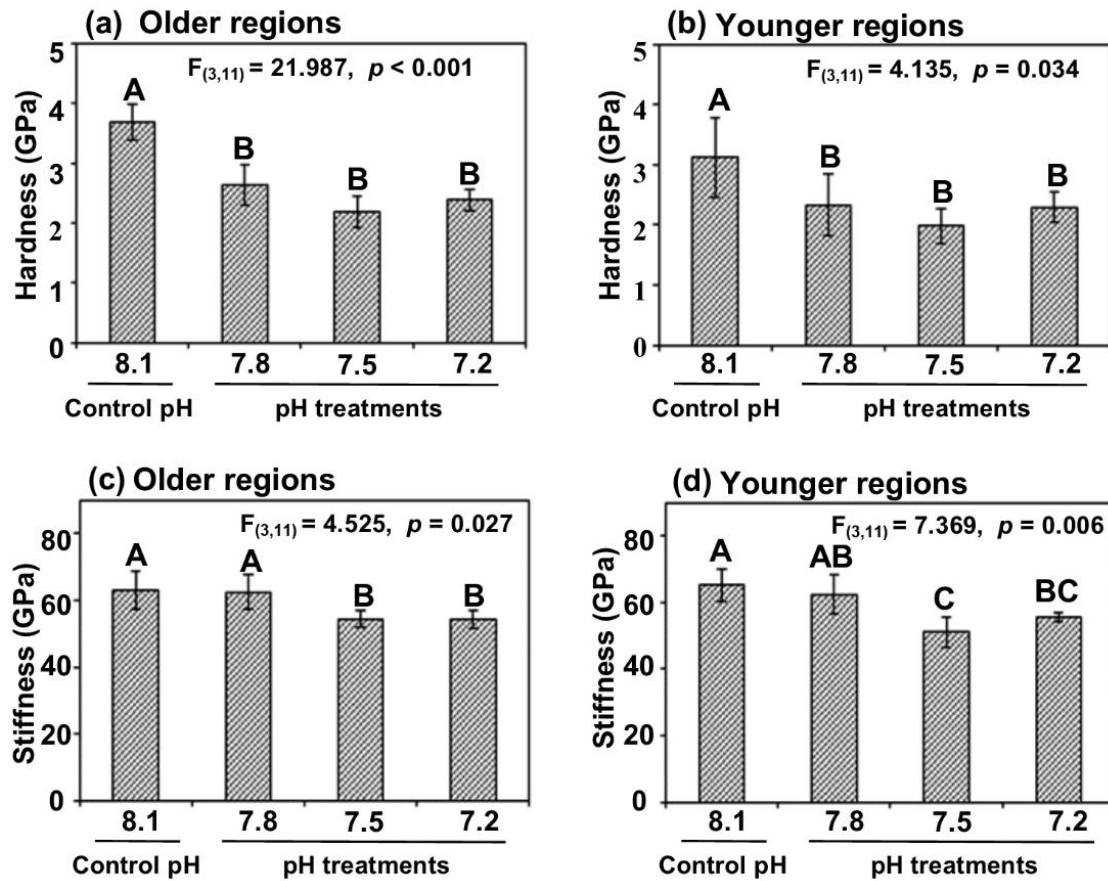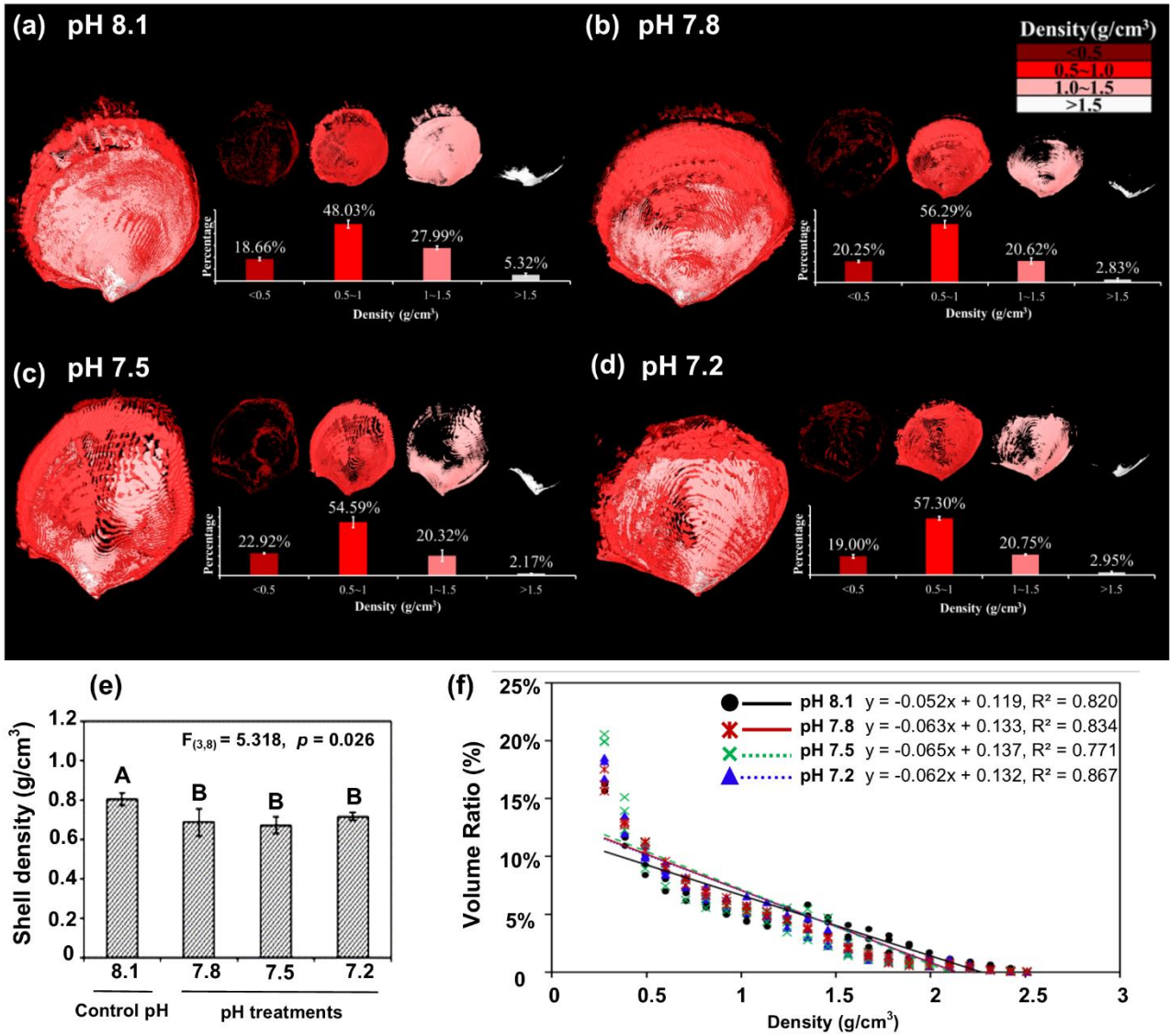
Figure 5: Shell mechanical properties in terms of hardness (a and b) and stiffness (c and d) with longer and shorter exposures in older hinge regions (a and c) and younger middle regions (b and d) in cross-sectional shell surfaces of *Magallana angulata* were compared. Data of mechanical properties are presented as mean ± SD of three to four replicates (n = 3 or 4).

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Figure 6: Effects of low pH on the shell density map (a-d), overall density (e), and shell density - volume ratio relationships (f) for the four experimental pH treatment groups were examined by micro-CT of shells of *Magallana angulata*. Three dimensional reconstructions represent the density distribution of the shells produced in ambient or control pH 8.1 (a), treatment pH 7.8 (b), pH 7.5 (c) and pH 7.2 (d). The volume ratios of density categories of <0.5 g/cm³, 0.5-1.0 g/cm³, 1.0-1.5 g/cm³, and > 1.5 g/cm³ were quantified. (e) The overall density was presented as mean ± SD of three replicates (n = 3). (f) shell density–volume ratio relationships for the four experimental pH treatment groups of *C. angulata*. Regression lines for the three low pH treatments closely overlap and are partly obscured.