

22 March 2018

Dear Prof. Treude,

Please consider our REVISED manuscript entitled “Effects of hypoxia and non-lethal shell damage on shell mechanical and geochemical properties of a calcifying polychaete” for publication as a research article in *Biogeosciences*.

We appreciate the anonymous reviewers for their suggestions to improve the impact of our manuscript. We have incorporated their suggestions into the manuscript and believe that the problems in the Materials and Methods have been solved. For those suggestions which are incorrect or obviously beyond the scope of study, we have fully explained in the Response. The changes have been stated in the Response with the line number and shown in the highlighted version of revised manuscript.

We would deeply appreciate an opportunity to respond to further review comments. Thank you very much for considering our manuscript again and we look forward to your positive reply at your earliest convenience.

Yours sincerely,



Dr. Jonathan Leung
Corresponding author
On behalf of Napo Cheung

Reviewer 1

Dear Editor,

The manuscript “Calcification and inducible defense response of a calcifying organism could be maintained under hypoxia through phenotypic plasticity” by Leung and Cheung, presents interesting questions about possible eco-physiological adaptations observed on a calcifying polychaete exposed to acute hypoxia, including changes in calcification rates, shell composition and metabolism.

Despite some interesting points, I think that the manuscript, in the present form is not acceptable for publication, because of a substantial lack of detail on the protocols used for the experiments and the analyses, very shallow description of the main results and some over-interpretation of the results. A (very) major revision is therefore suggested.

RESPONSE: We thank this reviewer for reviewing and providing useful suggestions for improving our manuscript. After reading the comments carefully, we found that most of them are related to the methods and can be clarified easily. However, there are some invalid arguments which are explained below. We have prepared a major revision of our manuscript by incorporating the good suggestions.

Here listed some of the **major comments**:

1) The materials and methods are too undetailed. Are the specimens at the T0 adult or juveniles? Were they exposed to a day/night light cycle? If so, how did you control algal proliferation during the experiment? If not, are you sure that this does not influence their physiology? Were added algae dead or alive? What basis the 20 ml algal concentration was chosen on? Before organic matter analyses, were the samples washed? If so, how? Also for statistical analyses more details are needed.

RESPONSE: The specimens were adults (Ln 78) and exposed to a day/light cycle of 14:10 hrs (Ln 115). Live algae were added (Ln 115), but there was no need to control their growth because the consumption rate of polychaetes is faster than the proliferation rate of algae. That is why we had to add algal suspension on a daily basis to sustain the growth of polychaetes (Ln 116). The feeding regime was based on our extensive experience in rearing *Hydroides* spp., which can ensure their normal growth under laboratory conditions. The shells were rinsed with deionized water before organic matter analysis (Ln 160-161). The above information has now been added in the revision and more details about statistical analysis have been provided (Ln 199-202).

2) I do not understand why in the materials and methods the authors say that they used 10 specimens per replicate (x 3 repl.) per treatment, but each observed response in the results is based on only n=3 or n=5 specimens (or replicates??). This is surprising if we consider that most of the performed analyses (e.g. respiration rates, growth rates, clearance rate) are not destructive and that just part of the shell is necessary for the rest of the analyses (e.g. Mg/Ca, organic matter, ACC...). So why did not the author use more than 3 or 5 specimens to do their analyses and calculate averages? Could they add more replicates?

RESPONSE: We adhere to the classic, unambiguous definition of “n” as “number of replicates” (i.e. not necessarily “number of individuals”). We have carefully clarified the exact sample size used for each of the measurements in our revised manuscript.

We initially had a total of 30 individuals per treatment (Ln 103). Since tube growth can be measured with sufficient accuracy and precision (Ln 128), the tube growth of each individual was analysed as one replicate, hence resulting in a sample size of 30 (Ln 125). The same concept applies to the measurements of organic matter and shell toughness, where accurate measurements could be made on individual shell (i.e. one shell from one individual as a replicate). However, due to the small size of the polychaetes and the limited quantity of the newly-formed shells, multiple individuals or their newly-

formed shells were pooled as composite samples for the remaining assays. Each of these composite samples (instead of the underlying individuals) was treated as a single statistical entity, i.e. a replicate. Based on our experience and preliminary test, this design provides adequate precision of measurement and statistical power. This is also evident from the small standard errors in our results (see Fig. 4 and 5).

Please note that although the respiratory rate and clearance rate were reported with a unit of “ind⁻¹ hr⁻¹”, this is merely a mathematical expression. For these measurements, 25 individuals were made into 5 composites (replicates) with 5 individuals per replicate. The measured rates reported for each replicate was simply expressed as an average of the pooled individuals (the units of these variables were expressed as “per individual”), while the effective sample size for the statistical analyses remains as “5 (composites)” instead of “25 (individuals)”.

3) The experimental design is quite weak. Maybe this impression derives also from the difficulty to understand how many individuals/measures/replicates were performed for each parameter. We lack fundamental information about the ability of these organisms to develop into cultures. All the results are discussed on the basis of comparison to normoxic conditions. How can the authors be sure that a “culturing effect” is not interfering with the results? This is particularly true for shell composition. Why did not the author compare the shells of individuals grown in normoxic conditions to pre-experiment portions of shells (grown into natural conditions) to see if any “culturing effect” is visible?

RESPONSE: We have now revised the experimental design section to clearly define replicates. As stated in the Introduction, this study aims to elucidate the effects of hypoxia on calcification and defence response of a calcifying polychaete, which can be experimentally tested under fully controlled laboratory conditions in order to isolate the effects of hypoxia and minimize the number of confounding factors. In contrast, the “culturing effect” cannot answer our research question because it only indicates the effects caused by the difference between laboratory and field conditions, but not the effects of hypoxia. As all the individuals were reared under laboratory conditions, the results were not influenced (or biased) by “culturing effect”.

4) The protocol used for respiration measurements is unusual to me. I cannot understand how the authors did measure the oxygen content of hypoxic waters into a syringe with a relatively thick probe tip and can be sure they avoided oxygenation during the measurement. Also, are they sure that the material the syringe is made of is impermeable to oxygen? Do they have any measure of blanks to estimate the possible gas exchange through the syringe walls during the analysis?

RESPONSE: We have to invalidate this comment. Our DO probe is thin (~2.4 mm in diameter) enough to perfectly insert into the hole of plastic syringe (~2.7 mm in diameter) for DO measurement. The syringes used were ordinary plastic syringes (Terumo Hypodermic Syringe without a needle) with a barrel wall which is definitely thick enough to be impermeable to air. Once the syringe is sealed (Ln 141), oxygenation cannot occur under such air-tight conditions. This simple method has been widely used before (e.g. Zhao et al., 2011; Leung et al., 2013).

Leung, Y.S., Shin, P.K.S., Qiu, J.W., Ang, P.O., Chiu, J.M.Y., Thiagarajan, V., and Cheung, S.G.: Physiological and behavioural responses of different life stages of a serpulid polychaete to hypoxia. *Mar. Ecol. Prog. Ser.*, 477, 135–145, 2013.

Zhao, Q., Cheung, S.G., Shin, P.K.S., and Chiu, J.M.Y.: Effects of starvation on the physiology and foraging behaviour of two subtidal nassariid scavengers. *J. Exp. Mar. Biol. Ecol.*, 409, 53–61, 2011.

5) In the experiment the authors consider hypoxic conditions to be reached at ~2.0 mg O₂/L. This concentration is the one which is normally considered to be the upper limit for hypoxia. Considering that the error associated to the probe used for O₂ survey is 0.1 mg/L (source: TauTheta manual) and that the average values the authors report in table S1 are always a slightly above 2.0 mg/L for hypoxic conditions, I wonder why the authors did not test a lower concentration to be sure to never exceed oxygen concentrations corresponding to namely hypoxic conditions. The results obtained should be carefully presented as the response to a case of acute (short time) and slight (upper limit) hypoxia and every over-interpretation or generalization to strong or long-term hypoxia should be avoided.

RESPONSE: There is no solid definition for the upper limit of hypoxia, but we used 2.8 mg O₂/L (~2.0 ml O₂/L) as the upper limit, which is defined in some influential papers and has been widely applied (Wu, 2002; Diaz and Rosenberg, 2008). Therefore, 2.0 mg O₂/L used in our study is definitely low enough to be considered hypoxic according to the conventional standard. We have also mentioned in the revision that the results indicate the impacts of short-term hypoxia on calcification.

Diaz, R.J., and Rosenberg, R.: Spreading dead zones and consequences for marine ecosystems. *Science*, 321, 926–929, 2008.

Wu, R.S.S.: Hypoxia: from molecular responses to ecosystem responses. *Mar. Pollut. Bull.*, 45, 35–45, 2002.

6) In lines 96-97 authors say that part of the individuals was left into hypoxic conditions during one week before the experiment (after damage and measures of the tubes). Can you please justify this choice? Does this mean that the T0 for tube sizes represents instead one week under treatment conditions? Did the authors measure the T0 size again after the acclimation and before the start of the experiment?

RESPONSE: This acclimation period is needed to remove the fight-or-flight response after shell breaking (Ln 99-101). The approximate tube length after shell breaking is provided (Ln 98), but it does not represent the initial tube length. We measured the tube length on Day 1 of the exposure period as the initial tube length (Ln 104, 125).

7) How did the authors get to have stable oxygen conditions during and just after water changes (every 3 days)? Did you measure oxygen into the culture solution before retiring old seawater? Could you please show these data somewhere in supplementary materials?

RESPONSE: Stable DO concentration at hypoxic level can be obtained by aerating the seawater continuously with a mixture of nitrogen and air, where the flow rates of these two gases were adjusted to achieve the target DO level (Ln 92-95). Stable equilibrium between gases in seawater can be achieved by this method quickly (< 5 min) so that the DO concentration in seawater can be very stable throughout the 3-week exposure period (Ln 109-113). This method has been widely used for hypoxia study (e.g. Leung et al., 2013; Mukherjee et al., 2013). The DO concentration data have been added in Figure A1. Leung, J.Y.S., Cheung, S.G., Qiu, J.W., Ang, P.O., Chiu, J.M.Y., Thiyagarajan, V., and Shin, P.K.S.: Effect of parental hypoxic exposure on embryonic development of the offspring of two serpulid polychaetes: Implication for transgenerational epigenetic effect. *Mar. Pollut. Bull.*, 74, 149–155, 2013. Mukherjee, J., Wong, K.K.W., Chandramouli, K.H., Qian, P.Y., Leung, P.T.Y., Wu, R.S.S., and Thiyagarajan, V.: Proteomic response of marine invertebrate larvae to ocean acidification and hypoxia during metamorphosis and calcification. *J. Exp. Biol.*, 213, 4580–4589, 2013.

8) Is edx analysis resolution enough for consistent Mg/Ca measurement in the shell? Can the authors give more information about the accuracy, precision, detection limits etc. of the analysis, please? Or add references if the protocol is routinely used.

RESPONSE: EDX (or EDS) has been extensively used to analyse Mg/Ca in the shell with good results. We have added references in the text (e.g. Ries, 2004; Zhang et al., 2010; Leung et al., 2017) (Ln 187).

Ries, J.B.: Effect of ambient Mg/Ca ratio on Mg fractionation in calcareous marine invertebrates: A record of the oceanic Mg/Ca ratio over the Phanerozoic. *Geology*, 32, 981–984, 2004.

Zhang, F., Xu, H., Konishi, H., Roden, E.E.: A relationship between d_{104} value and composition in the calcite-disordered dolomite solid-solution series. *Am. Mineral.*, 95, 1630–1656, 2010.

Leung, J.Y.S., Connell, S.D., Nagelkerken, I., and Russell, B.D.: Impacts of near-future ocean acidification and warming on the shell mechanical and geochemical properties of gastropods from intertidal to subtidal zones. *Environ. Sci. Technol.*, 51, 12097–12103, 2017.

9) Some of the conclusions/discussions are inferential and not supported by the data. For example paragraph from line 249 to 258 should be deleted, in my opinion, as it is not supported by the presented data.

RESPONSE: This paragraph is a general discussion on the mechanism affecting calcification and our results (e.g. seawater carbonate chemistry and shell growth) can support this discussion. This paragraph is important to reshape the concept of calcification and to improve the readership of our manuscript.

10) The hypothesis of relaxed magnesium regulation to explain higher Mg/Ca in the calcite produced under hypoxia is based on benthic foraminifera. These organisms are known to strongly discriminate against magnesium. This does not seem the case for polychaetes, which seem to contain very high concentrations of Mg in the shell. Authors should base their hypotheses on more adapted literature.

RESPONSE: We cannot validate this comment. The Mg/Ca in calcite varies greatly among foraminifera species, where many of them have Mg/Ca greater than 15 mol %, such as *Spiroloculina clara*, *Planispirinella exigua*, *Peneroplis proteus*, *Alveolinella quooii* and *Mychostomina revertens* (Blackmon and Todd, 1959). Please also note that the transport and sequestration of Mg are heavily regulated in eukaryotic cells and the underlying mechanisms are likely conserved among eukaryotic species. Thus, it is reasonable to conjecture that similar mechanism can be shown in our tested calcifying polychaete. In fact, the currently acknowledged regulatory mechanism of Mg in foraminifera is partially inferred from the combined biological knowledge on *Paramecium*, mollusks and cultured rodent cells (see Bentov and Erez, 2006).

Blackmon, P.D., and Todd, R.: Mineralogy of some foraminifera as related to their classification and ecology. *J. Paleontol.*, 33, 1–15, 1959.

Bentov, S. and Erez, J.: Impact of biomineralization processes on the Mg content of foraminiferal shells: A biological perspective. *Geochem. Geophys. Geosyst.*, 7, Q01P08, 2006.

Minor comments/suggestions:

Line 30: use “increase” instead of “augment”

RESPONSE: Suggestion adopted (Ln 30).

Line 51: replace “It” by “This”

RESPONSE: Suggestion adopted (Ln 52).

Line 53: What does “or other physiological processes via energy trade-off” mean? Can you explain what other processes you’re talking about please?

RESPONSE: Based on the energy budget model, other processes can include growth, reproduction, somatic maintenance, etc (Ln 54-55).

Line 58: Please delete “and defense response”.

RESPONSE: “Defense response” has a specific meaning in this study, which refers to the response following non-lethal shell damage.

Materials and methods: Please add titration protocol for alkalinity measures presented in table S1.

RESPONSE: It is unnecessary to add a protocol for alkalinity measurement in the manuscript because it is far too technical and general readers are not interested in how to operate a particular model of titrator. We have never seen research articles, except method papers, describing the operating procedures for a particular model of equipment. We have added the website for the protocol in Table S1.

Lines 93-97: Please add more details about the procedure used to measure the specimens, associated errors and discuss the potential stress that this manipulation may represent and the effect it could have on the final results

RESPONSE: We have now rewritten the procedure and the discuss the potential stress in section 2.3.

The order of paragraphs in the Material and methods section is, at present, a bit confusing and should follow a more logical pattern. I would suggest that the paragraph on experimental design should show the setting, the replication, times etc., for all type of analyses. Then a paragraph on procedures for physiological analyses should explain all the used methods for respiration rates, survival rates, feeding rates and shell growth. Then a final paragraph on the shell composition should follow.

RESPONSE: We appreciate reviewer’s suggestions and have changed the order accordingly.

Lines 99-100: Why is the color of new shell very different from the ancient one? Is it normal? Maybe you should discuss it somewhere. One would think that it is a culturing effect on shell structure...

Figure S1 should be in the main text.

RESPONSE: The colour of new shells should be white due to the presence of calcium carbonate. Yet, the colour will turn slightly yellowish over time because of the biofilm (e.g. bacteria, algae, etc.) growing on the surface (Fig. 1). It is very normal and important because the biofilm allows polychaete larvae to settle and form a colony. We have now put Figure S1 in the main text as Fig. 1.

Lines 103-112 should be part of the “Experimental set up” paragraph

RESPONSE: We have changed the order of paragraphs as suggested above.

Line 123: The specimens used for organic matter composition are the same measured for toughness? Please detail this kind of information

RESPONSE: It cannot be, unfortunately, because removing the organic matter in the shell at 550°C can substantially affect shell toughness. We have now clarified this (Ln 170-171).

Paragraph 123-129: 1) for the composite shell power did you mix calcite from different specimens, isn’t it? Did they come from a same replicate for a treatment or even replicates were mixed? How did you prepare the powder exactly? Did you wash the shell before to avoid contamination (from algae given as food for example)? How? Why did you choose Mg/Ca ratio as a parameter to be measured?

RESPONSE: For each composite sample, shells from 3-5 individuals in the same treatment were used (Ln 173-174) and we had 3 composite samples as replicates per treatment for the geochemical properties. The powder was prepared by removing the newly-produced shells, rinsing them with deionized water (to remove the microalgae and other debris), drying them at room temperature and finally grinding them

using a mortar and pestle (Ln 175-177). Many calcifying organisms can change Mg/Ca in their calcitic shells in response to the changing environment (e.g. ocean acidification). The underlying mechanism remains largely unknown, but may be associated with metabolic energy that can be greatly affected by hypoxia. Therefore, we expected that Mg/Ca would change in response to hypoxia.

Paragraph 148-155: How did you calibrate oxygen probes before the analysis?

RESPONSE: Before the analysis, the probe was calibrated by inputting the value of calibration slope in the software. Then, we have to validate the DO concentration using another DO meter (e.g. handheld DO meter), which is calibrated by measuring the DO concentration of oxygen-saturated seawater. We may need to change the value of calibration slope until the DO concentrations between DO probes are the same. We have added the website for the operation manual of the oxygen probe in Table A1.

Line 152: “The air inside the syringe...”: what air? Why is it there during the first measure? This part is not clear to me.

RESPONSE: The air is atmospheric air (Ln 140). For the initial measurement, the individuals were allowed to rest in the syringe for 15 min and the small pocket of air can help buffer the change in DO concentration during this period. We have added this information for clarity (Ln 140-141).

Line 154: “by gently stirring the FSW inside the syringe”. How did you avoid oxygenation at this step?

RESPONSE: Such gently stirring can only help homogenize the DO concentration in the water body. Oxygenation of water is caused by dissolving atmospheric oxygen in water. However, this process cannot occur because atmospheric air cannot interact with the water inside the syringe, which is under air-tight conditions (Ln 141).

Line 156: Please specify the unit used for consumed oxygen. Normally mL or μmol are used. In your figure 4a you use $\mu\text{g O}_2$, which is quite unusual as a unit for oxygen.

RESPONSE: For the unit of consumed oxygen, mg O_2 is frequently used, while $\mu\text{g O}_2$ is just a conversion for the magnitude to avoid many decimal places (Ln 144-145).

Lines 159-160: Why did you only used one algal species for this experiment? Can you add fundamental details such as if the experiment was performed under light conditions, please? Also you say that 5 replicated bottles were used per treatment. In the first materials and methods section you say you have 3 replicated bottles with 10 specimens for the experiments. I'm lost... Are these different bottles? How many individuals per bottle per treatment do you have then?

Statistical paragraph: Where data transformed before the analyses to homogenize the magnitudes? How many permutations were performed? Which distance parameter was used and why? Can you specify the “aforementioned parameters” of line 168, please?

RESPONSE: One algal species should be used for the feeding experiment to avoid the selective feeding of filter feeders (due to different sizes and/or textures between microalgae), which can complicate the calculation and interpretation. We have now stated that the experiment was performed under light conditions (Ln 151). The experimental design (e.g. number of replicates) has been revised in a more explicit way in the Materials and Methods section. For univariate analysis, data are on the same scale (i.e. carrying the same unit), therefore transformation is unnecessary. The number of permutation is 999 and Euclidean distance was used which is commonly applied (Ln 199-200). We have listed the parameters (e.g. respiration rate, clearance rate, shell toughness, etc.) in the revision (Ln 200-202).

Line 172: You say hypoxia slightly hindered, but your statistics say this difference is significant, so maybe this should be emphasized a bit more.

RESPONSE: Please note that “statistically significant” does not necessarily imply “biologically significant”. We can emphasize the statistical significance a bit more (Ln 205), but it is more important to compare the two factors with respect to the magnitude of change, which is of biological importance.

Line 174: Please replace “negligible” with “no”.

RESPONSE: “no” is too absolute. We prefer to be conservative by using “no significant” (Ln 207).

Line 180: You say “but only slightly by non-lethal shell damage”. It looks like a significant difference, visually (fig. 4a). Is it confirmed by statistical analyses? Yes, so you should say it!

RESPONSE: It is statistically significant (Ln 214).

Lines 181-182: specify whether statistical differences are visible and where.

RESPONSE: We have specified it (Ln 215).

Line 187: what do you mean by “ramifications”?

RESPONSE: It can mean changes in species populations, community structure and ecosystem functioning (Ln 220-221).

Line 188: “tolerant to hypoxia”, at what temporal scale?

RESPONSE: Short-term scale (Ln 222).

Line 194: I would suggest not to use “unthreatened conditions” as a general for “shell damage”, because hypoxia also is an unthreatened condition, so it can result confusing.

RESPONSE: We appreciate this suggestion, but we have explicitly defined “unthreatened conditions” as “individuals without shell damage” in the methods and mentioned the definition again in this sentence. There should be no confusion.

Line 195: “hypoxia slightly hinders”... again, is it significant or not?

RESPONSE: Significant (see Table S2).

Lines 201-202: redundant concept, already said.

RESPONSE: We have deleted this sentence.

Lines 205-206: Please replace “shell growth” with “inorganic components of the shell”. You suggest that under hypoxia the production of organic matter compensate for diminished quality of inorganic components (>ACC). Palmer (1992) on the contrary suggests that organic matter production is costly and that would be the reason why high-organic calcareous microstructures became rare with evolution. How do you explain this incoherence?

RESPONSE: Suggestion for the word choice have been adopted (Ln 240-241). However, we never suggest that “under hypoxia the production of organic matter compensate for diminished quality of inorganic components (>ACC)”. In this paragraph, we discussed the effects of hypoxia on shell growth and shell strength under unthreatened conditions. As the organic matter content was not affected by hypoxia, we suggest that the polychaetes still allocate similar amount of energy to maintain shell strength at the expense of shell growth under hypoxia.

Line 211-212: Could the overproduction of organic matter be related to higher calcification rates?

RESPONSE: Although we cannot rule out this possibility, we think it is still premature to make this speculation because we found that organic matter content does not necessarily increase with calcification rate (Normoxia vs. Hypoxia without shell damage).

Lines 219-221: I do not understand what you mean

RESPONSE: This sentence means when the individual is under life-threatening conditions and chance of survival becomes very low, it has to prioritize defence response as the last resort to maximize survival rate. We have rephrased this sentence to better illustrate the idea (Ln 255-257).

Line 224: You say the effect of hypoxia on defense response is not discernible. Although toughness is not affected, I would say that reduced shell growth rates (visible in figure 1) should be taken into account, to discern the effects of hypoxia, as well.

RESPONSE: We have revised this sentence by considering the effect of hypoxia on shell growth (Ln 260-261).

Line 234: please replace “signifies” with “may suggest”.

RESPONSE: Suggestion adopted (Ln 271).

Line 241: At the end of the sentence you should add “.. can generally be maintained at least under slight hypoxia, on a short timescale.”

RESPONSE: Suggestion adopted (Ln 278).

Line 248: please delete “and therefore *H. diramphus* prioritized defense response”.

RESPONSE: Suggestion adopted.

Line 259: please add “open” between marine and waters (because “costal” are also marine waters!)

RESPONSE: Suggestion adopted (Ln 294).

Line 264: when you say that the defense response can be sustained you should specify “on a short timescale”, because your results are based on short-term experiments.

RESPONSE: We appreciate this suggestion and have specified this in the text (Ln 299).

Lines 330-339: Please order the references on a chronological basis.

RESPONSE: Suggestion adopted (Ln 372-384).

Line 338 and 368: add authors to the list

RESPONSE: Authors added (Ln 403, 416).

Survival rate figure (S2) should not be in supplementary material, as this is an important result to take into account in the analysis of all the others.

RESPONSE: We have now described the results in the text (Ln 118-120).

Reviewer 2

The manuscript by Leung and Cheung provides information regarding how calcification processes in polychaete worms could be influenced by future hypoxia. The results are pretty straight forward, and I consider these types of studies are important, although not ground-breaking. I have a few concerns that should be addressed before this manuscript could be accepted.

RESPONSE: We are pleased to see that the reviewer recognizes the importance of this work.

The grammar and style should be improved in the introduction and discussion of this m/s before it could be published in any outlet. There are too many examples of this for me to highlight every one, but for example the use of the term “defence response”. I suggest the authors ask a senior colleague who is a native English speaker to read over and correct for them. In general there is also a lot of speculation for a 21 day long study.

RESPONSE: Being senior English writers, we believe that the overall quality of English writing is good enough for publication based on our experience. Yet, we have polished the writing in the revision to maximize readability. The term “defence response” is widely used in the literature to describe the defensive behaviours of organisms at both individual (e.g. anti-predator response) and tissue/cellular (e.g. immune response) levels.

Specific comments: Line 40: In general I agree that calcification costs energy, but in some organisms the energy-dependence has been postulated as low (e.g. in corals – see McCulloch et al. (2012)). So this may be true for gastropods, but not necessarily so for some other organisms. So this sentence needs to be balanced somewhat.

RESPONSE: Please note that McCulloch et al. (2012) examined the effect of ocean acidification, where the “low energy cost” only refers to the additional energy for pH regulation at the calcification site, while the energy cost of calcification (i.e. production of calcified structures) is not taken into consideration. Thus, this “low energy cost” is irrelevant to our study because hypoxia does not affect the acid-base balance of organisms.

Line 70: The hypotheses around phenotypic plasticity needs to be strengthened and clarified. What exactly is the phenotype that is plastic here? The capacity to form different types of mineral in the shell? Or simply that responses will differ between control and reduced O₂ concentrations? Reading the discussion, I think the authors are misusing the term phenotypic plasticity. Demonstrating variability in responses of individuals within the same population to a stressor is not demonstrating phenotypic plasticity, nor is demonstrating a different response under different treatments between different individuals.

RESPONSE: In ecology, “phenotypic plasticity” means that individuals can change their phenotypic traits (e.g., growth, behaviour, shell properties, etc.) in response to altered environmental and biological conditions, which has adaptive values (Malaua et al., 2005). In this study, the mineralogical properties of shells were proven to be plastic phenotypic traits as they were modified in response to hypoxia. The term “phenotypic plasticity” may be a bit general because the phenotypic traits in this study were only associated with mineralogical properties. To be more explicit, we will replace “phenotypic plasticity” with “mineralogical plasticity” (Leung et al., 2017) or similar wordings in the revision.

Leung, J.Y.S., Russell, B.D., and Connell, S.D.: Mineralogical plasticity acts as a compensatory mechanism to the impacts of ocean acidification. *Environ. Sci. Technol.*, 51, 2652–2659, 2017.

Malausa, T., Guillemaud, T., and Lapchin, L.: Combining genetic variation and phenotypic plasticity in tradeoff modelling. *Oikos*, 110, 330–338, 2005.

Line 82: How was pH measured, and on what scale, using what buffers? More information needed here. How was salinity and temperature measured? I see some of these details in table S1, but there are required in the methods section.

RESPONSE: The pH was measured on NBS scale by a pH meter, calibrated using NBS buffers (Table S1). Temperature and salinity were measured using a thermometer and refractometer, respectively (Table S1). Since they are not the key parameters in this study, it is better to keep them in the Supplementary Information.

Statistical analysis: why use a permanova? I would expect each parameter to be separated analysed using univariate analyses as a first step. A justification for using permanova over an anova or linear model needs to be justified here.

RESPONSE: Please note that PERMANOVA can be used for univariate analysis, as applied in our study. With the use of Euclidean distance matrix, PERMANOVA can produce the same F-statistics as traditional ANOVA (Anderson, 2001).

Anderson, M.J.: A new method for non-parametric multivariate analysis of variance. *Aust. Ecol.*, 26, 32–46, 2001.

Line 191-192: But this statement is at odds with the findings of the permanova and the figures, that calcification was impacted by hypoxia in this study. Also, the end of the sentence that this could be due to phenotypic plasticity needs to be explained, as this makes no sense to me.

RESPONSE: At this point of discussion (1st paragraph), we only make a general summary of the findings, which can help readers quickly grasp the key message of this study. The detailed explanation for each finding is provided in the subsequent paragraphs. We have revised the statement about the impact of hypoxia on calcification for clarity (Ln 224-226).

References used in the review: McCulloch MT, Falter J, Trotter J, Montagna P (2012) Coral resilience to ocean acidification and global warming through pH up-regulation. *Nature Climate Change*, 2, 623-627.

Reviewer 3

Dear Editor,

The manuscript “Calcification and inducible defense response of a calcifying organism could be maintained under hypoxia through phenotypic plasticity” by Leung and Cheung raises a few interesting questions regarding organismal adaptations to hypoxia. Although authors present a few interesting ideas, I do not find the manuscript in its current form suitable for publication due to a significant shortfall in methods description, exaggerated interpretation of results and an incoherent discussion. However, if the Discussion paper manuscript progresses for publication in Biogeosciences, I suggest that the authors consider some major revisions that I have described in detail in the supplement. Please find attached my suggestions for major and minor revisions.

RESPONSE: We thank this reviewer for reviewing and giving suggestions for our manuscript. We found that most of the comments are related to the methods and can be clarified easily. The only two comments for the discussion section are minor and can be easily addressed as well. Therefore, we are happy to incorporate the good suggestions into the revision.

Major Comments:

Title: I do not find this title representative of the authors results/discussion. Please describe which aspects of the phenotype are considered plastic, since there is no change in mechanical strength and authors discuss less crystallographic control and magnesium regulation under hypoxic conditions.

RESPONSE: The plastic traits are the mineralogical properties of shells. We have now made the title more descriptive (Ln 1-2).

Lines 34-38: Authors seem to go around a point here. Please state exactly what drives calcification instead of pH and seawater chemistry.

RESPONSE: The proposed main driver for calcification is the energetics of organisms (Ln 40).

Line 54: Energy costs can increase rather than reduce, if organic content is modified such that it increases.

RESPONSE: We have revised the sentence for clarity (Ln 56-57).

Line 76: Mean size of polychaetes?

RESPONSE: The tube length of polychaetes mostly ranged from 35 to 45 mm (Ln 78).

Line 102: Diameter of hole drilled?

RESPONSE: About 2 mm (Ln 105).

Line 103: Tubes were glued using what?

RESPONSE: Hot-melt adhesive (Ln 107).

Line 114: How was the shell fragment acquired and cleaned of organic tissue?

RESPONSE: Shell fragments were obtained by breaking the newly-produced shells using a pair of forceps and they were then cleaned by rinsing with deionized water (Ln 160-161). Please note that the flesh of polychaetes does not attach to the shell.

Line 116: Was the same surface (e.g: inner shell surface) always used for indentation?

RESPONSE: Yes, we always used the inner shell surface for indentation (Ln 164).

Line 122: Organic content of shell or whole polychaete? It is of the shell I assume, but is unclear.

RESPONSE: Only the newly-produced shells were used for analysis of organic matter content (Ln 170).

Line 126: How was the shell powder acquired?

RESPONSE: Shell powder was obtained by grinding the newly-produced shells using a mortar and pestle (Ln 175-177).

Line 130: Please provide information on how these polymorphs are typically distributed in the organism. Are the polymorphs specific to the outer/inner layer of the shell?

RESPONSE: We do not have information on the distribution of these carbonate polymorphs. Yet, it is not necessary to know that because it is irrelevant to our research question.

Line 139: Were ACC, aragonite and calcite standards measured? Please explain why, if not.

RESPONSE: Standards are used for determination of absolute quantity, but only relative quantity of these parameters is needed in our study. Specifically, since relative ACC content is indicated by the peak ratio in the IR spectrum, it is not necessary to measure the standard as long as background calibration for the baseline is made (Chan et al., 2012; Leung et al., 2017). As for the calcite to aragonite ratio, we apply the calibration equation in a method paper (Kontoyannis and Vagenas, 2000), which is derived by using pure calcite and aragonite.

Chan, V.B.S., Li, C., Lane, A.C., Wang, Y., Lu, X., Shih, K., Zhang, T. and Thiyagarajan, V: CO₂-driven ocean acidification alters and weakens integrity of the calcareous tubes produced by the serpulid tubeworm, *Hydroides elegans*. PLoS ONE, 7, e42718, 2012.

Leung, J.Y.S., Connell, S.D., Nagelkerken, I. and Russell, B.D.: Impacts of near-future ocean acidification and warming on the shell mechanical and geochemical properties of gastropods from intertidal to subtidal zones. Environ. Sci. Technol., 51, 12097–12103, 2017.

Kontoyannis, C.G. and Vagenas, N.V.: Calcium carbonate phase analysis using XRD and FT-Raman spectroscopy. Analyst, 125, 251–255, 2000.

Line 140: Diameter of the KBR-shell powder disc?

RESPONSE: 13 mm (Ln 193).

FTIR: FTIR is a bulk measurement and ideally should not be used to infer "relative" proportions of carbonate polymorphs. Typically, the presence of a 713 cm⁻¹ peak is indicative of crystalline calcium carbonate comprising the bulk of shell carbonates. However, I am aware that this interpretation has been used before and if authors proceed with the analyses, could they please clarify if the spectra were scaled so that 713 cm⁻¹ peaks had the same heights as described in Weiss et al (2002)? In addition, please specify the typical size of crystallites in shell since such ratios have been demonstrated to be influenced by particle size (Kristova et al 2015).

RESPONSE: FTIR has been widely used to indicate the relative ACC content by measuring the peak ratio between 856 cm⁻¹ and 713 cm⁻¹ (e.g. Beniash et al., 1997; Chan et al., 2012; Leung et al., 2017). Since relative ACC content is indicated by this ratio rather than an absolute peak height, it is not necessary to rescale the peak height at 713 cm⁻¹ to that in Weiss et al. (2002). The particle size of shell powder was ~5 µm (Ln 177).

Beniash, E., Aizenberg, J., Addadi, L., and Weiner, S.: Amorphous calcium carbonate transforms into calcite during sea urchin larval spicule growth. *Proc. R. Soc. B*, 264, 461–465, 1997.

Line 148: What were the syringes made to of?

RESPONSE: Polypropylene plastic (Ln 135).

Line 156: Hunger is only standardised if individuals were at the same start point.

RESPONSE: Therefore, all individuals were starved for 1 day prior to feeding trials. This is more than enough for them to clear their gut content.

Lines 156-166: This doesn't represent clearance rates during the experiment.

RESPONSE: We disagree. This clearance method has been widely applied for determination of clearance/filtering/feeding rate of feeding feeders (e.g. Riisgård, 2001; Contreras et al., 2012; Leung et al., 2013; Leung and Cheung, 2017) (Ln 147-148).

Contreras, A.M., Marsden, I.D., and Munro, M.H.G.: Effects of short-term exposure to paralytic shellfish toxins on clearance rates and toxin uptake in five species of New Zealand bivalve. *Mar. Freshw. Res.*, 63, 166–174, 2012.

Leung, J.Y.S. and Cheung, N.K.M.: Feeding behaviour of a serpulid polychaete: Turning a nuisance species into a natural resource to counter algal blooms? *Mar. Pollut. Bull.*, 115, 379–382, 2017.

Leung, Y.S., Shin, P.K.S., Qiu, J.W., Ang, P.O., Chiu, J.M.Y., Thiyagarajan, V., and Cheung, S.G.: Physiological and behavioural responses of different life stages of a serpulid polychaete to hypoxia. *Mar. Ecol. Prog. Ser.*, 477, 135–145, 2013.

Riisgård, H.U.: On measurement of filtration rates in bivalves — the stony road to reliable data: review and interpretation. *Mar. Ecol. Prog. Ser.*, 211, 275–291, 2001.

Line 170-180: Please provide full FTIR spectra as a supplementary figure.

RESPONSE: We have now provided FTIR spectra in Fig. A2.

Lines 267-268: Can inferences be made regarding whether inner/outer layers were calcified if the polymorphs are specific to a layer of the polychaete shell?

RESPONSE: We cannot make this inference based on our results. As mentioned above, further investigation on structural properties is needed to answer this question, which is beyond the scope of this study.

Line 233-234: This is a strong statement. Regulation of Mg may be interpreted but the authors results do not signify that it is relaxed under hypoxia.

RESPONSE: We have toned down this statement (Ln 271-272).

Line 256-257: Please delete this final sentence. It is a very strong statement and the whole paragraph does not explain why hypoxia the key stressor in the future (which is debatable anyway).

RESPONSE: We have deleted this sentence.

Minor Comments:

Line 10-11: Sentence like this needs a reference.

RESPONSE: In the Abstract, citations should be avoided.

Line 25: change "shells" to skeletons.

RESPONSE: We have replaced "shells" with "shells or skeletons" (Ln 26).

Line 32: Delete "however".

RESPONSE: Suggestion adopted.

Line 368: Please provide full reference.

RESPONSE: Suggestion adopted (Ln 403).

Figure 5 (SEM): Are these images of the aragonite or calcitic parts of the shell? The legend needs more descriptive text. It is not obvious to me how these images indicate shell integrity.

RESPONSE: We cannot identify the type of carbonate mineral based on these images and this is beyond the scope of this imaging analysis. We have elaborated the figure legend to indicate shell integrity in terms of crystal thickness and density.

Table A1: Please include other calculated parameters such as HCO_3^- , CO_3^{2-} and CT.

RESPONSE: Suggestion adopted (Table A1).

References used for review:

Weiss et al (2002) Mollusc larval shell formation: amorphous calcium carbonate is a precursor phase for aragonite. DOI: 10.1002/jez.90004

Kristova et al (2015) The effect of the particle size on the fundamental vibrations of the $[\text{CO}_3^{2-}]$ anion in calcite. DOI: 10.1021/acs.jpca.5b02942.

Effects of hypoxia and non-lethal shell damage on shell mechanical and geochemical properties of a calcifying polychaete

Jonathan Y.S. Leung^{1,2}, Napo K.M. Cheung^{2,3}

¹School of Biological Sciences, The University of Adelaide, Adelaide, Australia

5 ²Department of Biology and Chemistry, City University of Hong Kong, Hong Kong SAR

³Department of Biological Sciences, Graduate School of Science, The University of Tokyo, Tokyo, Japan

Correspondence to: Jonathan Y.S. Leung (jonathan_0919@hotmail.com)

Abstract. Calcification is a vital biomineralization process where calcifying organisms construct their calcareous shells for protection. While this process is expected to deteriorate under hypoxia which reduces the metabolic energy yielded by aerobic respiration, some calcifying organisms were shown to maintain normal shell growth. The underlying mechanism remains largely unknown, but may be related to **changing shell mineralogical properties**, whereby shell growth is sustained at the expense of shell quality. Thus, we examined whether such plastic response is exhibited to alleviate the impact of hypoxia on calcification by assessing the shell growth and shell properties of a calcifying polychaete in two contexts (life-threatening and unthreatened conditions). Although hypoxia substantially reduced respiration rate (i.e. less metabolic energy), shell growth was only slightly hindered without weakening mechanical strength under unthreatened conditions. Unexpectedly, hypoxia did not undermine defence response (i.e. enhanced shell growth and mechanical strength) under life-threatening conditions, which may be attributed to the changes in mineralogical properties (e.g. increased calcite/aragonite) to reduce the energy demand for calcification. While more soluble shells (e.g. increased Mg/Ca in calcite) were produced under hypoxia as the trade-off, our findings suggest that **mineralogical** plasticity could be fundamental for calcifying organisms to maintain calcification under metabolic stress conditions.

1 Introduction

25 Calcification is a biomineralization process where many marine organisms, such as corals, molluscs, polychaetes and echinoderms, deposit carbonate minerals and form their calcareous shells **or skeletons**. This process is highly associated with the fitness and survival of calcifying organisms because shell growth not only allows continuous somatic growth, but also strengthens protection against physical and chemical damages. The protective role of shells is particularly important under life-threatening conditions (e.g. following non-lethal shell damage), where many calcifying organisms are able to produce stronger shells at a higher rate to **increase** physical protection (Cheung et al., 2004; Brookes and Rochette, 2007; Hirsch et al., 2014). Indeed, such inducible defence response via enhanced calcification plays an important role in the survival of calcifying organisms (Harvell, 1990).

In view of the accelerated anthropogenic emission of carbon dioxide, calcification and hence defence response of calcifying organisms may be dampened by climate change stressors, such as ocean acidification and hypoxia (Bijma et al., 2013). While ocean acidification was expected to retard calcification (Orr et al., 2005), it is now realized that calcification is not primarily driven by the pH and carbonate saturation state of seawater (Roleda et al., 2012), meaning that the impact of ocean acidification on calcifying organisms through the changes in seawater carbonate chemistry is less deleterious than previously thought (e.g. Garilli et al., 2015; Ramajo et al., 2016; Leung et al., 2017a,b). Indeed, calcification is an energy-dependent physiological process actively regulated by calcifying organisms (Roleda et al., 2012); therefore, this process is likely determined by the energetics of calcifying organisms. As such, hypoxia (i.e. dissolved oxygen concentration in seawater $\leq 2.8 \text{ mg O}_2 \text{ L}^{-1}$ or $\leq 63 \text{ } \mu\text{mol L}^{-1}$, Wu, 2002) can probably compromise calcification through its direct, adverse effect on aerobic metabolism and hence production of metabolic energy (Wu, 2002; Leung et al., 2013a). Since calcification is an energy-demanding process (Palmer, 1992), the impaired aerobic metabolism under hypoxia could be the underlying mechanism causing the reduced calcification as previously observed (e.g. Cheung et al., 2008; Findlay et al., 2009; Wijgerde et al., 2014). As the occurrence of hypoxia is predicted to become more prevalent in future marine ecosystems owing to ocean warming and human-induced eutrophication (Diaz and Rosenberg, 2008; Keeling et al., 2010; Bijma et al., 2013), the impact of hypoxia on calcifying organisms would be continuously escalated.

However, few previous studies showed that some calcifying organisms are able to maintain calcification under hypoxia (Mukherjee et al., 2013; Frieder et al., 2014; Keppel et al., 2016), and even anoxia (Nardelli et al., 2014). These unexpected results suggest potential mechanisms which can help compensate for the reduced metabolic energy under hypoxia in order to sustain calcification. This could be mediated by phenotypic plasticity, which involves trade-offs between phenotypic traits in response to altered conditions (Malausa et al., 2005). For example, shell growth may be maintained under hypoxia at the expense of shell quality or other physiological processes (e.g. soft tissue growth, reproduction and somatic maintenance) via energy trade-offs (Nisbet et al., 2012; Sokolova et al., 2012). Alternatively, energy demand for calcification may be reduced by changing geochemical properties of shells (e.g. reducing organic matter content in the shell) and thus favours shell growth when metabolic energy is reduced (Ramajo et al., 2015; Leung et al., 2017a). Whether calcifying organisms can exhibit such plastic response to alleviate the impact of hypoxia-induced metabolic depression on calcification and defence response remains largely unknown and deserves a comprehensive investigation.

In this study, we examined how hypoxia affects calcification and defence response of a common calcifying polychaete (*Hydroides diramphus*), which is tolerant to hypoxia (Vaquer-Sunyer and Duarte, 2008; Leung et al., 2013b). Calcification was indicated by shell growth, while defence response by both shell growth and fracture toughness. We analysed the mineralogical properties of shells (organic matter content, calcite to aragonite ratio, magnesium-to-calcium ratio in calcite and relative amorphous calcium carbonate content) to indicate the possible changes in calcifying mechanism in response to hypoxia. Respiration rate and feeding rate were measured to reflect aerobic metabolism and energy gain, respectively. Given the possible impact of hypoxia on aerobic metabolism, we hypothesized that (1) the mineralogical properties of newly-produced shells would be modified to reduce the energy

70 demand for calcification so that shell growth can be sustained; (2) defence response would be undermined as the reduced metabolic energy is possibly insufficient to enhance both shell growth and fracture toughness. If **changing mineralogical properties of shells** can help alleviate the impact of hypoxia on calcification and even defence response without causing significant adverse effects by trade-offs, this suggests that some calcifying organisms would be more robust to metabolic stress conditions than previously thought.

75 2 Materials and methods

2.1 Collection and maintenance of specimens

A calcifying polychaete *Hydroides diramphus* was selected as the study species, which lives on hard substrate and is widely distributed within circumtropical regions (Çinar, 2006). **Adult** polychaetes (**tube length: 35 – 45 mm**) were collected from a fish farm at Yung Shue O (22°25'N, 114°16'E), Hong Kong, in summer when hypoxia is commonly observed (Leung et al., 2013a). Other fouling organisms on the calcareous tube of *H. diramphus*, such as mussels and tunicates, were carefully removed. Then, the polychaetes were temporarily reared in plastic tanks (50 cm × 40 cm × 30 cm) filled with natural seawater under laboratory conditions (dissolved oxygen concentration: $6.00 \pm 0.10 \text{ mg O}_2 \text{ L}^{-1}$, pH: 8.10 ± 0.05 , temperature: $28.0 \pm 1.0^\circ\text{C}$ and salinity: $33.0 \pm 0.5 \text{ psu}$). Algal suspension containing **live** *Isochrysis galbana* and *Dunaliella tertiolecta* (1:1, v/v) was daily provided as food. The polychaetes were allowed to acclimate under these laboratory conditions for one week before experimentation.

2.2 Experimental design and rearing method

The impact of hypoxia on the calcification and defence response of adult *H. diramphus* was examined using a full factorial experimental design, involving two dissolved oxygen levels (normoxia vs. hypoxia) and two contexts (unthreatened vs. threatened). Thus, there were four treatment conditions based on their crossed combinations: (1) normoxia and unthreatened, (2) normoxia and threatened, (3) hypoxia and unthreatened, and (4) hypoxia and threatened. Normoxia ($\sim 6.0 \text{ mg O}_2 \text{ L}^{-1}$, i.e. control) and hypoxia ($\sim 2.0 \text{ mg O}_2 \text{ L}^{-1}$) were achieved by continuously aerating seawater with air and a mixture of nitrogen and air, respectively (Leung et al., 2013b). Digital flow meters (Vögtlin Instruments, Switzerland) were used to adjust the flow rate of each gas (i.e. nitrogen and air) so that the desired dissolved oxygen concentration for hypoxia was maintained. To induce life-threatening condition for the polychaetes, non-lethal shell damage was made by carefully trimming the calcareous tube until the radioles were exposed, while the body was still fully covered. The polychaetes with “intact” (tube length: $\sim 40 \text{ mm}$; body length: $\sim 20 \text{ mm}$) and “damaged” (tube length: $\sim 20 \text{ mm}$; body length: $\sim 20 \text{ mm}$) tubes were then allowed to acclimate under either normoxia or hypoxia for another week before experimentation, **which can particularly help the “damaged” polychaetes to recover from the stress induced by tube trimming (i.e. fight-or-flight response) so that they were only subject to the stress induced by non-lethal shell damage in the following experiments.**

A total of 120 adult polychaetes were evenly and randomly assigned to each of the four treatment conditions (i.e. $n = 30$ polychaetes per treatment). The rearing method for the polychaetes was previously described (Leung and Cheung, 2017). Briefly, polychaetes with their initial tube length measured (see the section below) were individually transferred into 2-mL labelled microcentrifuge tubes with the radioles pointing upward. A small hole (~2 mm) was drilled at the bottom of each microcentrifuge tube to allow water exchange. The microcentrifuge tubes were glued together by hot-melt adhesives to maintain an upright position and put into a lidded glass bottle (10 polychaetes per bottle; 3 bottles per treatment) containing 180 mL filtered seawater (FSW) (pore size: 0.45 μm). Bottles assigned to the same dissolved oxygen level (i.e. normoxia or hypoxia) were connected to the same gas inlet and had the target dissolved oxygen concentration manipulated as described above. Stable equilibrium between gases in seawater was achieved rapidly by this constant aeration (< 5 min) and thus the target dissolved oxygen concentration in seawater, which was daily recorded using an optical dissolved oxygen probe (SOO-100, TauTheta Instruments, USA), was very stable over time (Fig. A1). To simulate the summer seawater temperature at the collection site, the whole setup was incubated in a water bath with temperature maintained at 28°C using a heating bath circulator. The polychaetes were reared under a day/light cycle of 14:10 h. Algal suspension (20 mL) containing live *I. galbana* and *D. tertiolecta* (1:1, v/v) at $\sim 1 \times 10^6$ cells mL^{-1} was provided daily as food to ensure adequate food supply for normal shell growth. The microcentrifuge tubes were cleaned and the seawater was gently renewed once every three days to prevent accumulation of excreted waste. The exposure lasted for 3 weeks, excluding the initial acclimation period. After the 3-week exposure period, only 4 out of 120 polychaetes died across treatments (2 from “Intact, Normoxia” and 2 from “Damaged, Hypoxia”), meaning that the treatment conditions *per se* did not cause fatality.

2.3 Shell growth

Shell growth was indicated by the increase in tube length over time, where the newly-produced shells can be easily identified by the difference in colour from the original shells (Fig. 1). The tube length of all individuals was measured on Day 1, Day 11 and Day 21 to estimate shell growth ($n = 30$ polychaetes per treatment). During the tube length measurement, a polychaete was temporarily placed in a Petri dish (diameter: 90 mm) filled with seawater at their respective dissolved oxygen concentration to avoid potential desiccation. Tube length was measured under a dissecting microscope with a scale to the nearest 0.1 mm, followed by putting the polychaete back to the respective glass bottle immediately (< 30 s for each measurement). Since tube growth can be measured with sufficient accuracy and precision under the dissecting microscope, the tube growth of each individual was analysed as a replicate.

2.4 Physiological performance

Following the 3-week exposure period, the respiration rate and feeding rate of polychaetes were measured using the method described in Leung et al. (2013a) with minor modifications. Briefly, 25 individuals from the same treatment were randomly sampled and evenly transferred into five airtight polypropylene syringes (Terumo® hypodermic syringe without needle, Terumo Corporation, Japan) each containing ~35 mL FSW with dissolved oxygen

concentration adjusted to the corresponding treatment level ($n = 5$ replicate syringes per treatment). They were allowed to rest in the syringe for 15 min. Then, the initial dissolved oxygen concentration of FSW was measured using an optical dissolved oxygen probe (SOO-100, TauTheta Instruments, USA), calibrated according to the manual of manufacturer. The atmospheric air inside the syringe, which helps buffer the change in dissolved oxygen concentration during the resting period, was then fully expelled and the tip of the syringe was sealed by Blu Tack to ensure an airtight condition. After one hour, the final dissolved oxygen concentration of FSW was recorded when it becomes steady by gently stirring the FSW inside the syringe. Blank samples without individuals were prepared to correct the background change in dissolved oxygen concentration, which fluctuated less than 1%. Respiration rate was expressed as $\mu\text{g O}_2 \text{ ind}^{-1} \text{ hr}^{-1}$.

To measure feeding rate, we determined the decrease in concentration of microalgae in a given period of time (i.e. clearance rate), as previously described (Riisgård, 2001; Contreras et al., 2012; Leung et al., 2013a; Leung and Cheung, 2017). For each treatment, 25 randomly selected individuals, which had been starved for one day to standardize their hunger level, were put into five glass vials (i.e. $n = 5$ replicate glass vials per treatment) each containing 80 mL FSW with an initial concentration of $\sim 1 \times 10^6 \text{ cell mL}^{-1}$ live *D. tertiolecta*. After feeding for one hour under light conditions, 1 mL seawater was taken from the bottle and the microalgae were enumerated using a haemocytometer (6 trials per bottle). Prior to counting, 1% Lugol's solution was used to fix the microalgae. Clearance rate was calculated using the following formula to represent feeding rate (Coughlan, 1969):

$$CR = \frac{V}{nt} \times \ln \frac{C_o}{C_t}$$

where CR is the clearance rate ($\text{mL ind}^{-1} \text{ hr}^{-1}$); V is the volume of seawater; n is the number of individuals; t is the feeding time; C_o and C_t are the initial and final concentrations of microalgae, respectively.

2.5 Shell properties

After measuring respiration rate and feeding rate, the newly-produced shells for the analyses of mechanical and geochemical properties were carefully removed using a pair of forceps and then rinsed with deionized water to remove the microalgae and other debris on the shell surface.

Fracture toughness was measured using a micro-hardness tester (Fischerscope HM2000, Fischer, Germany) to indicate mechanical strength. For each treatment, five shell fragments from five randomly selected individuals were mounted firmly onto a metal disc with the inner shell surface facing upwards using cyanoacrylate adhesives ($n = 5$ fragments per treatment). Then, the fragment was indented by a Vickers 4-sided diamond pyramid indenter for 10 s in the loading phase (Peak load: 300 mN; Creep: 2 s). In the unloading phase, the load decreased at the same rate as the loading phase until the loading force became zero. At least five random locations on each fragment were indented. Vickers hardness (H) and elastic modulus (E) were calculated based on the load-displacement curve using software WIN-HCU (Fischer, Germany). Vickers hardness to elastic modulus ratio (H/E) was calculated to indicate the fracture

170 toughness of shells (Marshall et al., 1982). Organic matter content of the newly-produced shells collected from another
five individuals was determined by mass loss upon ignition at 550°C in a muffle furnace for six hours ($n = 5$ replicates
per treatment).

Given the limited amount of newly-produced shells, shells from three to five individuals from the same
treatment was powdered to make one composite shell powder sample as a replicate for the analyses of the following
175 geochemical properties. Shell powder was prepared by removing the newly-produced shells using a pair of forceps,
rinsing them with deionized water to remove the microalgae and other debris, drying them at room temperature and
finally grinding them into powder (particle size: $\sim 5 \mu\text{m}$) using a mortar and pestle. Carbonate polymorphs were
analysed using an X-ray diffractometer (D4 ENDEAVOR, Bruker, Germany). A small quantity of shell powder was
transferred onto a tailor-made sample holder and then scanned by Co K α radiation (35 kV and 30 mA) from 20° to
180 70° 2 θ with step size of 0.018° and step time of 1 s ($n = 3$ replicates per treatment). Carbonate polymorphs were
identified based on the X-ray diffraction spectrum using the EVA XRD analysis software (Bruker, Germany). Calcite
to aragonite ratio was calculated using the following equation (Kontoyannis and Vagenas, 2000):

$$\frac{I_C^{104}}{I_A^{221}} = 3.157 \times \frac{X_C}{X_A}$$

where I_C^{104} and I_A^{221} are intensity of the calcite 104 peak (34.4° 2 θ) and aragonite 221 peak (54.0° 2 θ), respectively;
185 X_C/X_A is the calcite to aragonite ratio.

Magnesium to calcium ratio was determined by energy dispersive X-ray spectroscopy under the Philips XL30
field emission scanning electron microscope (Ries, 2004; Zhang et al., 2010; Leung et al., 2017b). A small quantity of
shell powder was transferred onto a stub and coated by carbon ($n = 3$ replicates per treatment; 3 trials per replicate).
The shell powder was irradiated by an electron beam with an accelerating voltage of 12 kV to obtain the energy
190 spectrum with background correction. Elements were identified and magnesium to calcium ratio was calculated using
software Genesis Spectrum SEM Quant ZAF (EDAX, USA). To determine relative amorphous calcium carbonate
(ACC) content, 1 mg shell powder was mixed with 10 mg potassium bromide, followed by compressing the mixture
into a disc (diameter: 13 mm) using a manual hydraulic press ($n = 3$ replicates per treatment) (Chan et al., 2012). An
infrared absorption spectrum ranging from 600 cm^{-1} to 1800 cm^{-1} with background calibration for the baseline was
195 obtained using a Fourier transform infrared spectrometer (Avatar 370 DTGS, Nicolet, USA). The relative ACC content
was estimated as the intensity ratio of the peak at 856 cm^{-1} to that at 713 cm^{-1} (Beniash et al., 1997).

2.6 Statistical analysis

Two-way permutational analysis of variance (PERMANOVA) was applied (number of permutations: 999;
200 Euclidean distance calculated) to test the effects of hypoxia and non-lethal shell damage on the shell growth, fracture
toughness, organic matter content, calcite to aragonite ratio, magnesium to calcium ratio, relative ACC content,
respiration rate and clearance rate using software PRIMER 6 with PERMANOVA+ add-on (Anderson, 2001).

3 Results

205 *H. diramphus* had continuous shell growth throughout the 3-week exposure period, but the growth was faster after non-lethal shell damage (Fig. 2, Table A2). Hypoxia slightly **but significantly** hindered shell growth in both contexts. The fracture toughness of newly-produced shells was enhanced after non-lethal shell damage (c.f. control), while hypoxia had **no significant** effect (Fig. 3, Table A2). As for the geochemical properties of newly-produced shells, organic matter content was elevated after non-lethal shell damage, whereas the effect of hypoxia was indiscernible (Fig. 4a, Table A2). Calcite was the dominant carbonate polymorph and its proportion increased under hypoxia (Fig. 210 4b, Table A2). *H. diramphus* produced high-Mg calcite (i.e. Mg/Ca > 0.04) and the magnesium content in calcite increased under hypoxia (Fig. 4c, Table A2). The relative ACC content was slightly elevated under hypoxia, meaning that less crystalline shells were produced (Fig. 4d, Table A2, **Fig. A2 for the IR spectra**). Calcite/Aragonite, Mg/Ca in calcite and relative ACC content were not significantly affected by non-lethal shell damage. Respiration rate was not only reduced by hypoxia, but also slightly **and significantly** by non-lethal shell damage (Fig. 5a, Table A2). Clearance rate decreased **significantly** not only under hypoxia, but also after non-lethal shell damage under normoxia (Fig. 5b, 215 Table A2).

4 Discussion

220 Hypoxia is expected to diminish the fitness and survival of marine organisms, probably leading to serious ramifications on marine ecosystems, **such as changes in species populations, community structure and ecosystem functioning** (Wu, 2002; Diaz and Rosenberg, 2008). Nevertheless, many less mobile marine organisms (e.g. molluscs, polychaetes and echinoderms) are generally tolerant to hypoxia **in the short term** (Vaquer-Sunyer and Duarte, 2008), suggesting their potential capacity to accommodate its impacts. Despite the substantial reduction in respiration rate and feeding rate under hypoxia, we found that calcification and defence response of a calcifying polychaete were 225 generally maintained, which could be associated with **mineralogical plasticity, such as increased calcite to aragonite ratio and magnesium to calcium ratio**.

230 Since energy demand for calcification is enormous (Palmer, 1992), the reduction in energy gain by feeding and energy production by aerobic respiration under hypoxia would undermine both quality and quantity of shells produced by calcifying organisms (Cheung et al., 2008; Wijgerde et al., 2014). Under unthreatened conditions (i.e. without shell damage), we found that hypoxia slightly **but significantly** hinders the shell growth of *H. diramphus*. However, hypoxia did not affect the fracture toughness (i.e. mechanical strength) of newly-produced shells. The retarded shell growth under hypoxia could be pertinent to the reduced feeding rate, and hence energy reserves for calcification. While energy gain by feeding is suggested to be fundamental for shell growth (Melzner et al., 2011; Thomsen et al., 2013; Leung et al., 2017a), aerobic respiration is necessary to efficiently convert energy reserves into 235 metabolic energy for various biological processes, including calcification. As such, the retarded shell growth is more likely ascribed to the hypoxia-induced metabolic depression, which reduces the amount of metabolic energy allocated to calcification. The quantity of organic matter (e.g. matrix proteins) occluded in the shell is the key factor affecting

mechanical strength (Weiner and Addadi, 1997; Addadi et al., 2006; Marin et al., 2008). Since the organic matter content was not affected by hypoxia, mechanical strength can be maintained. Our results imply that similar amount of metabolic energy is allocated to the production of organic matter for the shell, while less to inorganic components (i.e. calcium carbonate) for shell growth under hypoxia. This strategy (i.e. shell quality over shell quantity) is favourable under energy-limiting conditions because there is no exigency to expedite shell growth when risk is not imminent and the shell can already offer sufficient protection.

Under life-threatening conditions (i.e. following non-lethal shell damage), *H. diramphus* exhibited defence response, indicated by the production of tougher shells at a higher rate. As *H. diramphus* is sessile, enhancing the protective function of shells is probably the most effective defence response. Therefore, more organic matter was produced and occluded in the newly-produced shell to augment mechanical strength. Additionally, the carbonate crystals in the shell appeared to be more compacted (Fig. 6), which could also strengthen the shell. Such inducible defence response is commonly exhibited by calcifying organisms because shell repair should be prioritized to restore and enhance protection (Cheung et al., 2004; Hirsch et al., 2013; Brom et al., 2015). However, trade-offs are involved to activate defence response, such as reduction in the less essential biological processes or activities (Rundle and Brönmark, 2001; Trussell and Nicklin, 2002; Hoverman and Relyea, 2009; Babarro et al., 2016). For example, Brookes and Rochette (2007) showed that the calcification rate of a grazing gastropod is promoted under predation risk at the expense of grazing activity and somatic growth. Here, similar trade-offs were observed in *H. diramphus* (i.e. enhanced shell growth against reduced feeding rate). Indeed, when animals are under life-threatening conditions and the chance of survival becomes very low, they have to prioritize defence response (e.g. production of stronger shells for calcifying organisms) as the last resort to maximize survival rate (Bourdeau, 2009).

We expected that defence response would deteriorate under hypoxia in view of the substantial energy demand for shell production. Contrary to this prediction, *H. diramphus* can still produce tougher shells at a higher rate (c.f. Intact), meaning that the effect of hypoxia on defence response is mild considering the slight impact on the shell growth. This unexpected finding not only reveals the strong tolerance of *H. diramphus* to hypoxia, but also suggests potential mechanisms that enable efficient calcification under hypoxia despite the reduced metabolic energy. We propose that changing mineralogical properties could help compensate for the reduced metabolic energy in order to sustain defence response. In fact, the mineralogical properties of *H. diramphus* were altered consistently in response to hypoxia, irrespective of context. For instance, hypoxia resulted in a greater proportion of calcite in the shell. When metabolic energy is reduced, precipitation of calcite is favourable because it requires less metabolic energy and allows faster shell growth than that of aragonite (Weiner and Addadi, 1997; Hautman, 2006; Ries, 2011). In addition, we found that more magnesium ions were incorporated into the newly-produced shell under hypoxia. It is evident that the incorporation of magnesium ions into calcite is actively regulated through various biological mechanisms, such as active extrusion of excess magnesium ions at the calcification site (Bentov and Erez, 2006). The elevated Mg/Ca in calcite under hypoxia may suggest that the energy-requiring regulation of magnesium ions is reduced to conserve energy, which warrants further investigation. Furthermore, crystallization of amorphous calcium carbonate was slightly reduced by hypoxia, indicated by the higher relative ACC content. Since crystallization requires metabolic

energy for the transport of carbonate ions (Addadi et al., 2006; Weiner and Addadi, 2011), our results suggest that metabolic energy allocated to crystallographic control also decreased. Given the aforementioned changes in mineralogical properties, the energy cost for sustaining shell growth could be lessened. Such plastic response, also shown in other calcifying organisms under metabolic stress conditions (Ramajo et al., 2015; Leung et al., 2017a), may explain why the defence response of *H. diramphus* can generally be maintained under mild hypoxia in the short term.

Despite the benefit of changing mineralogical properties as the plastic response, trade-offs against other phenotypic traits are inevitably incurred (Malausa et al., 2005; Leung et al., 2013b). For instance, shell solubility increases due to the higher relative ACC content and Mg/Ca in calcite (Fernandez-Diaz, 1996; Ries, 2011; Fitzer et al., 2014). In other words, while the changes in mineralogical properties may allow sustained shell growth and mechanical strength under hypoxia, the chemical stability of shells may be weakened. Nevertheless, our results suggest that the benefit of defence response probably outweighs the cost of this trade-off under life-threatening conditions.

Based on the present findings, we support the paradigm that calcification is mainly driven by the physiology of calcifying organisms rather than the seawater carbonate chemistry (Pörtner, 2008; Roleda et al., 2012). For example, the shell growth of *H. diramphus* decreased when the carbonate saturation state slightly increased under hypoxia. This is contradictory to the paradigm that calcification generally increases with carbonate saturation state, *vice versa* (Orr et al., 2005). Indeed, most calcifying organisms do not directly utilize carbonate ions, but bicarbonate ions, as the substrate for calcification, meaning that formation of calcareous shells is not a chemical reaction between calcium and carbonate ions (Pörtner, 2008; Roleda et al., 2012; Bach, 2015). This concept based on physiology explains why many calcifying organisms can maintain or even enhance calcification when carbonate saturation state is reduced (e.g. Ries et al., 2009; Garilli et al., 2015; Ramajo et al., 2016; Leung et al., 2017a).

Hypoxia can last for a long period of time (e.g. month) as observed in many coastal and marine open waters worldwide (Helly and Levin, 2004; Diaz and Rosenberg, 2008), and is predicted to be more prevalent in future due to ocean warming and human-induced eutrophication (Bijma et al., 2013). In order to maintain populations under hypoxia, calcifying organisms have to counter its impact on calcification. Despite the impaired aerobic metabolism, this study revealed that hypoxia only mildly hampers the shell growth of a calcifying polychaete, whereas its defence response (i.e. harder shells produced at a higher rate) can be sustained in the short term. This is likely mediated by modifying mineralogical properties of shells to reduce the energy demand for calcification. While some potential trade-offs are incurred, such plastic response could be the cornerstone of calcifying organisms to acclimate to metabolic stress conditions, and hence sustain their populations and ecological functions in coastal and marine ecosystems.

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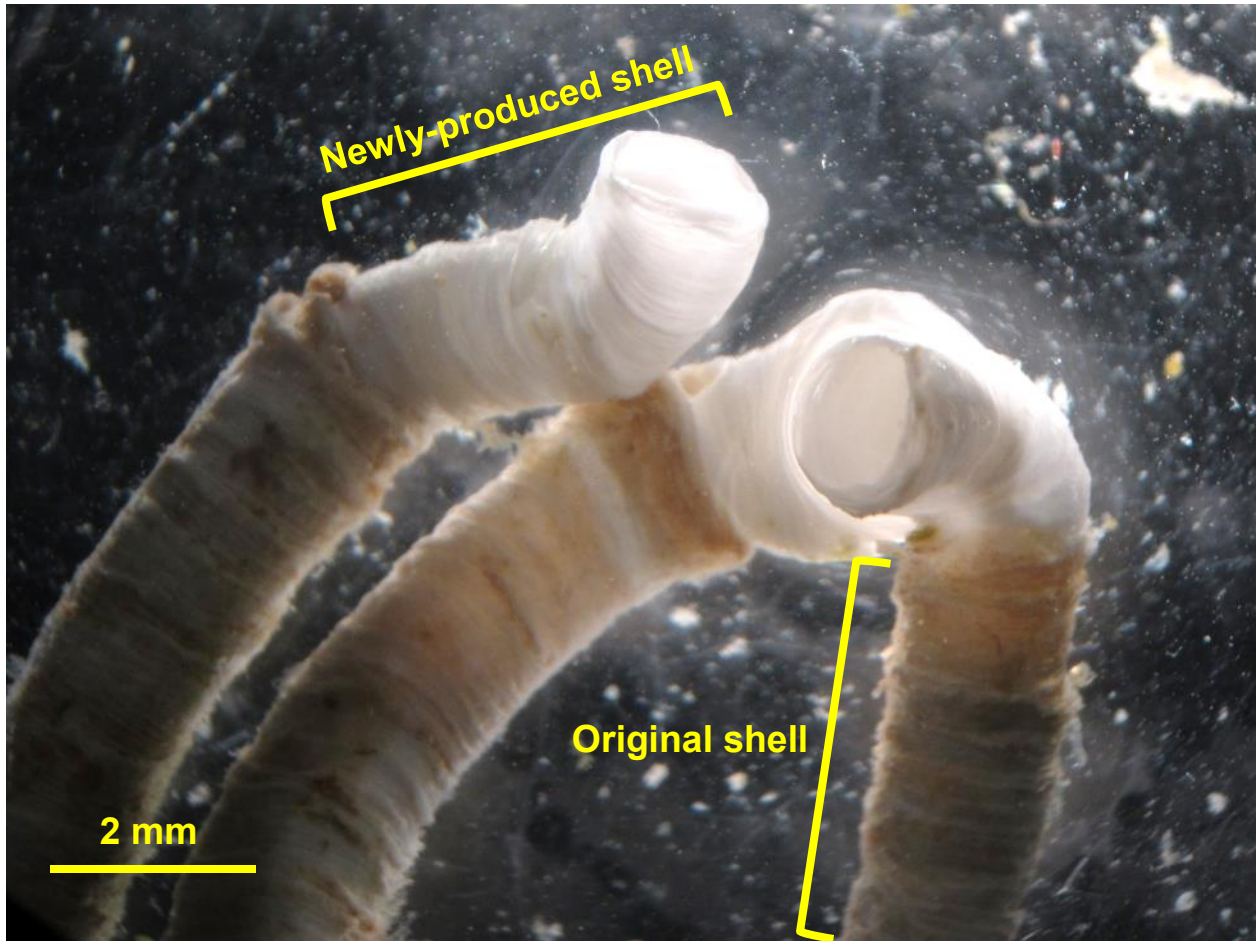
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Figure 1 A micrograph showing the newly-produced shell and original shell of *H. diramphus*, where the former is easily distinguished from the latter by the white colour. The original shell appears slightly coloured due to the biofilm (e.g. bacteria, algae, etc.) growing on the surface in the field.

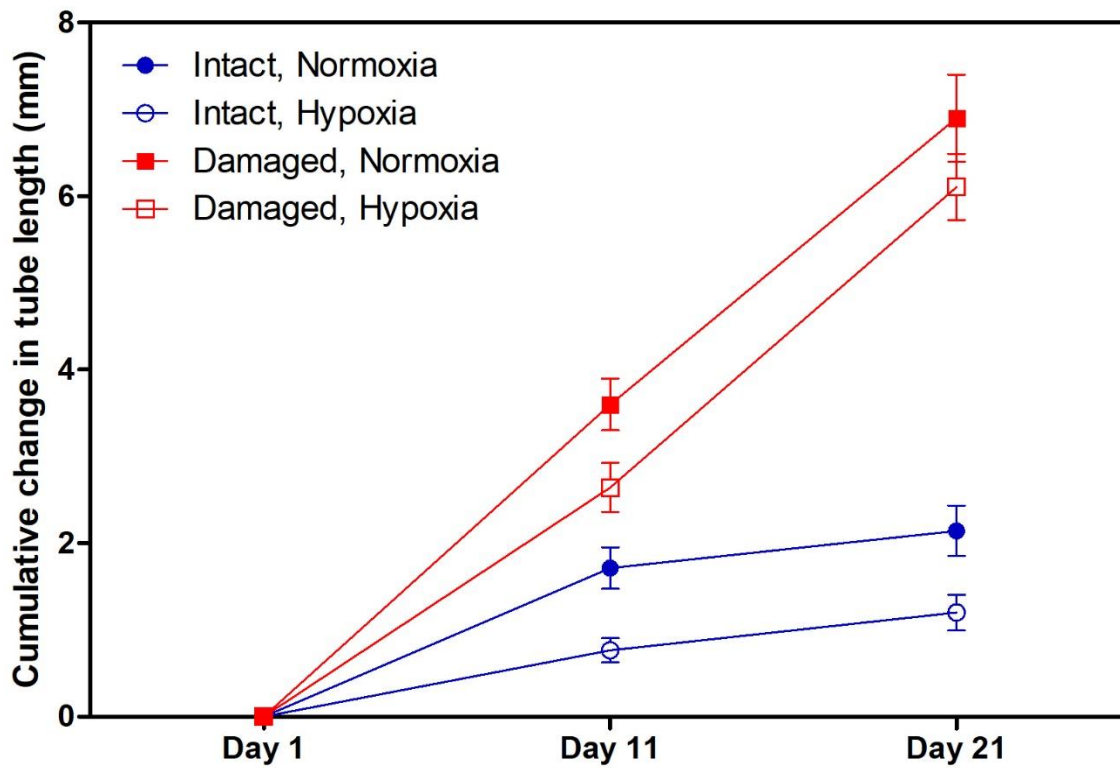


Figure 2 Cumulative change in the tube length of *H. diramphus* in different treatments across the 3-week exposure period (mean \pm S.E.; $n = 30$ for “Intact, Hypoxia” and “Damaged, Normoxia”; $n = 28$ for “Intact, Normoxia” and “Damaged, Hypoxia” due to the mortality).

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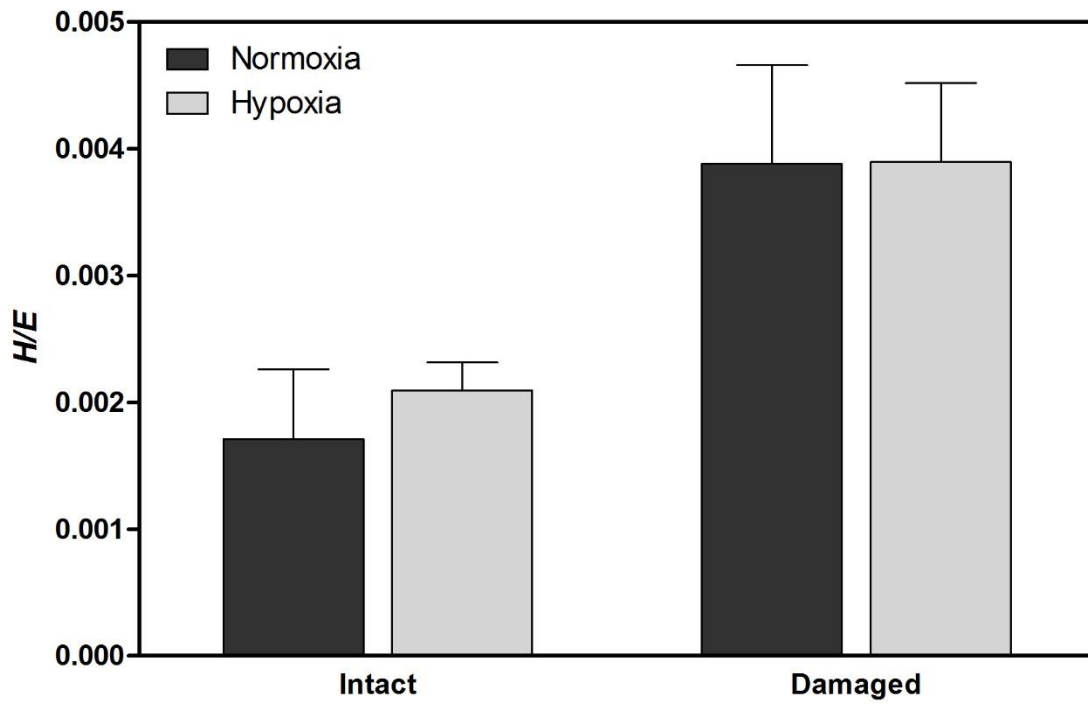


Figure 3 Vickers hardness to elastic modulus ratio (H/E), indicating fracture toughness, of *H. diramphus* shells produced in different treatments (mean + S.E.; $n = 5$).

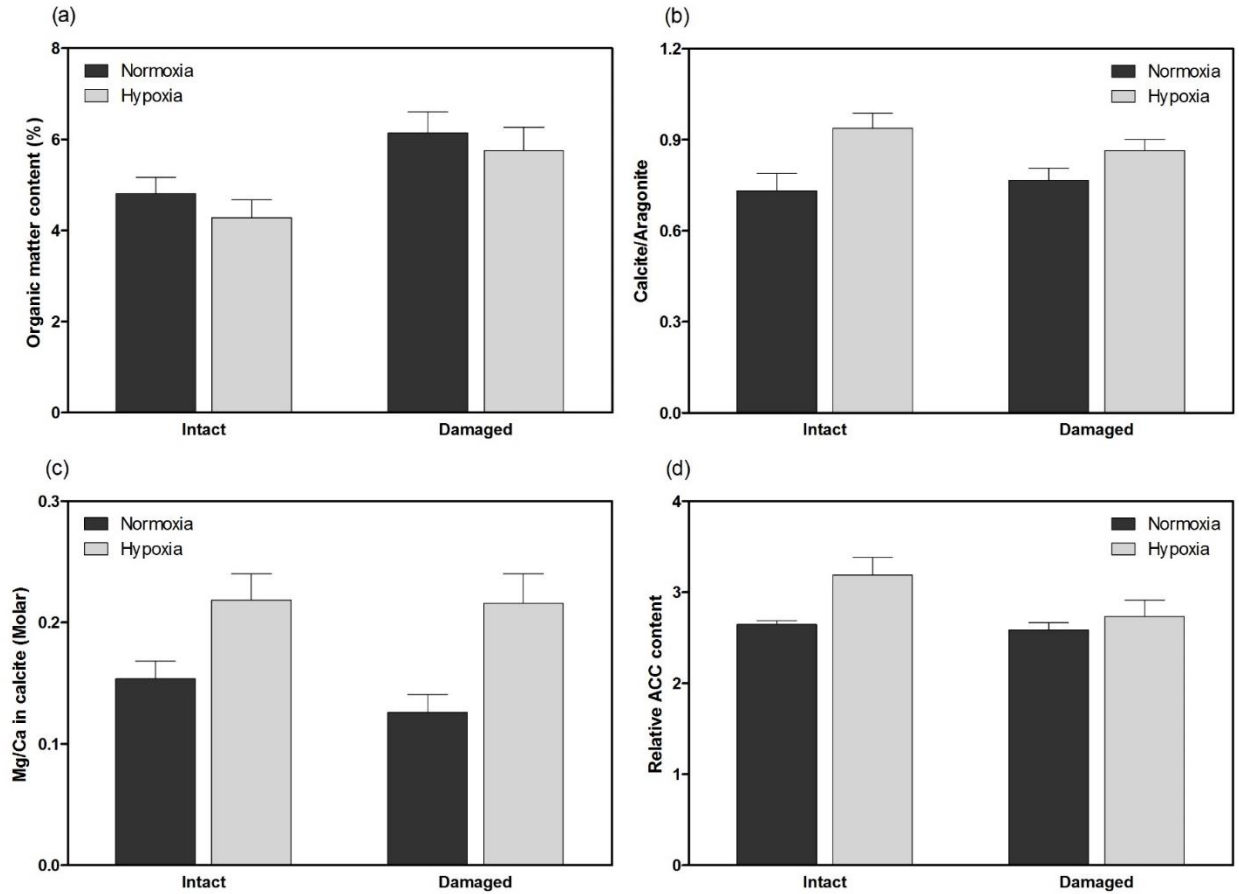


Figure 4 Geochemical properties of *H. diramphus* shells, including (a) organic matter content, (b) calcite to aragonite ratio, (c) magnesium to calcium ratio in calcite and (d) relative amorphous calcium carbonate content, in different treatments (mean + S.E.; $n = 3$, except $n = 5$ for organic matter content).

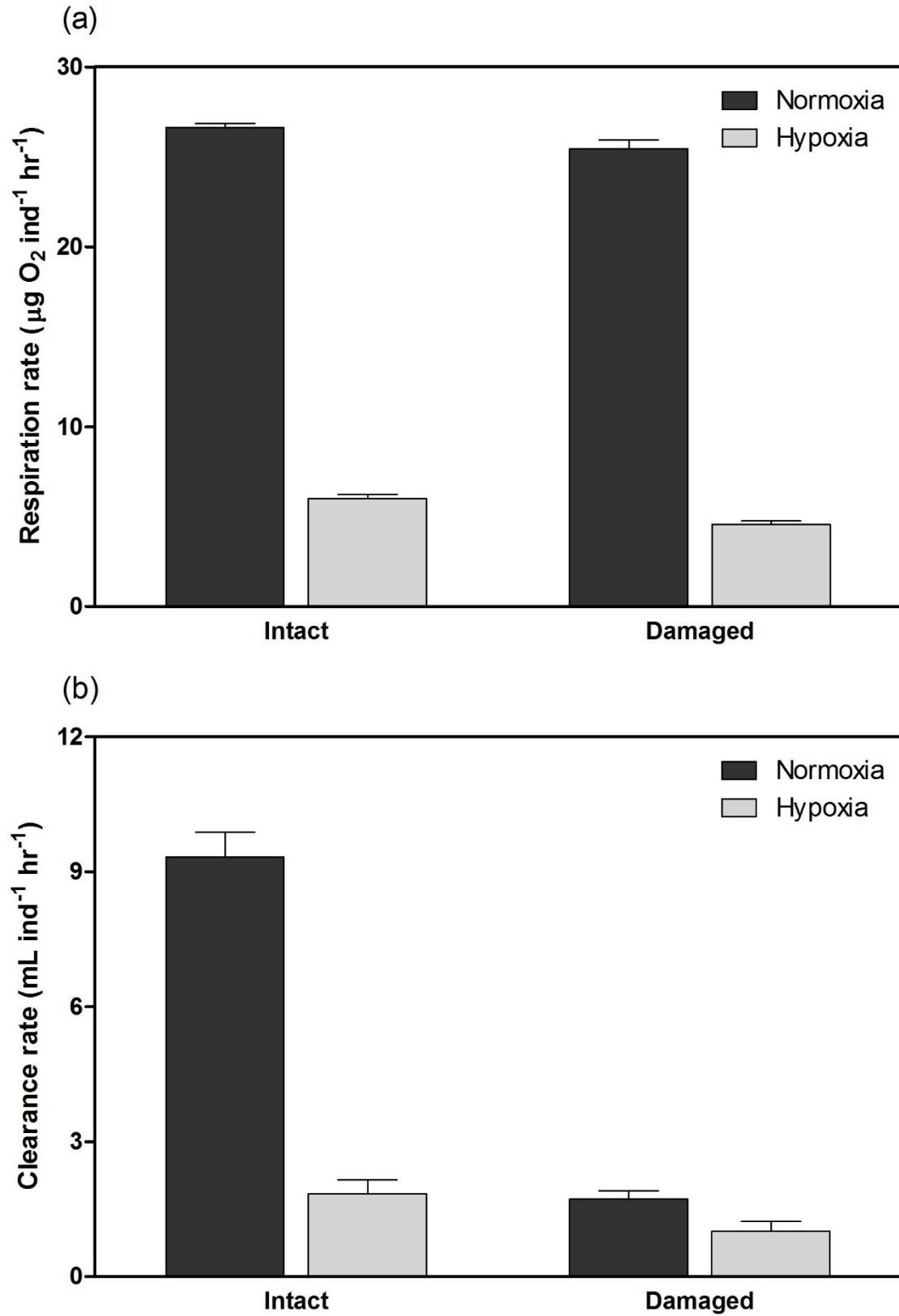


Figure 5 (a) Respiration rate and (b) clearance rate of *H. diramphus* in different treatments (mean + S.E.; $n = 5$).

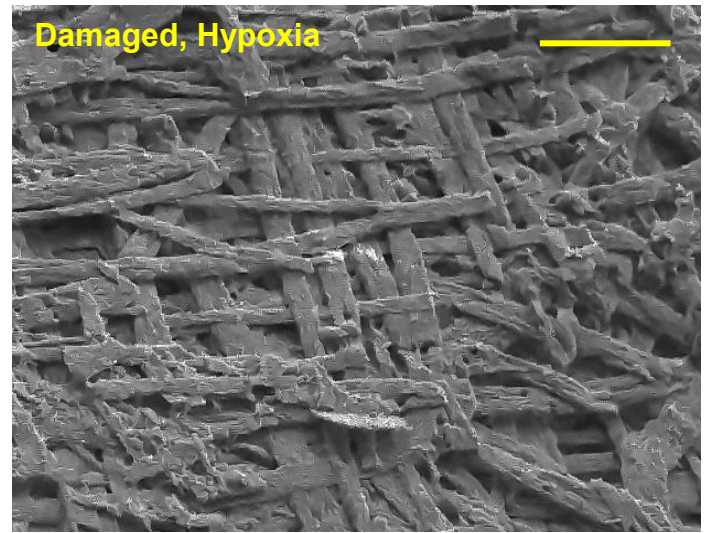
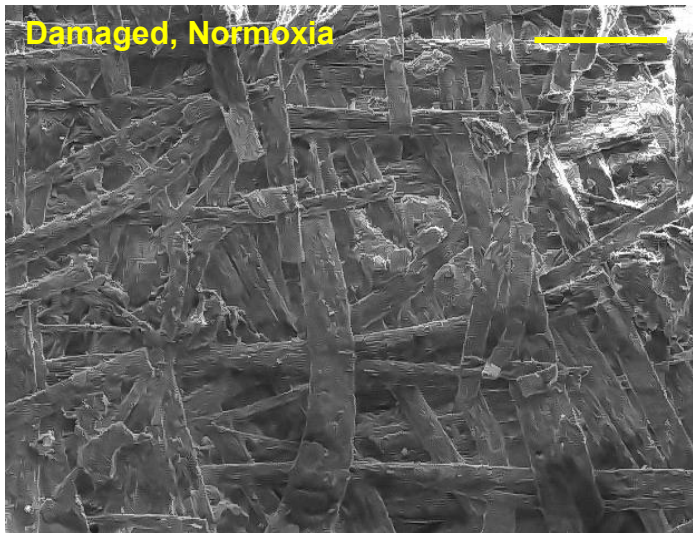
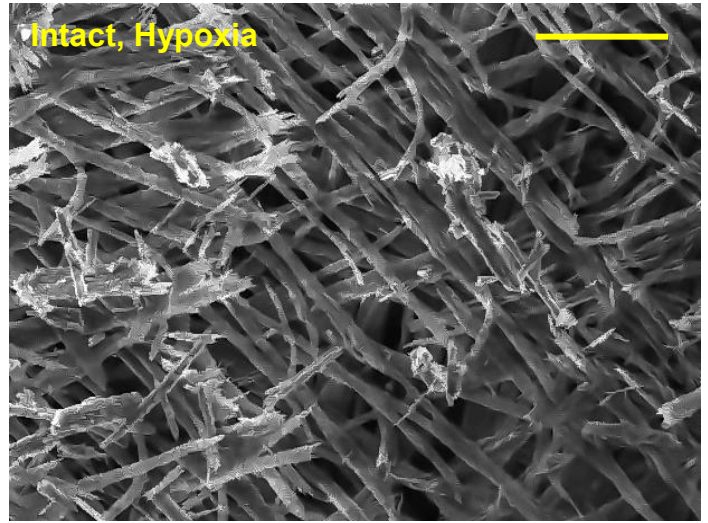
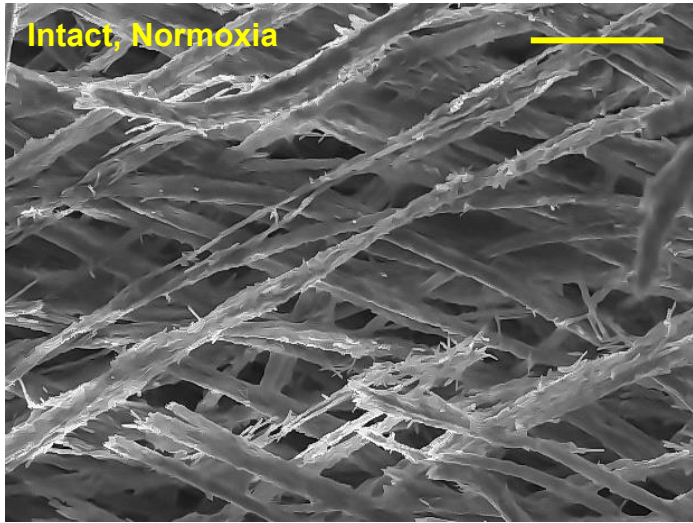


Figure 6 SEM images of the inner surface of *H. diramphus* shells produced in different treatments, indicating the shell integrity. The carbonate crystals of newly-produced shells appear to be thicker and more compact following non-lethal shell damage, regardless of the dissolved oxygen level. Scale bar: 20 μm .

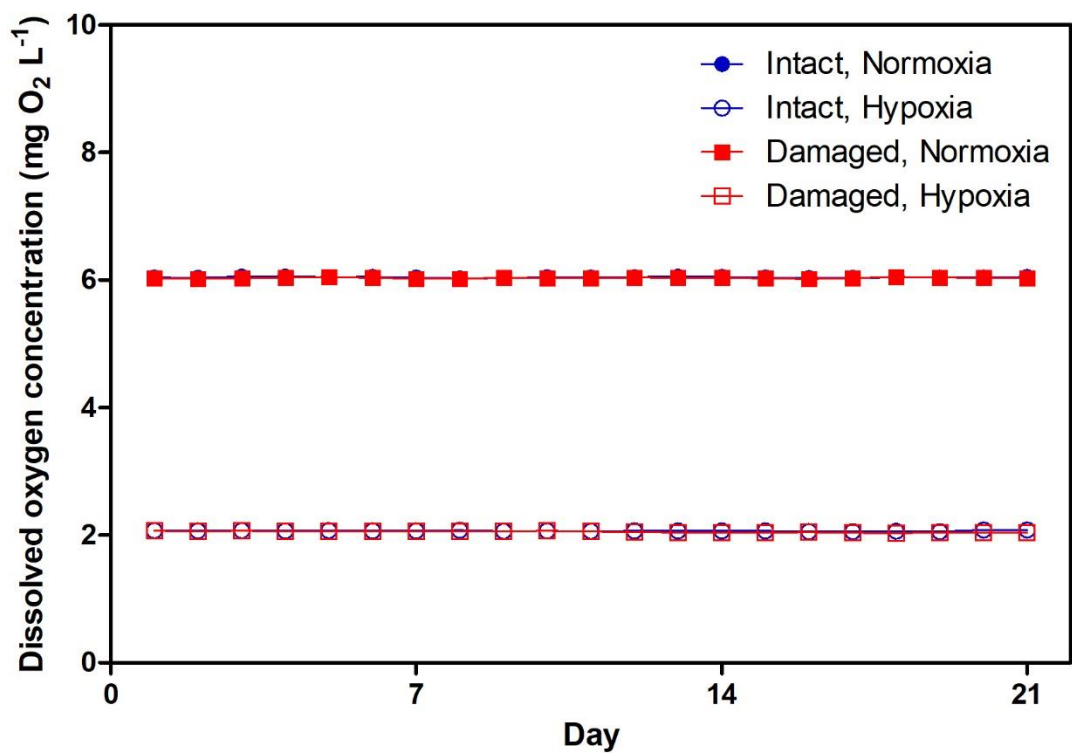


Figure A1 Dissolved oxygen concentration of seawater in different treatments across the 3-week experimental period (mean \pm S.D., $n = 3$).

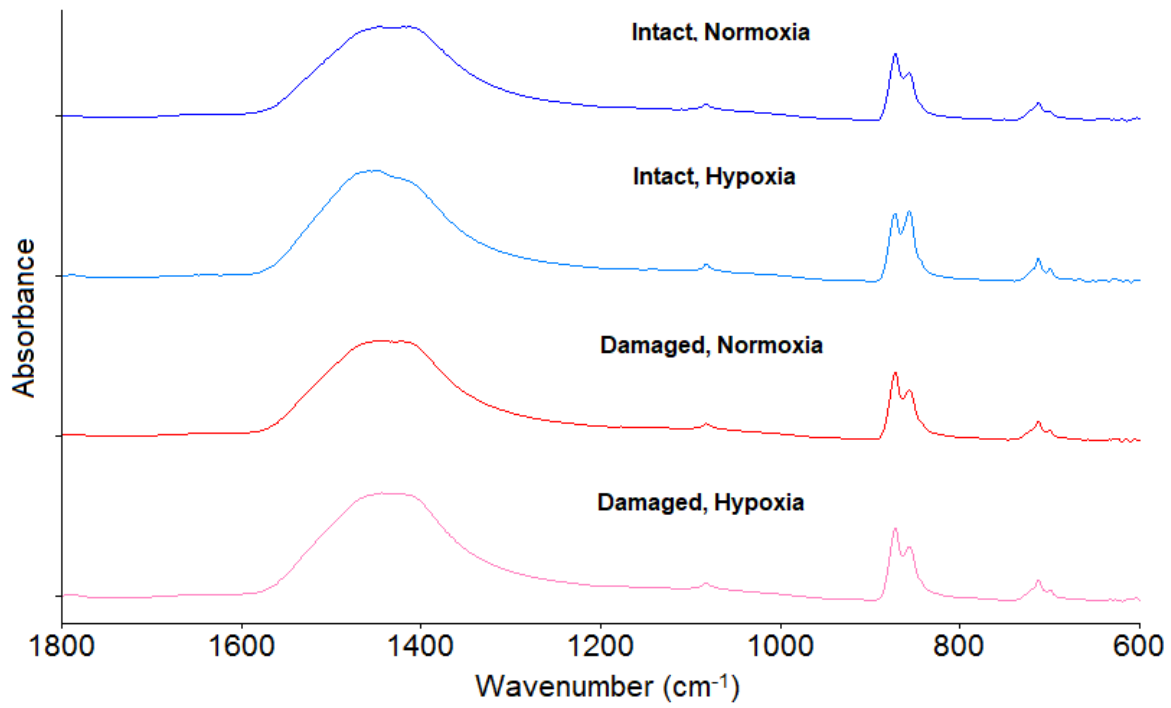


Figure A2 Infrared spectra for the newly-produced shells of *H. diramphus* growing under different treatment conditions.

Table A1 The seawater parameters under different treatment conditions throughout the exposure period (mean \pm S.D.). Dissolved oxygen concentration was daily measured using an optical dissolved oxygen probe (SOO-100, TauTheta Instruments, USA). pH was daily measured using a pH meter (HI 9025, HANNA Instruments, USA). Temperature and salinity were daily measured using a thermometer and refractometer, respectively. Total alkalinity was weekly measured using a titrator (HI 84431, HANNA Instruments, Germany). Saturation states (Ω) of calcite and aragonite were calculated using the CO2SYS program (Pierrot et al., 2006), with dissociation constants from Mehrbach et al. (1973) refitted by Dickson and Millero (1987).

	Intact, Normoxia	Intact, Hypoxia	Damaged, Normoxia	Damaged, Hypoxia
<u>Measured parameters</u>				
Dissolved oxygen (mg O ₂ L ⁻¹)	6.04 \pm 0.02	2.07 \pm 0.03	6.03 \pm 0.01	2.05 \pm 0.03
pH (NBS scale)	8.10 \pm 0.05	8.26 \pm 0.04	8.09 \pm 0.05	8.26 \pm 0.04
Temperature (°C)	28.2 \pm 0.08	28.2 \pm 0.09	28.2 \pm 0.08	28.2 \pm 0.10
Salinity (psu)	32.9 \pm 0.35	33.0 \pm 0.25	33.0 \pm 0.42	33.1 \pm 0.25
Total alkalinity (μ mol kg ⁻¹)	2241 \pm 8.96	2231 \pm 12.2	2241 \pm 9.11	2243 \pm 9.44
<u>Calculated parameters</u>				
CT (μ mol kg ⁻¹)	1984 \pm 26.1	1885 \pm 27.9	1988 \pm 29.0	1895 \pm 24.3
HCO ₃ ⁻ (μ mol kg ⁻¹)	1784 \pm 40.2	1632 \pm 44.3	1790 \pm 45.0	1641 \pm 38.5
CO ₃ ²⁻ (μ mol kg ⁻¹)	187 \pm 16.1	244 \pm 17.6	184 \pm 18.3	246 \pm 15.4
Ω_{calcite}	4.61 \pm 0.40	6.01 \pm 0.43	4.54 \pm 0.45	6.05 \pm 0.38
$\Omega_{\text{aragonite}}$	3.04 \pm 0.26	3.98 \pm 0.29	3.01 \pm 0.30	4.01 \pm 0.25

Operation manual of titrator for total alkalinity:

<https://www.manualslib.com/manual/530078/Hanna-Instruments-Hi-84431.html#manual>

Operation manual of optical dissolved oxygen probe for dissolved oxygen concentration:

https://in-situ.com/wp-content/uploads/2015/05/Stable_Optical_Oxygen_System_-SOO-100_Manual.pdf

Table A2 PERMANOVA table showing the effects of dissolved oxygen (DO) and context on shell growth, fracture toughness, organic matter content, calcite/aragonite, Mg/Ca in calcite, relative ACC content, respiration rate and clearance rate.

	<i>df</i>	Mean square	Pseudo-F	<i>p</i>	Comparison of means
<u>Shell growth (Day 21)</u>					
DO	1	21.8	5.66	0.019	Normoxia > Hypoxia
Context	1	676	175	0.001	Damaged > Intact
DO × Context	1	0.163	0.042	0.838	
<u>Fracture toughness</u>					
DO	1	2.00×10^{-7}	0.119	0.734	
Context	1	1.97×10^{-5}	11.7	0.004	Damaged > Intact
DO × Context	1	1.69×10^{-7}	0.101	0.755	
<u>Organic matter content</u>					
DO	1	1.04	1.10	0.309	
Context	1	9.83	10.5	0.005	Damaged > Intact
DO × Context	1	0.022	0.024	0.880	
<u>Calcite/Aragonite</u>					
DO	1	0.070	11.0	0.017	Hypoxia > Normoxia
Context	1	1.14×10^{-3}	0.178	0.623	
DO × Context	1	8.84×10^{-3}	1.39	0.249	
<u>Mg/Ca in calcite</u>					
DO	1	0.018	16.0	0.004	Hypoxia > Normoxia
Context	1	6.92×10^{-4}	0.618	0.455	
DO × Context	1	4.83×10^{-4}	0.431	0.530	
<u>Relative ACC content</u>					
DO	1	0.355	6.02	0.047	Hypoxia > Normoxia
Context	1	0.199	3.37	0.104	
DO × Context	1	0.116	1.97	0.206	
<u>Respiration rate</u>					
DO	1	2.15×10^{-3}	4.36×10^3	0.001	Normoxia > Hypoxia
Context	1	8.52×10^{-6}	14.2	0.001	Intact > Damaged
DO × Context	1	7.40×10^{-8}	0.150	0.715	
<u>Clearance rate</u>					
DO	1	84.0	140	0.001	Within Intact: Normoxia > Hypoxia Within Damaged: Normoxia > Hypoxia
Context	1	89.1	148	0.001	Within Normoxia: Intact > Damaged Within Hypoxia: N.S.
DO × Context	1	57.2	95.5	0.001	