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Subject: Favorable recommendation to the defense of the PhD thesis of Justyna Maria Swiezak **"Impact of potential CO2 leakage from sub-seabed storage site in the southern Baltic Sea on the Baltic clam *Limecola balthica* under relevant hydrostatic pressure".**

REPORT

The continuous increase of atmospheric CO_2 emissions represents an increasing concern due to their predicted impact on wildlife biodiversity, ecosystems functioning and human life. Among the mitigation strategies, CO_2 capture and storage into marine deep geological formations has been suggested as an option for reducing emissions. However, concerns have been raised from possible leakage from such storage sites, and the associated elevated levels of CO_2 could locally impact marine organisms nearby these areas. The literature on this topic has been recently increasing, but still scarce information is available on the impacts induced to marine invertebrate species, especially bivalves, inhabiting these areas. In this context, the present thesis aimed to evaluate the effects of CO_2-induced seawater acidification on the key benthic species *Limecola balthica*, under laboratory conditions.

The main achievements of this study included: the identification of the clam *species Limecola balthica* as a good bioindicator species to acidification events due to leakage

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from sub-seabed CO_2 storage areas; determination of the most suitable biomarkers to assess impacts of pH decrease.

In this context, the study conducted is relevant, innovative and the results obtained will clearly advance the state of the art. At this stage my **recommendation** is that the candidate should highlight in the thesis possible publications resulting from this study. Although PhD thesis have nowadays a wider impact, scientific publications are the best vehicles to disseminate science and spread original data. I would suggest that the candidate could clearly identify possible publications from the results achieved.

I would also **recommend** that the main achievements should be put in context of the current *Sustainable Development Goals* (SDGs) by UN, that are the blueprint to achieve a better and more sustainable future for all, addressing the global challenges we face, including those related to climate change and preservation marine biodiversity. Few lines relating the aims of the present study with the SDGs should be added to the thesis.

During her work, the PhD candidate conducted field sampling campaigns, performed laboratory experiments and measured a wide variety of biological responses in the clam species *Limecola balthica*. The selection on this species as model organisms was clearly justified by the candidate and put into context, since is one of the most abundant and wide spread species in the Balthic sea and largely used as bioindicator species. Nevertheless, at some point the PhD candidate reports in the thesis experiments conducted with polychaetes but the results were not presented in the document. Therefore, **my comment/suggestion** is that it would be very interesting to compare the responses given by species from two distinct biological groups, with a different behavior and, probably, different responses to acidification. If not possible to add these data I would suggest to remove all the information along the thesis, mentioning the use of polychates in certain experiments.

Considering the available information on the impacts of CO_2 towards marine bivalves, I also **suggest/recommend** to add few lines at the conclusion section on comparative threats to bivalves' populations derived from different CO_2 sources (ocean acidification derived from CO_2 storage or from atmospheric CO_2) and species sensitivity. It is missing a broader scale interpretation of the results concerning the use of this species in particular and the impacts to this population.

The biomarkers selected for this study were sound and appropriate. Nevertheless, I would **recommend** that in the introduction chapter the rationale behind these biomarkers could be added, explaining what they measure and how they relate between each other. Final research reports, as a PhD thesis, should represent complex and integrative information, constituting a baseline literature for future research. In this sense, it should have clear explanations of the options taken in what concerns to objectives and tasks to develop, methods used, interpretation of results and comparison with available information.

Overall, the dissertation is very well written, in a proper English language, with a good organization along each chapter. The reading was interesting and easy and I just have identified minor errors/typos along the text, that I will point out latter in this report.

Considering each chapter in more detail: the doctoral dissertation consisted of 6 chapters/sections, including *Introduction, Material and Methods, Results, Discussion, Conclusions, References*. The use of Figures and Tables in each section was appropriate. I have few **suggestions/comments: i)** at each Table or Figure caption, it must be given the full name of each acronym or letter used in the Table of Figure. For example, Table 2.1 should include in the legend the meaning of T, S (or write the full name as in Table 3.1.5 where it is written Temperature, Salinity); Tables 3.1.2, 3.1.3, 3.1.6 add meaning of *H, df, N, P*; **ii)** in Figures always give full name of the species name (Figure 1.3 vs 2.6); **iii)** in figures legends (for example Figures 3.1.9 and 3.1.10) replace acclimatization by acclimation; **iv)** in Figures and Tables legends it should be always Balthic clam or Balthic clams but not different among legends (see for example Tables 3.2.5, 3.2.6, 3.2.7, 3.2.8 legends; Figure 3.2.6, 3.2.7, 3.2.8 and 3.2.9).

CHAPTER 1: *Introduction* corresponds to a state of the art, where the topics under research are put in context and a rationale behind the experimental approach conducted is presented. Nevertheless, as mentioned before, a rationale behind the biomarkers selected for this study should be given and the work should be put in a broader perspective considering the UN SDGs.

Major comments/questions

The Introduction is well written, interesting and easy to read. The main topics of this study were here included and well addressed. However, since it has been mentioned that

CO_2 may impact bottom habitats, changing for example metal behavior and bioavailability and consequently acting as an extra impact factor, it would be interesting to have in this section background information on pollution levels of the study area. This information could be also in the Material and Methods section. Did the candidate look for this information? Could pollution act as a confounding factor at the study area, interacting with CO_2? What can the candidate discuss about this?

Typos

P18: It is missing a space between 2 and °C

P25: Replace glutathione S-transferase (GST) by glutathione S-transferases (GSTs) since it is a group of enzymes (isoforms)

P27: After writing for the first time the full name of a given species than just abbreviates it. For example, use *L. balthica* on page 27 and not *Limecola balthica* since the full name was given previously; the same for *Mytilus trossulus* and *Cerastoderma edule*.

P32: Replace *Limecola balthica* by L. *balthica*

Always avoid to start sentences with abbreviations of chemical symbols. Example page 16: "CO_2 emission…"

CHAPTER 2: *Material and Methods* although well written and clear, to my point of view, this section needs some improvement, namely in terms of missing details/information (see my comments below).

Major comments/questions

My main concern regarding the work performed and the methods followed is the fact that experiments under different pH levels were conducted at different periods, using animals collected at different sampling times and, consequently, during different seasons and biological stages. How the candidate justifies this situation? What can be the influence on the results achieved? The candidate briefly mentions this situation at the Discussion but a better interpretation of the results should be given considering this fact.

Considering that at each time a different pH level was tested, a CTL condition should be always used in each experiment to guarantee the quality of the data. How this was guaranteed? Were 10 days results always comparable? How acclimation organisms were compared among different experiments?

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Did the experimental setup mimic the environmental conditions along the exposure period? Was the change on pH level gradual, resembling a possible leakage?

Null hypotheses should be given in this section. What were the H0?

It would be worthy to have a rationale behind the use of the selected biomarkers.

Minor issues

It was missing a deeper characterization of the field conditions during sampling, namely in terms of pollution levels (pollutants types and concentration) that could act as confounding factors. How could the candidate overcome this situation?

The use of sediment in the experiments should be better explained since most of the studies described in the literature have been conducted only with water.

Organic matter content should be given for the sediments used. What could be the influence of these properties on the results? What is the relevance of these properties for the interpretation of the results?

Could the preservation of the sediment prior to the experiments influence the results?

The candidate should justify the use of a Titanium pressure tank instead of injection of CO_2. Was this related with depth pressure? What is the range of depth where *L. balthica* can inhabit? What is the correspondent pressure?

Why clams were immersed in calcein solution prior to the experiments?

Why polychaetes were included here? If not presented in the results section than all the information regarding polychaetes should be removed from the thesis.

The number of days used for the experiment (40 days of exposure) should be better explained. Why 40 and not less or more?

The objective of determining water alkalinity should be given.

For each sampling period along the experiments it is not clear the number of individuals per tray and the number of trays used.

It is not clear if the whole soft tissue of clams was used or different organs and if pools were made. It is not clear the number of true replicates used per condition and sampling period used for the biochemical measurements.

The candidate should explain why enzymes activities were expressed by protein content and not by fresh weight. What are the advantages and disadvantages?

Typos

P46: A space is missing between 10 and °C as well as between 90 and °C.

P47: A space is missing between 10 and °C, between 50 and °C, and between 100 and °C.

P49: In table 2.3 Replace Glytathione S-transferase (GST) by Glutathione S-transferases (GSTs)

P54: Replace glutathione S-transferase (GST) by glutathione S-transferases (GSTs)

P55: Replace GST activity by GSTs activity

CHAPTER 3: *Results* section was clear and each biological response presented in a proper detail.

Major comments/questions

In my opinion, the significant differences observed among pH treatments in terms of salinity and oxygen saturation (Table 3.1.1) could greatly influence the results. How can the candidate discuss this? Changes in salinity over time could also influenced the results as it is well-known that salinity is one of the most important factors affecting aquatic organisms.

The number of replicates used in each biomarker and for each pH treatment was not always the same. How this situation could influence the results interpretation and the statistics? In some analyses the number of replicates was 2 (See Figure 3.1.3, Figure 3.1.5, Figure 3.1.7, Figure 3.1.9). Was this enough?

The alkalinity levels differed significantly among pH levels. How this could influence the results?

How could the total inorganic carbon influence the results, namely at the lowest pH treatment where the it influenced the saturation of calcium? How discuss this results on light of such changes?

In general: what were the factors that could act as confounding factors?

What are the biomarkers that could be more affected by these water changes/differences among pH treatments?

For all biomarkers comparison between pH treatments at each sampling period should be conducted and statistics provided. The general pattern along time for each pH treatment should be given. Was the sampling periods selected adequate? What could be improved in this experimental setup?

I would expect higher CA values at lower pH level. Why this was not observed? Any reason?

Total cytosolic protein used to express enzymes activities could influence the general enzymes patter. What was the variation in protein content and how this could influence enzymes data?

Why CAT and GPx enzymes were not close related with SOD? Any reason for this?

Could the number of replicates used influence the biomarkers results along sampling periods and between pH treatments?

Could LPO levels be related with electron transport system activity since there were no significant differences in enzymes activities among pH treatments? What could ETS measurements bring to this study?

Any reason why clams tend to increase AChE with pH decrease at day 10 and have an opposite pattern at day 40?

Minor issues

In my opinion differences between sampling periods for a given pH treatment could be campaigned by differences among pH levels at each sampling period. In this case Tables with statistics could be moved to thesis Appendix. Tables with statistics along the text breaks the reading.

What could be the most reliable marker: CI 1 or CI 2?

Typos

P85: Remove the word *the* before pH 7.7 ("with significant differences between the pH 7.7...")

P85: Replace *the* by *between* at: "...and the pH 7.0 and 6.3...) and add an *s* after treatment ("...and 6.3 treatment...)

P88: harmonize the format of presenting units. For example, sometimes it is written 26.4 mm ± 0.4 mm (page 85), while sometimes it is written 26.8 ± 6.2 mm (pages 86 and 88).

P93: Replace 6.3 by 7.0 ("...incubates at pH 7.7 and 6.3...").

P112: Remove *of* from "...pairwise comparison of revelaed...".

P113: Remove the word *clam* from the figure leged ("..Baltic clams *clam Limecola balthica*).

P115: add s to GST

P123: Add a space before "In the control treatment...."

P131: add *est* to the word low ("...exposed to the *low* pH condition...").

CHAPTER 4: *Discussion* could benefit from improvements since general patterns for each biomarker could be clearer highlighted for differences among pH treatments and along sampling periods. Although the results were very well discussed and interpreted, due to the amount of data and the fact that responses for all biomarkers were not always clear and easy to interpret along the sampling periods, this section could benefit from the addition of some paragraphs referring to general patterns of response to each pH treatment.

In general, given the results obtained from all biomarkers, it seems that at pH 7.0 clams were more responsive than clams at 6.3. Any reason for this?

Major comments/questions

What was the best set of biomarkers to study pH variation should be highlighted, considering probable confounding factors and comparing with literature?

What should be the best compromise between the number of sampling periods and the best set of results? The huge sampling periods along the experiments was necessary to reveal the impacts?

If feeding could influence the results what should be the amount of food that best could resemble environmental conditions? Could food supply greatly influence biological responses? What responses could be more affected?

It is not clear in the discussion how depth and associated pressure could influence the biological responses since the candidate compares the obtained studies with others conducted at different pressure values and with clams inhabiting different depth levels.

P153: I disagree with the candidate when she says: "Increased levels of GSH in response to strongly reduced seawater pH 6.3 is likely caused by lipid peroxide detoxification via glutathione peroxidase". Under stressful conditions organisms oxidize GSH into GSSG by using GPx and for this reason, when under stress, organisms decrease GSH content (increasing GSSG content) and a decrease in the ration GSH/GSSH is commonly used to identify stress and loss of redox homeostasis.

P155: How this response: "decrease of MDS activity suggests temporal slowdown of metabolic rate" relates with the energy reserves contents obtained?

Minor issues

Results obtained could be also influenced by the presence of metals in sediments, as highlighted by the candidate. Thus, why metals were not measured in the sediments?

Typos

P138: replace *clam* by *cockles* ("… more *clams* were found shallower…").

P138: Replace *Ruditapes* by *R.* ("… Manila clam *Ruditapes philippinarum*…").

P141: A space is missing between 72 and h^{-1}

P145: Remove the word *that* ("… suggests in turn that *that* the MDH…").

P151: Add $_2$ to the word H_2O ("…to H_2O and O_2 whereas CAT…").

P153: Add an *s* to GST

P154: Add an *s* to GST

P155: Replace *Mytilus* by *M.*

P156: Add an *s* to GST

P157: eliminate *heavy* before *metals* since the term heavy metal is no longer used.

P158: eliminate *heavy* before *metals* since the term heavy metal is no longer used.

P158: Replace *Ruditapes* by *R.* ("… Japanese carpet shell *Ruditapes philippinarum*…").

CHAPTER 5: *Conclusions* section present the main achievements of this study but in my opinion it lacks of a major take home message, not only regarding impacts of pH towards this species but a broader message regarding the influence that CO_2 storage areas could have on marine wildlife biodiversity preservation (relate this with SDGs).

What could be changed to improve this study could be here presented. What went wrong? The candidate should be able to identify the main drawbacks of the study.

Typos

P160: Add an *s* to GST

CHAPTER 6: *References* list was long, updated and well presented. With minor errors.
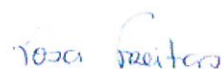

Correct the following references:

Carstensen et al.: replace , by : after volume

Compton et al.: remove italic from journal name

Huang et al.: remove italic from journal name

Despite the above mentioned concerns/comments, I found this PhD thesis very good. I consider the work conducted by Justyna Maria Świeżak as excellent, and I am supportive and agree with the PhD defense.

Rosa Freitas

Aveiro, 4th September 2020