Nash et al.

Mineralogical response of the Mediterranean crustose coralline alga *Lithophyllum cabiochae* to near-future ocean acidification and warming

Response to reviewers

We thank the reviewers for their positive comments and suggestions. We think the MS has been substantially improved by the consequent edits.

Reviewer 2

R2 *Specific comments: While I am not familiar with the technique used for the MgCO3 concentration determination I feel the whole approach would benifit from additional analyses providing spatially resolved data on Mg distribution (e.g. EMPA, LA-ICPMS, SIMS). I am aware this might not be practical for the current work but would like to encourage the authors to extend the work into that direction. Without such information we can hardly evaluate any effects (e.g. seasonal changes, internal and interindividual variation) which until now could be hidden by pooling the sample amount needed for the respective analyses. I*

Response

One of the advantages of powder X-ray diffraction is that all the variation is pooled, so to find significant differences across treatments, despite internal and seasonal variation is an indicator as to the strength of the temperature response. But, we consider these results just a good starting point. The big question is how does Mg change at the cellular scale? As referee #2 suggests we do, we already had planned for further interrogation of these samples to understand changes at the ultrastructure scale. Following referee #2 suggestion we undertook exploratory SEM-EDS to see if more detailed Mg study could be incorporated into a revised MS within a reasonable timeframe. However, as happens so often with the corallines, the first results are more complicated than expected and it will take some time to carry out detailed analyses and interpretation. This work will be well beyond the scope of the present manuscript but will be undertaken in a follow-up study.

No changes made

Referee #2: *In the discussion I do miss details regarding the role of photosynthesis which could compensate for the changes in pH caused by the pCO2 treatment.* Respiration, photosynthesis and calcification rate data would be extremely helpful.
Referee #1 also mentioned metabolic costs.

Response

All physiological parameters request were measured and reported in the original papers (Martin et al 2009, 2013) referenced at line 82. This sentence has been edited to note specifically respiration, photosynthesis and calcification rate data reported in Martin et al. (2009 and 2013).

Already in the MS at line 244: 'A biological control of mineralization by coralline algae has already been inferred in *L. cabiochae* because its rate of calcification is maintained or even enhanced under elevated pCO_2 (Martin et al., 2013a).

We are hesitant to enter into a discussion about photosynthesis ameliorating for pH or metabolic costs. Using the term 'compensate' infers that there must be a negative influence of CO2 on calcification processes that drives the Mg down and the CCA is responding by actively working to maintain Mg- and that is why there was no change in Mg. The fact that there is no pH-related change in Mg in this experiment does not necessarily mean Mg would be declining but for the organism doing something to compensate. It may just mean there is no effect. Unfortunately, little is known about the cellular scale controls on Mg uptake, thus speculating about compensatory metabolic responses under differing pCO_2 treatments is fraught with chasm-sized knowledge gaps. This also applies to referee #2's comment about investigating the spatial distribution of Mg. We consider even more than that is needed, the beautiful NanoSIMS work of Ragazzola et al. (2016) starts to show the complexity of Mg distribution within a cell wall of just one species. But without a parallel understanding of the distribution and types of organic molecules within the cell walls, and how these do or do not control Mg content, the ability to reliably extend chemical analytical results to a metabolic context are limited.

We have added a paragraph before the final paragraph addressing the above issues and some from the next comment. See paragraph inserted below next comment.

Referee #2**:** *I finally miss any reference to some recent papers (e.g. Ragazzola et al. 2013 and 2016). Those papers should be included into the discussion as they provide direct evidence for the CCA's response to OA (while carried out using a different species from a different habitat) on the microscale, chemically and structurally.*

Response
Ragazzola et al. (2016) came out after this paper was prepared for submission. It is now cited, along with Ragazzola et al. (2013), in the discussion regarding Mg changes. There is already discussion on the Mg and physiological response. As we did not carry out SEM to investigate structural changes or microscale distribution of Mg, we do not have a discussion of this where the Ragazzola structural work could seamlessly fit. Our hypothesis was to test for changes in Mg content ranther than structural changes. However, we have included Ragazzola's papers and added Hoffman et al. (2012) into the discussion on compensatory mechanisms.

Added as second last paragraph
It is worth considering whether there is a compensatory mechanism enabling Mg-content maintenance in the elevated pCO_2 treatment. This consideration implies that the Mg content automatically declines with lower pH (the hypothesis we tested) and the organism must therefore have compensated because the results showed no difference with pCO_2.

While there have been many studies on Mg content responses to elevated $_pCO_2$ treatments (e.g. Ries, 2011; Egilsdottir et al., 2013; Kamenos et al., 2013; Diaz-Pulido et al., 2014; Nash et al., 2015; Ragazzola et al., 2016) as yet, there has been no study on the internal cellular-scale metabolic controls on Mg uptake in coralline algae. That is, the controls of Mg uptake are unknown. Is it the internal carbonate chemistry, the type of organic substrate or a combination of both? Without an understanding of the physiological mechanisms that control Mg uptake, it is impossible to do more than speculate about potential compensatory metabolic processes. This inhibition to carrying out an informed analysis of potential metabolic controls on Mg uptake highlights the need for basic scientific investigation into how coralline algae calcify and what role the anatomy and organic substrates play in calcification and Mg uptake. Then, we could start to understand how these processes react to external environmental changes. Ragazzola et al. (2013, 2016) and Hoffman et al. (2012) have shown anatomical changes in response to pCO_2 that may be ameliorated over longer time periods but the exact controls on those changes are not known. Here, rather than attributing a complicated compensatory response in a poorly understood cellular scale process, probably the simplest explanation is the most logical, that within this range of pCO_2 for the *L. cabiochae* there is no influence of carbonate chemistry on the Mg content of the CCA Mg-calcite and the hypothesis of a pCO_2 driven decline in Mg is not supported.

Referee #2: *As this topic directly relates to the use of Mg in CCA as environmental proxy for paleo temperature reconstruction the discussion could extend further into that particular direction. This is meant twofold: 1) with respect to the primary incorporation of Mg and 2) the preservation of the proxy signal in the fossil record.*

Response- Good suggestion. Thank you. The following two paragraphs have been added in the discussion.

The consistent shift of ~0.33 mol% $MgCO_3$/°C across both the control and CO_2 treatments suggests the magnesium change is a robust temperature response and this is of interest for CCA paleo temperature proxies (e.g. Halfar et al. 2000, Kamenos et al. 2008, Hetzinger et al., 2009). A similar ratio was found experimentally in *P. onkodes* [0.37 mol% $MgCO_3$/°C (Diaz-Pulido et al. 2014)]. This ratio is also in agreement with results obtained by XRD of articulate corallines [0.286 – 0.479 mol% $MgCO_3$/°C (Williamson et al. 2014)] and a variety of species [0.36 mol% $MgCO_3$/°C using only XRD results in Chave (1954)] collected across a geographical temperature range. The reports of ratios of up to 2 mol% $MgCO_3$/°C (Halfar et al., 2000: Kamenos et al., 2008, Hetzinger et al., 2009; Caragnano et al., 2014) may be due to different analytical methods or species-specific effects. The similarity of the results obtained using XRD for both experimental and *in situ* corallines supports using a ratio of ~0.3 to 0.4 mol% $MgCO_3$/°C as a paleo thermometer when the analytical methods return an effective spatial average for Mg-calcite and the absence of other carbonates; aragonite, low-Mg-calcite, dolomite and magnesite, has been confirmed.

Prior to utilizing CCA as a temperature proxy, it is necessary to verify that the Mg in the Mg calcite is the primary Mg incorporated during calcification and not the result of diagenesis. The depletion of Mg in the dissolution experiment crusts over eight months indicates this change can be relatively rapid once the organism is no longer protected by living tissue. This is likely to be more of a problem when using fossil branching corallines than thick crusts that retain a living surface layer. Indeed, Kamenos et al. (2008) noted their sub-fossil *Lithothamnion glaciale* had significantly lower Mg in the summer season than their living samples. The likelihood of remineralization was considered by Kamenos et al. but rejected, as remineralization was presumed to be to either low Mg calcite or aragonite. The possibility of remineralization to a lower phase of Mg-calcite, as occurred in this experiment and noted at the base of CCA *Porolithon onkodes* (Nash et al. 2013b) had not previously been reported. The present study experimentally confirms that diagenesis does not necessarily mean a change to aragonite or low Mg-calcite, but instead can be to a lower phase of Mg-calcite thus making it more difficult to detect post-mortem diagenesis from Mg measurements or mineralogy alone. High magnification SEM work would be required to check for remineralization.

Referee #1
The pigmented zone on many corallines does not delimit the living tissue. However, on the thin pieces illustrated in Fig. 1 they may. Nevertheless, the "pink" thallus simply has pigments and it can vary in depth into the thallus. I did not see anywhere, where the thickness of the pigmented tissue was reported for the upper and for the lower surface of these crusts. Given the attention given on mineralization that occurs in the pigmented region, that thickness seems to me to be important. (e.g., see lines 200 – 202)

Response
The following sentences added to methods
The depth of the pink-pigmented crust was ~200- 500 μm but only the surface is sampled by scraping on the top with the aim of collecting predominantly epithallial material. However, we do not refer to it as epithallus because by this sampling method we cannot confirm that no sub-perithallial crust has been included, hence surficial pink crust is the most accurate description of this subsample. Our development of this method has shown that if too much pressure is applied during the scraping then substantial amounts of perithallial crust, that also can be pink-pigmented, may be unintentionally sampled.

Referee #1 *Line 71: replace "preferentially with differentially.*

Response – changed

Referee #1 *Line 108: Fragments 2 – 3 mm are extremely small with considerable exposed thallus relative to the entire photosynthetic surface area. Could this have had an effect on the outcome of the experiment?*

These fragments grew entirely during the experiment and it is likely the small size did not affect the outcome. Noting that without a true control, I.e. a piece of similar size grown

over the same duration in the natural environment, it is not possible to confirm or deny a potential size effect due to the experimental conditions. The situation would be different had these been pieces of crust removed from the main crust and placed into the experiment, in which case the greater exposure may be a problem.

No change to MS

Referee #1 *Line 108: I suggest you replace the second use of the word "diameter" with "thickness".*

Response –changed

Reviewer #3

Reviewer #3
Specific comments L76: an increase in Mg content under increased temperatures is well established in the literature and does not need to be tested here

Response
The original experiment was designed to test a range of physiological responses to anticipated future changes of rising temperature and pCO_2, as well as a combination of both. To give the results of the combined treatment context, necessarily a 'control' elevated temperature comparison needs to be made.
We agree with r**eviewer #3** that an increase in Mg is well established, but disagree that this means it no longer needs testing experimentally. As the added discussion on temperature proxies shows, there is a substantial difference in the range of Mg changes in response to temperature across species, i.e. a ten-fold difference ranging from ~0.2 to 2 mol% $MgCO_3$. Consequently there is very much a need to test the Mg response to temperature in controlled experimental conditions across a range of species in order to build a more precise paleo thermometer.
No changes made

Reviewer #3
L78: typo
Fixed

Reviewer #3
L103: I am not familiar with the gross growth morphology of this species of alga. It is difficult to understand the difference between the new crust and the surficial crust. I appreciate that the authors included visualization in figure one of the different crusts but I would suggest they include a cartoon or sketch to better show the different crusts and how they differ.
Response
Black and white sketch added to figure and caption edited

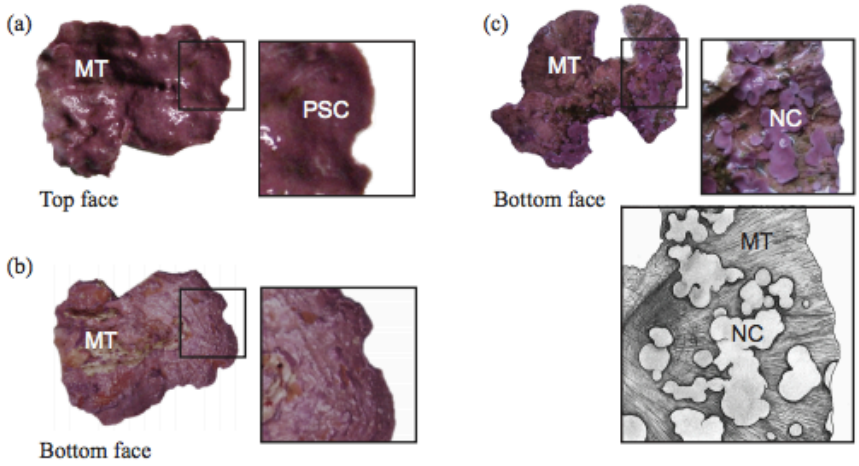

Figure 1: (a) Top face of the main thallus (MT) of *L. cabiochae* showing the pink surficial crust (PSC) and bottom faces (b) free of crusts at the time of collection and (c) with new crusts grown during the experimental period (photos and drawing S. Martin).

Reviewer #3

L105: Is there any evidence that this new material was grown during the experiment? Was growth not measured or a skeletal stain used to mark new growth? Much of the results depend on this presumption and I think evidence to support is needed.

Response: The surfaces were cleaned of epiphytes at the time of collection and photographed. The new crusts used for analyses were not there when collected from the field and no staining was required to visibly identify the new crust growth.

Reviewer #
L 109: Again, please confirm that material sampled for XRD was grown under cultured conditions

Response: As discussed above and the following sentence added to MS in materials and methods:

> These crusts were confirmed to have grown during the experiment as the pre-existing crust was cleaned and photographed at the time of collection and these growths were not present at that time.

Reviewer #3
L130-156 Present results here in the same order as presented in the methods L103 – 107
Response- The order presented in methods L103-107 has been changed to reflect the order in the results.

Reviewer #3
L171 – 175: It would be useful if this discussion was expanded to be consistent with depth of discussion elsewhere, or the differences between these crusts types and calcification processes better introduced earlier

Response The discussion has been expanded and another reference added. See revised paragraph below.

> The pink surficial crust also trended up with temperature and while no statistical analyses could be carried out, these results are consistent with the increase in Mg measured for pink surficial crust as a function of increasing temperature reported in previous work (Diaz-Pulido et al., 2014). The lower Mg content recorded for the pink surficial crust relative to the bulk crust is in agreement with previous studies on CCA *Porolithon onkodes* (Diaz-Pulido et al., 2014; Nash et al., 2015). Sampling of the surface aims to capture predominantly epithallial carbonate. The epithallial cells of corallines are typically different in shape to the perithallial cells, being shorter and flattened or ovoid shape (e.g. Pueschel and Keats 1997). It is not presently known why the epithallus has a lower Mg than the bulk perithallus or if this offset is common to all species. However, the close agreement of the temperature response for the *P. onkodes* surficial crust of 0.37 mol% $MgCO_3$/°C (Diaz-Pulido et al. 2014) with the 0.33 mol% $MgCO_3$/°C measured for the new crust in this experiment does suggest the controls on Mg uptake are similar for both the perithallial and epithallial cells.

Reviewer #3 *Analyses of the pre-existing thalli (main thalli) provides a baseline Mg content only if analyzed prior to the experiment, otherwise, analysis of the thalli provides baseline Mg content for experimental conditions.*

Response
As XRD is a destructive process, the exact piece cannot be analysed prior to the experiment. However, it is possible to take subsamples prior to the experiment for analysis and if the mineralogy matches the post-experiment analysis, then this is usually sufficient to establish similarity (e.g. as done in Nash et al. 2013a). Unfortunately where the focus of the experiment is on biological responses, not mineral, to the frustration of the mineralogist this early subsampling is not usually done. As r**eviewer #3** has noted, this is a problem. We do note in that paragraph that the values for one of the treatments suggest there has been alteration during the experimental duration.

Sentence edited and now reads

> Analyses of the pre-existing thalli (main thalli) provide a baseline Mg content for pre-experimental *L. cabiochae* with the assumption that this has not changed during the experiment.

Further, we have added to this paragraph to highlight experimental design problems and solutions.

'Ideally when carrying out experiments where it is planned to analyse the crust, for either mineral or structural changes, it is best if a subsample is taken from of each piece prior to being placed into the experimental tanks. This way it can be established that post-experiment crust features are truly representative of the environmental sample (e.g. Nash et al. 2013a) and have not been altered by virtue of being placed in tanks for the duration. Problematically some CCA exhibit changes in growth unrelated to treatment after being placed in tanks (Nash et al. 2015). Thus best practice would be to keep aside subsamples, particularly where a species has not already been well studied at the cellular scale, so that it can be determined if the control tanks result in growth and mineral composition comparable to in-situ growth.'

Reviewer #3
L209: What does NBS and NCC stand for?

Reponse
NBS – National Bureau of Standards
NCC was a typo and has been removed.

Reviewer #3
L212 – 214 this could be tested using SEM

Response
As already mentioned above, SEM work is ongoing but beyond the scope of this part of the study.