Biogeosciences Discuss.,
https://doi.org/10.5194/bg-2018-11-RC2, 2018

Biogeosciences Discussions
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Interactive comment on "Interactive effects of seawater carbonate chemistry, light intensity and nutrient availability on physiology and calcification of the coccolithophore *Emiliania huxleyi*" *by* Yong Zhang et al.

Anonymous Referee #2

Received and published: 27 February 2018

Review of Zhang et al. "Interactive effects of seawater carbonate chemistry, light intensity and nutrient availability on physiology and calcification of the coccolithophore Emiliania huxleyi" submitted to Biogeosciences.

The study by Zhang et al. is an important effort to address multifactorial control over the response to acidification of an important phytoplankton species, using an ambitious matrix of treatments. However, there are some major problems that must be resolved, as currently I am unsure of a major portion of the data or results interpretations as presented.

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1. In the Introduction the authors plant the study as if it aims to mimic the natural environment presently or in the future in the laboratory, that is, that the nutrient, light, and CO2 conditions they chose are truly representative. I think this is not unnecessary and risks setting up an incorrect context for interpreting the study results. For example, the authors justify the choice of light regimes in the first paragraph by claiming that phytoplankton in the future ocean will be exposed to higher light levels in the mixed layer, citing two studies. I note also that neither of the studies cited (Gao et al. 2012 and Hutchins and Hu 2017) is relevant to cite, as one is a lab study and the other is a review of lab studies, and neither is a model study predicting average light fields at which phytoplankton will be exposed in the future ocean. In any case, it is difficult to imagine that changes in the stratification of the central ocean basins can only lead to an increase in light exposure. Light exposure is highly dynamic and depends on mixing regime, so yes, the light regime should change, but to model that in the lab with constant light levels is not reasonable. My comment here does not at all negate the study design: Even though we can never mimic the ocean in the lab, it still serves to understand how factors may interact. In the case of trying to predict the response to acidification, it at least serves us to understand how robust the lab results might be for predicting the direction of possible responses, and often as well helps provide insight into mechanisms underlying the responses. I do suggest that they consider revising the Intro.

2. There is at least one major problem with the growth rates reported, possibly many more:

a. It makes no sense to report a single growth rate as the response to nutrient-limitation in batch culture experiments. At inoculation of cultures, cells should be nutrient replete even in the LP and LN conditions. If they have been “acclimated” to growing previously in the same media, the inoculums likely are from cultures that have already exhausted the phosphate (in LP) or nitrate (in LN), so the cells will have to re-configure nutrient uptake and metabolism, begin to grow, then exhaust the nutrients, re-configuring nu-

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trient and connected metabolism again. The growth rate most certainty will NOT be constant. A recent study where these effects can be seen would be that of Rokitta et al. (2014). The authors report only a single growth rate, not changes in cell density over time, no indication of when nutrient limitation may start nor how long cells have been in nutrient limited conditions. In this sense, a good study to look at would be the recent one by Müller et al. (2017) using a continuous culture approach to understand the interaction/independence of nutrient limitation and acidification effects (curiously, the authors cite the study in the Intro but do not discuss at all, despite its central relevance!). The results presented in the current manuscript are therefore completely uninterpretable.

b. The growth rate presented appears to be calculated only from an initial cell concentration and a final one, which is generally not adequate even in batch culture experiments when nutrient limitation is avoided, because it is necessary to understand if growth rate changes or not during the experiment.

c. The initial cell concentration appears not to have been measured, but to have been calculated, which causes many errors.

d. The growth rates provided seem high in comparison to most previous studies of this species. Most authors report that the maximum growth rate of Emiliania huxleyi in batch cultures under “optimum” nutrient and light conditions and a day:night lighting is in the range of 0.7-0.9, a little more than one doubling per day (for just a sampling of studies, see van Bleisjwiik et al. 1994; Zondervan et al. 2002; Rokitta et al. 2014; Müller et al. 2015). Higher growth rates are occasionally reported, but under longer light cycles, e.g. Langer et al. 2009, or a very nice study by the same first author (Zhang et al. 2014). The rates here seem quite high for a 12:12 light:dark cycle, and for that reason it’s important to see the data (at least in supplementary), to have full confidence in the methods, and to have at least a brief mention of this.

e. I’m especially concerned in the Methods when they say that 4-6 days corresponds

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to 14 generations. That would correspond to growth rates between 1.62 day-1 (at a "low" light level previous studies have found to nearly saturate growth rate) or 2.42 day-1, a level unachievable even for most diatoms (and not readily believable for a coccolithophore, even E. huxleyi). Perhaps this is a typographical error?

f. For cell counts they use a particle counter (presumably based on the Coulter principle, although the information provided is inadequate to identify the type of instrument). This is potentially very problematic particularly in the case of E. huxleyi. How can living cells be distinguished from detached coccoliths, agglomerations of detached coccoliths, and/or empty coccospheres, all of which are very abundant in E. huxleyi cultures? In limited conditions these other particles can actually dominate the suspended particles found in cultures and it can be difficult to distinguish cells. With all these issues, I really am not sure from the information provided that they are actually measuring cells. Cells should be counted under a microscope or with a flow cytometer (a Coulter-type particle counter can be used, if it is being checked, compared, calibrated with microscope or cytometer counts throughout the experiment). Details are needed.

Because of these unresolved methodological issues in measuring cell abundance, at the present time I cannot trust growth rate data or cell elemental quotas reported.

3. There is no way to know when nutrients became depleted. In the case of nitrate, it is not clear if that nutrient became limiting or sampling occurred when cells were just about to use up the last uM. In this sense, it is essentially impossible to interpret differences in any of the measured parameters between HNHP, LN, and LP conditions. The Fv/Fm data in Fig. 3 heightens my suspicion that cells never truly reached P starvation under LP conditions, as Fv/Fm doesn't show any clear drop in LP compared to HNHP condition at any light or CO2 treatment (compare to Rokitta et al. 2016, for example). In the case of phosphate, perhaps they became limiting at the end, but when? The fact that the increase in PIC/cell reported in many previous studies wasn't observed, but occurred under LN instead, is consistent with the suspicions that the presumed nutrient status was not limiting (and that cell abundance was not being

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measured properly).

4. I think the approach for analyzing and interpreting the data could be more powerful:

a. The 3-way ANOVA ignores differences between LP and LN conditions

b. The 3-way ANOVA approach followed by a posthoc test to identify pairwise differences can be valid, but it doesn't help for identifying patterns. In this case, the Eilers and Peeters model they fit would help, but they only look at the fit of the alpha parameter, when the curves shown in their figures clearly indicate that the other fitted parameters (a, b, c) may be interesting as well.

5. What about cell volume effects? As reported recently by Müller et al. (2017), these could be crucial. If I understood that previous study correctly, nutrient limitation seemed to act independently rather than synergistically with ocean acidification when cell volume was accounted for. Of course, that study used continuous culture rather than batch culture/starvation conditions, but still it seems relevant at least to consider. Currently the Discussion seems to ignore some relevant studies such as Müller et al. 2017 that I previously cited, as well as Olson et al. 2016. Further, it needs to be much clearer. Finally, some revision of the English is suggested.

6. The Discussion focuses a lot on ETR and photophysiology (Fv/Fm, Fv'/Fm'), which doesn't make a lot of sense. Effects both of high CO2 and of supposed nutrient limitation on photophysiological parameters seem to be subtle in comparison to what they report on growth rates and cell quotas. The light dependence of photosynthesis in E. huxleyi has actually been comparatively well studied, and much of the discussion seems overly speculative and not to focus on some of the curious differences with what has been reported previously (e.g., Houdan et al. 2005 reporting that calcified cells are especially resistant to high PAR).

The doubts I have about the study are quite serious, and hopefully my major comments (above) and minor comments (below) help the authors determine where to clar-

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ify. Nevertheless, I think the study design may not be appropriate for investigating an interaction between nutrient limitation and acidification. The only way such an experimental design could potentially work for the question planted is with daily and trusted cell counts and nutrient measurements showing when nutrient depletion occurred.

There are many minor points through the manuscript to address as well. I mention a few:

Line 27 “and exposing phytoplankton to increased light intensities” and lines 52-53 later. I think this is too much over-simplification. At the base of the mixed layer and within the pycnocline, nutrients can be obtained by diffusion across the pycnocline, so phytoplankton will grow and increase in biomass until they compete for light. I do not see how the average light exposure of phytoplankton will necessarily increase. The references cited (Gao et al. 2012 and Hutchinson and Fu 2017) do not explain this (as I mentioned earlier).

Lines 101-103 “Interaction of rising CO2 with light appears to affect differentially coccolithophores when grown under different experimental setups.” The sentence is not clear. What does “differentially affect coccolithophores” mean? Do those factors affect coccolithophores differently than other phytoplankton or do these factors have contrasting effects or ??

Lines 142-144: “added by 2200 μmol L–1 bicarbonate (as opposed to 2380 μmol L–1 in the original recipe), in order to reflect the alkalinity in the South and East China Seas of about 2200 μmol L–1” First, I don’t understand why it’s important to match the South and East China Seas if they are not specifically using strains isolated from those seas and trying to predict how organisms there will respond. Second, I don’t see how bicarbonate concentration is equated with alkalinity, as CO32- also contributes to alkalinity, and for alkalinity every unit of CO32- counts twice. I think carbonate usually can contribute about a fifth or a fourth or so of total alkalinity (see Zeebe and Wolfgladrow 2001 or other references).

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Lines 161-165: I mentioned above the problems with these lines.

Line 172: How often were nutrients measured?

Lines 182-184: Was pH measured immediately or after storage? pH should be measured immediately, as I understand (within a couple hours is best).

Lines 210-215: I already mentioned the major problems I have with the methodology as described here. Perhaps they can fix that.

Line 225: Do they mean “difference” instead of “variance”

Lines 251-255: It would be invaluable to know when nutrients were depleted. Do they have data on this?

Lines 256-264: This whole paragraph is basically redundant with Table 1. Also, it seems “(mean values for the beginning and end of incubations)” means that the beginning and ending values have been averaged together, while in Table S2 the beginning and ending values are given separately. Table S2 is far more useful, especially for assessing the changes in carbonate parameters during the experiment. I would place that table (S2) in the main text, using it to replace the current Table 1. Then in this text the focus should be more on how consistent were carbonate parameters over time and across treatments within the LC and within the HC treatments. Furthermore, when I calculate the averages using the values given in the text immediately before, I get different values (405 for LC and 918 for HC). What is happening? Were some replicates not used?

Lines 368-373: This is not a very good description. It seems to exaggerate small differences between HC and LC.

Line 467: I can’t find any reference to Fig. 5 in the Results. Why does it appear suddenly in the Discussion? Further, I have problems with this Michaelis-Menten fit: It does not make any sense to fit growth rate in a batch culture (measured from initial and final concentrations) to the initial phosphate concentration. This seems to ignore

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understanding of phytoplankton macronutrient physiology since Droop. But, as there isn't a clear description of this experiment, I am not sure. Finally, the ability to calculate a half-saturation from the data in the graph would be very limited because there is no value in an intermediate range of growth (growth rate is either 0 or saturated or nearly saturated). For this entire paragraph, the study was not designed to address the details of phosphate metabolism, which has already been fairly extensively studied in this species, and their discussion of the previous work is unclear.

Line 529: Do the authors consider the ballast effect to be completely irrelevant? Also, I have an issue with considering E. huxleyi as representative of the biogeochemically most important coccolithophores. It is the most numerically abundant, most widespread, and most easy to culture in the laboratory. However, E. huxleyi is definitely not the coccolithophore responsible for most sinking inorganic carbon and it may not be an appropriate model for the responses of other principal groups of coccolithophores, as it (and it's close relatives in the Gephyrocapsa genus) is different from most coccolithophores. For example, E. huxleyi does not require Si for calcification while most do.

In general, I have a hard time following the Discussion. It lacks clarity and focus, and seems to stray into inadequate review of important but peripheral themes. It's difficult for me to provide more detailed comments as I am not convinced that they know what state of nutrient limitation (or not) the cells were in when harvested.

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