

Interactive comment on “Phosphorus limitation and heat stress decrease calcification in *Emiliana huxleyi*” by Andrea C. Gerecht et al.

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Authors' reply to reviewers' comments:

We would like to thank all five reviewers for their positive and supportive evaluation of the manuscript and for their suggestions on improvements. We have carefully considered all comments and hope to have addressed each comment in a satisfactory manner. Here, we first address some common points that were raised by most/several reviewers, before responding to the reviewer's general comments, followed by the specific comments to the text.

Common points raised:

1) Several reviewers requested more information on the particular strain used in the

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current study (referees #2, #4, #5).

Authors' response: Additional information on the origin and isolation of this strain has been added to the methods section, including date of isolation and the conditions under which the stock culture is maintained in the laboratory. The strain is not axenic and has been deposited at the NIVA culture collection (niva-cca.com). This strain belongs to morphotype A, which is widespread in the Northeast Atlantic.

2) Several reviewers required further details on how the coccolith morphology was assessed (referees #1, #2), especially in regard to distinguishing between “incomplete” and “dissolved” coccoliths (referees #1, #2, #3).

Authors' response: We have defined three categories of coccolith morphology: normal, malformed and incomplete. We have added a table to the methods section to outline the characteristics of these categories. We do not differentiate between different kinds of malformations, only between incomplete (considered to be of normal morphology, but are uniformly unfinished) and malformed (of overall irregular morphology or with irregular morphological features). Calcite content of single malformed coccoliths can be expected to be lower, the same or higher than normal coccoliths depending on the kind of malformations, whereas incomplete coccoliths can be expected to contain less calcite (though we did not measure this). We could clearly distinguish between incomplete and malformed coccoliths on the SEM images.

Our data set is complicated by the occurrence of high amounts of dissolved coccoliths in P-limited batch cultures, the features of which we describe in the new table. This dissolution affects all categories of coccoliths (normal, malformed, incomplete). However, the features of dissolved coccoliths are similar to those of incomplete coccoliths and we were not able to make an unambiguous distinction between the two possible origins of “incomplete coccolith morphology” i.e. “incompletely produced” or “incomplete because of secondary dissolution”. We therefore only have one category of “incomplete coccoliths” in our Fig. 3, most of which are a result of dissolution in P-limited

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stationary phase batch. We have added an upfront description of these differences in the methods section as well as additional images to Fig. 1.

3) Referee #2 questioned the validity of using 24°C as supraoptimal temperature and most reviewers required further justification for the choice of the specific culture conditions (referees #2, #3, #4, #5).

Authors' response: The main evidence that these cultures were heat-stressed is provided by the decrease in growth rate at 24°C compared to 19°C. The decrease is slight (6-9%, Table 1), and only observed in semi-continuous, not batch cultures. However, measurements of growth rate in semi-continuous cultures are more robust because growth rate is measured as an average of numerous dilution cycles. A decrease in growth rate at a higher temperature is the definition of above-optimum growth. According to Eppley (1972), phytoplankton growth rates increase with increasing temperature as long as this temperature is below the optimum for growth, whereas above optimum temperature, growth rates decline. As growth rates decline more sharply at above-optimum temperatures (i.e. heat stress is more detrimental than below-optimum temperature), it is often difficult to culture phytoplankton at above-optimum temperature. Therefore, obtaining a stronger difference in growth rate at even higher temperature would have been technically difficult and would likely have resulted in the crash of the culture. The isolation temperature and increased presence of malformations at 24°C are additional indications that this temperature was above optimum. These motivations and definitions have now been mentioned upfront in the methods section.

We agree with referee #5 that it would have been beneficial to rigorously test growth rates over a broad range of temperatures before choosing the applied temperatures of 19 and 24°C. The fact that we did not do this, however, does not detract from the fact that 24°C was above the optimum for growth.

4) Several reviewers expressed concern about possible DIC-limitation in P-limited batch cultures (referees #2, #3, #5).

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Authors' response: As outlined in the text, we discuss that P-limited batch cultures may have been co-limited by P and DIC at the time of harvest. However, entry into stationary phase was due to P-limitation for the following reasons: There was a drastic decrease (3.3-3.7-fold; Table 1) in the POP content of "P-limited stationary phase cultures", which is a sign of strong P-limitation and would not be expected under DIC limitation. Secondly, POC fixation continued in stationary phase leading to a strong increase in cell size/POC content, which again argues against DIC limitation. The recent publication by Wördenweber et al. (2017) gives mechanistic support for this observation. By analyzing the metabolome, these authors observed that under P-starvation metabolites such as lipids are accumulated i.e. enzymatic functionality is preserved, whereas enzymatic functionality ceases under nitrogen starvation. This continuing fixation of POC by non-dividing cells led to the high consumption of DIC in P-limited batch cultures. Thirdly, Buitenhuis et al. (1999) calculated a minimum DIC-threshold for calcification in *E. huxleyi* of ca. 600 μM . So our DIC-values were arguably only at the threshold for DIC –limitation.

5) Several reviewers questioned the validity of using stationary phase as an "end-of-bloom" scenario (referees #2, #3).

Authors' response: It is difficult to recreate natural situations in the laboratory and we do not pretend to have faithfully recreated an "end-of-bloom" scenario as bloom demise is likely regulated by many factors, such as viral lysis. However, "stationary phase batch cultures" are the closest approximation we could achieve in the laboratory to an "end-of-bloom" scenario and it would now be interesting to test this hypothesis in the field.

Anonymous Referee #1:

1) The environmental significance of the data is presented with appropriate caution in the main text, but becomes over-stated in the abstract and conclusions sections.

Authors' response: We agree that general statements on the effects of P-limitation and

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heat stress on *E. huxleyi* or coccolithophores as a whole, require further investigation of additional strains and/or species to account for strain- and species-specific variability. We have therefore modified the relevant section of the abstract (coccolithophores now reads *E. huxleyi*) and conclusions (*E. huxleyi* now reads “a temperate strain of *E. huxleyi*”) to specify that our interpretations are specific to *E. huxleyi*/the strain under study. We have also elaborated on which physiological effects may be more general and which require further studies as outlined below.

The main interpretations of the data presented are:

- A future rise in global temperature, accompanied by a decrease in nutrient availability may decrease CO₂ sequestration by coccolithophores through lower overall carbon production.

Authors' response: As an additional justification of the above hypothesis, we would like to point out the following. An increase in phosphorus requirements at higher temperature has been observed also for *Coccolithus pelagicus* (Gerecht et al., 2014) and can be inferred from the data presented in Satoh et al. (2009) and Feng et al. (2008) for two additional strains of *E. huxleyi*, as discussed on p. 8, l. 30-33. As in the present study, in Gerecht et al. (2014), higher P-requirements led to lower final cell concentrations in P-limited stationary phase cultures of *C. pelagicus* i.e. lower biomass under P-limitation. Increased phosphorus requirements leading to lower overall carbon production in a future warmer ocean may therefore be a general feature of coccolithophores. We have slightly rewritten the relevant section of the discussion and conclusions to better highlight this point.

- The export of carbon may be diminished by a decrease in calcification and a weaker coccolith ballasting effect.

Authors' response: A decrease in coccolith coverage at high temperature has also been observed for *C. pelagicus* (Gerecht et al., 2014) as stated on p. 9, l. 8-9. However, this is to our knowledge the first study to describe a decrease in calcification under

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weak (i.e. not affecting growth rate) P-limitation. On the contrary, in *C. pelagicus* weak P-limitation decreases POC over PIC production (Gerecht et al., 2015). So further studies on other strains and species are necessary to confirm how widespread this physiological response is. We have stated this accordingly in the conclusions.

For example, it would be beneficial to assess how widespread/dominant this strain of *E. huxleyi* is in comparison to those strains/species cited from other publications.

Authors' response: Although we have no information on the dominance of this particular strain, it belongs to morphotype A that is widespread in the Northeast Atlantic, while morphotype B dominates in the North Sea (van Bleijswijk et al., 1991). However, considering the physiological variability within a morphotype, e.g. Langer et al. (2009), we cannot make any conclusions about the general physiological responses based only on information of the morphotype.

Further recognition of the potential for acclimation is also important.

Authors' response: Cultures were acclimated to experimental conditions (temperature, P-concentrations) for ca. 10 generations before starting the experiment (this has been added to the methods section). Ten generations should be sufficient for acclimation to experimental conditions, according to the model of Aloisi (2015). Longer-term adaptation to changing environment is a factor that needs to be considered and we have included a short discussion about the potential for adaptation in the discussion.

2) Assessing coccolith morphology

The criteria for classifying coccolith morphology as 'normal, incomplete, and malformed' should be included in Section 2.4 (Methods). At present, the significance of 'incomplete' coccoliths as those that have undergone secondary dissolution (rather than being 'incomplete' due to incomplete primary formation) is only discussed in Section 4.2. This is an important distinction, particularly for fellow scientists who attempt to apply the same morphologic criteria in other experiments.

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Authors' response: Please see common point (2).

An additional image of a representative coccosphere from the cultures that had higher levels of malformation would also be a useful addition to Fig. 1.

Authors' response: We have added additional images to illustrate the different classes of coccoliths in more detail in Fig. 1, and have included a representative coccosphere with high levels of malformed coccoliths.

3) Semi-continuous and batch culture experiments. . . In this regard, does the experimental approach have any relevance for the strain of *E. huxleyi* used? I.e., would the approach that most closely replicates its original natural environment in the Oslo fjord be more representative for this strain?

Authors' response: *Emiliania huxleyi* blooms can occur in the Oslo fjord, usually after the diatom spring bloom. So a bloom scenario is certainly relevant for carbon production by this strain in this area. However, during the rest of the year, *E. huxleyi* is likely present in low numbers so that also a simulated "continuous low nutrient" environment is relevant for this strain. Here, we would like to note that we wanted to test the physiological limits of this particular strain in our simulated growth scenarios (batch and semi-continuous culture). These scenarios need to be applied with caution to the more complex, natural system.

Other minor comments:

- The uncertainty for the number of coccoliths per cell is much higher for the P-limited batch cultures at both temperatures (+/- 20 and 15). Is there a reason for this?

Authors' response: P-limited batch cultures were harvested in stationary phase in which the number of coccoliths per cell had increased considerably. At the same time, the variability in coccolith coverage was higher in the stationary growth phase whereas the number of coccoliths per cell was more constrained in exponentially growing cultures. The higher uncertainty is therefore not methodological, but due to higher

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biological variability in coccolith coverage in stationary phase.

- Table 2 (page 5 line 16) is referred to before Table 1 (page 5, line 38)

Authors' response: The numbering of the tables has been changed.

- The significance of the red and blue colors in Figure 2 is missing

Authors' response: This information has been added to the figure legend.

- There is no reference to the error bars in Figure 4.

Authors' response: Reference to the error bars has been added to the figure legend.

M. Hermoso, Referee #2:

(1) More information is needed on the cultured strain of *Emiliana huxleyi*, and the conditions under which the stock culture is maintained in the laboratory.

Authors' response: Please see common point (1).

(2) There are no details given of the culture technique per se apart from strategy (batch vs. semi-continuous) adopted. Were the cells acclimated to the target phosphorus concs and temperature conditions when proper experiments began?

Authors' response: Cultures were acclimated for ca. 10 generations to the two phosphate concentrations and temperatures before starting the experiments. This information has been added to the methods section.

This is all the more important as changing temperature is conceived a stressing factor in the study. We know that coccolithophores, and singularly *E. huxleyi* is fast adapting to changing environments so this methodological aspect is crucial for the implications of culture study to wild specimens and at timescales compatible with adaptation in the natural environment.

Authors' response: The *E. huxleyi* strain used in this study was isolated from the Oslo Fjord in the summer of 2011 and was kept in the lab as stock culture at 12°C until

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running the experiments in the fall of 2013. Cultures were acclimated for about ca. 10 generations at 24°C before starting the semi-continuous cultures which ran for 10 dilution cycles with ca. 3-4 generations between dilutions so another 30-40 generations. As the growth rate was ca. 8% lower at 24°C than at 19°C we observed no indications for short-term acclimation to this higher growth temperature. This growth temperature may therefore have been at the physiological limit for this particular strain. As other strains of *E. huxleyi* grow at higher temperature it is likely that this strain could adapt to higher growth temperatures over longer time periods.

(3) It would be valuable to elaborate on the malformations of the coccospheres/coccoliths observed by the Authors. The rationale behind the discrete class of malformation features and the implications for biomineralisation are elusive in the manuscript. ... Likewise, it is not entirely clear to me how the Authors are able to distinguish between malformation and dissolution features.

Authors' response: Please see common point (2).

On this note the argument that 24°C represents a heat stress for this strain as it was isolated from waters measured at 21°C or lower, and that more malformations were observed (p. 8 lines 23-25) does not appear as a strong argument to me.

Authors' response: Please see common point (3).

(4) I feel that at places the discussion is too descriptive and lacks a better attempt to understand the cellular mechanisms at play for the environmentally-driven change in carbon fixation. An integration of P acquisition strategy by *E. huxleyi* (a species with the ability to excrete ligands to increase P supply to the cell) with growth dynamics and organic and inorganic carbon fixation for each condition would be extremely useful and add value to the paper.

Authors' response: The reviewer points out that *E. huxleyi* has a particularly high capacity for obtaining P from its environment, e.g. Riegman et al. (2000). This char-

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acteristic makes it even more intriguing that a weak P-limitation, as imposed by our semi-continuous set-up, in which P is still readily available to the cells, should have an effect on the calcification rate. The few studies that have addressed the effect of P-limitation in a continuous setup observed either an increase in calcification (Riegman et al., 2000; Paasche, 1998; Paasche et al., 1996) or no change (Borchard et al., 2011). Please note that the increase in calcification was observed in co-occurrence with the decrease in growth rate in the continuous cultures so there is the overriding effect of growth rate. To our knowledge we present, for the first time, growth rate independent data on changes in calcification in *E. huxleyi* to show that calcification can decrease under P-limitation. We have some mechanistic understanding of the increase in calcification in stationary phase, mostly based on the work of Müller et al. (2008) i.e. cells are kept in the G1 (assimilation) phase of the cell cycle as P is lacking for cell division and therefore coccolith production (and to some extent also POC production) continues. However, we find it difficult to speculate about possible cellular mechanisms regarding the decrease in calcification under weak P-limitation as there is no knowledge base in the literature to explain why calcification would have specific P-requirements. On the contrary, most data point to a stronger dependence of POC than PIC production on P-resources, as exemplified by the increase in PIC/POC in stationary phase cultures. We elaborate on this in the revised discussion.

Specific comments:

(1) Page 3 Lines 6-7: In my opinion, looking at Table 3 it is not nutrient limitation that limits further algal growth in thus set-up, but rather the drift in the carbonate chemistry of the medium (see e.g. Hermoso 2014 in *Cryptogamie, Algologie* 35(4): 323-351).

Authors' response: If the reviewer is referring to the change in pH as "drift in the carbonate chemistry" than the change in pH was not great (lowest pH=7.7) due to the opposing effects of photosynthesis and calcification on medium pH. This pH-value is unlikely to have affected growth rates. If the reviewer is referring to the decrease of DIC as "drift" than DIC is also a nutrient that may have been limiting at the end of the

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experiment. However, although P-limited batch cultures at the time of harvest were possibly co-limited by P and DIC as outlined in the text, entry into stationary phase was due to P-limitation for the reasons outlined in common point (4).

(2) More broadly, I do not believe that the stationary phase represents an end-of-bloom scenario.

Authors' response: Please see common point (5).

(3) Page 3 Line 19: inter alia?

Authors' response: This expression has been changed to "among other things".

(4) Page 3 Line 22: Carbon "fixation" rather than "production".

Authors' response: Carbon production is a technical term referring to the calculation of production based on cellular elemental content (e.g. POC) and growth rate, e.g. Langer et al. (2013). This term is widely used in the literature and is the term we are referring to here.

(5) Page 3 Lines 22-24: There are many other references (Bollmann et al. 2010 in Protist 161:78–90; McClelland et al. 2016 in SciReport 6:34263 etc etc of which some cited at the end of the discussion should be also mentioned here).

Authors' response: Although numerous studies, such as those mentioned by the reviewer, have studied the effect of temperature on carbon production and coccolith shape and size, only a handful of studies have specifically examined the effect of temperature on coccolith malformations (as defined in this manuscript). We have specified that we are referring to this aspect of temperature effects in the text.

(6) Page 3 Line 30: I still think that "heat stress" is not appropriate here for the reasons outlined in general comments. The effect of changing temperature from 19 to 24 °C on growth rate is not very detrimental (by 10 percent) compared to the effect of other manipulations of the culture medium in literature. *E. huxleyi* has a broad tolerance and

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adaptability to temperature change compared other taxa, such as *C. pelagicus*.

Authors' response: Please see comment point (3).

(7) Page 6 Line 26-27: I disagree with this statement. Also Table 3 should be given the starting conditions.

Authors' response: This statement has been removed and only the DIC values measured in the cultures are left as information in the text. Starting conditions for the cultures was the growth medium and this was the same for all cultures grown in either 0.5 and 10 μ M medium. Therefore, we have added this information to the methods section rather than to table 3 to preserve clarity.

(8) Page 7 Line 1-2: How about the number of layers of coccoliths forming the spheres? This could be useful to put in the context of the dynamics of cell division.

Authors' response: We agree that this would be useful information. However, it is difficult to unambiguously determine the number of layers of coccoliths under SEM. This would require a cross section of the cell, see e.g. Hoffmann et al. (2015), which is a method that was not available to us.

(9) Page 7 Lines 35-36: The Authors should add a discussion on the mechanisms for this observation. There are a few studies on cells being stuck in the haploid phase due to the lack of N and P provision to replicate DNA and allow further division.

Authors' response: Although the role of N and P in switching between haploid (non-calcified) and diploid (calcified) phases in coccolithophores is intriguing, to our knowledge no data has been published so far to unequivocally show the role of nutrient limitation in phase switches. As we did not observe phase switches or address the haploid phase in the manuscript we feel that including this theory is too far removed from the main focus of this paper. We do discuss the lack of P-provision to replicate DNA, blocking further cell division in the diploid phase, which is the reason for the cultures entering stationary phase under P-limitation.

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(10) Page 8 Lines 8-9: Please refer to recent study on this particular point by Aloisi in *Biogeosciences* 15: 4665-4692, and incorporate suitable discussion on the mechanisms.

Authors' response: We have elaborated on the mechanisms for the cell size increase under P-limitation based on the reference suggested by the reviewer and the work of Müller et al. (2008).

(11) Page 9 Lines 6-7: I do not follow the argument being made here. Please clarify.

Authors' response: The two possible reasons for the discrepancy between coccolith numbers per cell and PIC content have now been separated into two paragraphs for clarity. The argument and discussion of partially dissolved coccoliths is now mentioned in the methods section instead of introducing it at this point.

(12) Page 10 Lines 1-2: I recommend that the Authors tone this down, as we know that such a conclusion at the scale of the global biogeochemical cycle requires longer-term and multi-strain investigation although I appreciate the "may" being used here.

Authors' response: This sentence has been reformulated.

Anonymous Referee #3: (1) They find that P-limitation can actually decrease the PIC quota, PIC production, and PIC/POC ratios in *E. huxleyi*, which is opposite that which has been most commonly reported in earlier literature, although some more recent studies are cited to report similar results. This contrast is little discussed.

Authors' response: Earlier experiments e.g. by Paasche used either batch or continuous (i.e. chemostat) cultures. As discussed in the text and illustrated by our data on P-limited batch cultures, there is an overriding effect of growth phase changes i.e. the change from exponential phase to stationary phase. In stationary phase, P-limitation blocks further cell division and the cells remain in the G1 (assimilation) phase of the cell cycle in which both photosynthesis and calcification continue, leading to, respectively, an increase in POC content/cell size and an overcalcification of the cells.

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A similar process may be at play in chemostats in which growth is continuous, but at a lower rate. Therefore cells will be in the assimilation phase longer which may lead to an overproduction of coccoliths such as reported in Paasche and Brubak (1994). Please note that the POC content also increased in their chemostat. Similar results have been observed by Müller et al. (2008) and Perrin et al. (2016). An exception is the study by Oviedo et al. (2014) who also reported a decrease in the PIC/POC ratio in five out of six *E. huxleyi* strains. However, also in this study the absolute PIC content increased, together with POC content/cell size in stationary phase.

To our knowledge, ours is the first study to address nutrient limitation without introducing the confounding factor of growth rate changes. So most of this contrast can be explained by methodological differences. The relevant parts of the discussion have been modified to more clearly illustrate this issue.

(2) Neither the P-stress nor the heat stress used is very clearly justified. Where are such changes predicted to occur? Why is P-stress chosen instead of N-stress, when much more of the world's ocean is thought to show N-limitation of primary production?

Authors' response: Although much of the world's ocean is thought to be N- rather than P-limited, P-limitation can be relevant locally. This particular strain of *E. huxleyi* for example was isolated from the inner Oslo Fjord where the load of N over P can exceed the Redfield ratio and winter N concentrations are usually high (<http://www.miljodirektoratet.no/old/klif/publikasjoner/2253/ta2253.pdf>). Furthermore, P-stress may be more relevant than N-stress for calcification as P-resources are necessary for energy storage and are a part of cellular membranes, two aspects that are relevant to coccolith production.

(3) It is especially not clear to me what natural conditions are mimicked in the P-limited batch cultures. Do *E. huxleyi* blooms naturally experience these chemical conditions (e.g., such low DIC and omega-calcite values)? If these are conditions arise in batch cultures at very high cell densities only reachable in lab monocultures, perhaps they

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must be more careful of extrapolating their results from stationary phase cultures to changes in carbon export.

Authors' response: The reviewer correctly points out that the low DIC values (due to high cell concentrations) are unlikely to occur in nature. However, we used P-limited batch cultures vs. semi-continuous cultures to test physiological limits i.e. what effect does P-limitation have once it is growth-limiting i.e. blocking further cell division vs. when cell division is continuous. Furthermore, we only extrapolated results for carbon export in the natural system from semi-continuous cultures (with more realistic cell concentrations and carbonate chemistry) and use P-limited batch cultures as a comparison.

(4) In terms of heat stress, it's not made very clear why the temperatures of 19°C and 24°C were chosen, although there is some justification given in the Discussion. Is 19°C a typical SST in the North Sea (assuming the clone here represents a North Sea population) or typical of the Oslo Fjord? With global warming is it expected to reach 24°C regularly? Authors' response: Please see common point (3). A temperature of 19°C can be considered a high summer temperature in the Oslo Fjord. As *E. huxleyi* often has maximum growth rates at temperatures above those found at the isolation site (Sett et al., 2014) we chose a temperature that was at the high end of the range that this strains is likely to encounter in nature as our "normal temperature".

It is unlikely that the Oslo Fjord will reach temperatures as high as 24°C regularly, at least on time scales that would preclude adaptation to higher temperature. The reason for choosing this temperature was that it was high enough to induce heat stress (defined as a decrease in growth rate) without causing the culture to crash. We were therefore focused on choosing a feasible culturing temperature that allowed us to observe the physiological effects of heat stress rather than recreating the natural environment. This has been stated up front in the methods section.

What about *E. huxleyi* populations currently found in 8-12°C waters, would the same

tendencies occur if grown at from 13°C to 19°C?

Authors' response: This is a difficult question to answer and would depend very much on whether the physiological range of that strain corresponds to its natural range. As the reviewer rightly cites in the specific comments, numerous studies have tested the effect of temperature on carbon production in *E. huxleyi* using similar or higher temperatures than we have (24°C). The temperature that is above the optimum will depend on the strain (and hence likely the place of isolation).

In this regard it is important to define "above-optimum temperature". This is defined as the temperature at which growth rate declines in respect to maximum growth rates obtained at the optimum temperature (please see common point (3)). This is precisely the reason why the temperature of 24°C did not induce heat stress in Feng et al. (2008) because the growth rate increased from 20 to 24°C. Incidentally, this strain was isolated from the Sargasso sea (strain CCMP 371) with a known temperature range of 17-26°C. Rosas-Navarro et al. (2016) report that growth rates decreased in three strains of *E. huxleyi* at 27.5°C. However, they do not present data for PIC and POC production, or occurrence of malformed coccoliths, at this above-optimum temperature, which is unfortunate as this would have been a very interesting data set for comparison.

(5) It's not necessary for all studies to try to replicate specific environmental conditions (often impossible), perhaps especially when the goal is to understand physiological limits or to take a first approximation.

Authors' response: As the reviewer rightly points out, our choice of experimental set-up was based on testing the physiological limits of P-limitation and heat stress, which defined also the temperatures to use as outlined above.

However, considering this lack of grounding of experimental design within an explicit environmental context, it appears that the Conclusions should be more cautious in extrapolating to biogeochemical effects.

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Authors' response: We have tried to outline our experimental design/motivations more clearly in the answers to the above comments and have also clarified these aspects in the text.

(6) There is a focus on biogeochemical effects, but nothing on ecological effects of the documented changes in PIC and coccoliths. What function do they serve? I might suggest the review by Monteiro et al. to look at, and think of some of the consequences.

Authors' response: A consideration of the possible functions of coccoliths and hence consequences of lower calcification rate/coccolith coverage has been added to the discussion.

(7) Finally, I'm not so sure of the extent of the novelty of this study. They say "To our knowledge, this study is the first to specifically test the impact of heat stress. . ." but then there actually are a few quite relevant studies (it depends on how "heat stress" is defined), some of which they cite. For that reason, a more rigorous study design in an explicit environmental context would have been much stronger.

Authors' response: We have now defined what we mean by the term "heat stress" i.e. a decrease in growth rate, both in the replies to the above comments and in the text. Considering this definition, we are not aware that other studies have examined the effect of heat stress on PIC production.

Specific comments:

(1) p. 1 Line 26, should probably cite something more recent as well, such as the metaanalysis by Meyer and Riebesell 2015.

Authors' response: This citation has been added.

(2) p. 2 Lines 6-9 : "Batch culture on the other hand represents an end-of-bloom scenario in which the lack of nutrients limits further cell division. . . production cannot be determined in the batch approach ". That's only true if the last part of a batch culture is analyzed, as growth becomes limited due to exhaustion of nutrients, build-up

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of metabolites, shading, limited gas exchange, etc. In fact, there are many published experiments where production rates were determined in dilute batch culture, in the early exponential phase of growth before DIC consumption or nutrient consumption was substantial.

Authors' response: The reviewer correctly points out that production rates can be determined in dilute batch cultures, in which growth is exponential. We could for example have examined the effect of heat stress in dilute batch cultures. However, it is not possible to test P-limitation in dilute batch cultures as these per definition will not be limited by nutrients such as P. We have added a sentence in the introduction to clarify this point.

(3) Line 29: "None of these studies, however, tested the effect of above-optimum temperature". I don't understand this unless one defines what is "above-optimum temperature". In the study presented here the temperatures used are 19°C and 24°C. The study of Feng et al. (2008) (which they cite) used 20°C and 24°C, so if 24°C is an above-optimum temperature in the present study, why wasn't it in Feng et al.? Rosas-Navarro et al. 2016), which they also cite, used 25°C as the highest temperature. How is "above-optimum temperature" defined, and wouldn't it possibly depend on the origin of the cultures? For example, *E. huxleyi* from lat. 50°C in the North Atlantic probably does not experience such temperature, *E. huxleyi* in the Mediterranean Sea will rarely experience temperatures that high, and those may be unexceptional surface temperatures for the tropical Pacific, where *E. huxleyi* can also be found. This is discussed much later on p. 8, lines 20-26, but I am not so convinced how relevant this temperature range is.

Authors' response: Please see common point (3).

(4) Lines 36-37: Should consider (and cite) also work of van Bleiswijk et al. 1994 and Rokitta et al. 2016 very relevant for theme of *E. huxleyi* response to P-limitation.

Authors' response: These references have been considered in the final version.

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(5) I have a problem with the use of K medium for nutrient experiments. K medium contains a mix of ammonium and nitrate as N-source, and it contains glycerophosphate as a P source. It's not clear from Gerecht et al. (2014) if they modified these components. They need to give the basal medium composition they used.

Authors' response: It is correct that K-medium usually contains glycerophosphate as a P-source, as well as ammonia. We, however, modified the medium to contain only nitrate as a N-source and KH_2PO_4 as a P-source. The full medium recipe has been added to the methods section.

What volume were cultures?

Authors' response: Batch cultures were 350 mL, whereas semi-continuous cultures were kept at 50 mL until the last dilution round where volumes were increased to 350 mL.

(6) p.4 For semi-continuous cultures, what was the dilution rate or growth rate?

Authors' response: The average growth rates for semi-continuous cultures are reported in Table 2. The dilution rate varied slightly depending on the cell concentrations reached in the cultures after two days as all cultures were diluted back down to 10.000 cells mL^{-1} every two days. A supplementary figure has been added to show the development of cell concentrations for each dilution cycle of semi-continuous cultures.

How was it determined or confirmed that the cultures in fact were limited by P-limitation in the semi-continuous cultures?

Authors' response: During the course of the experiment we could not confirm whether the cultures were limited, as the growth rate was not affected. We could confirm that the cultures were limited only after harvesting the cultures and determining phosphorus (POP) content which was lower in cultures grown on 0.5 μM initial phosphate medium than at 10 μM .

How could maximum cell concentrations have reached 170000 cells/ml if cultures were

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diluted back to 10000 cells/ml every second day? To have reached 170000 cells/ml from 10000 cells/ml in only 2 days would require approx. 4 cell divisions per day, which has never been reported for this species.

Authors' response: To reach a maximum of 170.000 from 10.000 cells mL⁻¹ in two days requires approx. 4 cell divisions in two days i.e. approx. 2 cell divisions per day, which is in the range of what has been reported for this species.

(7) Line 7: "P-limited cultures were harvested in stationary phase, ..." for how long in stationary phase? This is not clear from Fig. 4.

Authors' response: P-limited cultures were harvested on the day of the last data point presented in Fig. 4. We have added arrows to the figure to illustrate this.

(8) p. 5 Line 10: Give manufacturer & city for "CASYS".

Authors' response: Manufacturer & city are given at first mention of the instrument (p. 4 line 6).

(9) Line 30: "Average values were compared by a t-test". Was this pairwise test performed after the two-way ANOVA? If so, with what correction for multiple comparison? They are testing two factors (T and P-limitation) so should be doing a two-way ANOVA, not t-tests.

Authors' response: The reviewer rightly points out that a two-way ANOVA is necessary to test the effect of two factors (T and P-limitation). We used t-tests when comparing the effect of only one of these factors. However, as we describe both factors together in the text, the reference to the t-test is obsolete and has been removed. As stated in the next sentence, we used a two-way ANOVA to compare the data ("The influence of P-availability and temperature on variables was determined by means of a two-way analysis of variance (ANOVA).").

(10) p. 6 Line 24 and Table 3: What limited the growth of control batch cultures?

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Authors' response: Nothing was limiting control batch cultures as they were harvested in exponential phase.

(11) p. 8 Lines 10-12: "These large "ready-to-divide" cells (Gibbs et al., 2013) not only accumulate POC, but also accumulate PIC, leading to the 2-3-fold increase in coccolith number per cell observed in stationary phase cultures (Fig. 2c,d)." How do you know these cells are "ready-to-divide"? If they really are "ready-to-divide", do you mean they are blocked in G2 or M phase of the cell cycle? That doesn't make much sense.

Authors' response: This sentence has been rephrased.

(12) Lines 10-12: This is an important justification for their selection of temperatures. Nevertheless, I'm not very convinced about how these temperatures are reoe. I would prefer them to explicitly give the range of temperatures experienced in the North Sea as well as the fjord. Why is 19 °C a "normal temperature"? What does that mean?

Authors' response: The natural temperature range that can be expected for this strain has been added to the text. As to the definition of the temperatures used please see common point (3).

(13) Lines 12-13: "Stationary phase can be likened to an end-of-bloom scenario in nature, during which *E. huxleyi* sheds numerous coccoliths, leading to the characteristic milky color of coccolithophore blooms". Maybe, but it's also well know that the end of *E. huxleyi* blooms involves infection by the virus EHV.

Authors' response: In this statement we are not referring to what causes the demise of *E. huxleyi* blooms in nature (for which there may be numerous reasons), but rather describing the characteristic "overproduction" of coccoliths.

(14) p. 9 Line 10 "The percentage of partially dissolved coccoliths was higher at normal temperature than under heat" Where is this shown? Data is presented on "incomplete", "malformed", and "normal" coccoliths in Fig. 3. They state "The high numbers of incomplete coccoliths observed in P-limited batch cultures were likely a result of secondary

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dissolution (Fig. 1d; Langer et al., 2007) due to the low calcite saturation state reached in stationary phase cultures.” I would like to see more examples of incomplete coccoliths. Perhaps they can show that the type of incompleteness that appears in P-limited batch cultures (when ω -calcite is less than 1) is distinct from what appears when ω -calcite is greater than one?

Authors’ response: Please see common point (2).

Anonymous referee #4:

(1) I feel that the discussion is weakened by an emphasis on comparing two culturing methods rather than comparing individual vs. interactive effects of the stressors in question.

Authors’ response: We would like to point out that the inclusion and discussion of the two culturing methods in the manuscript not only serves as a “methodological comparison”. It also serves to compare two differing environmental scenarios. Whereas batch culture represents a strong P-limitation as may be encountered at the end of blooms, the semi-continuous culture tests the effect of a more continuous low-P environment. We agree that the discussion would benefit from a comparison of individual vs. interactive effects of the two stressors and this has been added.

(2) The specific choice of experimental conditions is also poorly justified and not placed into context.

Authors’ response: Please see common point (3) and reply to general comment (2) of referee #3.

Specific Comments:

(1) In the broader interpretations of their calcification results, the authors state in the Abstract, Introduction and Conclusions that decreases in calcification rates in *E. huxleyi* could “lessen the ballasting effect of coccoliths and weaken carbon export out of the photic zone”, or similar wording. In support, they reference Ziveri et al. (2007), who

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conclude that, despite the high abundance of *Emiliana* relative to other coccolithophore species, the small size and very low species-specific carbonate mass of their coccoliths means that they consequently export far less carbonate than expected. Baumann et al. (2004) similarly concluded that *Emiliana* plays only a relatively minor role in carbonate export in the Equatorial and South Atlantic. Can the authors support their statements of alterations to the carbon cycle more quantitatively using their PIC production values and abundances of *Emiliana* in the field? Or provide references of studies that better support these statements?

Authors' response: The reviewer correctly points out that the overall contribution of *E. huxleyi* to pelagic carbonate flux is small compared to other species. Nevertheless, coccolith ballasting (or lack thereof) can be considered relevant locally e.g. during blooms of *E. huxleyi*. To support the above statement i.e. weaker ballasting due to decreased calcification rates, it is necessary to examine whether and to what extent these physiological responses are applicable to coccolithophores as a whole. We have some indication that other species react similarly, including those arguably more (regionally) relevant to carbonate export such as *C. pelagicus*. For example, we have previously reported that *C. pelagicus* increases P-requirements and decreases coccolith coverage under heat stress (Gerecht et al., 2014). The relevant parts of the text have been modified to reflect this.

(2) Considerable emphasis is made on the short-comings of the batch culturing technique compared to the semi-continuous culture technique. Comparison of these two methods is present throughout the results and discussion, and, in my opinion, obscures a clear and explicit evaluation of contrasting individual (warming only, P-limitation only) vs. interactive (warming and P-limitation) effects and evidence (or not) for positive interactions.

Authors' response: Please see reply to general comment (1).

It is stated that batch culture experiments can only represent a severely nutrient de-

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pleted scenario whereas a semi-continuous set-up provides an acclimated low-nutrient population (p3, ln 7; p7 lns 12-17). I think that these statements are somewhat misleading for the following reason: A batch culture experiment experiences exponential growth at whatever the starting nutrient concentrations until these nutrients become sufficiently depleted that exponential rates of growth can no longer be maintained and growth rate rapidly falls to zero. A semi-continuous culture is just a batch culture that is subcultured/diluted (typically) around mid-exponential-phase cell concentrations several times. I therefore find it strange that the authors did not just sample their P-limited batch culture experiment at mid-exponential phase (as they did with the control experiments) well before the 'severe' nutrient depletion of stationary phase began, which based on Fig. 4 would have meant sampling on day 3 or 4. Would this not have been a more realistic comparison of control and P-limited conditions during exponential phase in all experiments?

Authors' response: Although the reviewer is correct insofar as there are similarities between an exponential batch culture and a semi-continuous culture, these two scenarios are not identical. The crucial difference is that a semi-continuous culture experiences stable limiting conditions over many generations, which leaves ample time for acclimation processes to re-structure the physiological machinery dealing with this environmental stress. In a batch culture, by contrast, the specific state of limitation equivalent to a semi-continuous scenario is a transient state experienced by the cells for a short time only (less than one generation). The whole point of comparing batch and semi-continuous culture was to compare the cumulative effect of a series of transient limitation states (increasingly severe; batch) to a single constant limitation state representing roughly the average of the many transient batch culture states (semi-continuous). Our experimental setup was designed to serve this purpose.

It would also allow a comparison of multiple generations of growth (semi-continuous) with far fewer generations of growth (batch to mid-exponential phase).

Authors' response: We do not see the benefit of this.

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It would also have removed the issues with calculating production rates, as only exponential growth phase populations would have been sampled.

Authors' response: True, but see comments above.

(3) I would like to see a broader context of the area where a similar degree of temperature and phosphorus stress is predicted to be experienced in the context of this strain isolated from a Norwegian fjord.

Authors' response: The aim of this study was not to test/evaluate specific predicted environmental factors relevant for our strain. The aim was to look at environmental stressors (high temperature, P-limitation) by testing the physiological limits, independently of whether and when this strain will encounter these conditions in nature. Therefore we chose a temperature that was above the optimum for growth (but that still allowed growth; please see common point (3)) to test the effect of heat stress. Similarly we tested the effect of strong and weak P-limitation on this strain by having one laboratory scenario in which P-limitation becomes limiting for cell replication (stationary phase batch culture) and one in which P-limitation is not strong enough to affect cell replication i.e. growth rate, but does affect calcification rate (semi-continuous culture). In this regard, see also reply to general comment (3) of referee #1 and to general comments (2) and (4) of referee #3.

Similarly, there is no discussion of the fact that physiological stress experience by one strain of this species under climate change is as likely to lead to its ecological replacement by another, more tolerant strain given recent studies presenting the large genetic pool of *Emiliania* (e.g., Read et al., 2013, which also discusses differences in genes for tolerance of low phosphorus conditions between strains).

Authors' response: This aspect has now been considered in the discussion.

(4) I was surprised to see that no figures of any POC, PIC, or POP data were presented, only data in the tables. Was there a reason for this? Given the two experimental

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approaches, two temperatures and two nutrient states, it made it difficult to quickly visualise the dataset.

Authors' response: There was no particular reason, apart from keeping the manuscript concise. We will add the relevant figures as a supplement to the final version.

(5) The strain used seems to be a new isolate – do the authors intend to deposit this strain into a culture collection for use by other researchers? Given it is not held in a culture collection, the authors must provide the essential ancillary information on the isolate and its maintenance in culture.

Authors' response: Please see common point (1).

(6) It is stated deep into the discussion that the temperature at the isolation location does not exceed 21 degrees and this is presumably how the authors decided that a temperature of 24 degrees was beyond the thermal optimum. Did the authors perform a systematic temperature optimum assessment by determining growth rates at a range of temperatures?

Authors' response: Please see common point (3).

Given that the exponential growth rates were not substantially different between temperature treatments (semi-continuous) and in fact were higher in the 24 degree treatment for the batch culture approach, this would perhaps suggest that (using exponential growth rate as a physiological indicator) this isolate has a relatively broad thermal tolerance.

Authors' response: Please see common point (3).

(7) The authors do not state whether there was any period of acclimation for populations experiencing low phosphate or high temperature treatments.

Authors' response: Cultures were acclimated for ca. 10 generations to low phosphate and high temperature culture conditions before starting the experiment. This informa-

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tion has been added to the methods section.

(8) How did the authors account for the tendency of *Emiliana* to form multi-layer coccospheres when counting the number of coccoliths from SEM images? Comparing the coccosphere size from CASY with the cell size from light microscopy (back calculated from the volume data) and considering the thickness of *Emiliana* coccoliths, would suggest that coccospheres were not mono-layer.

Authors' response: The majority of multilayered coccospheres found in our study collapsed during the filtration process, which allowed us to count the coccoliths from all layers. The non-collapsed multilayered coccospheres commonly had an only partially covered first layer i.e. an incomplete second layer. Thus, it was possible to count the visible coccoliths in the first and second layer and estimate the number of covered coccoliths of the first layer. In very rare cases when we could not estimate the number of layers due to a complete outer layer, we estimated the number of layers and the number of coccoliths, which could fit under the outermost layer. This approach was consistently used throughout the SEM analysis to minimize error arising from the inability to see all coccoliths on the coccosphere.

(9) Is there any reason why the authors refer to both light microscope and CASY cell size measurements as “cell size” when CASY gives coccosphere size measurements? There are huge differences in volumes between the two methods due to the coccosphere and whilst cell size directly relates to cell carbon, coccosphere size does not and therefore this unnecessarily confuses the reading in parts.

Authors' response: The CASY system does not actually provide accurate coccosphere size measurements (Gerecht et al., 2015), but gives an intermediate value between cell and coccosphere size. We therefore use CASY measurements as a proxy for “cell size” to be able to observe and visualize the size increase during the development of the batch culture.

(10) The discussion would benefit from a discussion of the physiological mechanisms

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behind the observed response to P-limitation and heat stress singularly and combined, and there is considerable literature on this species, other coccolithophore species and other phytoplankton groups that would support such a discussion.

Authors' response: We do discuss the possible physiological mechanism behind the cell size increase under P-limitation, as well as the possible explanations for the increased P-requirements under heat stress. As this is the first observation of decreased calcification rate under heat stress and P-limitation in this species, we find it difficult to speculate further about the possible physiological mechanisms unless the referee would like to point us towards relevant literature. In this regard, please also see reply to general comment (4) of referee #2.

p6, In 37-39 – referred to changes in size but “twice as large in stationary phase” refers to cell volume, so this should be changed to reflect this.

Authors' response: The reference to cell volume has been added.

p8, In 7-9 – The reference to Sheward et al. (2016) on line 7 should be changed to Gibbs et al. (2013) as the latter presented *Emiliana* data. This sentence could also include *C. braarudii*, *Calcidiscus* and *Helicosphaera* from Sheward et al. (2017). The Sheward et al. (2016) at the end of line 9 should be changed to 2017 (I think you have referred to the discussion paper rather than the finally-published article).

Authors' response: The relevant references have been changed.

Technical Corrections:

Throughout the paper, there are inconsistencies with the author order in your references. Sometimes they are ordered by date, other times alphabetically, and several times I can find no logic to the order! (e.g., p2, In 37-38).

Authors' response: This has been resolved.

Anonymous referee #5:

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...the choice of the cultures conditions, P limitation and temperature stress, need to be express with more details in the introduction or in the methodology part concerning the choice of the values.

Authors' response: Please see common point (3).

Specific comments

p. 2; Ln 9: You need to add the word particulate to present for the first time in your manuscript the PIC and POC terms.

Authors' response: This has been added.

p. 3; 19: What does inter alia mean?

Authors' response: Latin for "among other things"; "inter alia" has been replaced with this term in the text.

p. 3; Ln 29 & 35 : Did you test different temperature on the strain (before these experiment) to find the optimal temperature at the selected light dose (12 :12, 100_mol photons.m-2.s-1)? Or did you have an idea (or reference) of the optimal temperature of your strain before selecting your two temperature conditions?

Authors' response: Please see common point (3).

p.3; Ln 34-39: What is the name of your strain?

Authors' response: Please see common point (1).

Have you done some acclimations of your strain to the temperature and P concentration conditions before starting your experiment? You should indicate in your method if you did.

Authors' response: Yes, cultures were acclimated for ca. 10 generations to the temperature and P concentration conditions before starting the experiment. This information has been added to the methods section.

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What is the initial nitrate concentration in your medium? Is it the standard concentration of NO₃ in a K medium? If not, the value of the concentration need to be indicate in this method part.

Authors' response: The basal composition of the medium has been added to the methods section.

You also need to add the value of the salinity of your medium.

Authors' response: This information has been added to the methods section.

p. 5; Ln 21-26: Did you take into account that *Emiliana huxleyi* can have multiple layers of coccoliths? It is clearly visible on your figure 1 that your strain can have several layers of coccoliths depending of the cultures conditions. How did you take this particularity in your coccoliths counts?

Authors' response: Please see reply to specific comment (8) of referee #4.

What is the standard deviation on your counts? At least, you have triplicate so you need to specify the standard deviation on your number.

Authors' response: The standard deviation of our counts of coccolith number per cell is listed for each culture condition in Table 1. This standard deviation is based on the total number of cells (n) analysed, which is the sum of all three replicate cultures for each condition. N is also listed for each culture condition in Table 1.

What about coccospheres diameter on your SEM images? Did you think about doing coccospheres measurements? If not, is it because of the high number of detached coccoliths on your filter?

Authors' response: As the reviewer points out, there was a high number of detached coccoliths on the filter so that coccosphere measurements under SEM would possibly have been underestimated. Additionally, most of the coccospheres in P-limited batch cultures had collapsed, making accurate estimates of coccosphere size based on SEM

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measurements difficult.

It should be interesting to discuss the PIC content related to your coccolith number and the thickness of your coccolith layer.

Authors' response: Yes, we agree with the reviewer that this would have been an interesting aspect to examine. However, determining the thickness of the coccolith layer or the number of coccolith layers around one cell with SEM was not possible; see also response to specific comment (8) of referee #2.

p. 6; Ln 24-34: Final cell density for batch cultures were being push really high in order to get the P-limitation. However, respect to LaRoche et al. 2010, inorganic carbon system changes need to be kept below 5% to avoid carbon system changing due to the high cell density. This means that nutrient limitation experiment for batch system have to attain a reasonable final cell density in order to keep a DIC system quasiconstant through the experiment and to avoid a inorganic carbon limitation before the P limitation in this case. In your P-limited cultures, DIC changes that you get at the end of the experiment led to a low calcite saturation constant. Therefore observations in morphology and calcite content may be due to this changes rather than the P-limitation and heat stress effect. You took into account this changes in your discussion but it will be useful to indicate why you did batch experiments in this way. Could you justify this choice in the methodology of your batch experiment? Did you think about carrying the experiment with a lower initial P concentration rather than push the cell density so high? Or did you have a target initial P concentration that you wanted to test? In this last case, a comment on the 2.1 part will be necessary.

Authors' response: We did not choose an initial phosphate concentration of 0.5 μM to recreate a specific field situation. Rather, this concentration was chosen based on prior considerations of collecting enough biomass for all analyses, while keeping cell concentrations of semi-continuous cultures well below stationary phase. The low DIC concentrations in P-limited stationary phase batch cultures do no affect the conclusions

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of the manuscript in regard to P-limitation, which are that P-limitation does not affect coccolith morphology per se, but decreases calcification rate in this strain of *E. huxleyi*.

p. 7; Ln 27: What is the normal temperature? Even if you explain it later (p.8 Ln 26), you should describe here that the normal temperature is 19°C if you want to use this term. It is not obvious for readers.

Authors' response: The terms (and motivations) of using 19°C as normal and 24°C as supraoptimal temperature have been now explained in the methods section.

p. 8; Ln 1-5: same comments that previously on the DIC system

Authors' response: See response to previous comments.

Figures & Tables

Figure 2: You need to add the color explanations: blue is your schematic initial cell, red is the schematic effect observed at the end of your experiment.

Authors' response: This information has been added.

Figure 4: You should add the error bar on triplicate somewhere in your legend.

Authors' response: This information has been added.

Tables 1, 2 & 3: You should add the standard deviation in your legend and your n number (for example, n = 3 if it is triplicate).

Authors' response: This has been added.

Table 2: You need to clarify in the legend of your table if the cell volume has been calculated with measurements of cell diameter from the harvest day or from an average of daily measurements.

Authors' response: The cell volume presented in the table was calculated from measurements of cell diameter from the harvest day. This information has been added to the table.

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