Point-by-point reply to reviewers' comments:

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.
We agree that general statements on the effects of P-limitation and 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 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 e.g. an increase in phosphorus requirements at higher temperature, observed also for *Coccolithus pelagicus* (Gerecht et al., 2014) and inferable from the data presented by Satoh et al. (2009) and Feng et al. (2008) for two additional strains of *E. huxleyi*. Whereas a decrease in coccolith coverage at high temperature has also been observed for *C. pelagicus* (Gerecht et al., 2014), to our knowledge this is the first study to describe a decrease in calcification under weak (i.e. not affecting growth rate) P-limitation. 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.

Further recognition of the potential for acclimation is also important.
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) 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. 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.

We have added a table to the methods section to outline the characteristics of the three morphological categories "normal, incomplete, and malformed". We have also added an upfront description in the methods section of the differences between the two possible origins of "incompleteness" of coccoliths and state that we could not distinguish between them. We have added additional images to Fig. 1 to illustrate the

different classes of coccoliths in more detail and have included a representative coccosphere with high levels of malformed coccoliths (new Fig. 2e).

Other minor comments:
5 - *Table 2 (page 5 line 16) is referred to before Table 1 (page 5, line 38)*
The numbering of the tables has been changed.

- *The significance of the red and blue colors in Figure 2 is missing*
This information has been added to the figure legend.
10
- *There is no reference to the error bars in Figure 4.*
Reference to the error bars has been added to the figure legend.

M. Hermoso, Referee #2:
15 *(1) More information is needed on the cultured strain of Emiliania huxleyi, including (where possible) the date of isolation, the morphotype of coccoliths, whether the strain is deposited in a Culture Collection (or in the process to be), and the conditions under which the stock culture is maintained in the laboratory (temperature, light irradiance, etc), is the strain axenic?*
20 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 non-axenic and belongs to morphotype A, which is widespread in the Northeast Atlantic. It has been deposited at the NIVA culture collection (niva-cca.com). Unfortunately, the strain has apparently
25 ceased to calcify after deposition at the culture collection.

(*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?*
30 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.

(3) It would be valuable to elaborate on the malformations of the
35 *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.*
We have defined three categories of coccolith morphology: normal, malformed and
40 incomplete. We have added a table to the methods section to better 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). We could clearly distinguish between incomplete

and malformed coccoliths on the SEM images. 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).

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.
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 isolation temperature and increased presence of malformations at 24°C are additional indications that this temperature was above optimum. This has now been mentioned in the methods section and specified in the discussion.

(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.
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 characteristic 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 an increase in calcification was observed in all studies in co-occurrence with a 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 this 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).

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 culture 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 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 following reasons. There was a drastic decrease (3.3-3.7-fold; Table 2) 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. This continuing fixation of POC by non-dividing cells led to the high consumption of DIC in P-limited batch cultures. We have elaborated on this for the final discussion.

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

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. 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.

(3) Page 3 Line 19: inter alia?

This expression has been changed to "among other things".

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

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).

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

Yes, the decrease in growth rate at 24 compared to 19°C is slight (6-9%, new Table 4), 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, even if slight, 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 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. These motivations and definitions have now been mentioned in the methods section.

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

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 µM medium. Therefore, we have added this information to the methods section (new table 1) 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.

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.

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.

(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.
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.
The two possible reasons for the discrepancy between coccolith numbers per cell and cellular PIC content have now been separated into two paragraphs for clarity. The occurrence 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.
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.
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 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.
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 obtained 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?
P- and heat stress are expected to co-occur in a future warmer ocean. Our aim with this study was to test the physiological limits of P-limitation and heat stress rather than testing specific predicted values.

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

5 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

10 observe the physiological effects of heat stress rather than recreating the natural environment. We now mention the motivations/definitions for choosing these temperatures in the methods section.

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

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

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

20 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 (Eppley, 1972). This is precisely why the

25 temperature of 24°C did not induce heat stress in Feng et al. (2008) as 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

30 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

35 *physiological limits or to take a first approximation.*

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.

40 *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.*

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.
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.
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.
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 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.
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 modified this sentence to read that nutrient-limited production cannot be determined in the batch approach.

(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
We have now defined "above-optimum temperature" upfront in the methods section.

(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.
The work of Wördenweber et al. 2017 is considered more relevant to this particular study and has been included in this manuscript.

5 *(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.*
10 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 (new table 1).

15 *What volume were cultures?*
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?
20 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 to 10.000 cells mL^{-1} every two days. A supplementary figure (1) has been added to show the development of cell concentrations for each dilution cycle of semi-continuous cultures.
25
How was it determined or confirmed that the cultures in fact were limited by P-limitation in the semi-continuous cultures?
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
30 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
35 *were 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 aprox. 4 cell divisions per day, which has never been reported for this species.*
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
40 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.
P-limited cultures were harvested on the day of the last data point presented in Fig. 4

(new Fig. 1). We have added arrows to the figure to illustrate this.

(8) p. 5 Line 10: Give manufacturer & city for "CASY".
Manufacturer & city are given at first mention of the instrument (p. 4 line 14).

(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.
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.

(10) p. 6 Line 24 and Table 3: What limited the growth of control batch cultures?
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.
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 aare 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?
The natural temperature range that can be expected for this strain has been added to the text.

(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.
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 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 omega-calcite is less than 1) is distinct from what appears when omega-calcite is greater than one?

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 a new table in the methods section. 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 stationary phase batch. We have added an upfront description of these differences in the methods section as well as additional images to (new) Fig. 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.

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 manuscript would benefit from a comparison of individual vs. interactive effects of the two stressors and a short discussion has been added to the conclusions section.

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

The choice of the experimental conditions is now described in the methods section.

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 conclude that, despite the high abundance of Emiliania 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 Emiliania 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 Emiliania in the field? Or provide references of studies that better support these statements?
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.
Please see reply to general comment (1).

It is stated that batch culture experiments can only represent a severely nutrient depleted 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?
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.

(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.
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) 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).

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).
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 approaches, two temperatures and two nutrient states, it made it difficult to quickly visualise the dataset.
There was no particular reason, apart from keeping the manuscript concise. The relevant figures have been added as a supplement to the final version (new supplementary Fig 2.).

(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.
This has been added.

(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?
We agree 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. We have mentioned the motivations/definitions for choosing the two temperatures upfront in the methods section.

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.

A decrease in growth rate, even slight, 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.

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

Cultures were acclimated for ca. 10 generations to low phosphate and high temperature culture conditions before starting the experiment. This information has been added to the methods section.

(8) How did the authors account for the tendency of Emiliania 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 Emiliania coccoliths, would suggest that coccospheres were not mono-layer.

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.

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 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.
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, ln 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.
The reference to cell volume has been added.

p8, ln 7-9 – The reference to Sheward et al. (2016) on line 7 should be changed to Gibbs et al. (2013) as the latter presented Emiliania 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).
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, ln 37-38).
This has been resolved.

Anonymous referee #5:
…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.
This has been added.

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.
This has been added.

p. 3; 19: What does inter alia mean?
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?
We agree 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. We choose these temperatures based on the natural temperature range for this strain. This motivation has been added to the text.

p.3; Ln 34-39: What is the name of your strain?
Please see reply to general comment (1) of referee #2.

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.
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.

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.
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.
This information has been added to the methods section (new table 1).

p. 5; Ln 21-26: Did you take into account that Emiliania 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?
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.
The standard deviation of our counts of coccolith number per cell is listed for each culture condition in (the new) table 3. This standard deviation is based on the total number of cells analysed (n), which is the sum of all three replicate cultures for each condition. N is listed for each culture condition in table 3.

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

It should be interesting to discuss the PIC content related to your coccolith number and the thickness of your coccolith layer.
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 meens 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.
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 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.
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
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.
This information has been added.

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

Cited references

Aloisi, G.: Covariation of metabolic rates and cell size in coccolithophores, Biogeosciences, 12, 4665-4692, 2015.

Borchard, C., Borges, A. V., Händel, N., and Engel, A.: Biogeochemical response of *Emiliania huxleyi* (PML B92/11) to elevated CO_2 and temperature under phosphorus limitation: a chemostat study, J. Exp. Mar. Biol. Ecol., 410, 61-71, 2011.

Buitenhuis, E. T., de Baar, H. J. W., and Veldhuis, M. J. W.: Photosynthesis and calcification by *Emiliania huxleyi* (Prymensiophyceae) as a function of inorganic carbon species, J. Phycol., 35, 949-959, 1999.

Eppley, R. W.: Temperature and phytoplankton growth in the sea, Fishery Bulletin, 70, 1063-1085, 1972.

Feng, Y., Warner, M. E., Zhang, Y., Sun, J., Fu, F.-X., Rose, J. M., and Hutchins, D. A.: Interactive effects of increased pCO_2, temperature and irradiance on the marine coccolithophore *Emiliania huxleyi* (Prymnesiophyceae), Eur. J. Phycol., 43, 87-98, 2008.

Gerecht, A. C., Šupraha, L., Edvardsen, B., Probert, I., and Henderiks, J.: High temperature decreases the PIC / POC ratio and increases phosphorus requirements in *Coccolithus pelagicus* (Haptophyta), Biogeosciences, 11, 3531-3545, 2014.

Gerecht, A. C., Šupraha, L., Edvardsen, B., Langer, G., and Henderiks, J.: Phosphorus availability modifies carbon production in *Coccolithus pelagicus* (Haptophyta), J. Exp. Mar. Biol. Ecol., 472, 24-31, 2015.

Hoffmann, R., Kirchlechner, C., Langer, G., Wochnik, A. S., Griesshaber, E., Schmahl, W. W., and Scheu, C.: Insight into *Emiliania huxleyi* coccospheres by focused ion beam sectioning, Biogeosciences, 12, 825-834, 2015.

Langer, G., Oetjen, K., and Brenneis, T.: Coccolithophores do not increase particulate carbon production under nutrient limitation: a case study using *Emiliania huxleyi* (PML B92/11), J. Exp. Mar. Biol. Ecol., 443, 155-161, 2013.

Müller, M. N., Antia, A. N., and LaRoche, J.: Influence of cell cycle phase on calcification in the coccolithophore *Emiliania huxleyi*, Limnol. Oceanogr., 53, 506-512, 2008.

Oviedo, A. M., Langer, G., and Ziveri, P.: Effect of phosphorus limitation on coccolith morphology and element ratios in Mediterranean strains of the coccolithophore *Emiliania huxleyi*, J. Exp. Mar. Biol. Ecol., 459, 105-113, 2014.

Paasche, E., and Brubak, S.: Enhanced calcification in the coccolithophorid *Emiliania huxleyi* (Haptophyceae) under phosphorus limitation, Phycologia, 33, 324-330, 1994.

Paasche, E., Brubak, S., Skattebøl, S., Young, J. R., and Green, J. C.: Growth and calcification in the coccolithophorid *Emiliania huxleyi* (Haptophyceae) at low salinities, Phycologia, 35, 394-403, 1996.

Paasche, E.: Roles of nitrogen and phosphorus in coccolith formation in *Emiliania huxleyi* (Prymnesiophyceae), Eur. J. Phycol., 33, 33-43, 1998.

Perrin, L., Probert, I., Langer, G., and Aloisi, G.: Growth of the coccolithophore *Emiliania huxleyi* in light- and nutrient-limited batch reactors: relevance for the BIOSOPE deep ecological niche of coccolithophores, Biogeosciences, 13, 5983-6001, 2016.

Riegman, R., Stolte, W., Noordeloos, A. A. M., and Slezak, D.: Nutrient uptake and alkaline phosphatase (EC 3:1:3:1) activity of *Emiliania huxleyi* (Prymnesiophyceae) during growth under N and P limitation in continuous cultures, J. Phycol., 36, 87-96, 2000.

Rosas-Navarro, A., Langer, G., and Ziveri, P.: Temperature affects the morphology and calcification of *Emiliania huxleyi* strains, Biogeosciences, 13, 2913-2926, 2016.
Satoh, M., Iwamoto, K., Suzuki, I., and Shiraiwa, Y.: Cold stress stimulates intracellular calcification by the coccolithophore, *Emiliania huxleyi* (Haptophyceae) under phosphate-deficient conditions, Mar.
5 Biotechnol., 11, 327-333, 2009.
Sett, S., Bach, L. T., Schulz, K. G., Koch-Klavsen, S., Lebrato, M., and Riebesell, U.: Temperature modulated coccolithophoris sensitivity of growth, photosynthesis and calcification to increasing seawater *p*CO2, PLOS ONE, 9, e88308, 2014.
Wördenweber, R., Rokitta, S. D., Heidenreich, E., Corona, K., Kirschhöfer, F., Fahl, K., Klocke, J. L.,
10 Kottke, T., Brenner-Weiß, G., Rost, B., Mussgnug, J. H., and Kruse, O.: Phosphorus and nitrogen starvation reveal life-cycle specific responses in the metabolome of *Emiliania huxleyi* (Haptophyta), Limnol. Oceanogr., 10.1002/lno.10624, 2017.

Phosphorus limitation and heat stress decrease calcification in *Emiliania huxleyi*

Andrea C. Gerecht[1,a], Luka Šupraha[2,b], Gerald Langer[3], Jorijntje Henderiks[1,2]

5 [1]Centre for Ecological and Evolutionary Synthesis, Department of Biosciences, University of Oslo, Oslo, 0316, Norway

[2]Department of Earth Sciences, Palaeobiology, Uppsala University, Uppsala, 75236, Sweden

[3]The Marine Biological Association of the United Kingdom, The Laboratory, Citadel Hill, Plymouth, Devon, PL1 2PB, UK

10 [a]present address: The Faculty of Biosciences, Fisheries and Economics, UiT – The Arctic University of Norway, Tromsø, 9037, Norway

[b]present address: Section for Aquatic Biology and Toxicology, Department of Biosciences, University of Oslo, Oslo, 0316, Norway

15 *Correspondence to*: Andrea C. Gerecht (andrea.gerecht@uit.no)

Abstract. Calcifying haptophytes (coccolithophores) sequester carbon in the form of organic and inorganic cellular components (coccoliths). We examined the effect of phosphorus (P) limitation and heat stress on particulate organic and inorganic carbon (calcite) production in the coccolithophore *Emiliania huxleyi*. Both environmental stressors are related to rising CO_2 levels and affect carbon production in marine microalgae, which in turn impacts biogeochemical cycling. Using semi-continuous cultures, we show that P-limitation and heat stress decrease the calcification rate in *E. huxleyi*. However, using batch cultures, we show that different culturing approaches (batch versus semi-continuous) induce different physiologies. This affects the ratio of particulate inorganic (PIC) to organic carbon (POC) and complicates general predictions on the effect of P-limitation on the PIC/POC ratio. Furthermore, heat stress increases P-requirements in *E. huxleyi*, possibly leading to lower standing stocks in a warmer ocean, especially if this is linked to lower nutrient input. In summary, the predicted rise in global temperature and resulting decrease in nutrient availability may decrease CO_2 sequestration by *E. huxleyi* through lower overall carbon production. Additionally, the export of carbon may be diminished by a decrease in calcification and a weaker coccolith ballasting effect.

Information Services 29/9/2017 12:45
Deleted: This could lessen the ballasting effect of coccoliths and weaken carbon export out of the photic zone.
Information Services 21/9/2017 08:50
Deleted: (POC)
Information Services 25/9/2017 09:24
Deleted: first of all
Information Services 21/6/2017 13:19
Deleted: *coccolithophores*
Information Services 25/9/2017 09:25
Deleted: Secondly

1 Introduction

Emiliania huxleyi is an abundant and ubiquitous phytoplankton species, belonging to the coccolithophores (Haptophyta), a group of calcifying microalgae. Coccolithophores fix CO_2 into organic matter by photosynthesis, contributing to the drawdown of atmospheric CO_2 (Raven and Falkowski, 1999). Calcification on the other hand, releases CO_2 in the short-term (Rost and Riebesell, 2004) and stores carbon in coccoliths in the long term (Sikes et al., 1980; Westbroek et al., 1993). In addition, coccolith ballast can accelerate the removal of organic carbon from upper water layers and aid long-term burial of carbon (Ziveri et al., 2007). Many studies have therefore addressed the production of organic and inorganic carbon (calcite) in *E. huxleyi*, as well as its modification by environmental factors such as carbonate chemistry (Riebesell et al., 2000; Meyer and Riebesell, 2015), nutrient availability (Paasche and Brubak, 1994; Langer and Benner, 2009), temperature (Watabe and Wilbur, 1966; Langer et al., 2010), salinity (Paasche et al., 1996; Green et al., 1998) and light (Paasche, 1968; Paasche, 1999).

This study investigates the physiological and morphological response of *E. huxleyi* to two environmental stressors, phosphorus (P) limitation and increased temperature. These are predicted to occur simultaneously as a rise in global temperature will increase the likelihood of nutrient limitation in the photic zone due to a stronger stratification of the water column (Sarmiento et al., 2004). The availability of macronutrients such as nitrogen and P have been shown to affect the production of particulate organic (POC) and inorganic carbon (PIC) in coccolithophores (reviewed by Zondervan 2007). Coccolith number per cell generally increases in P-limited cultures, often leading to an increase in the PIC/POC ratio (Paasche and Brubak, 1994; Paasche, 1998; Müller et al., 2008; Perrin et al., 2016). However, five out of six Mediterranean *E. huxleyi* strains showed a decreased PIC/POC ratio in response to P-limitation, and one strain displayed no change (Oviedo et al., 2014). While this demonstrates that there are strain-specific responses to P-limitation, some differences between studies

Information Services 25/9/2017 09:29
Deleted: In nature, organisms are exposed to a combination of changing environmental factors, which can modify their response to single parameters (e.g. Borchard et al., 2011; Milner et al., 2016).
Information Services 25/9/2017 09:27
Deleted: therefore combines
Information Services 25/9/2017 09:27
Deleted: possible future

on PIC and POC production are due to differences in experimental methods, notably batch culture and (semi)-continuous culture approaches (Langer et al., 2013b). We used both set-ups in this study to examine the difference between strong, yet brief P-limitation (stationary phase batch culture) against weak, but continuous P-limitation (semi-continuous culture). The latter method best represents areas with permanently low nutrient availability such as the eastern Mediterranean (Krom et al., 1991; Kress et al., 2005), while stationary phase batch culture can be approximated to an end-of-bloom scenario in which the lack of nutrients limits further cell division. Both approaches are relevant in ecological terms, but for methodological reasons (i.e. non-constant growth rates), nutrient-limited production cannot be determined in the batch approach (e.g. Müller et al., 2008; Langer et al., 2012; Langer et al., 2013b, Gerecht et al., 2014; Oviedo et al., 2014; Perrin et al., 2016). In a (semi)-continuous culturing set-up, growth rate is constant over the course of the experiment and production rates can be calculated (Paasche and Brubak, 1994; Paasche, 1998; Riegman et al., 2000; Borchard et al., 2011). Ratio data such as coccolith morphology, on the other hand, should be comparable between batch and (semi)-continuous culture experiments (Langer et al., 2013b) as has been shown for *C. pelagicus* (Gerecht et al., 2014; Gerecht et al., 2015). However, the only strain of *E. huxleyi* (B92/11) that was tested in both batch and continuous culture was not analyzed for coccolith morphology and the PIC/POC ratio showed a markedly different response to P-limitation in batch and in continuous culture (Borchard et al., 2011; Langer et al., 2013b). In this study we therefore tested another strain of *E. huxleyi* in both semi-continuous and batch culture and analyzed among other things, coccolith morphology and the PIC/POC ratio.

In addition to P-limitation we studied the effect of temperature on coccolith morphology and carbon production. Only a few studies have specifically dealt with the effect of temperature on the occurrence of coccolith malformations. These studies suggest that higher than optimum temperature leads to an increase in malformations (Watabe and Wilbur, 1966; Langer et al., 2010). Although the effect of temperature on carbon production in *E. huxleyi* has been addressed in numerous studies (Sorrosa et al., 2005; Feng et al., 2008; Satoh et al., 2009; De Bodt et al., 2010; Borchard et al., 2011; Sett et al., 2014; Matson et al., 2016; Milner et al., 2016; Rosas-Navarro et al., 2016), none of these studies tested the effect of above-optimum temperature. To our knowledge, this study is the first to specifically test the impact of heat stress on carbon production in this species.

2 Materials & Methods

2.1 Cultures

We grew a strain of *E. huxleyi* isolated from the Oslo fjord (22.06.2011 by S. Ota) in triplicate semi-continuous and batch cultures in replete (control) and P-limiting medium at two temperatures (19, 24 °C). The *E. huxleyi* strain used in this study was isolated from the Oslo fjord which experiences high summer temperatures of 19-21 °C with winter lows of down to 0 °C (Aure et al., 2014). As *E. huxleyi* often has maximum growth rates at temperatures above those found at the isolation site (Sett et al., 2014), we chose 19 °C as our control temperature, which is towards the high end of the temperature range this strain is likely to encounter in nature. We used a 5 °C temperature increase to induce heat stress. This temperature (24 °C) was above the optimum for growth i.e. the cultures grew

Information Services 25/9/2017 09:31
Deleted: . When studying nutrient limitation two fundamentally different approaches are available, namely

Information Services 20/9/2017 10:57
Deleted: a steady-state scenario with constant seawater nutrient concentrations and constant growth rates and is representative of

Information Services 25/9/2017 11:08
Deleted:

Information Services 21/9/2017 08:58
Deleted: B

Information Services 25/9/2017 09:33
Deleted: on the other hand represents

Information Services 25/9/2017 09:34
Deleted: in a

Information Services 25/9/2017 09:34
Deleted: a

Information Services 25/9/2017 09:35
Deleted: . This hypothesis is supported by data on

Information Services 19/6/2017 14:27
Deleted: inter alia

Information Services 15/9/2017 10:37
Deleted: coccolith

Information Services 15/9/2017 10:38
Deleted: morphology

Information Services 15/9/2017 10:38
Deleted: ,

Information Services 15/9/2017 10:38
Deleted: ing

Information Services 25/9/2017 13:27
Deleted: The

Information Services 25/9/2017 09:36
Deleted: and

Information Services 25/9/2017 13:58
Deleted: Rosas-Navarro et al. (2016) determined that the PIC/POC ratio in *E. huxleyi* is lowest at optimal temperature with an increase in PIC over POC at suboptimal temperature. N

Information Services 25/9/2017 13:27
Deleted: , however,

Information Services 20/9/2017 10:42
Moved down [2]: To our knowledge, this study is the first to specifically test the impact of heat stress on carbon production in this species.

Information Services 29/9/2017 12:15
Deleted: (Sett et al., 2014)(Eppley, 1972)

Information Services 20/9/2017 10:42
Moved (insertion) [2]

Information Services 23/6/2017 13:51
Deleted: clone

exponentially, but at a lower rate (Eppley, 1972). Cultures were grown in modified K/2 medium (Table 1), at a salinity of 34 ppm and an initial phosphate concentration of 10 µM (control) or 0.5 µM (P-limiting). This strain belongs to morphotype A and was kept in stock culture at 12 °C under low light in K/2 medium prior to the start of the experiment. The strain has been deposited at the NIVA Culture Collection of Algae (niva-cca.no) as strain UIO 265. The cultures were grown in an environmental test chamber (MLR-350, Panasonic, Japan), on a 12:12 h light:dark cycle at an irradiance of ~100 µmol photons m^{-2} s^{-1}. Cultures were acclimated for ca. 10 generations to the two initial P-concentrations and temperatures before starting the experiment.

Information Services 21/9/2017 09:05
Deleted: (Gerecht et al., 2014)

Information Services 25/9/2017 09:43
Deleted: kept at 19 or 24 °C

Semi-continuous cultures were inoculated at an initial cell concentration of ~10.000 cells mL^{-1} and diluted back to this cell concentration with fresh medium every second day. Cell concentrations were determined daily using an electronic particle counter (CASY, Roche Diagnostics, Switzerland). Maximum cell concentrations (<170.000 cells mL^{-1}) were well below stationary phase so that all cultures were kept continuously in the exponential growth phase (supplementary Fig. 1). Semi-continuous cultures were harvested after 10 dilution cycles. For batch cultures, the initial inoculum was also ~10.000 cells mL^{-1}. P-limited cultures were harvested in stationary phase, whereas control cultures were harvested in exponential phase at similar cell concentrations (see Gerecht et al., 2014; Fig. 1). Exponential growth rates (μ_{exp}) were calculated by linear regression of log-transformed cell concentrations over time. For batch cultures, only the exponential part of the growth curve was considered. For semi-continuous cultures, μ_{exp} was calculated as an average of μ_{exp} of all dilution cycles.

2.2 Medium chemistry

2.2.1 Phosphate concentrations

Residual phosphate concentrations were determined in the culture media upon harvest of the cultures. The medium was sterile filtered (0.2 µm) into plastic scintillation vials (Kartell, Germany) and stored at -20 °C until analysis. Phosphate concentrations were determined colorimetrically on a spectrophotometer (UV 2550, Shimadzu, Japan) as molybdate reactive phosphate following Murphy and Riley (1962) with a precision of ± 4 %.

2.2.2 Carbonate chemistry

Total alkalinity (A_T) and pH of the medium were determined upon harvest of the cultures. The initial carbonate chemistry of the culture media is presented in Table 1. Samples for A_T were filtered through GF/F-filters (Whatman, GE Healthcare, UK), stored airtight at 4 °C and analyzed within 24 h. A_T was calculated from Gran plots (Gran, 1952) after duplicate manual titration with a precision of ± 50 µmol kg^{-1}. The pH was measured with a combined electrode (Red Rod, Radiometer, Denmark) that was two-point calibrated to NBS scale (precision ± 0.03). Dissolved inorganic carbon (DIC) concentrations and saturation state of calcite (Ω_{Ca}) were calculated using CO2sys (version 2.1 developed for MS Excel by D. Pierrot from E. Lewis and D. W. R. Wallace) using A_T and pH as input parameters and the dissociation constants for carbonic acid of Roy et al. (1993).

2.3 Elemental composition

2.3.1 Particulate organic phosphorus

Samples for particulate organic phosphorus (POP) were filtered onto precombusted (500 °C, 2 h) GF/C-filters (Whatman) and stored at -20 °C. POP was converted to orthophosphate by oxidative hydrolysis with potassium persulfate under high pressure and temperature in an autoclave (3150EL, Tuttnauer, Netherlands) according to Menzel and Corwin (1965). Converted orthophosphate was then quantified as molybdate reactive phosphate as described in Sect. 2.2.1.

2.3.2 Particulate organic and inorganic carbon

Samples for total particulate carbon (TPC) and particulate organic carbon (POC) were filtered onto precombusted GF/C-filters, dried at 60 °C overnight in a drying oven, and stored in a desiccator until analysis on an elemental analyzer (Flash 1112, Thermo Finnegan, USA; detection limit 2 µg; precision ± 8 %). Particulate inorganic carbon (PIC) was removed from POC filters by pipetting 230 µL of 2 M HCl onto the filters before analysis (Langer et al., 2009) and calculated as the difference between TPC and POC.

2.4 Cell geometry

Cell volume was calculated from cell diameters measured both visually from light microscopy images (LM) and automatically with an electronic particle counter (CASY). With LM, cell diameters of live cells were measured at 200 times magnification after dissolving the coccoliths with 0.1 M HCl (19 µL to 1 mL sample; Gerecht et al., 2014) after harvesting the cultures. CASY cell diameters were recorded during daily measurements of cell concentrations (see 2.1) without removing coccoliths. Cell volume derived from CASY data therefore overestimates actual cell volume, because part of the coccosphere is included. However, volume estimates from CASY data are based on the measurement of many cells, leading to robust data i.e. a low standard deviation compared to LM measurements (Table 2). They are therefore useful for comparative purposes and for following the development of cell size during culture growth (Fig. 1; see also Gerecht et al., 2015).

A Zeiss Supra35-VP field emission scanning electron microscope (Zeiss, Germany) was used to capture images for morphological analyses. The number of coccoliths per coccosphere was estimated from these images by counting visible, forwards facing coccoliths, multiplying this number by two to account for the coccoliths on the back side of the coccosphere, and adding the number of partially visible coccoliths along its edge (Gerecht et al., 2015). Coccolith morphology was classified into three categories: normal, incomplete, and malformed (Table 3; Fig. 2). Due to the low calcite saturation state reached in stationary phase batch cultures, we observed a high number of partially dissolved coccoliths in these cultures (the features of this secondary dissolution are described in Table 3 and Fig. 2). As it was not possible to unambiguously distinguish incomplete morphology due to secondary dissolution from incompletely produced coccoliths, only one class of incomplete coccoliths is presented in Fig. 3.

Information Services 22/9/2017 15:09
Deleted: Note that average cell volumes for CASY measurements reported in Table 2 for semi-continuous cultures are averages of all dilution cycles and cannot be directly compared to LM measurements which are from the last (harvest) day only.

2.5 Statistical treatment of the data

The average value of parameters from triplicate cultures is given as the statistical mean together with standard deviation. The influence of P-availability and temperature on variables was determined by means of a two-way analysis of variance (ANOVA). For discrete data (DIC, coccolith morphology), a non-parametric test (Mann-Whitney U test) was used. All statistical treatment of the data was preformed using Statistica (release 7) software (StatSoft, USA).

Information Services 15/9/2017 11:19
Deleted: Average values were compared by a t-test.

3 Results

3.1 Semi-continuous cultures

Particulate organic phosphorus (POP) cellular content (F-value=24.46, p<0.001) and production (F-value=20.92, p<0.001) were significantly lower in P-limited than in control cultures (Table 4; supplementary Fig. 2). P-limitation, however, had no effect on exponential growth rate (μ_{exp}) (F-value=0.54, p=0.47), POC content (F-value=4.16, p=0.055), POC production (F-value=3.71, p=0.09) or cell size (Table 2; F-value=0.21, p=0.65). PIC production, on the other hand, was significantly decreased in P-limited cultures (Table 4; F-value=13.25, p=0.0066) and P-limited cells were covered by one to two fewer coccoliths (Table 2; Fig. 4a,b), which led to a decrease in the PIC/POC ratio (Table 4; F-value=19.01, p=0.0024). Coccolith morphology was unaffected by P-limitation (Table 2, Fig. 3; Z-value=-0.40, p=0.69).

The 5 °C temperature increase from 19 to 24 °C decreased μ_{exp} by 10 % in control cultures and by 7 % in P-limited cultures (Table 4; F-value=20.74, p<0.001). POC production, however, was unaffected by temperature (F-value=0.38, p=0.55) as there was a significant increase in POC content (supplementary Fig. 2; F-value=8.52, p=0.0085) and cell size (Table 2; F-value=10.36, p=0.0029) at 24 °C. PIC production was significantly lower at 24 °C (Table 4; F-value=19.73, p=0.0022) and the cells were covered by three to four fewer coccoliths (Table 2; Fig. 4b). The lowest PIC/POC ratio (0.81 ± 0.06) and coccolith numbers per cell (15 ± 5) were therefore observed in P-limited cultures at 24 °C. There was a strong increase in the occurrence of malformed coccoliths at 24 compared to 19 °C (Table 2; Z-value=-2.88, p=0.0039).

There was no direct effect of temperature on POP content (Table 4; supplementary Fig. 2; F-value=2.66, p=0.12). There was, however, a combined effect of temperature and P-limitation (F-value=4.49, p=0.047) so that the lowest POP content was measured in P-limited cultures at 24 °C. These cultures had taken up most of the phosphate from the medium by the time of harvest (Table 5).

3.2 Batch cultures

Cells from control batch cultures were overall smaller than those from semi-continuous cultures (Table 2) and consequently contained less POP and POC (Table 4; supplementary Fig. 2). POC/POP-values of control batch and control semi-continuous cultures, however, were similar.

Initial phosphate availability did not affect μ_{exp} (F-value=3.19, p=0.11). At 19 °C, cultures growing in P-limiting medium stopped dividing at a cell concentration of ~740,000 cells mL^{-1}. At 24 °C, final cell concentrations in stationary phase were significantly lower at ~620,000 cells mL^{-1} (t-value=13.77, df=16, p<0.001). Final DIC concentrations were significantly lower at 19 °C (400 ± 50 µmol kg^{-1}) than at 24 °C (550 ± 50 µmol kg^{-1}; Table 5; Z-value=-2.62, p<0.01), whereas DIC concentrations remained

Information Services 15/9/2017 10:44
Deleted: Most of the dissolved inorganic carbon (DIC) had been consumed by P-limited cultures at both temperatures (Table 3). At 19 °C,

at ~1000 µmol kg^{-1} in control cultures. The pH of the culture medium in P-limited batch cultures was also significantly different between the two temperatures. At 19 °C, the final pH-value was 7.70 ± 0.02 compared to 7.85 ± 0.01 at 24 °C. In control cultures, the pH stayed close to normal seawater values (~8.2) at both temperatures. P-limited cultures were undersaturated in calcite (Ω_{Ca}<1) at the time of harvest with a significantly stronger undersaturation at 19 °C (Ω_{Ca} = 0.40 ± 0.03) than at 24 °C (Ω_{Ca} = 0.77 ± 0.05; Z-value=-2.61, p<0.01).

POP content was ~3-4 times lower at both temperatures in P-limited than in control cultures (Table 4; supplementary Fig. 2). However, POP content was significantly higher in cultures grown at 24 °C (83 ± 3 ng cell^{-1}) than at 19 °C (71 ± 9 ng cell^{-1}; t-value=-3.24, df=10, p<0.01). Cells from P-limited cultures increased in size as cell division rates slowed down (Fig. 1) and cell volume was twice as large in stationary phase than in control cultures in exponential phase (Table 2, Fig. 4c,d). This coincided with a 2.7- and 2.1-fold increase in POC content in P-limited cultures at 19 and 24 °C, respectively (Table 4; supplementary Fig. 2).

In P-limited cultures, the average number of coccoliths per cell tripled at 19 °C (from ~15 to ~45 coccoliths cell^{-1}) and doubled (from ~16 to ~34 coccoliths cell^{-1}) at 24 °C (Table 2). The PIC content, on the other hand, increased by ~150 % at both temperatures (Table 4; Fig. 4c,d). Coccolith morphology was obscured in P-limited cultures by secondary dissolution with 77 % of all coccoliths showing incomplete morphology at 19 °C and 52 % of coccoliths at 24 °C (Table 2; Fig. 3). The percentage of incomplete coccoliths was negligible in control cultures. Coccolith malformations were twice as common in control cultures at 24 °C than at 19 °C (Table 2; Fig. 3; Z-value=-1.96, p=0.049). Temperature had no effect on μ_{exp} (F-value=3.19, p=0.11) or on production rates in control cultures (Table 4).

Information Services 9/8/2017 13:17
Deleted: were

Information Services 9/8/2017 13:17
Deleted: as those harvested from

Information Services 25/9/2017 13:54
Deleted: I

Information Services 25/9/2017 13:54
Deleted: ,

Information Services 25/9/2017 13:54
Deleted: were

Information Services 25/9/2017 13:54
Deleted: , whereas

Information Services 25/9/2017 13:54
Deleted: were incomplete

Information Services 29/9/2017 13:04
Moved (insertion) [3]

Information Services 29/9/2017 13:04
Deleted:

Information Services 29/9/2017 13:04
Moved up [3]: Coccolith malformations were twice as common in control cultures at 24 °C than at 19 °C (Table 2; Fig. 3; Z-value=-1.96, p=0.049).

4 Discussion

4.1 The effect of P-limitation on PIC and POC production

When testing nutrient limitation in a laboratory setting, it is important to consider the putative physiological difference between cells growing exponentially at lower nutrient availability (continuous or semi-continuous culture) and cells entering stationary phase once the limiting nutrient has been consumed (batch culture) (Langer et al., 2013b; Gerecht et al., 2015). While the former allows for acclimation to lower nutrient availability, the latter creates a strong limitation of short duration that leads to a cessation of cell division. A good parameter to assess this potential physiological difference is the PIC/POC ratio, because, in contrast to PIC and POC production, it can be determined in both batch and continuous culture (Langer et al., 2013b). Despite the considerable body of literature on carbon production under P-limitation in *E. huxleyi* (see Introduction), only one strain (B92/11) has been examined in a comparative study showing that the PIC/POC response to P-limitation varies with the approach chosen (Borchard et al., 2011; Langer et al., 2013b). The case of *E. huxleyi* B92/11 suggests that the physiological state induced by P-limitation in batch culture indeed differs from the one induced by P-limitation in continuous culture. In this strain P-limitation decreased the PIC/POC ratio in batch culture (Langer et al., 2013b), while no change occurred in continuous culture (Borchard et al., 2011). In the strain used in this study the opposite is true, i.e. the PIC/POC ratio decreased in

semi-continuous culture and remained constant in batch culture at normal temperature. The highly variable PIC/POC response to P-limitation observed here and in B92/11 (Borchard et al., 2011; Langer et al., 2013b) shows that the physiological state under P-limitation depends on the experimental approach, and that there is no clear trend in the response pattern among different strains. Consequently it is difficult to formulate a common scenario with respect to carbon allocation under P-limitation. However, our semi-continuous culture experiment shows that in this strain under P-limitation POC production remains unchanged and PIC production decreases. The 14 % decrease in PIC production observed here is quite remarkable, because the limitation imposed by our semi-continuous setup was weak as can be inferred from the maintained growth rate and the weak (11 %) decrease in POP production. Hence in this strain of *E. huxleyi* the calcification rate is particularly sensitive to P-limitation. As this is the first report of P-limitation decreasing coccolith production in *E. huxleyi*, it would be beneficial to test further strains in a similar set-up to observe how common this physiological response is in *E. huxleyi*. Ecological benefits of coccoliths are likely to be various (Monteiro et al., 2016). Protection from UV-radiation (Xu et al., 2011), for example, may be relevant as this species grows at high light intensities. Furthermore, the consumption of coccoliths by grazers in addition to organic cell material may decrease overall grazing rates (Monteiro et al., 2016). A decrease in coccolith coverage may therefore constitute a loss in overall fitness of an *E. huxleyi* population. Coccolith morphogenesis, on the other hand, was unaffected by P-limitation. This reflects the potentially wide spread insensitivity of coccolith morphogenesis to P-limitation (Langer et al., 2012; Oviedo et al., 2014) with the exception of *C. pelagicus* (Gerecht et al. 2015).

In a recent study, Bach et al. (2013) determined that POC production in *E. huxleyi* is DIC-limited at concentrations <1000 µmol kg^{-1}. Final DIC concentrations in our stationary phase cultures were well below that value and these cultures were possibly limited in both P and DIC at the time of harvest. DIC-limitation, however, was not the trigger for entering stationary phase as POC production continued for several days after cessation of cell division. Wördenweber et al. (2017) have recently shown that although the cell cycle is arrested by P-starvation, enzymatic functionality is widely preserved. P-starvation blocks the synthesis of DNA and membrane phospholipids, necessary for cell replication, arresting the cells in the G1 (assimilation) phase of the cell cycle (Müller et al., 2008). The assimilation phase is thus prolonged and the cell continues assimilating POC, presumably in the form of non-essential lipids and carbohydrates (Sheward et al., 2017), leading to an increase in cell size (Aloisi, 2015). A similar increase in cell size as observed in this study has been previously described by others for *E. huxleyi* (Paasche and Brubak, 1994; Riegman et al., 2000; Müller et al., 2008; Gibbs et al., 2013; Oviedo et al., 2014) and recently also for other species, such as *C. pelagicus*, *Helicosphaera carteri* and two *Calcidiscus* species (Gerecht et al., 2015; Sheward et al., 2017) and may thus be a common feature of coccolithophores.

Cells that are arrested in the G1 (assimilation) phase of the cell cycle (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. 4c,d). 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 (Balch et al., 1991; Holligan et al., 1993). Though these blooms

Information Services 15/9/2017 10:30
Deleted: presumably

Information Services 20/9/2017 12:47
Deleted: This led to a strong increase in cell size,

Information Services 20/9/2017 12:40
Deleted: an effect that has been

Information Services 20/9/2017 12:43
Deleted: previously

Information Services 28/6/2017 13:00
Deleted: In the absence of cell division, the cell is able to continue accumulating POC in the cell, presumably in the form of non-essential lipids and carbohydrates (Sheward et al., 2017)(Aloisi, 2015).

Information Services 20/9/2017 12:48
Deleted: These large "ready-to-divide" cells

are important contributors to the sequestration of atmospheric CO_2 and carbon export, they are short-lived phenomena. The present data set is unique in providing information on PIC production under P-limitation without the confounding factor of changes in growth rate. By using semi-continuous cultures in which cell division rates remained constant between control and P-limited cultures, we could show that the likely outcome of diminished P-availability will be a long-term decrease in PIC production in *E. huxleyi*, which may weaken carbon export from surface waters (Ziveri et al., 2007).

4.2 The effect of heat stress on calcification

The decrease in growth rate at 24 °C, observed in semi-continuous cultures, confirmed that this temperature was indeed above the optimum for growth for this particular strain (Eppley, 1972). Although a similar decrease in growth rate was not observed in batch culture, measurements of growth rate in semi-continuous cultures are more robust because growth rate is measured as an average of numerous dilution cycles. The doubling in coccolith malformations provides further evidence that 24 °C cultures were indeed heat-stressed (Watabe and Wilbur, 1966; Langer et al., 2010; Milner et al., 2016).

The POP content of (P-limited) stationary phase cultures can be used as an indicator for minimum P-requirements (Šupraha et al., 2015). These increased by ~17 % under heat stress. Increased P-requirements led to lower final biomass, both in terms of final cell numbers and lower cellular POC content in heat-stressed cultures. An increase in P-requirements at higher temperature has previously been described for the coccolithophore *C. pelagicus*, which also led to lower final cell numbers in stationary phase P-limited cultures (Gerecht et al., 2014). Higher P-requirements at higher temperature can be furthermore inferred for two additional strains of *E. huxleyi* from the studies carried out by Feng et al. (2008) and Satoh et al. (2009). Increased P-requirements at higher temperature may therefore be a general feature of coccolithophores with the potential to decrease coccolithophore carbon production in a future warmer ocean. A similar increase was not observed in heat-stressed, exponentially growing cultures i.e. control batch and semi-continuous cultures because P-uptake was 3-4 times higher than the minimum requirement. The low residual phosphate concentrations of P-limited semi-continuous cultures are also indicative of increased P-uptake under heat stress. This was not reflected in the POP content, which was actually lower under heat stress. A possible explanation for these conflicting results may be an increased production of exudates due to heat stress with a concomitant loss of organic P from the cell (Borchard and Engel, 2012). Higher P-requirements may be due either to increased energy demands under heat stress or to an upregulation of heat stress related genes as much of cellular P can be found in RNA (Geider and LaRoche, 2002).

Heat stress had a stronger effect than P-limitation on coccolith number in semi-continuous cultures. Whereas P-limited cells were covered by one to two fewer coccoliths, heat stress decreased the number of coccoliths per cell by three to four coccoliths (Fig. 4a,b). In *C. pelagicus*, heat stress has also been described to decrease the coccolith coverage of the cell (Gerecht et al., 2014). Also in P-limited batch cultures, fewer coccoliths accumulated around the cells under heat stress (Fig. 4d). This was not, however, reflected by a lower PIC content of these cells. There are two possible mechanisms to explain this incongruence. One reason may be the partial dissolution of coccoliths in P-limited stationary phase

Information Services 20/9/2017 12:49
Deleted: Based on t

Information Services 20/9/2017 12:51
Deleted: his study

Information Services 20/9/2017 12:53
Deleted: The *E. huxleyi* clone used in this study had been isolated from the Oslo fjord, in which annual maximum temperatures do not exceed 21 °C (Aure et al., 2014).

Information Services 20/9/2017 12:53
Deleted: clone

Information Services 20/9/2017 12:55
Deleted: t

Information Services 20/9/2017 12:55
Deleted: In the following, 24 °C will therefore be referred to as heat stress and 19 °C as normal temperature.

Information Services 23/6/2017 09:16
Moved (insertion) [1]

Information Services 25/9/2017 15:59
Deleted: *occolithus*

Information Services 23/6/2017 10:48
Deleted: and

Information Services 25/9/2017 10:47
Deleted: and Feng et al. (2008)

Information Services 29/9/2017 13:11
Deleted: is incongruence

Information Services 23/6/2017 09:16
Moved up [1]: Increased P-requirements led to lower final biomass, both in terms of final cell numbers and lower cellular POC content.

Information Services 22/9/2017 15:20
Deleted: quota

Information Services 28/6/2017 13:12
Deleted: did

Information Services 28/6/2017 13:12
Deleted: correspond

Information Services 28/6/2017 13:12
Deleted: to

Information Services 28/6/2017 13:13
Deleted: reasons

Information Services 28/6/2017 13:15
Deleted: for this discrepancy are

cultures. High numbers of partially dissolved coccoliths were observed in P-limited batch cultures at both temperatures due to the low calcite saturation state reached in stationary phase cultures. However, the occurrence of secondary dissolution was higher at normal temperature than under heat stress as these cultures reached higher final biomass and consequently were less saturated in calcite. These partially dissolved coccoliths likely contained less calcite, which may explain why the cellular PIC content was similar at both temperatures even if the coccolith number per cell differed. Due to this secondary dissolution, the PIC quota and PIC/POC ratio measured in P-limited batch cultures are most likely underestimated, especially at normal temperature, and need to be interpreted with caution.

Another possible reason for the discrepancy between PIC and coccolith quota between the two temperatures is a difference in the ratio of attached to loose coccoliths. Possibly, more coccoliths were shed under heat stress, underestimating the coccolith number of these cells. As *E. huxleyi* in general sheds many coccoliths, this effect can be considerable (Milner et al., 2016). We therefore cannot conclusively determine whether the effect of P-limitation on the PIC/POC ratio was modified by heat stress in batch culture. Despite the high percentage of partially dissolved coccoliths in P-limited batch culture, the detrimental effect of heat stress on morphogenesis is evident. As all *E. huxleyi* strains tested so far show this response, it could be widespread if not ubiquitous (Watabe and Wilbur, 1966; Langer et al., 2010, this study). Interestingly, we observed similar malformations e.g. merged distal shield elements in field samples collected from the Oslo fjord (Fig. 2d) at a time when *E. huxylei* was abundant in the water column (Gran-Stadniczeñko et al., 2017). The percentage of malformed coccoliths in field samples was lower (ca. 6%) than in our control cultures (ca. 20%), lending support to the hypothesis that coccolith malformations occur more frequently in culture (Langer et al., 2013a). The types of malformations, however, appear to be similar, indicating that the affected physiological mechanisms are the same.

De Bodt et al. (2010) described a decrease in the PIC/POC ratio at higher temperature in *E. huxleyi*. Several studies have contrastingly reported the PIC/POC ratio to be insensitive to temperature (Feng et al., 2008; Matson et al., 2016; Milner et al., 2016) or to increase with rising temperatures (Sett et al., 2014). In all of the above studies, however, growth rate increased from low to high temperature and none of the tested temperatures were therefore above the optimum for growth (Eppley, 1972). To our knowledge, this study is the first to show that heat stress is not only detrimental for coccolith morphology (Watabe and Wilbur, 1966; Langer et al., 2010; Milner et al., 2016), but also for coccolith production in *E. huxleyi*. Certainly, the potential for long-term adaptation needs to be considered, as temperature increases are unlikely to occur on time scales short enough to preclude adaptation in a rapidly growing species. The species *E. huxleyi* is present also at higher temperatures in nature (Feng et al., 2008) so a physiological constraint to adaptation to higher temperatures is not probable. Similarly, considering the metabolic diversity among different *E. huxleyi* strains (Langer et al., 2009; Read et al., 2013), this strain could be replaced by a more heat-tolerant strain.

5. Conclusions

By employing semi-continuous cultures, we show that both P-limitation and heat stress decrease calcification rate in a temperate strain of *E. huxleyi*. Considering that these stressors are likely to co-

Information Services 28/6/2017 13:15
Deleted: and

Information Services 28/6/2017 13:15
Deleted: an increase in the number of shed coccoliths. The h

Information Services 20/9/2017 13:30
Deleted: incomplete

Information Services 20/9/2017 13:30
Deleted: were likely a result of secondary dissolution (Fig. 1d; Langer et al., 2007)

Information Services 20/9/2017 13:32
Deleted: T

Information Services 20/9/2017 13:32
Deleted: percentage

Information Services 20/9/2017 13:32
Deleted: of partially dissolved coccoliths

Information Services 20/9/2017 13:34
Deleted: T

Information Services 28/6/2017 13:19
Deleted: were

Information Services 28/6/2017 13:19
Deleted: therefore

Information Services 22/9/2017 15:21
Deleted: quota

occur in a future ocean (Sarmiento et al., 2004), it is important to consider this additive effect. The increase in cellular P-requirements under heat stress may intensify nutrient limitation, decreasing the standing stock of *E. huxleyi* in a warmer ocean, which would have a negative feedback on carbon sequestration. An increase in P-requirements and decrease in coccolith production under heat stress have also been described for *C. pelagicus* and may be a general feature of coccolithophores. To what extent a decrease in calcification under weak P-limitation is a general feature of *E. huxleyi* needs to be verified by additional studies, considering that the response of the PIC/POC ratio to P-limitation is both strain and method dependent. The method dependency is due to the determining effect of cell size and cell division rate i.e. growth phase on the PIC/POC ratio. This high variability of the PIC/POC ratio, one of the most important parameters in biogeochemical terms, makes it difficult to predict the impact of P-limitation in *E. huxleyi* on the carbon cycle. However, we have shown that lower phosphorus input and higher global temperature can have an additive negative effect on calcification. Decreased calcification rates weaken carbon export due to less coccolith ballasting (Ziveri et al., 2007). It is therefore fundamental to understand how environmental factors interact in their effect on calcification in coccolithophores – from the cellular to the ecological level.

Author contributions

AG, JH, and GL designed the experiments. AG and LS carried out the experiment. All authors interpreted the findings. AG prepared the manuscript with contribution from all co-authors.

The authors declare that they have no conflict of interest.

Acknowledgments

This research was funded by the Research Council of Norway (FRIMEDBIO project 197823 to JH) and the Royal Swedish Academy of Sciences through a grant from the Knut and Alice Wallenberg Foundation (KAW 2009.0287 to JH). The authors would like to acknowledge T. Claybourn (Department of Earth Sciences, Palaeobiology, Uppsala University) who collected the morphology images using scanning electron microscopy. The authors would furthermore like to thank Prof. B. Edvardsen (Section for Aquatic Biology and Toxicology, Department of Biosciences, University of Oslo) for fruitful discussions of the presented data.

References

Aloisi, G.: Covariation of metabolic rates and cell size in coccolithophores, Biogeosciences, 12, 4665-4692, 2015.

Aure, J., Danielssen, D. S., and Naustvoll, L.-J.: Miljøundersøkelser i norske fjorder: Ytre Oslofjord 1937-2011, Havforskningsinstituttet, 2014.

Bach, L. T., Mackinder, L. C. M., Schulz, K. G., Wheeler, G., Schroeder, D. C., Brownlee, C., and Riebesell, U.: Dissecting the impact of CO_2 and pH on the mechanisms of photosynthesis and calcification in the coccolithophore *Emiliania huxleyi*, New Phytol., 199, 121-134, 2013.

Information Services 29/9/2017 12:21
Deleted: Furthermore, t

Information Services 29/9/2017 12:22
Deleted: e

Information Services 28/6/2017 13:31
Deleted: T

Information Services 28/6/2017 13:32
Deleted: nutrient

Information Services 28/6/2017 13:32
Deleted: may

Information Services 20/9/2017 13:14
Deleted: which may lead to weakened carbon export due to less coccolith ballasting (Ziveri et al., 2007).

Balch, W. M., Holligan, P. M., Ackleson, S. G., and Voss, K. J.: Biological and optical properties of mesoscale coccolithophore blooms in the Gulf of Maine, Limnol. Oceanogr., 36, 629-643, 1991.
Borchard, C., Borges, A. V., Händel, N., and Engel, A.: Biogeochemical response of *Emiliania huxleyi* (PML B92/11) to elevated CO_2 and temperature under phosphorus limitation: a chemostat study, J.
5 Exp. Mar. Biol. Ecol., 410, 61-71, 2011.
Borchard, C., and Engel, A.: Organic matter exudation by *Emiliania huxleyi* under simulated future ocean conditions, Biogeosciences, 9, 3405-3423, 2012.
De Bodt, C., Van Oostende, N., Harlay, J., Sabbe, K., and Chou, L.: Individual and interacting effects of pCO$_2$ and temperature on *Emiliania huxleyi* calcification: study of the calcite production, the
10 coccolith morphology and the coccosphere size, Biogeosciences, 7, 1401-1412, 2010.
Eppley, R. W.: Temperature and phytoplankton growth in the sea, Fishery Bulletin, 70, 1063-1085, 1972.
Feng, Y., Warner, M. E., Zhang, Y., Sun, J., Fu, F.-X., Rose, J. M., and Hutchins, D. A.: Interactive effects of increased pCO$_2$, temperature and irradiance on the marine coccolithophore *Emiliania huxleyi*
15 (Prymnesiophyceae), Eur. J. Phycol., 43, 87-98, 2008.
Geider, R., and LaRoche, J.: Redfield revisited: variability of C:N:P in marine microalgae and its biochemical basis, Eur. J. Phycol., 37, 1-17, 2002.
Gerecht, A. C., Šupraha, L., Edvardsen, B., Probert, I., and Henderiks, J.: High temperature decreases the PIC / POC ratio and increases phosphorus requirements in *Coccolithus pelagicus* (Haptophyta),
20 Biogeosciences, 11, 3531-3545, 2014.
Gerecht, A. C., Šupraha, L., Edvardsen, B., Langer, G., and Henderiks, J.: Phosphorus availability modifies carbon production in *Coccolithus pelagicus* (Haptophyta), J. Exp. Mar. Biol. Ecol., 472, 24-31, 2015.
Gibbs, S. J., Poulton, A. J., Bown, P. R., Daniels, C. J., Hopkins, J., Young, J. R., Jones, H. L.,
25 Thiemann, G. J., O'Dea, S. A., and Newsam, C.: Species-specific growth response of coccolithophores to Palaeocene-Eocene environmental change, Nat. Geosci., 6, 218-222, 2013.
Gran, G.: Determination of the equivalence point in potentiometric titrations of seawater with hydrochloric acid, Oceanol. Acta, 5, 209-218, 1952.
Gran-Stadniczeñko, S., Šupraha, L., Egge, E. D., and Edvardsen, B.: Haptophyte diversity and vertical
30 distribution explored by 18S and 28S ribosomal RNA gene metabarcoding and scanning electron microscopy, J. Eukaryot. Microbiol., 64, 514-532, 2017.
Green, J. C., Heimdal, B. R., Paasche, E., and Moate, R.: Changes in calcification and the dimensions of coccoliths of *Emiliania huxleyi* (Haptophyta) grown at reduced salinities, Phycologia, 37, 121-131, 1998.
35 Holligan, P. M., Fernández, E., Aiken, J., Balch, W. M., Boyd, P. W., Burkill, P. H., Finch, M., Groom, S. B., Malin, G., Muller, K., Purdie, D. A., Robinson, C., Trees, C. C., Turner, S. M., and van der Wal, P.: A biogeochemical study of the coccolithophore, *Emiliania huxleyi*, in the North Atlantic, Global Biogeochem. Cy., 7, 879-900, 1993.

Kress, N., Thingstad, F., Pitta, P., Psarra, S., Tanaka, T., and Zohary, T.: Effect of P and N addition to oligotrophic Eastern Mediterranean waters influenced by near-shore water: a microcosm experiment, Deep-Sea Res. Pt. II, 52, 3054-3073, 2005.
Krom, M. D., Kress, N., Brenner, S., and Gordon, L. I.: Phosphorus limitation of primary productivity in the eastern Mediterranean Sea, Limnol. Oceanogr., 36, 424-432, 1991.
Langer, G., and Benner, I.: Effect of elevated nitrate concentration on calcification in *Emiliania huxleyi*, J. Nannoplankton Res., 30, 77-80, 2009.
Langer, G., Nehrke, G., Probert, I., Ly, J., and Ziveri, P.: Strain-specific responses of *Emiliania huxleyi* to changing seawater carbonate chemistry, Biogeosciences, 6, 2637-2646, 2009.
Langer, G., de Nooijer, L. J., and Oetjen, K.: On the role of the cytoskeleton in coccolith morphogenesis: the effect of cytoskeleton inhibitors, J. Phycol., 46, 1252-1256, 2010.
Langer, G., Oetjen, K., and Brenneis, T.: Calcification of *Calcidiscus leptoporus* under nitrogen and phosphorus limitation, J. Exp. Mar. Biol. Ecol., 413, 131-137, 2012.
Langer, G., Oetjen, K., and Brenneis, T.: On culture artefacts in coccolith morphology, Helgol. Mar. Res., 67, 359-369, 2013a.
Langer, G., Oetjen, K., and Brenneis, T.: Coccolithophores do not increase particulate carbon production under nutrient limitation: a case study using *Emiliania huxleyi* (PML B92/11), J. Exp. Mar. Biol. Ecol., 443, 155-161, 2013b.
Matson, P. G., Ladd, T. M., Halewood, E. R., Sangodkar, R. P., Chmelka, B. F., and Iglesias - Rodríguez, D. M.: Intraspecific differences in biogeochemical responses to thermal change in the coccolithophore *Emiliania huxleyi*, PLOS ONE, 11, e0162313, 10.1371/journal.pone.0162313, 2016.
Menzel, D. W., and Corwin, N.: The measurement of total phosphorus in seawater based on the liberation of organically bound fractions by persulfate oxidation, Limnol. Oceanogr., 10, 280-282, 1965.
Meyer, J., and Riebesell, U.: Reviews and Syntheses: Responses of coccolithophores to ocean acidification: a meta-analysis, Biogeosciences, 12, 1671-1682, 2015.
Milner, S., Langer, G., Grelaud, M., and Ziveri, P.: Ocean warming modulates the effects of acidification on *Emiliania huxleyi* calcification and sinking, Limnol. Oceanogr., 61, 1322-1336, 2016.
Monteiro, F. M., Bach, L. T., Brownlee, C., Bown, P. R., Rickaby, R. E. M., Poulton, A. J., Tyrrell, T., Beaufort, L., Dutkiewicz, S., Gibbs, S. J., Gutowska, M. A., Lee, R., Riebesell, U., Young, J. R., and Ridgwell, A.: Why marine phytoplankton calcify, Science Advances, 2, e1501822, 10.1126/sciadv.1501822, 2016.
Müller, M. N., Antia, A. N., and LaRoche, J.: Influence of cell cycle phase on calcification in the coccolithophore *Emiliania huxleyi*, Limnol. Oceanogr., 53, 506-512, 2008.
Murphy, J., and Riley, J. P.: A modified single solution method for the determination of phosphate in natural waters, Anal. Chim. Acta, 27, 31-36, 1962.
Oviedo, A. M., Langer, G., and Ziveri, P.: Effect of phosphorus limitation on coccolith morphology and element ratios in Mediterranean strains of the coccolithophore *Emiliania huxleyi*, J. Exp. Mar. Biol. Ecol., 459, 105-113, 2014.

Paasche, E.: The effect of temperature, light intensity, and photoperiod on coccolith formation, Limnol. Oceanogr., 13, 178-181, 1968.
Paasche, E., and Brubak, S.: Enhanced calcification in the coccolithophorid *Emiliania huxleyi* (Haptophyceae) under phosphorus limitation, Phycologia, 33, 324-330, 1994.
5 Paasche, E., Brubak, S., Skattebøl, S., Young, J. R., and Green, J. C.: Growth and calcification in the coccolithophorid *Emiliania huxleyi* (Haptophyceae) at low salinities, Phycologia, 35, 394-403, 1996.
Paasche, E.: Roles of nitrogen and phosphorus in coccolith formation in *Emiliania huxleyi* (Prymnesiophyceae), Eur. J. Phycol., 33, 33-43, 1998.
Paasche, E.: Reduced coccolith calcite production under light-limited growth: a comparative study of
10 three clones of *Emiliania huxleyi* (Prymnesiophyceae), Phycologia, 38, 508-516, 1999.
Perrin, L., Probert, I., Langer, G., and Aloisi, G.: Growth of the coccolithophore *Emiliania huxleyi* in light- and nutrient-limited batch reactors: relevance for the BIOSOPE deep ecological niche of coccolithophores, Biogeosciences, 13, 5983-6001, 2016.
Raven, J. A., and Falkowski, P. G.: Oceanic sinks for atmospheric CO_2, Plant Cell Environ., 22, 741-
15 755, 1999.
Read, B. A., Kegel, J., Klute, M. J., Kuo, A., Lefebvre, S., Maumus, F., Mayer, C., Miller, J., Monier, A., Salamov, A., Young, J. R., Aguilar, M., Claverie, J.-M., Frickenhaus, S., Gonzalez, K., Herman, E. K., Lin, Y.-C., Napier, J., Ogata, H., Sarno, A. F., Shmutz, J., Schroeder, D. C., de Vargas, C., Verret, F., von Dassow, P., Valentin, K., Van de Peer, Y., Wheeler, G., Dacks, J. B., Delwiche, C. F.,
20 Dyhrman, S. T., Glöckner, G., John, U., Richards, T., Worden, A. Z., Zhang, X., and Grigoriev, I. V.: Pan genome of the phytoplankton *Emiliania* underpins its global distribution, Nature, 499, 209-213, 2013.
Riebesell, U., Zondervan, I., Rost, B., Tortell, P. D., Zeebe, R. E., and Morel, F. M. M.: Reduced calcification of marine plankton in response to increased atmospheric CO_2, Nature, 407, 364-367,
25 2000.
Riegman, R., Stolte, W., Noordeloos, A. A. M., and Slezak, D.: Nutrient uptake and alkaline phosphatase (EC 3:1:3:1) activity of *Emiliania huxleyi* (Prymnesiophyceae) during growth under N and P limitation in continuous cultures, J. Phycol., 36, 87-96, 2000.
Rosas-Navarro, A., Langer, G., and Ziveri, P.: Temperature affects the morphology and calcification of
30 *Emiliania huxleyi* strains, Biogeosciences, 13, 2913-2926, 2016.
Rost, B., and Riebesell, U.: Coccolithophores and the biological pump: responses to environmental changes, in: Coccolithophores - from molecular processes to global impact, edited by: Thierstein, H. R., and Young, J. R., Springer, Berlin, 99-125, 2004.
Roy, R. N., Roy, L. N., Vogel, K. M., Porter-Moore, C., Pearson, T., Good, C. E., Millero, F. J., and
35 Campbell, D. M.: The dissociation constants of carbonic acid in seawater at salinities 5 to 45 and temperature 0 to 45 °C, Mar. Chem., 44, 249-267, 1993.
Sarmiento, J. L., Slater, R., Barber, R., Bopp, L., Doney, S. C., Hirst, A. C., Kleypas, J., Matear, R., Mikolajewicz, U., Monfray, P., Soldatov, V., Spall, S. A., and Stouffer, R.: Response of ocean ecosystems to climate warming, Global Biogeochem. Cy., 18, GB3003, 10.1029/2003GB002134,
40 2004.

Satoh, M., Iwamoto, K., Suzuki, I., and Shiraiwa, Y.: Cold stress stimulates intracellular calcification by the coccolithophore, *Emiliania huxleyi* (Haptophyceae) under phosphate-deficient conditions, Mar. Biotechnol., 11, 327-333, 2009.

Sett, S., Bach, L. T., Schulz, K. G., Koch-Klavsen, S., Lebrato, M., and Riebesell, U.: Temperature modulates coccolithophorid sensitivity of growth, photosynthesis and calcification to increasing seawater pCO_2, PLOS ONE, 9, e88308, 10.1371/journal.pone.0088308, 2014.

Sheward, R. M., Poulton, A. J., Gibbs, S. J., Daniels, C. J., and Bown, P. R.: Physiology regulates the relationship between coccosphere geometry and growth-phase in coccolithophores, Biogeosciences, 14, 1493-1509, 2017.

Sikes, C. S., Roer, R. D., and Wilbur, K. M.: Photosynthesis and coccolith formation: Inorganic carbon sources and net inorganic reaction of deposition, Limnol. Oceanogr., 25, 248-261, 1980.

Sorrosa, J. M., Satoh, M., and Shiraiwa, Y.: Low temperature stimulates cell enlargement and intracellular calcification of coccolithophorids, Mar. Biotechnol., 7, 128-133, 2005.

Šupraha, L., Gerecht, A. C., Probert, I., and Henderiks, J.: Eco-physiological adaptation shapes the response of calcifying algae to nutrient limitation, Sci. Rep.-UK, 5, 16499, 10.1038/srep16499, 2015.

Watabe, N., and Wilbur, K. M.: Effects of temperature on growth, calcification, and coccolith formation in *Coccolithus huxleyi* (Coccolithineae), Limnol. Oceanogr., 11, 567-575, 1966.

Westbroek, P., Brown, C. W., van Bleijswijk, J. D. L., Brownlee, C., Brummer, G. J., Conte, M., Egge, J. K., Fernández, E., Jordan, R. W., Knappertsbusch, M., Stefels, J., Veldhuis, M. J. W., and van der Wal, P.: A model system approach to biological climate forcing. The example of *Emiliania huxleyi*, Global Planet. Change, 8, 27-46, 1993.

Wördenweber, R., Rokitta, S. D., Heidenreich, E., Corona, K., Kirschhöfer, F., Fahl, K., Klocke, J. L., Kottke, T., Brenner-Weiß, G., Rost, B., Mussgnug, J. H., and Kruse, O.: Phosphorus and nitrogen starvation reveal life-cycle specific responses in the metabolome of *Emiliania huxleyi* (Haptophyta), Limnol. Oceanogr., 10.1002/lno.10624, 2017.

Xu, K., Gao, K., Villafañe, V. E., and Helbling, E. W.: Photosynthetic responses of *Emiliania huxleyi* to UV radiation and elevated temperature: roles of calcified coccoliths, Biogeosciences, 8, 1441-1452, 2011.

Ziveri, P., de Bernardi, B., Baumann, K.-H., Stoll, H. M., and Mortyn, P. G.: Sinking of coccolith carbonate and potential contribution to organic carbon ballasting in the deep ocean, Deep-Sea Res. Pt. II, 54, 659-675, 2007.

Zondervan, I.: The effects of light, macronutrients, trace metals and CO_2 on the production of calcium carbonate and organic carbon in coccolithophores - A review, Deep-Sea Res. Pt. II, 54, 521-537, 2007.

Table 1: Basal composition of the culture media (modified K/2), including salinity and carbonate chemistry (total alkalinity, pH, dissolved inorganic carbon, calcite saturation state). * For trace metal composition please refer to the recipe available for K/2 Ian at roscoff-culture-collection.org/basic-page/culture media.

5

Final conc. [µM]	"Control, replete medium"	"P-limited medium"
$NaNO_3$	288	288
KH_2PO_4	10	0.5
(Na)FeEDTA	5.85	5.85
Trace metals	*	*
Vitamins	"f/2"	"f/2"
Salinity [ppm]	34	34
A_T [µmol kg^{-1}]	2250	2100
pH (NBS)	8.14	7.89
DIC [µmol kg^{-1}]	1800	1800
Ω_{Ca}	4.7	2.7

Table 2: Cell volume calculated from light microscopy (LM) and electronic particle counter (CASY) measurements at the time of harvest, number of coccoliths cell^{-1} and number of coccoliths analyzed by scanning electron microscopy (SEM) and classified into normal, incomplete and malformed coccoliths in semi-continuous and batch control and P-limited cultures of *Emiliania huxleyi* grown at 19 and 24 °C. The number of cells (n) analysed for each measurement is presented as the sum of three replicates, except for CASY cell volume measurements for which n=3; ± standard deviation.

	Semi-continuous cultures		Batch cultures	
	Control	P-limited	Control	P-limited
Cell volume [µm^3] (LM)				
19 °C	34.3 ± 17.7 (n=111)	19.9 ± 9.5 (n=116)	29.7 ± 12.1 (n=346)	57.6 ± 22.7 (n=205)
24 °C	24.6 ± 12.8 (n=117)	34.3 ± 17.7 (n=194)	24.7 ± 14.1 (n=352)	64.3 ± 31.7 (n=217)
(CASY)				
19 °C	74.4 ± 8.9	75.5 ± 7.4	62.7 ± 7.3	106.9 ± 9.8
24 °C	94.0 ± 4.5	92.1 ± 6.8	67.1 ± 5.8	115.1 ± 3.0
Coccoliths cell^{-1}				
19 °C	20 ± 9 (n=148)	18 ± 6 (n=149)	15 ± 5 (n=151)	45 ± 20 (n=149)
24 °C	16 ± 7 (n=145)	15 ± 5 (n=146)	16 ± 6 (n=149)	34 ± 15 (n=145)
Number of coccoliths analysed for morphology				
19 °C	821	824	693	3496
24 °C	731	721	691	2010
Normal [%]				
19 °C	81.5	79.5	77.6	21.3
24 °C	51.0	54.7	57.1	33.8
Incomplete [%]				
19 °C	1.3	0.8	1.8	76.7
24 °C	2.0	0.7	4.4	52.4
Malformed [%]				
19 °C	17.2	19.7	20.6	2.0
24 °C	46.9	44.7	38.5	13.7

Information Services 18/9/2017 15:02
Deleted: 1

Information Services 29/9/2017 13:20
Deleted: , and number of coccoliths cell^{-1}

Information Services 25/9/2017 14:09
Deleted: c

Information Services 25/9/2017 14:09
Deleted: c

Information Services 25/9/2017 14:09
Deleted: c

Information Services 25/9/2017 14:19
Deleted: c

Information Services 25/9/2017 14:11
Deleted: c

Information Services 25/9/2017 14:13
Deleted: (n)

Information Services 25/9/2017 14:25
Deleted: n

Information Services 25/9/2017 14:25
Deleted: i

Information Services 25/9/2017 14:25
Deleted: m

Information Services 25/9/2017 14:19
Deleted: coccoliths cell^{-1} ... [1]

Table 3: Characteristics of the three classes (normal, incomplete, malformed) used to describe coccolith morphology, including a description of "dissolution features"

Coccolith type	Description
Normal	Central area, proximal and distal shield fully developed; distal shield elements clearly separated by slits with complete outer rim of the distal shield (Fig. 2a).
Incomplete	Central area, proximal and/or distal shield not fully developed; incomplete or absent outer rim of the distal shield (Fig. 2b), but without visible malformations of distal shield elements (as defined below).
Malformed	Several types of malformations were observed (Fig. 2c-g): 1) more than two merged distal shield elements (Fig. 2c) 2) tips of distal shield elements forming triangular thickening with outer rim (Fig. 2e) 3) increased gaps between distal shield elements (Fig. 2c) 4) missing central area (Fig. 2d) 5) irregular outgrowth of calcite (Fig. 2e) 6) strongly malformed coccoliths of irregular shape (Fig. 2e)
Signs of secondary dissolution	Distal shield elements thinning or detaching (Fig. 2f, g); incomplete outer rim with "hammer-like" distal shield elements (Fig. 2f); thinning central area (Fig. 2g); thinning of the proximal shield with exposed shield elements separated by slits (Fig. 2g); coccoliths lose their structural integrity and coccospheres are mostly collapsed (Fig. 2g).

Table 4: Exponential growth rate (μ_{exp}), particulate organic phosphorus (POP), carbon (POC) and inorganic carbon (PIC) cellular content, production and ratios in semi-continuous and batch control and P-limited cultures of *Emiliania huxleyi* grown at 19 and 24 °C; n=3 ± standard deviation.

	Semi-continuous cultures		Batch cultures	
	Control	P-limited	Control	P-limited
μ_{exp}				
19 °C	1.32 ± 0.05	1.31 ± 0.02	1.08 ± 0.07	1.15 ± 0.03
24 °C	1.20 ± 0.07	1.23 ± 0.07	1.15 ± 0.02	1.18 ± 0.04
POP [pg cell^{-1}]				
19 °C	0.42 ± 0.03	0.38 ± 0.03	0.26 ± 0.03	0.071 ± 0.009
24 °C	0.43 ± 0.03	0.33 ± 0.05	0.27 ± 0.02	0.083 ± 0.003
POP [pg cell^{-1} d^{-1}]				
19 °C	0.56 ± 0.04	0.50 ± 0.04	0.28 ± 0.02	n/a
24 °C	0.51 ± 0.03	0.40 ± 0.06	0.32 ± 0.02	n/a
POC [pg cell^{-1}]				
19 °C	13.5 ± 0.9	14.8 ± 0.7	8.1 ± 0.7	21.5 ± 0.8
24 °C	15.1 ± 1.2	15.3 ± 0.5	8.9 ± 0.3	18.3 ± 0.4
POC [pg cell^{-1} d^{-1}]				
19 °C	17.8 ± 1.2	19.3 ± 1.0	8.8 ± 0.4	n/a
24 °C	18.1 ± 1.4	18.8 ± 0.6	10.5 ± 0.1	n/a
POC/POP [mol mol^{-1}]				
19 °C	82.8 ± 5.2	101 ± 8	79.9 ± 1.8	792 ± 93
24 °C	91.1 ± 7.0	123 ± 16	85.3 ± 6.9	572 ± 17
PIC [pg cell^{-1}]				
19 °C	14.7 ± 0.9	12.8 ± 0.6	6.6 ± 0.6	16.5 ± 0.4[a]
24 °C	13.6 ± 1.3	12.4 ± 0.7	7.3 ± 0.3	18.7 ± 0.9[a]
PIC [pg cell^{-1} d^{-1}]				
19 °C	19.4 ± 1.2	16.7 ± 0.8	7.1 ± 0.3	n/a
24 °C	16.3 ± 1.5	15.3 ± 0.9	8.6 ± 0.3	n/a
PIC/POC				
19 °C	1.09 ± 0.07	0.87 ± 0.07	0.81 ± 0.03	0.77 ± 0.02[a]
24 °C	0.90 ± 0.08	0.81 ± 0.06	0.82 ± 0.03	1.02 ± 0.04[a]

[a]presumably underestimated because of calcite undersaturation (see Table 5)

Information Services 18/9/2017 15:03
Deleted: 2

Information Services 25/9/2017 14:57
Deleted: c

Information Services 25/9/2017 14:57
Deleted: c

Information Services 18/9/2017 15:15
Deleted: 3

Table 5: Cell concentrations, residual phosphate, total alkalinity, pH, dissolved inorganic carbon, and saturation state of calcite in the culture medium at the time of harvest of semi-continuous and batch control and P-limited cultures of *Emiliania huxleyi* grown at 19 and 24 °C; n=3 ± standard deviation.

	Semi-continuous cultures		Batch cultures	
	Control	P-limited	Control	P-limited
×10^4 cells mL^{-1}				
19 °C	8.29 ± 0.54	7.87 ± 0.46	78.32 ± 16.38	73.78 ± 2.26
24 °C	14.26 ± 1.50	14.98 ± 0.66	79.99 ± 1.16	61.63 ± 1.37
PO_4^{3-} [µM]				
19 °C	6.41 ± 0.37	0.50 ± 0.05	3.58 ± 1.03	0.18 ± 0.09
24 °C	6.65 ± 0.95	0.06 ± 0.03	2.93 ± 0.39	0.06 ± 0.04
A_T [µmol kg^{-1}]				
19 °C	2000 ± 50	2100 ± 50	1450 ± 100	500 ± 50
24 °C	1950 ± 50	2000 ± 50	1250 ± 50	700 ± 50
pH (NBS)				
19 °C	8.01 ± 0.01	8.05 ± 0.04	8.21 ± 0.06	7.70 ± 0.02
24 °C	8.13 ± 0.06	8.16 ± 0.11	8.22 ± 0.02	7.85 ± 0.01
DIC [µmol kg^{-1}]				
19 °C	1650 ± 50	1700 ± 50	1050 ± 100	400 ± 50
24 °C	1550 ± 100	1550 ± 100	950 ± 50	550 ± 50
Ω_{Ca}				
19 °C	3.14 ± 0.08	3.66 ± 0.29	3.20 ± 0.16	0.40 ± 0.03
24 °C	3.91 ± 0.35	4.38 ± 0.69	2.93 ± 0.10	0.77 ± 0.05

Information Services 18/9/2017 15:03
Deleted: 3

Information Services 25/9/2017 15:01
Deleted: c

Information Services 25/9/2017 15:01
Deleted: c

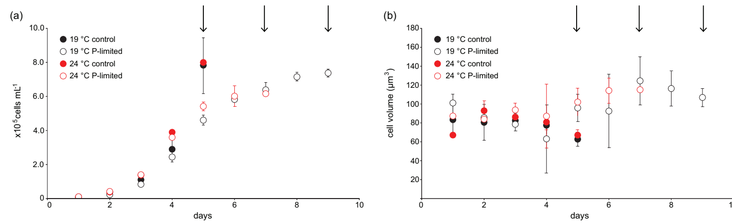

Figure 1: (a) Cell concentrations and (b) cell volume over time in batch cultures of *Emiliania huxleyi* grown at 19 and 24 °C in control and P-limited medium. Error bars denote the standard deviation of mean triplicate measurements of triplicate cultures. Arrows indicate when cultures were harvested.

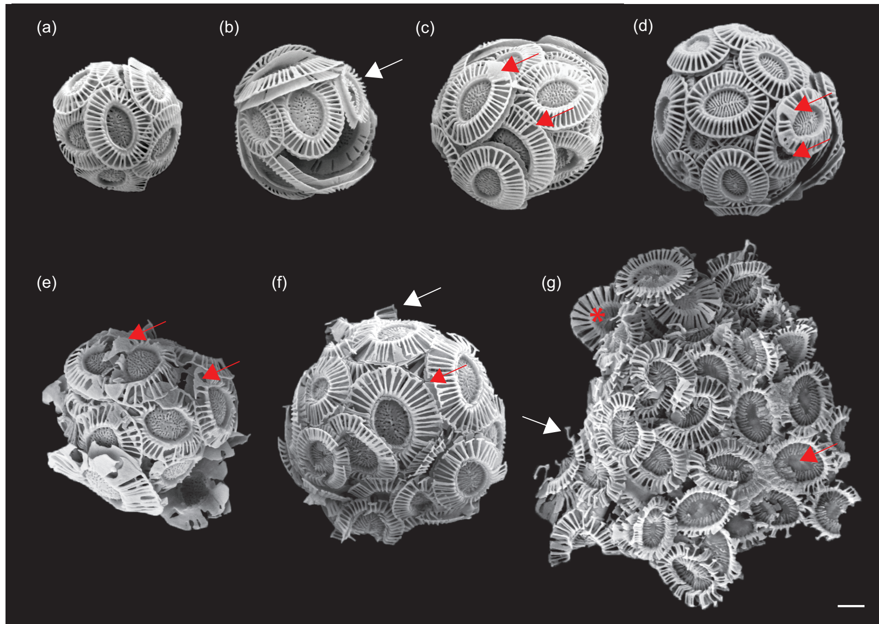

Figure 2: Representative scanning electron micrographs of normal, incomplete and malformed coccoliths, including dissolution features: (a) coccosphere bearing normal coccoliths; (b) arrow: an incomplete coccolith in control batch culture; (c) arrows: malformed coccoliths with merged distal shield elements/increased gaps (d) a coccosphere from an Oslo fjord field sample; arrows highlight the same type of malformations (merged distal shield elements, missing central area) as observed in culture; (e) coccosphere with many malformed coccoliths showing merged distal shield elements, triangular thickening of the elements and irregular calcite growth; (f) partially dissolved coccosphere; white arrow: detached distal shield elements; red arrow: "hammer-like" distal shield elements; (g) strongly dissolved coccosphere; white arrow: detached distal shield elements; red arrow: dissolved central area; asterisk: exposed proximal shield elements. Scale bar = 1µm.

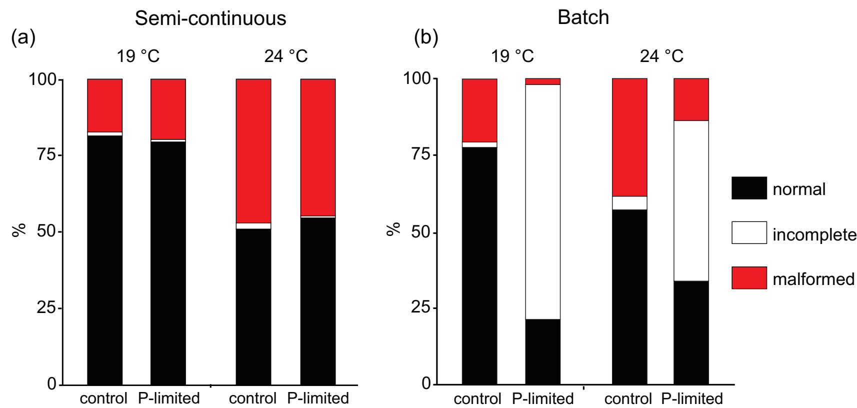

Figure 3: Coccolith morphology of *Emiliania huxleyi* grown at 19 and 24 °C in control and P-limited medium in semi-continuous and batch culture. Coccoliths were classified into the categories normal, incomplete, and malformed; see Table 3, Fig. 2.

Information Services 22/9/2017 14:37
Deleted: 1

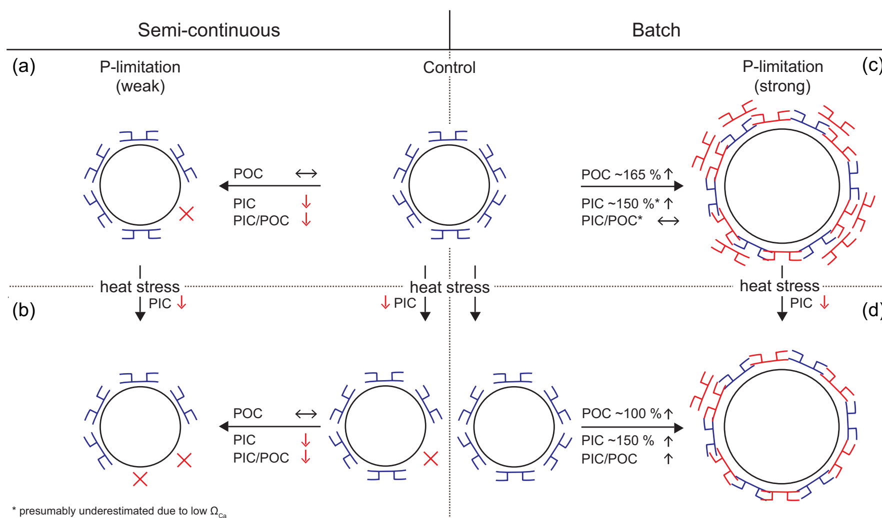

Figure 4: Schematic of the combined effect of P-limitation and heat stress in semi-continuous (a,b) and batch culture (c,d) of *Emiliania huxleyi*. Blue coccoliths represent coccoliths covering cells of control cultures, whereas red coccoliths/crosses denote new/missing coccoliths. The asterisk (*) indicates cultures that were strongly undersaturated in calcite.

Information Services 25/9/2017 10:20
Deleted: 2