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

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

[1]Department of Biosciences, University of Oslo, Oslo, 0316, Norway

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

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

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

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

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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*. This could lessen the ballasting effect of coccoliths and weaken carbon export out of the photic zone. However, using batch cultures, we show that different culturing approaches (batch versus semi-continuous) induce different physiologies. This affects the ratio of inorganic (PIC) to organic (POC) carbon 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 first of all decrease CO_2 sequestration by coccolithophores through lower overall carbon production. Secondly, the export of carbon may be diminished by a decrease in calcification and a weaker coccolith ballasting effect.

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, 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), 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).

In nature, organisms are exposed to a combination of changing environmental factors, which can modify their response to single parameters (e.g. Milner et al., 2016; Borchard et al., 2011). This study therefore combines two possible future environmental stressors, phosphorus (P) limitation and increased temperature. These are predicted to occur simultaneously because 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; Müller et al., 2008; Paasche, 1998; 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

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Discussion started: 5 May 2017

responses to P-limitation, some differences between studies on PIC and POC production are due to differences in experimental method. When studying nutrient limitation two fundamentally different approaches are available, namely batch culture and (semi)-continuous culture. The latter method represents a steady-state scenario with constant seawater nutrient concentrations and constant growth rates and is representative of areas with permanently low nutrient availability such as the eastern Mediterranean (Kress et al., 2005; Krom et al., 1991). Batch culture on the other hand represents 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), production cannot be determined in the batch approach (e.g. Gerecht et al., 2014; Langer et al., 2012; Langer et al., 2013, Müller et al., 2008; 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 in a batch and a continuous culture experiment (Langer et al., 2013). This hypothesis is supported by data on *C. pelagicus* (Gerecht et al., 2015; Gerecht et al., 2014). 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., 2013). In this study we therefore tested another strain of *E. huxleyi* in both semi-continuous and batch culture and analyzed inter alia 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 dealt with the effect of temperature on coccolith morphology, suggesting that higher than optimum temperature leads to an increase in malformations (Watabe and Wilbur, 1966; Langer et al., 2010). The effect of temperature on carbon production in *E. huxleyi* has been addressed in numerous studies (Sorrosa et al., 2005; De Bodt et al., 2010; Feng et al., 2008; Borchard et al., 2011; Milner et al., 2016; Matson et al., 2016; Rosas-Navarro et al., 2016; Sett et al., 2014; Satoh et al., 2009) and 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. None of these studies, however, 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 clone of *E. huxleyi* isolated from the Oslo fjord in triplicate semi-continuous and batch culture in replete (control) and P-limiting medium at two temperatures (19, 24 °C). Cultures were grown in modified K/2 medium (Gerecht et al., 2014) at an initial phosphate concentration of 10 µM (control) or 0.5 µM (P-limiting). The cultures were kept at 19 or 24 °C 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}.

Biogeosciences Discuss., doi:10.5194/bg-2017-126, 2017
Manuscript under review for journal Biogeosciences
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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. 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).

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. 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

Biogeosciences Discuss., doi:10.5194/bg-2017-126, 2017
Manuscript under review for journal Biogeosciences
Discussion started: 5 May 2017

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). 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 (see also Gerecht et al., 2015). 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.

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 (Fig. 1).

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. Average values were compared by a t-test. 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).

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 1). 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-

Biogeosciences Discuss., doi:10.5194/bg-2017-126, 2017
Manuscript under review for journal Biogeosciences
Discussion started: 5 May 2017

value=0.21, p=0.65). PIC production, on the other hand, was significantly decreased in P-limited cultures (Table 1; F-value=13.25, p=0.0066) and P-limited cells were covered by one to two fewer coccoliths (Table 2; Fig. 2a,b), which led to a decrease in the PIC/POC ratio (Table 1; 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 1; 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 (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 1; F-value=19.73, p=0.0022) and the cells were covered by three to four fewer coccoliths (Table 2; Fig. 2b). 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 1; 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 3).

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 1). 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). Most of the dissolved inorganic carbon (DIC) had been consumed by P-limited cultures at both temperatures (Table 3). At 19 °C, DIC concentrations were significantly lower (400 ± 50 µmol kg^{-1}) than at 24 °C (550 ± 50 µmol kg^{-1}; Z-value=-2.62, p<0.01). DIC concentrations remained 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 1). 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. 4) and were twice as large in stationary phase as those harvested from control cultures in exponential phase (Table 2, Fig. 2c,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 1).

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 1; Fig. 2c,d). In P-limited cultures, 77 % of all coccoliths were incomplete at 19 °C, whereas 52 % of coccoliths were incomplete at 24 °C (Table 2; Fig. 3). The percentage of incomplete coccoliths was negligible in control cultures.

Temperature had no effect on μ_{exp} (F-value=3.19, p=0.11) or on production rates in control cultures (Table 1). 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., 2013; 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., 2013). 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., 2013). 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., 2013), 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., 2013) 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. 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 presumably limited in both P and DIC. DIC-limitation, however, was not the trigger for entering stationary phase as POC production continued for several days after cessation of cell division. This led to a strong increase in cell size, an effect that has been described previously for *E. huxleyi* (Müller et al., 2008; Riegman et al., 2000; Paasche and Brubak, 1994; Oviedo et al., 2014; Sheward et al., 2016) and recently also for *C. pelagicus* (Gerecht et al., 2015). 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., 2016).

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). 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 (Holligan et al., 1993; Balch et al., 1991). Though these blooms are important contributors to the sequestration of atmospheric CO_2 and carbon export, they are short-lived phenomena. Based on this study, 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 *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). The decrease in growth rate at 24 °C, observed in semi-continuous cultures, confirmed that this temperature was indeed above the optimum for this clone. Although a similar decrease in growth rate was not observed in batch culture, the doubling in coccolith malformations provides further evidence that 24 °C cultures were indeed heat-stressed (Langer et al., 2010; Watabe and Wilbur, 1966; Milner et al., 2016). In the following, 24 °C will therefore be referred to as heat stress and 19 °C as normal temperature.

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. An increase in P-requirements at higher temperature has previously been described for the coccolithophore *Coccolithus pelagicus* (Gerecht et al., 2014) and can be inferred for *E. huxleyi* from the studies carried out by Satoh et al. (2009) and Feng et al. (2008). 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 this incongruence 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). Increased P-requirements led to lower final biomass, both in terms of final cell numbers and lower cellular POC content.

Heat stress had a stronger effect than P-limitation on coccolith quota 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. 2a,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. 2d). This did not, however, correspond to a lower PIC content of these cells. Two possible reasons for this discrepancy are partial dissolution and an increase in the number of shed coccoliths. 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. The percentage of partially dissolved coccoliths was higher at normal temperature than under heat stress as these cultures reached higher final biomass and consequently were less saturated in calcite. The PIC quota and PIC/POC ratio measured in P-limited batch cultures were therefore 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 quota 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).

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; Milner et al., 2016; Matson 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. To our knowledge, this study is the first to show that heat stress is not only detrimental for coccolith morphology (Milner et al., 2016; Watabe and Wilbur, 1966; Langer et al., 2010), but also for coccolith production in *E. huxleyi*.

5. Conclusions

By employing semi-continuous cultures, we show that both P-limitation and heat stress decrease calcification rate in *E. huxleyi*. Furthermore, the increase in P-requirements under heat stress may decrease the standing stock of *E. huxleyi* in a warmer ocean, which would have a negative feedback on carbon sequestration. 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, lower nutrient input and higher global temperature may

have an additive negative effect on calcification which may lead to weakened carbon export due to less coccolith ballasting (Ziveri et al., 2007).

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.

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Biogeosciences Discuss., doi:10.5194/bg-2017-126, 2017
Manuscript under review for journal Biogeosciences
Discussion started: 5 May 2017

Table 1: 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.

	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
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]presumably underestimated because of calcite saturation (see Table 3)

Biogeosciences Discuss., doi:10.5194/bg-2017-126, 2017
Manuscript under review for journal Biogeosciences
Discussion started: 5 May 2017

Table 2: Cell volume calculated from light microscopy (LM) and electronic particle counter (CASY) measurements, number of coccoliths analyzed by scanning electron microscopy (SEM) and classified into normal, incomplete and malformed coccoliths, and number of coccoliths cell^{-1} in semi-continuous and batch control and P-limited cultures of *Emiliania huxleyi* grown at 19 and 24 °C.

	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 (n)				
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
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)

Biogeosciences Discuss., doi:10.5194/bg-2017-126, 2017
Manuscript under review for journal Biogeosciences
Discussion started: 5 May 2017

Table 3: Cell concentrations, residual phosphate, total alkalinity, pH, dissolved inorganic carbon, and saturation 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.

	Semi-continuous cultures		Batch cultures	
	control	P-limited	control	P-limited
$\times 10^4$ 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

Biogeosciences Discuss., doi:10.5194/bg-2017-126, 2017
Manuscript under review for journal Biogeosciences
Discussion started: 5 May 2017

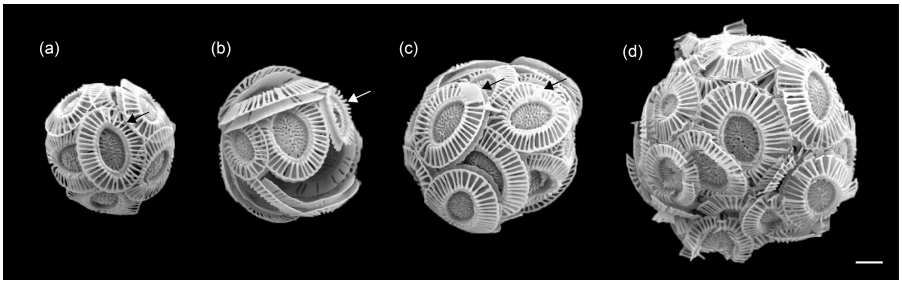

Figure 1: Scanning electron micrographs of the coccolith classes used in morphological analysis (identified by the arrows): (a) Normal (b) Incomplete (c) Malformed. (d) Representative coccosphere from P-limited batch culture, showing partial dissolution of the coccoliths. Scale bar = 1µm.

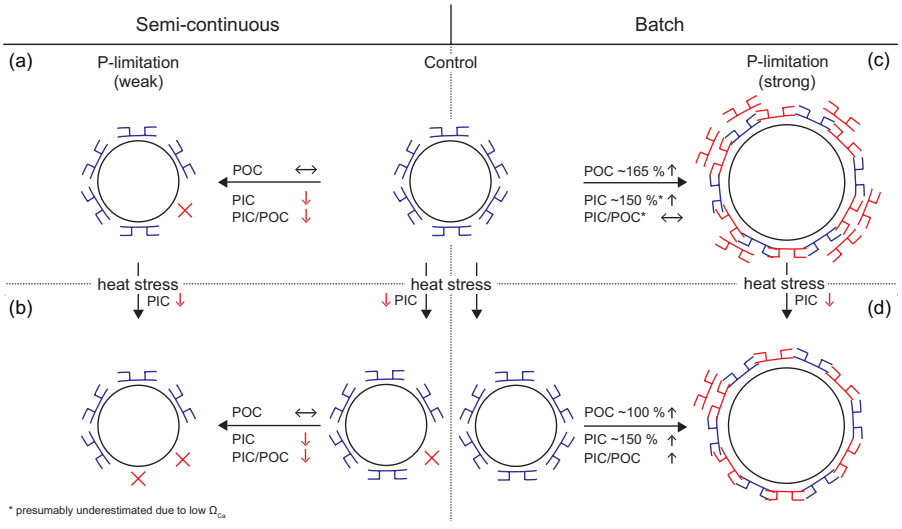

Figure 2: Schematic of the combined effect of P-limitation and heat stress in semi-continuous (a,b) and batch culture (c,d) of *Emiliania huxleyi*. The asterisk (*) indicates cultures that were strongly undersaturated in calcite.

Biogeosciences Discuss., doi:10.5194/bg-2017-126, 2017
Manuscript under review for journal Biogeosciences
Discussion started: 5 May 2017

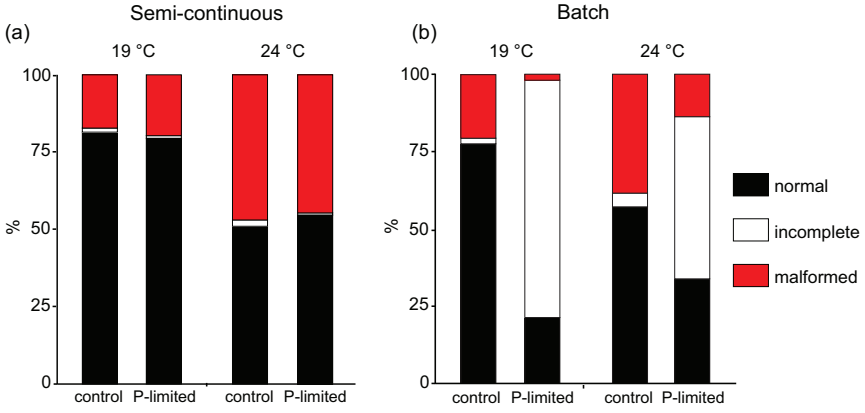

Figure 3: Coccolith morphology of *Emiliania huxleyi* grown at 19 and 24 °C in control and P-limited medium in semi-continuous and batch culture. Coccoliths were classified into the categories normal, incomplete, and malformed; see Fig. 1.

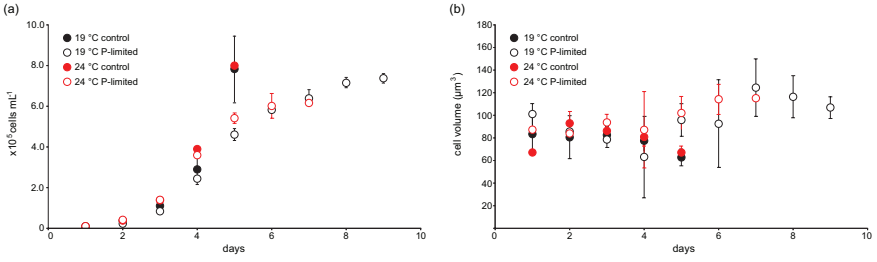

Figure 4: (a) Cell concentrations and (b) cell volume over time in batch cultures of *Emiliania huxleyi* grown at 19 and 24 °C in control and P-limited medium.