https://doi.org/10.5194/bg-2019-179
Preprint. Discussion started: 27 May 2019

Biogeosciences Discussions
Open Access
EGU

How will the key marine calcifier *Emiliania huxleyi* respond to a warmer and more thermally variable ocean?

Xinwei Wang[1], Feixue Fu[2], Pingping Qu[2], Joshua D. Kling [2], Haibo Jiang[3], Yahui Gao[1,4], David A. Hutchins[2*]

1. School of Life Sciences, Xiamen University, Xiamen, 361005, China

2. Department of Biological Sciences, University of Southern California, Los Angeles, California, 90089, USA

3. School of Life Sciences, Central China Normal University, Wuhan, Hubei, China.

4. Key Laboratory of the Ministry of Education for Coastal and Wetland Ecosystems, Xiamen University, Xiamen 361005, China

*Corresponding author: David Hutchins, tel: 1-213-7405616, fax: 1-213-7408123, Email address: dahutch@usc.edu

Key words: thermal variation, *Emiliania huxleyi*, coccolithophore, calcification, growth rate, elemental composition, global warming

https://doi.org/10.5194/bg-2019-179
Preprint. Discussion started: 27 May 2019

Biogeosciences Discussions
Open Access
EGU

Abstract

Global warming will be combined with predicted increases in thermal variability in the future surface ocean, but how temperature dynamics will affect phytoplankton biology and biogeochemistry is largely unknown. Here, we examine the responses of the globally important marine coccolithophore *Emiliania huxleyi* to thermal variations at two frequencies (one-day and two-day) at low (18.5 ºC) and high (25.5 ºC) mean temperatures. Elevated temperature and thermal variation decreased growth, calcification and physiological rates, both individually and interactively. One-day thermal variation frequencies were less inhibitory than two-day variations under high temperature, indicating that high frequency thermal fluctuations may reduce heat-induced mortality and mitigate some impacts of extreme high temperature events. Cellular elemental composition and calcification was significantly affected by both thermal variation treatments relative to each other, and to the constant temperature controls. The negative effects of thermal variation on *E. huxleyi* growth rate and physiology are especially pronounced at high temperatures. These responses of the key marine calcifier *E. huxleyi* to warmer, more variable temperature regimes have potentially large implications for ocean productivity and marine biogeochemical cycles under a future changing climate.

https://doi.org/10.5194/bg-2019-179
Preprint. Discussion started: 27 May 2019

Introduction

Climate-driven changes such as ocean warming alter the productivity and composition of marine phytoplankton communities, thereby influencing global biogeochemical cycles (Boyd et al., 2018; Hutchins & Fu, 2017; Thomas, et al., 2012). Increasing sea surface temperatures have been linked to global declines in phytoplankton concentration (Boyce, et al., 2010), changes in spring bloom timing (Friedland et al., 2018), and biogeographic shifts in harmful algal blooms (Gobler et al., 2017). Warming and acidification may drive shifts away from dinoflagellate or diatom dominance, and towards nanophytoplankton (Hare et al., 2007; Keys, et al., 2018). Similarly, Morán et al. (2010) predicted that a gradual shift will occur towards smaller primary producers in a warmer ocean.

Effects of temperature increases on phytoplankton diversity are uncertain. Warming and phytoplankton biodiversity were found to be inversely correlated in a coastal California diatom assemblage, at least on short timescales (Tatters et al., 2018). In contrast, a five-year long mesocosm experiment found that elevated temperature can modulate species coexistence, thus increasing phytoplankton species richness and productivity (Yvon-Durocher et al. 2015). Globally, rising temperatures may result in losses of phytoplankton biodiversity in the tropics, but gains in the polar regions (Thomas et al., 2012). It is thought that ocean warming will lead to a poleward range expansion of warm-water species at the expense of cold-water species (Boyd et al., 2010; Gao et al., 2018; Hallegraeff, 2010; Hutchins & Fu, 2017; Thomas et al., 2012). It is evident that rising ocean temperatures will benefit some groups, while having

https://doi.org/10.5194/bg-2019-179
Preprint. Discussion started: 27 May 2019

detrimental consequences for others (Boyd et al., 2010, 2015, 2018; Feng, et al., 2017; Fu et al., 2014). For example, recent decades of satellite observations show a striking poleward shift in the distribution of blooms of the coccolithophore *Emiliania huxleyi*, a species that was previously virtually absent in polar waters (Boyd et al., 2010; Neukermans et al., 2018).

Coccolithophores are the most successful calcifying phytoplankton in the ocean, and contribute almost half of global marine calcium carbonate production. They play crucial biogeochemical roles by performing both photosynthesis and calcification, and facilitate carbon export to the deep ocean through the ballasting effects of their calcium carbonate shells (Klaas & Archer, 2002; Krumhardt et al., 2017; Monteiro et al., 2016). *E. huxleyi* (Lohm.) is the most abundant and cosmopolitan coccolithophore, forming prolific blooms in many regions (Holligan, et al., 1983; 1993; Iglesias-Rodríguez et al., 2002; Westbroek et al., 1993).

The responses of *E. huxleyi* to global change factors have been intensively investigated. Many *E. huxleyi* strains are sensitive to ocean acidification, which negatively affects their growth rates and calcification (Feng et al., 2018; Hoppe et al., 2011). However, among the many currently changing environmental drivers, temperature may be among the most important in regulating coccolithophore physiology (Boyd et al., 2010). Feng et al. (2008) reported that the growth rate of *E. huxleyi* was improved by elevated temperature at low irradiance. Furthermore, temperature was the most important driver controlling both cellular particulate organic and inorganic carbon content of a Southern Hemisphere *E. huxleyi* strain (Feng et al.,

https://doi.org/10.5194/bg-2019-179
Preprint. Discussion started: 27 May 2019

2018).

Most research about the effects of global warming on *E. huxleyi* and phytoplankton in general has focused on predicted increases in mean temperatures. However, in the natural environment, seawater temperatures fluctuate over timescales ranging from hours, to days, to months (Bozinovic et al., 2011; Jiang et al., 2017). Future climate models predict not only in an increase in mean temperature, but also an increase in temperature variability (frequency and intensity), as well as a higher probability of extreme events (IPCC 2014).

The impacts of climatic variability and extremes have been best studied in metazoans, where they may sometimes have a larger effect than increases in climatic averages alone (Vázquez et al., 2017; Vasseur et al., 2014; Zander et al., 2017). Variability can promote greater zooplankton species richness, compared with long-term average conditions (Cáceres 1997; Shurin et al. 2010). In corals, temperature variability could buffer warming stress, elevate thermal tolerance and reduce the risk of bleaching (Oliver & Palumbi, 2011; Safaie et al., 2018).

In comparison, we still lack a thorough understanding of how thermal variation affects phytoplankton growth and physiology. Unlike zooplankton, the few available studies suggest increasing thermal variation may decrease phytoplankton biomass and biodiversity, and shift the community towards small phytoplankton (Burgmer & Hillebrand, 2011; Rasconi et al., 2017). Two studies have shown that plastic responses play a key role in acclimation and adaptation to thermal fluctuations in algae (Kremer et al., 2018; Schaum & Collins, 2014). Population growth rates of phytoplankton in

https://doi.org/10.5194/bg-2019-179
Preprint. Discussion started: 27 May 2019

fluctuating thermal environments have been quantitatively modeled based on data from thermal response curves obtained under constant temperatures (Bernhardt et al., 2018).

In view of this relative lack of information on the effects of non-steady state temperatures on biogeochemically important phytoplankton, we carried out a thermal variability study using the Sargasso Sea *E. huxleyi* isolate CCMP371. Our experiments combined ocean warming with thermal variations, with a focus on the increasing frequency of temperature variations under global climate change. We examined growth rates, photosynthesis, calcification and elemental composition under constant, one-day and two-day temperature variations. This study is intended to provide insights into how different frequencies of thermal variation may influence the physiology and biogeochemistry of this important marine calcifying phytoplankton species under both current and future sea surface temperatures.

Materials and methods

The marine coccolithophore *E. huxleyi* (Lohm.) Hay and Mohler stain CCMP371 (isolated from the Sargasso Sea) was maintained in the laboratory as stock batch cultures in Aquil medium (100 µmol L^{-1} NO_3^- , 10 µmol L^{-1} PO_4^{3-}) made with 0.2 µM-filtered coastal seawater collected from the California region (Sunda et al., 2005). Cells were grown at 22 ºC under 120 µmol photons m^{-2} s^{-1} cool white fluorescent light with a 12 h/12 h light/dark cycle.

Experimental set-up

An aluminum thermal gradient block with a range of 13 temperatures was used to perform the thermal response curve and temperature variation experiments. For the

https://doi.org/10.5194/bg-2019-179
Preprint. Discussion started: 27 May 2019

thermal curve experiment, the extreme temperatures of the thermal-block were set to 8.5 ºC and 28.6 ºC, with intermediate temperatures of 10.5 ºC, 12 ºC, 13.5 ºC, 15.5 ºC, 17.5 ºC, 18.5 ºC, 21.3 ºC, 22.6 ºC, 24.5 ºC, 26.6 ºC, and 27.6 ºC. The *E. huxleyi* cells were transferred from the stock cultures into triplicate 120 ml acid washed polycarbonate bottles in the thermal block under a 12 h light /12h dark cycle at 180 µmol photons m^{-2} s^{-1}.

Semi-continuous culturing methods were used for all experiments. Cultures were diluted every two days to keep them in exponential growth stage while acclimating to the treatment temperatures for two weeks. Dilution volumes were calculated to match growth rates of each individual replicate, as measured using in vivo chlorophyll a (Chl *a*) fluorescence. Once steady-state growth rates were recorded for 3–5 consecutive transfers, the cultures were sampled (Zhu et al., 2017). To estimate the negative growth rates observed at 28.6 ºC, these cultures were diluted from 22 ºC stock cultures, and sampled after 4-6 days for growth rates and elemental stoichiometry.

Six treatments were used to determine the responses of *E. huxleyi* growth, photosynthesis and calcification to different frequencies of temperature fluctuation. Temperature fluctuation treatments included: 1) Low temperature, constant (18.5 ºC). 2) Low temperature, one-day fluctuation cycle (16-21ºC, mean = 18.5ºC). 3) Low temperature, two-day fluctuation cycle (16-21ºC, mean =18.5ºC). 4) High temperature, constant (25.5 ºC). 5) High temperature, one-day fluctuation cycle (23-28ºC, mean = 25.5ºC). 6) High temperature, two-day fluctuation cycle (23-28ºC, mean = 25.5ºC). The experimental *E. huxleyi* cultures were grown in triplicate in 120 ml acid washed

https://doi.org/10.5194/bg-2019-179
Preprint. Discussion started: 27 May 2019

polycarbonate bottles using the thermal-block under a 12 h light /12h dark cycle at 180 μmol photons m^{-2} s^{-1}.

For the variable temperature experiment, cultures were diluted semi-continuously every two days for constant and one-day variation treatments, and every four days for two-day variation treatments. 100 μmol L^{-1} nitrate and 10 μmol L^{-1} phosphate was added every two days. Cultures were grown for at least eight dilutions (~16 days for constant and one day variation treatments; ~32 days for two-day variation treatments) to acclimate to the different experimental conditions before final sampling. All variation treatments were sampled twice across the thermal variation cycle, once during the cool phase and once during the warm phase.

Growth rates

In vivo fluorescence was measured daily for the one-day variation treatment and every two days for the constant and two-day variation treatments using a Turner 10-AU fluorometer (Turner Designs, CA). In vivo-derived growth rates were subsequently verified using cell samples counted with a nanoplankton counting chamber on an Olympus BX51 microscope. Specific growth rates (d^{-1}) were calculated using the in vivo fluorescence and cell count data as: $\mu=\ln[N(T_2)/N(T_1)]/(T_2-T_1)$, in which $N(T_1)$ and $N(T_2)$ are the in vivo fluorescence values or cell counts at T_1 and T_2.

Chl *a* analysis

Twenty ml culture samples were filtered onto GF/F glass fiber filters (Whatman GFC, Maidstone, UK) for Chl *a* analysis. In vitro Chl *a* was extracted with 90% aqueous acetone for 24 hours at -20 °C, and then measured using a Turner 10-AU

https://doi.org/10.5194/bg-2019-179
Preprint. Discussion started: 27 May 2019

fluorometer (Turner Design, USA). (Fu et al., 2007).

Elemental analysis

Elemental composition sampling included Total Particulate Carbon (TPC), Particulate Organic Carbon (POC), Particulate Organic Nitrogen (PON), Particulate Inorganic Carbon (PIC) and Particulate Organic Phosphorus (POP), allowing calculation of cellular elemental stoichiometry and calcite/organic carbon rations (PIC/POC) (Feng et al.; 2008). Culture samples for TPC, POC and PON, were collected onto pre-combusted GF/F glass fiber filters (Whatman) and dried in a 60 ºC oven overnight. For POC analysis, filters were fumed for 24 hours with saturated HCl to remove all inorganic carbon prior to analysis. TPC, PON and POC were then measured by a 440 Elemental Analyzer (Costech Inc, CA) following Fu et al. (2007). PIC was calculated as the difference between TPC and POC. For POP measurement, culture samples were filtered on onto pre-combusted GF/F filters (Whatman) and analyzed using a molybdate colorimetric method according to Fu et al. (2007).

Total carbon fixation, photosynthetic and calcification rates & ratios

Total carbon fixation, photosynthetic carbon fixation and calcification rates were measured using ^{14}C incubation techniques (Feng et al., 2008). Sixty mL culture samples from each treatment were spiked with 0.2 μCi $NaH^{14}CO_3$ and then incubated for 4 h under their respective experimental conditions. After incubation, samples were filtered on two Whatman GF/F filters (30mL each) for total carbon fixation and photosynthetic rate separately. The filters for photosynthetic rate measurement were fumed with saturated HCl before adding scintillation fluid. Thirty mL from each

https://doi.org/10.5194/bg-2019-179
Preprint. Discussion started: 27 May 2019

treatment (10 mL from each replicate bottle) was filtered immediately, after adding equal amounts of $NaH^{14}CO_3$ for procedural filter blanks. Filters were then placed in 7 mL scintillation vials with 4 mL scintillation fluid overnight in the dark. To determine the total radioactivity (TA), 0.2 μCi $NaH^{14}CO_3$ together with 100 μL phenylalanine was placed in scintillation vials with the addition of 4 mL scintillation solution. All samples were counted on a Perkin Elmer Liquid Scintillation Counter to measure the radioactivity. Total carbon fixation and photosynthetic rate were calculated from TA, final radioactivity and total dissolved inorganic carbon (DIC) values. Calcification rate was then calculated as the difference between total carbon fixation and photosynthetic rate for each sample.

Model for population growth of *E. huxleyii*

Growth rates measured under constant temperatures in the thermal block were fitted to the Eppley thermal performance curve or TPC (Eppley, 1972; Norberg, 2004; Thomas et al., 2012). This function quantifies parameters of growth temperature effects, including the temperature optimum for growth (T_{opt}), and high and low temperature limits (T_{max} and T_{min} respectively) in our strain of *E. huxleyii*. A modified version of this equation was also plotted to predict the impact that fluctuating temperatures might have on growth rates at present-day and future mean temperatures (Bernhardt et al., 2018, Kling et al. in review, Qu et al. in review).

Statistical analysis

The mean values of most parameters measured under the variation treatments were calculated by averaging the values from the cool and warm phases, including all the

https://doi.org/10.5194/bg-2019-179
Preprint. Discussion started: 27 May 2019

elemental content and ratios, photosynthetic and calcification rates and ratios. All statistical analyses, including student t-tests and ANOVA were conducted using the open source statistical software R version 3.5.0 (R Foundation).

Results

Responses of *E. huxleyi* to warming

The growth rates of *E. huxleyi* at constant temperature increased significantly with warming from 0.09±0.01 d^{-1} at 8.5 ºC to a maximum value of 0.90±0.02 d^{-1} at 21.3 ºC. Growth was optimal up to 24.5 ºC, and then decreased rapidly to -0.46±0.05 d^{-1} at 28.6 ºC ($p<0.05$, Fig. 1).

The elemental ratios of the cells in the different temperature treatments were compared to the average elemental ratios across the entire temperature range (Fig. 2). The thermal trends of TPC/PON ratios were generally similar with those of growth rates, in that ratios increased from 8.5 to 17.5 ºC, and then decreased from 24.5 to 27.6 ºC. The TPC/PON ratios at 8.5, 10.5 and 27.6 ºC were significantly lower than the average level of all the temperature points ($p<0.05$, Fig 2A). The POC/PON ratios of most temperature points were very close to the mean value of 6.3, except at 27.6 ºC (7.1) and 28.6 ºC (7.4), which were significantly higher than the average ($p<0.05$, Fig 2B). The highest PIC/POC ratio was 0.49±0.07 at 22.6 ºC, and the lowest PIC/POC ratio was 0.05±0.04 at 27.6 ºC, a value that was almost 90% less than the highest value. The PIC/POC ratios at the lowest temperature tested (10.5 ºC) and at the high end of the temperature range (26.6 and 27.6 ºC) were significantly lower than the average level (Fig. 2C). Chl *a*/POC ratios were significant lower at 8.5, 10.5 and 27.6 ºC than

https://doi.org/10.5194/bg-2019-179
Preprint. Discussion started: 27 May 2019

the mean, and at 17.5, 21.3, 22.6 and 24.5 °C were significantly higher than the average ($p<0.05$, Fig. 2C). The trends of PIC/POC and Chl *a*/POC ratio were similar, in that they gradually increased from low temperature and to the highest value at 22.6 °C, and then dropped rapidly as temperature increased further. (Fig. 2C, D).

Responses of *E. huxleyi* to temperature variations

Growth rate

In low temperature experiments, both one-day and two-day temperature variations had a negative effect on growth rate. The mean growth rates of the one-day (0.71±0.01 d^{-1}) and two-day (0.72±0.01 d^{-1}) variation treatments were not significantly different from each other ($p>0.05$), but both were lower than that of the constant 18.5 °C treatment (0.76±0.01, $p < 0.05$) (Fig. 3A). Growth rates were low during the cool phase (16 °C) of the experiment (~0.5-0.6 d^{-1}), but those of the two-day variation cycle were not significantly different from the constant control at this temperature ($p>0.05$). However, the cool phase of the one-day variation cycle had growth rates were lower than those of the constant 16 °C treatment ($p<0.05$). During the warm phase of the thermal cycle (21°C), there were no significant differences in the elevated growth rates (~0.85-0.9 d^{-1}) of the constant control and those of either variable treatment ($p>0.05$, Fig. 3A).

In the high temperature experiments, as in the low temperature experiments, both temperature variation frequencies had a negative effect on mean growth rates. The growth rates in the two-day variation treatment were (0.20±0.02 d^{-1}), a decrease of ~74% compared with the constant 25.5 °C ($p<0.05$), and ~62% of the one-day

https://doi.org/10.5194/bg-2019-179
Preprint. Discussion started: 27 May 2019

variation treatment value ($p<0.05$, Fig. 3B). During the cool phase (23 °C), the growth rate of the one-day variation treatment was slightly lower ($p<0.05$) than the constant 23 °C, but there were no significant changes between two-day variations and the constant 23 °C treatment ($p > 0.05$, Fig. 3B). During the warm phase (28 °C), the constant 28 °C and two-day variation treatment both had negative growth rates of -0.45±0.05 d^{-1} and -0.45±0.04 d^{-1}, respectively. However, the one-day variation treatment had a low but positive warm phase growth rate at 0.25±0.02 d^{-1} (Fig. 3B).

Cellular PIC and POC contents and ratios

In low temperature experiments, the cellular PIC content of the constant 18.5 °C treatment was 3.5±0.3 pg/cell, and there were no significant differences with temperature variation treatments ($p> 0.05$, Table 1). However, the cellular POC content of the constant 18.5 °C treatment was 8.0±0.6 pg/cell, which was lower than in the two-day variation treatment, but significantly higher than in the one-day variation treatment ($p<0.05$).

Like POC, the PIC/POC ratio was significantly affected by temperature variations (Fig. 4A). The lowest PIC/POC ratio was found in the one-day variation treatment (0.38 ±0.07), which was significantly lower than the two-day variation treatment value ($p < 0.05$), but close to that in the constant 18.5 °C ($p > 0.05$). A similar trend was found in both the cool (16 °C) and warm phases (21 °C) of the two variation treatments, in that the PIC/POC ratio of the one-day variation treatment was lower than of the two-day variation treatment ($p < 0.05$, Fig. 4A). Both variation treatments had lower PIC/POC ratios during the warm phase than during the cool phase, although these

https://doi.org/10.5194/bg-2019-179
Preprint. Discussion started: 27 May 2019

differences were not significant (p>0.05).

High temperature experiments showed particulate carbon trends that were contrary to those of the low temperature treatments. The PIC content and PIC/POC ratios were significantly decreased by temperature variation. The cellular PIC content of the constant treatment (25.5 ^{o}C) was 5.5±0.3 pg/cell, which was ~ 200% higher than that of the one-day variation and ~ 160% higher than in the two-day variation treatments ($p<0.05$, Table 1). The same trend was found for PIC/POC ratios in one-day variation and two-day variation treatments, which decreased ~ 67% and 33% compared with the constant 25.5 ^{o}C treatment, respectively ($p<0.05$, Fig. 4B). However, the POC content of one-day and two-day variation treatments was higher than in the constant 25.5 ^{o}C treatment ($p < 0.05$,Table 1). During the cool phase (23 ^{o}C), the PIC content and PIC/POC ratio of the one-day variation treatment was significantly lower than in the two-day variation treatment, but contrary to PIC content, the POC content of the one-day variation treatment was significantly higher than that in the two-day variation treatment. During the warm phase (28 ^{o}C), there were no significant differences of PIC content, POC content, or PIC/POC ratio between the one-day and two-day variation treatments (Fig. 4B, Table 1).

Photosynthetic and calcification rates and ratios

In low temperature treatments, there were no differences between total carbon fixation rates (photosynthesis plus calcification) for the two variable treatments relative to the constant control (Fig. 5A). However, during the cool phase total carbon fixation rates were higher in the one-day variation than in the two-day variation

https://doi.org/10.5194/bg-2019-179
Preprint. Discussion started: 27 May 2019

(p<0.05, Fig 5A), while this rate was the same in both variation treatments during the warm phase (p > 0.05, Fig. 5A). In high temperature experiments, the total carbon fixation rates of the one-day and two-day variation treatments were significantly decreased by about ~20% and ~18% respectively, compared with the constant 25.5 ^{o}C treatment (p<0.05, Fig. 5 B).

The photosynthetic and calcification rates of the constant 18.5 ^{o}C treatment were 0.04±0.00 pmol C cell^{-1} hr^{-1} and 0.02±0.00 pmol C cell^{-1} hr^{-1}, respectively, which were not significantly different from both of the temperature variation treatments (p > 0.05, Fig. 5 C,E). Photosynthetic rates changed within the thermal cycle for both one-day and two-day variation treatments, with a decrease of 22% and 28% from the warm phase to the cool phase, respectively (Fig. 5C). However, there were no significant changes in calcification rates under either variation frequency treatment between the cool and warm phases of the thermal cycles (p > 0.05).

In the mean 25.5 ^{o}C experiment, photosynthetic rates were not significantly different between the one-day variation and constant treatments (p > 0.05), while the photosynthetic rate of the two-day variation was slightly higher than that of the constant 25.5 ^{o}C treatment (p<0.05, Fig. 5D). In contrast, calcification rates of one-day and two-day variation treatments at a mean temperature of 25.5 ^{o} were significantly decreased by about ~46% and ~51%, respectively, relative to the constant control (p<0.05, Fig. 5F). There were no significant differences in total carbon fixation, photosynthetic and calcification rates between the one-day variation and two-day variation treatments during both the cool (23 ^{o}C) and warm (28 ^{o}C) phases (p>0.05,

https://doi.org/10.5194/bg-2019-179
Preprint. Discussion started: 27 May 2019

Fig. 5 B,D,F).

In the low temperature treatments, there were no significant differences in Cal/Photo ratios between the constant and the two variable treatments ($p > 0.05$, Fig 6A). In contrast, in the high temperature experiments, the Cal/Photo ratio of the one-day variation and two-day variation treatments were decreased by ~40% and 49%, respectively, compared with the constant 25.5 ^{o}C treatment ($p<0.05$, Fig. 6B). For both low and high temperature experiments, there were no significant differences between the one-day and two-day variation treatments in either the cool or warm phases of the thermal cycle ($p > 0.05$, Fig. 6B). However, in both temperature treatments the lower photosynthetic rates during the cool phase (Fig. 5C,D) resulted in an increase in the Cal/Photo ratio during the cool phase for both the one-day and two-day variation treatments ($p<0.05$ Fig. 6A,B).

Elemental content and stoichiometry

In the low temperature experiments, the one-day variation and two-day thermal variations had different effects on cellular elemental contents and ratios, relative to the constant 18.5 ^{o}C treatment. One-day variation increased most of the cellular elemental and biochemical contents (TPC, PON, and Chl *a*) but with no significant difference ($p>0.05$), except for POP content ($p<0.05$), compared with the constant 18.5 ^{o}C treatment (Table 1). In contrast, the two-day variation treatment decreased all the measured cellular elemental and biochemical contents (TPC, PON, POP and Chl *a*, $p<0.05$) in relation to the constant 18.5 ^{o}C treatment (Table 1). However, the

https://doi.org/10.5194/bg-2019-179
Preprint. Discussion started: 27 May 2019

TPC/PON and Chl *a*/POC ratios of the two-day variation treatment were higher than those of the one-day variation and constant 18.5 ºC treatments ($p<0.05$, Fig. 7A,E), while the PON/POP ratio was lower than in the one-day variation and constant 18.5 ºC treatments ($p<0.05$, Fig. 7C). There were no significant differences in TPC/PON, PON/POP and Chl *a*/POC ratios between the constant 18.5 ºC and the one-day variation treatments ($p > 0.05$, Fig. 7A).

In high temperature experiments, the highest cellular TPC, PON and POP contents were all obtained under the one-day variation treatment, which was significantly higher than under constant 25.5 ºC conditions ($p<0.05$, Table 1). However, there were no significant differences in cellular Chl *a* content between the constant 25.5 ºC and both variation treatments ($p > 0.05$, Table 1). The TPC/PON ratio of the constant 25.5 ºC treatment was ~22% and ~35% higher than that of the two-day variation and one-day variation treatments, respectively ($p<0.05$, Fig. 7B), while the PON/POP ratio was highest in the day variation, followed by the two-day variation and finally by the constant control (Fig. 7D). The Chl *a*/POC ratio of the one-day variation treatment was significantly lower than that of the constant 25.5 ºC and two-day variation treatments ($p<0.05$), but there were no significant differences between the constant 25.5 ºC and two-day variation treatments ($p > 0.05$, Fig. 7F).

During the cool phase of the high temperature experiments (23 ºC), the cellular TPC, PON, POP and Chl *a* content of two-day variation were all significantly lower than in the one-day variation treatment ($p<0.05$). Similar decreasing trends during the cool phase were observed for the TPC/PON ratios (Fig. 7B), but not the Chl *a*/POC

https://doi.org/10.5194/bg-2019-179
Preprint. Discussion started: 27 May 2019

ratio, which was ~32% higher than in the one-day variation treatment ($p<0.05$, Fig. 7F). During the warm phase (28 ^{o}C), there were no significant differences of cellular TPC, PON and POP contents between one-day and two-day variation treatments ($p > 0.05$, Table 1) as well as the TPC/PON ratio (Fig 7B). However, the Chl *a* content of the one-day variation treatment was ~20% lower than that of the two-day variation treatment ($p<0.05$). The Chl *a*/POC ratio was not significantly different between the one-day and two-day variation treatments at the warm phase ($p > 0.05$, Table 1, Fig. 7F).

Experimental constant temperature performance curves and measured and modeled fluctuating temperature TPCs

The experimentally-determined constant condition TPCs and the predicted fluctuating temperature condition TPCs based on the Eppley thermal performance curve are shown in Fig. 8 for *E. huxleyi*. Compared with the measured TPC under constant thermal conditions, the modeled TPC of the fluctuating temperature condition showed a leftward shift towards lower temperatures at optimum temperatures and above. The maximum and optimal temperature of the modeled fluctuating temperature TPC were all lower than those of the measured constant condition TPC. In particular, the optimal temperature for growth decreased from 22°C in constant conditions to 21 ^{o}C under fluctuating temperature conditions. At the same time, the maximum growth rate (μ_{max}) of the fluctuating temperature condition was 0.8 d^{-1}, which was lower than the constant condition value of 0. 9 d^{-1}. The measured growth rates of experimental one-day (0.71 d^{-1}) and two-day (0.72 d^{-1}) variation treatments at the relatively low

https://doi.org/10.5194/bg-2019-179
Preprint. Discussion started: 27 May 2019

mean temperature of 18.5 °C closely matched the model-predicted fluctuating temperature growth rate at this temperature (0.74^{-1}, Fig. 8). However, measured and predicted growth rates did not match as well at the higher mean temperature. At 25.5 °C, the measured growth rate of the one-day variation was 0.52 d^{-1}, 30% higher than the predicted fluctuating temperature growth rate of 0.40 d^{-1}. In contrast, the measured growth rate of the experimental two-day variation treatment was 0.20 d^{-1}, a decrease of 50% compared to the model-predicted fluctuating temperature growth rate of 0.40 d^{-1} at this temperature (Fig. 8).

Discussion

Effects of warming on *Emiliania huxleyi* growth rates and elemental ratios

Thermal response curves and optimum growth temperatures describe the importance of temperature as a control on the distribution of *E. huxleyi* strains in the ocean (Buitenhuis et al., 2008; Paasche, 2001). The optimal temperature range of 21.3-24.5 °C found in our study is similar to that of some other *E. huxleyi* strains (De Bodt et al., 2010; Feng et al., 2017; Rosas-Navarro et al., 2016). Most studies have focused on the lower part of the temperature curve where growth rates increase with rising temperatures, with relatively few examining stressfully warm temperatures where growth is inhibited (Feng et al., 2017; Matson et al., 2016). In our study, the descending portion of the upper TPC ranged from 24.5 °C to 28.6 °C, at which point growth rates became negative. This *E. huxleyi* strain was isolated from the Sargasso Sea where the sea surface temperature can reach 29 °C in the summer, and will be higher in the future with global warming (https://seatemperature.info/sargasso-sea-water-

https://doi.org/10.5194/bg-2019-179
Preprint. Discussion started: 27 May 2019

temperature.html). This suggests that this strain may be currently living near its upper thermal limit for part of the year, as are many other tropical and subtropical phytoplankton (Thomas et al. 2012), and that it may therefore be vulnerable to further warming.

Calcification is the key biogeochemical functional trait of this species, and the PIC/POC ratio of *E. huxleyi* can influenced by factors that include CO_2 concentration, nutrient status, irradiance and temperature (Feng et al., 2008, 2017; Raven & Crawfurd, 2012). The cellular PIC/POC of *E. huxleyi* has been reported to decrease as irradiance and CO_2 concentration rises, but increase under nitrate and phosphate limitation (Feng et al., 2017; Paasche, 1999; Riegman et al., 2000). The effect of temperature on *E. huxleyi* cellular PIC/POC ratio is however more complex. De Bodt et al. (2010) and Gerecht et al. (2014) observed that higher cellular PIC/POC ratios were obtained at lower temperatures for both *E. huxleyi* and *Coccolithus pelagicus*. Sett et al. (2014), however, found an opposite trend, whereby the PIC/POC ratio increased with temperature in another strain of *E. huxleyi*. Feng et al. (2017) reported that the cellular PIC/POC of *E. huxleyi* was increased as the temperature rose from 4 ^{o}C to 11 ^{o}C, but decreased with warming from 11 ^{o}C to 15 ^{o}C and remained steady afterwards.

In our study, the cellular PIC/POC ratio of *E. huxleyi* was positively correlated to growth rate (R^2=0.73), and increased with warming from 8.5 ^{o}C to a maximum at 22.6 ^{o}C, and then decreased with further warming to 27.6 ^{o}C. In a meta-analysis of studies using different coccolithophore subgroups, Krumhardt et al. (2017) found that the highest PIC/POC ratios were observed between 15 °C and 20 °C, in the same thermal

https://doi.org/10.5194/bg-2019-179
Preprint. Discussion started: 27 May 2019

range where the highest growth rates of *E. huxleyi* are found, as seen here and in Sett et al. (2014). In contrast, Rosas-Navarro et al. (2016) reported that the cellular PIC/POC ratio showed a minimum at optimal growth temperature (between 20 and 25 °C) for three strains of *E. huxleyi*. However, the *E. huxleyi* strain used here was isolated from a warmer area (the Sargasso Sea) compared with isolates from coastal Japan and New Zealand in previous studies (Rosas-Navarro et al. 2016; Feng et al. 2017). The growth temperature for our stock cultures was 22-24ºC, higher than that of the other two *E. huxleyi* strains. Feng et al. (2017) also found that the optimal temperature for calcification was close to the stock culture maintenance temperature in their study. Our results also support suggestions that stressful high temperatures may lead to decreases in cellular PIC/POC ratios and calcification (De Bodt et al., 2010; Feng et al., 2017; Gerecht et al., 2014; Krumhardt et al., 2017).

The cellular Chl *a*/POC ratio of *E. huxleyi* showed a similar pattern with the PIC/POC ratio, as it was also positively correlated to growth rate. Zhu et al. (2017) reported the cellular Chl *a*/POC ratio of a Southern California diatom was also correlated to growth rate across a very similar temperature range. In contrast, Feng et al. (2017) found that the cellular Chl *a*/POC ratio of *E. huxleyi* dramatically decreased with warming. However, in our experiments, the cellular Chl *a*/POC ratio was lower at 27.6 ºC than at 28.6 ºC, likely due to the negative growth rates and consequent lack of acclimation of the cultures maintained at the highest temperature. Traits such as PIC/POC ratios, Chl *a*/POC ratios and TPC/PON ratios also showed some evidence for possible carryover from the stock cultures (22-24 ºC) in this 28.6 ºC treatment, as we

https://doi.org/10.5194/bg-2019-179
Preprint. Discussion started: 27 May 2019

were forced to sample before the cells died completely, after only 2-3 cycles of dilution.

Effect of thermal variation on *Emiliania huxleyi* growth and physiology

Constant vs variable temperature

Thermal variability in the surface ocean is becoming an increasingly relevant topic as global warming proceeds. In our study, we found that the growth rates of a subtropical *E. huxleyi* strain were quite sensitive to temperature variation under both low (18.5 ºC, "winter") and high (25.5 ºC, "summer") mean temperatures. In both low and high temperature experiments, growth rates always decreased under temperature variation, compared with the constant mean temperature. This result agrees with previous studies showing that temperature variation slowed the growth rates of the fresh water green alga *Chlorella pyrenoidosa* and the marine diatom *Cyclotella meneghiniana*, as observed in laboratory work but also during long-term field observations (Zhang et al., 2016).

This growth rate inhibition under temperature variation was more pronounced at high temperature than at low temperature, indicating that variability at the warm range boundary will have a stronger negative effect on population growth rate than variability near the lower thermal limits (Bernhardt et al., 2018). This trend suggests that high temperatures, whether constant or variable, can in general irreversibly damage key cellular biochemical pathways and so inhibit growth rate. However, following Jensen's inequality model to predict the thermal performance curve, there should be an inflection point where the transfer between positive and negative effects of temperature variability will occur compared with the constant thermal curve.

https://doi.org/10.5194/bg-2019-179
Preprint. Discussion started: 27 May 2019

Conversely, for phytoplankton living in regions of suboptimal temperatures, thermal variation can enhance growth (Bernhardt et al., 2018). Thus, for some polar phytoplankton or for temperate species extending their ranges poleward, such as *E. huxleyi* (Neukermans et al., 2018), not only warming but also thermal variability may need to be taken into consideration in order to understand changes in high latitude microbial communities and biogeochemistry cycles.

Temperature variation affected the physiology of *E. huxleyi* differently compared with constant temperature. Physiological traits that were affected by thermal fluctuations also differed at low temperature ("winter") and high temperature ("summer"), suggesting different response mechanisms. Under low temperature variations (16-21 ^{o}C), photosynthesis and calcification were correlated with temperature, leading to rates similar to those observed with constant temperature. However, elemental contents and ratios under thermal variations differed from constant temperature. For instance, the cellular POC, PON, POP and Chl *a* contents increased during one-day variations but decreased during two-day variations, compared with constant temperature.

These cellular quota changes were reflected in elemental ratio differences (PIC/POC, Chl a/POC and TPC/POC) between the thermal variation treatments and constant temperature. However, the changes between thermal variation and constant treatments were not significant under low temperature ("winter"), indicating that the thermal variation wouldn't significantly influence biogeochemical cycles under these conditions. Unlike constant temperature treatments where selection may favor a higher

https://doi.org/10.5194/bg-2019-179
Preprint. Discussion started: 27 May 2019

growth rate, the trade-off for the thermal variation treatments may involve sacrificing increased growth rate in order to adjust cellular stoichiometry to adapt to the fluctuating environment.

In contrast, photosynthetic and calcification rates under high temperature thermal variations (23-28 ^{o}C) were significantly different from those seen under constant temperature (25 ^{o}C), especially the calcification rate. Thermal variation treatments transiently but repeatedly experienced the extreme high temperature point (28 ^{o}C), leading to extremely low calcification rates and PIC contents, and thus relatively low PIC/POC and Cal/Photo ratios. Previous *E. huxleyi* studies agree that high temperature decreases PIC content, PIC/POC ratios and Cal/Photo ratios (Feng et al., 2017; 2018; Gerecht et al., 2014). The two different patterns of responses to thermal variation we observed under low and high temperatures imply a seasonal pattern in the ways that thermal variations will affect the elemental stoichiometry of *E. huxleyi* .

Under other stresses such as nutrient limitation, trade-offs between growth rates and resource affinities may be necessary to adapt to thermal changes. For instance, nitrate affinity declines in cultures of the large centric diatom *Coscinodiscus* acclimated to warmer temperatures (Qu et al. 2018), while warming decreases cellular requirements for iron in the nitrogen-fixing cyanobacterium *Trichodesmium* (Jiang et al. 2018). In nitrogen-limited cultures of the marine diatom *Thalassiosira pseudonana*, long-term thermal adaptation acted most strongly on systems other than those involved in nitrate uptake and utilization (O'Donnell et al., 2018). Thus, it is possible that our thermal response results with *E. huxleyii* might have differed under nutrient-limited

https://doi.org/10.5194/bg-2019-179
Preprint. Discussion started: 27 May 2019

growth conditions.

One-day vs two-day thermal variation

As temperature fluctuations in the surface ocean increase along with climate change, phytoplankton will be influenced by the frequencies and intensities of these thermal excursions. We found that the responses of *E. huxleyi* to one-day versus two-day temperature variations were different at both low and high temperature. For instance, under low temperature the transition from the warm phase to the cool phase during the thermal variation could be treated as a low temperature stress leading to a lag phase in growth. The growth rate of the one-day variation treatment at the cool phase was lower than that of the two-day variation, suggesting that physiological acclimation is not rapid enough to accommodate to the shorter variation treatment, while the two day variation allows enough time for growth to recover. However, at the warm phase (21 ^{o}C) there was no difference in growth rates between the one-day and two-day variations compared with the constant 21-degree treatment. These results imply that there was a shorter lag phase after transfer at the optimal temperature point (21 ^{o}C at the warm phase) than during low temperature stress (16 ^{o}C at the cool phase).

There was no significant difference in photosynthetic rates between the one-day and two-day variation during the warm phase (21 ^{o}C), but both were higher than during the cool phase, indicating the photosynthetic rate was correlated to the thermal variation cycle. However, for the calcification rate there was no significant difference between one-day and two-day variations during either the cool or warm phases. These results suggested that photosynthesis was more responsive to temperature variations

https://doi.org/10.5194/bg-2019-179
Preprint. Discussion started: 27 May 2019

than calcification, and so ultimately determined the growth rate in both cool and warm phases. Feng et al. (2017) reported a similar relationship between growth and photosynthetic rates of a Southern Hemisphere *E. huxleyi* cultured at different temperatures.

Temperature variation frequencies also strongly influenced elemental composition. In low temperature experiments, the cellular contents of PON, POP and POC in the one-day variation treatment were all higher than under two-day variations. A notable exception to this trend was the cellular PIC content, which was not significantly different between one-day and two-day variation treatments. The PIC content was positively correlated to calcification and relatively stable, indicating that coccolith production and storage of *E. huxleyi* was relatively independent of the frequency of thermal variation.

Unlike the photosynthetic rate, the cellular elemental content of one-day and two-day variations were significantly different, but were not changed during temperature variation when transitioning from the warm phase to the cool phase or vice versa. The temperature dependent photosynthetic enzyme activity likely determined the similar photosynthetic rate of one-day and two-day variation treatments at both cool and warm phase in our short-term experiment, but the divergent responses of cellular stoichiometry in one-day and two-day thermal variations indicated different mechanisms of rapid acclimation to different thermal fluctuation frequencies. Our results imply that the responses of *E. huxleyi* to one-day and two-day thermal variations have different patterns, but both reach stable states during extended periods

https://doi.org/10.5194/bg-2019-179
Preprint. Discussion started: 27 May 2019

of temperature fluctuation. Due to decreasing POC content, the PIC/POC ratio increased in the two-day variation compared with the one-day variation, suggesting that more rapid thermal fluctuations might lead to a decrease in calcite ballasting of sinking organic carbon.

Under the high temperature scenario, thermal variation forces the microalgae to intermittently deal with a lethal high temperature during the warm phase (28 ^{o}C), with potentially irreversible damage to the cells. In the "summer" experiments, the mean growth rate of the two-day variation was much lower than that of the one-day variation. This mainly resulted from the negative growth rate of two-day variation cultures during the warm phase (28 ^{o}C), whereas the growth rate of the one-day variation was >0.20 d^{-1}. This result demonstrates that high frequency temperature variations (one-day) can partly mitigate growth inhibition by high temperatures in *E. huxleyi*, and so allow tolerance to extreme thermal events relative to longer exposures. This observation agrees with previous studies of other marine organisms such as corals (Oliver & Palumbi, 2011; Safaie et al., 2018). In the case of our experiments, the lag phase and metabolic inertia would help to maintain the microalgae during short exposures (one-day) to high temperature when transitioning from the cool phase (23 ^{o}C) to the warm phase (28 ^{o}C).

Likewise, the particulate organic element contents (PON, POP and POC) of *E. huxleyi* were more stable in one-day than in two-day temperature variation treatments. The relatively steady status of cellular particulate organic matter content in the high frequency temperature variation treatment may conserve energy, compared to the

https://doi.org/10.5194/bg-2019-179
Preprint. Discussion started: 27 May 2019

energy-intensive redistribution of major cellular components under lower frequency temperature variations. This differential energetic cost may help to explain the differences in growth rates between the two treatments. Adaptation to high temperature may also require higher investment in repair machinery, such as heat shock proteins, leading to an increased demand for nitrogen and other nutrients, thus increasing cellular POC, PON and POP contents (O'Donnell et al., 2018).

Prediction and modelling of *E. huxleyi* responses to thermal variation

Mathematical curves based on population growth rates from laboratory studies have been used to predict future population abundance, persistence or fitness in a changing world (Bernhardt et al., 2018; Deutsch et al., 2008; Jiang et al. 2017). We applied a modified version of the Eppley thermal performance curve to predict the influence of thermal variation on the growth rate of *E. huxleyi* (Fig. 8). *E. huxleyi* growth rates were predicted to be much lower at warmer temperatures under variable conditions compared to constant conditions, but there were no significant differences at cooler temperatures. Thus, the effect of thermal variation on population growth at the upper thermal limit was predicted to be stronger than that in the lower portion of the thermal range (Bernhardt et al., 2018; Sunday et al., 2012). This phenomenon has been widely observed in ectothermic taxa (Dell et al., 2011), but this model for the effect of thermal variation on population growth rate may lack the ability to predict species responses at the extreme edges of their ranges (Bernhardt et al., 2018).

Our results showed that the measured effects of a variable thermal regime on *E. huxleyi* growth rate fitted well with model-predicted values at a relatively low

https://doi.org/10.5194/bg-2019-179
Preprint. Discussion started: 27 May 2019

temperature (mean=18.5 ^{o}C), but differed considerably at high temperature (mean=25.5 ^{o}C). This was especially evident under the two-day variation conditions at a mean of 25.5 ^{o}C, where the growth rate was sharply lower than predicted from the constant TPCs-based model. This result suggests that transient heat waves may erode thermal tolerances of *E. huxleyi* populations already growing near their upper thermal limits, and that the frequency and duration of such extreme events is critically important in determining the magnitude of this stress. This sensitivity to increased thermal variability may reduce the fitness of *E. huxleyi* in the future subtropical and tropical oceans.

Although thermal variation at high temperature negatively impacted the growth rate of *E. huxleyi* in our experiment, our relatively short-term study didn't address the potential for *E. huxleyi* to evolve under selection by frequent extreme heat events. Evolutionary change in the thermal optimum and the maximum growth temperature in response to ocean warming may reduce heat-induced mortality, and mitigate some ecological impacts of global warming (O'Donnell et al., 2018, Thomas et al., 2012). For example, Schlüter et al. (2014) found that after one year of experimental adaptation to warming (26.3°C), the marine coccolithophore *E. huxleyi* evolved a higher growth rate when assayed at the upper thermal tolerance limit. Similar results were reported for the marine diatom *Thalassiosira pseudonana* in recent studies (O'Donnell et al., 2018; Schaum et al., 2018). Schaum et al. (2018) also found that the evolution of thermal tolerance in marine diatoms can be particularly rapid in fluctuating environments. Furthermore, populations originating from more variable environments are generally

https://doi.org/10.5194/bg-2019-179
Preprint. Discussion started: 27 May 2019

more plastic (Schaum & Collins, 2014; Schaum et al., 2013). Long-term evolutionary experiments with *E. huxleyi* will be necessary to determine how the thermal performance curve of this important marine calcifier may diverge under selection by different frequencies and durations of extreme thermal variation events.

Understanding the combination of ocean warming and magnified thermal variability may be a prerequisite to accurately predicting the effects of climate change on the growth and physiology of the key marine calcifier *E. huxleyi.* This information will help to inform biogeochemical models of the marine and global carbon cycles, and ecological models of phytoplankton distributions and primary productivity. How changing thermal variation frequencies will affect marine phytoplankton remains a relatively under-explored topic, but one that is likely to become increasingly important in the future changing ocean.

https://doi.org/10.5194/bg-2019-179
Preprint. Discussion started: 27 May 2019

Biogeosciences Discussions
Open Access
EGU

Acknowledgements

Support was provided by U.S. National Science Foundation Biological Oceanography grants OCE1538525 and OCE1638804 to F-XF and DAH. XW was supported by a grant from the China Scholarship Council.

https://doi.org/10.5194/bg-2019-179
Preprint. Discussion started: 27 May 2019

References

Bernhardt, J. R., Sunday, J. M., Thompson, P. L., and O'Connor, M. I.: Nonlinear averaging of thermal experience predicts population growth rates in a thermally variable environment, bioRxiv, 247908, 2018.

Boyce, D. G., Lewis, M. R., and Worm, B.: Global phytoplankton decline over the past century, Nature, 466, 591, 2010.

Boyd, P., Dillingham, P., McGraw, C., Armstrong, E., Cornwall, C., Feng, Y.-y., Hurd, C., Gault-Ringold, M., Roleda, M., and Timmins-Schiffman, E.: Physiological responses of a Southern Ocean diatom to complex future ocean conditions, Nature Climate Change, 6, 207-216, https://doi.org/10.1038/nclimate2811, 2015.

Boyd, P. W., Strzepek, R., Fu, F., and Hutchins, D. A.: Environmental control of open-ocean phytoplankton groups: Now and in the future, Limnol. Oceanogr., 55, 1353-1376, https://doi.org/ 10.4319/lo.2010.55.3.1353, 2010.

Boyd, P. W., Collins, S., Dupont, S., Fabricius, K., Gattuso, J. P., Havenhand, J., Hutchins, D. A., Riebesell, U., Rintoul, M. S., and Vichi, M.: Experimental strategies to assess the biological ramifications of multiple drivers of global ocean change—A review, Glob. Chang. Biol., 24, 2239-2261, https://doi.org/ 10.1111/gcb.14102, 2018.

Bozinovic, F., Bastías, D. A., Boher, F., Clavijo-Baquet, S., Estay, S. A., and Angilletta Jr, M. J.: The mean and variance of environmental temperature interact to determine physiological tolerance and fitness, Physiol. Biochem. Zool., 84, 543-552, https://doi.org/ 10.1086/662551, 2011.

https://doi.org/10.5194/bg-2019-179
Preprint. Discussion started: 27 May 2019

Buitenhuis, E. T., Pangerc, T., Franklin, D. J., Le Quéré, C., and Malin, G.: Growth rates of six coccolithophorid strains as a function of temperature, Limnol. Oceanogr., 53, 1181-1185, https://doi.org/ 10.4319/lo.2008.53.3.1181, 2008.

Burgmer, T., and Hillebrand, H.: Temperature mean and variance alter phytoplankton biomass and biodiversity in a long-term microcosm experiment, Oikos, 120, 922-933, https://doi.org/2011.

Cáceres, C. E.: Temporal variation, dormancy, and coexistence: a field test of the storage effect, Proceedings of the National Academy of Sciences, 94, 9171-9175, https://doi.org/ 10.2307/42576, 1997.

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 coccolith morphology and the coccosphere size, Biogeosciences, 7, 1401-1412, https://doi.org/ 10.5194/bg-7-1401-2010, 2010.

Dell, A. I., Pawar, S., and Savage, V. M.: Systematic variation in the temperature dependence of physiological and ecological traits, Proceedings of the National Academy of Sciences, 108, 10591-10596, https://doi.org/ 10.2307/27978658, 2011.

Deutsch, C. A., Tewksbury, J. J., Huey, R. B., Sheldon, K. S., Ghalambor, C. K., Haak, D. C., and Martin, P. R.: Impacts of climate warming on terrestrial ectotherms across latitude, Proceedings of the National Academy of Sciences, 105, 6668-6672, https://doi.org/ 10.1073/pnas.0709472105, 2008.

Eppley, R. W.: Temperature and phytoplankton growth in the sea, Fish. bull, 70, 1063-

https://doi.org/10.5194/bg-2019-179
Preprint. Discussion started: 27 May 2019

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, https://doi.org/10.1080/09670260701664674, 2008.

Feng, Y., Roleda, M. Y., Armstrong, E., Boyd, P. W., and Hurd, C. L.: Environmental controls on the growth, photosynthetic and calcification rates of a Southern Hemisphere strain of the coccolithophore *Emiliania huxleyi*, Limnol. Oceanogr., 62, 519-540, https://doi.org/10.1002/lno.10442, 2017.

Feng, Y., Roleda, M. Y., Armstrong, E., Law, C. S., Boyd, P. W., and Hurd, C. L.: Environmental controls on the elemental composition of a Southern Hemisphere strain of the coccolithophore *Emiliania huxleyi*, Biogeosciences, 15, 581-595, https://doi.org/10.1002/lno.10442, 2018.

Friedland, K. D., Mouw, C. B., Asch, R. G., Ferreira, A. S. A., Henson, S., Hyde, K. J., Morse, R. E., Thomas, A. C., and Brady, D. C.: Phenology and time series trends of the dominant seasonal phytoplankton bloom across global scales, Global Ecol. Biogeogr., 27, 551-569, https://doi.org/10.1111/geb.12717, 2018.

Fu, F.-X., Yu, E., Garcia, N. S., Gale, J., Luo, Y., Webb, E. A., and Hutchins, D. A.: Differing responses of marine N_2 fixers to warming and consequences for future diazotroph community structure, Aquat. Microb. Ecol., 72, 33-46, https://doi.org/10.5194/bgd-12-4273-2015, 2014.

Fu, F. X., Warner, M. E., Zhang, Y., Feng, Y., and Hutchins, D. A.: Effects of Increased

https://doi.org/10.5194/bg-2019-179
Preprint. Discussion started: 27 May 2019

temperature and CO_2 on photosynthesis, growth, and elemental ratios in marine *Synechococcus* and *Prochlorococcus* (cyanobacteria) 1, J. Phycol., 43, 485-496, https://doi.org/ 10.1111/j.1529-8817.2007.00355.x, 2007.

Gao, K., Zhang, Y., and Häder, D.-P.: Individual and interactive effects of ocean acidification, global warming, and UV radiation on phytoplankton, J. Appl. Phycol., 30, 743-759, https://doi.org/ 10.1007/s10811-017-1329-6, 2018.

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, https://doi.org/ 10.5194/bg-11-3531-2014, 2014.

Gobler, C. J., Doherty, O. M., Hattenrath-Lehmann, T. K., Griffith, A. W., Kang, Y., and Litaker, R. W.: Ocean warming since 1982 has expanded the niche of toxic algal blooms in the North Atlantic and North Pacific oceans, Proceedings of the National Academy of Sciences, 201619575, https://doi.org/ 10.1073/pnas.1619575114, 2017.

Hallegraeff, G. M.: Ocean climate change, phytoplankton community responses, and harmful algal blooms: a formidable predictive challenge, J. Phycol., 46, 220-235, https://doi.org/ 10.1111/j.1529-8817.2010.00815.x, 2010.

Hare, C. E., Leblanc, K., Ditullio, G. R., Kudela, R. M., Zhang, Y., Lee, P. A., Riseman, S., and Hutchins, D. A.: Consequences of increased temperature and CO_2 for phytoplankton community structure in the Bering Sea, Marine Ecology Progress, 352, 9-16, https://doi.org/ 10.3354/meps07182, 2007.

https://doi.org/10.5194/bg-2019-179
Preprint. Discussion started: 27 May 2019

Holligan, P., Viollier, M., Harbour, D., Camus, P., and Champagne-Philippe, M.: Satellite and ship studies of coccolithophore production along a continental shelf edge, Nature, 304, 339, https://doi.org/ 10.1038/304339a0, 1983.

Holligan, P. M., Fernández, E., Aiken, J., Balch, W. M., Boyd, P., Burkill, P. H., Finch, M., Groom, S. B., Malin, G., and Muller, K.: A biogeochemical study of the coccolithophore, *Emiliania huxleyi,* in the North Atlantic, Global Biogeochem. Cycles, 7, 879-900, https://doi.org/ 10.1029/93GB01731, 1993.

Hoppe, C., Langer, G., and Rost, B.: *Emiliania huxleyi* shows identical responses to elevated pCO_2 in TA and DIC manipulations, J. Exp. Mar. Bio. Ecol., 406, 54-62, https://doi.org/ 10.1016/j.jembe.2011.06.008, 2011.

Hutchins, D. A., and Fu, F.: Microorganisms and ocean global change, Nat Microbiol, 2, 17058, https://doi.org/ 10.1038/nmicrobiol.2017.58, 2017.

Iglesias-Rodríguez, M. D., Brown, C. W., Doney, S. C., Kleypas, J., Kolber, D., Kolber, Z., Hayes, P. K., and Falkowski, P. G.: Representing key phytoplankton functional groups in ocean carbon cycle models: Coccolithophorids, Global Biogeochem. Cycles, 16, 47-41-47-20, https://doi.org/ 10.1029/2001GB001454, 2002.

Jiang, L., Sun, Y.-F., Zhang, Y.-Y., Zhou, G.-W., Li, X.-B., McCook, L. J., Lian, J.-S., Lei, X.-M., Liu, S., and Cai, L.: Impact of diurnal temperature fluctuations on larval settlement and growth of the reef coral *Pocillopora damicornis*, Biogeosciences, 14, 5741-5752, https://doi.org/ 10.5194/bg-14-5741-2017, 2017.

Keys, M., Tilstone, G., Findlay, H. S., Widdicombe, C. E., and Lawson, T.: Effects of elevated CO_2 and temperature on phytoplankton community biomass, species

https://doi.org/10.5194/bg-2019-179
Preprint. Discussion started: 27 May 2019

composition and photosynthesis during an experimentally induced autumn bloom in the western English Channel, Biogeosciences, 15, 3203-3222, https://doi.org/ 10.5194/bg-15-3203-2018, 2018.

Klaas, C., and Archer, D. E.: Association of sinking organic matter with various types of mineral ballast in the deep sea: Implications for the rain ratio, Global Biogeochem. Cycles, 16, 63-61-63-14, https://doi.org/ 10.1029/2001GB001765, 2002.

Kremer, C. T., Fey, S. B., Arellano, A. A., and Vasseur, D. A.: Gradual plasticity alters population dynamics in variable environments: thermal acclimation in the green alga *Chlamydomonas reinhartdii*, Proceedings Biological Sciences, 285, 20171942, https://doi.org/ 10.1098/rspb.2017.1942, 2018.

Krumhardt, K. M., Lovenduski, N. S., Iglesias-Rodriguez, M. D., and Kleypas, J. A.: Coccolithophore growth and calcification in a changing ocean, Prog. Oceanogr., 159, 276-295, https://doi.org/ 10.1016/j.pocean.2017.10.007, 2017.

Matson, P. G., Ladd, T. M., Halewood, E. R., Sangodkar, R. P., Chmelka, B. F., and Iglesias-Rodriguez, M. D.: Intraspecific differences in biogeochemical responses to thermal change in the coccolithophore *Emiliania huxleyi*, PLoS One, 11, e0162313, https://doi.org/10.1371/journal.pone.0162313, 2016.

Monteiro, F. M., Bach, L. T., Brownlee, C., Bown, P., Rickaby, R. E., Poulton, A. J., Tyrrell, T., Beaufort, L., Dutkiewicz, S., and Gibbs, S.: Why marine phytoplankton calcify, Science Advances, 2, e1501822, https://doi.org/ 10.1126/sciadv.1501822, 2016.

https://doi.org/10.5194/bg-2019-179
Preprint. Discussion started: 27 May 2019

Morán, X. A. G., LÓPEZ-URRUTIA, Á., CALVO-DÍAZ, A., and Li, W. K.: Increasing importance of small phytoplankton in a warmer ocean, Glob. Chang. Biol., 16, 1137-1144, https://doi.org/ 10.1111/j.1365-2486.2009.01960.x, 2010.

Neukermans, G., Oziel, L., and Babin, M.: Increased intrusion of warming Atlantic water leads to rapid expansion of temperate phytoplankton in the Arctic, Glob. Chang. Biol., https://doi.org/ 10.1111/gcb.14075, 2018.

Norberg, J.: Biodiversity and ecosystem functioning: a complex adaptive systems approach, Limnol. Oceanogr., 49, 1269-1277, https://doi.org/ 10.4319/lo.2004.49.4_part_2.1269, 2004.

O'Donnell, D. R., Hamman, C. R., Johnson, E. C., Kremer, C. T., Klausmeier, C. A., and Litchman, E.: Rapid thermal adaptation in a marine diatom reveals constraints and tradeoffs, Glob. Chang. Biol., doi:10.1111/gcb.14360, 2018.

Oliver, T., and Palumbi, S.: Do fluctuating temperature environments elevate coral thermal tolerance?, Coral Reefs, 30, 429-440, https://doi.org/ 10.1007/s00338-011-0721-y, 2011.

Paasche, E.: Reduced coccolith calcite production under light-limited growth: a comparative study of three clones of *Emiliania huxleyi* (Prymnesiophyceae), Phycologia, 38, 508-516, 1999.

Paasche, E.: A review of the coccolithophorid *Emiliania huxleyi* (Prymnesiophyceae), with particular reference to growth, coccolith formation, and calcification-photosynthesis interactions, Phycologia, 40, 503-529, https://doi.org/ 10.2216/i0031-8884-40-6-503.1, 2001.

https://doi.org/10.5194/bg-2019-179
Preprint. Discussion started: 27 May 2019

Rasconi, S., Winter, K., and Kainz, M. J.: Temperature increase and fluctuation induce phytoplankton biodiversity loss–Evidence from a multi-seasonal mesocosm experiment, Ecology and evolution, 7, 2936-2946, https://doi.org/ 10.1002/ece3.2889, 2017.

Raven, J. A., and Crawfurd, K.: Environmental controls on coccolithophore calcification, Mar. Ecol. Prog. Ser., 470, 137-166, https://doi.org/ 10.3354/meps09993, 2012.

Riegman, R., Stolte, W., Noordeloos, A. A., 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, https://doi.org/ 10.1046/j.1529-8817.2000.99023.x, 2000.

Rosas-Navarro, A., Langer, G., and Ziveri, P.: Temperature affects the morphology and calcification of *Emiliania huxleyi* strains, Biogeosciences, 13, 2913-2926, https://doi.org/10.5194/bg-13-2913-2016, 2016.

Safaie, A., Silbiger, N. J., McClanahan, T. R., Pawlak, G., Barshis, D. J., Hench, J. L., Rogers, J. S., Williams, G. J., and Davis, K. A.: High frequency temperature variability reduces the risk of coral bleaching, Nat Commun, 9, https://doi.org/ 10.1038/s41467-018-04074-2, 2018.

Schaum, C. E., and Collins, S.: Plasticity predicts evolution in a marine alga, Proceedings of the Royal Society B: Biological Sciences, 281, 20141486, https://doi.org/ 10.1098/rspb.2014.1486, 2014.

https://doi.org/10.5194/bg-2019-179
Preprint. Discussion started: 27 May 2019

Schaum, C. E., Buckling, A., Smirnoff, N., Studholme, D., and Yvondurocher, G.: Environmental fluctuations accelerate molecular evolution of thermal tolerance in a marine diatom, Nat Commun, 9, 1719, https://doi.org/ 10.1038/s41467-018-03906-5, 2018.

Schaum, E., Rost, B., Millar, A. J., and Collins, S.: Variation in plastic responses of a globally distributed picoplankton species to ocean acidification, Nature Climate Change, 3, 298-302, https://doi.org/ 10.1038/NCLIMATE1774, 2013.

Schlüter, L., Kai, T. L., Gutowska, M. A., Gröger, J. P., Riebesell, U., and Reusch, T. B. H.: Adaptation of a globally important coccolithophore to ocean warming and acidification, Nature Climate Change, 4, 1024-1030, https://doi.org/ 10.1038/nclimate2379, 2014.

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, https://doi.org/ 10.1371/journal.pone.0088308, 2014.

Shurin, J. B., Winder, M., Adrian, R., Keller, W., Matthews, B., Paterson, A. M., Paterson, M. J., Pinel-Alloul, B., Rusak, J. A., and Yan, N. D.: Environmental stability and lake zooplankton diversity–contrasting effects of chemical and thermal variability, Ecol. Lett., 13, 453-463, https://doi.org/ 10.1111/j.1461-0248.2009.01438.x, 2010.

Sunda, W. G., Price, N. M., and Morel, F. M.: Trace metal ion buffers and their use in culture studies, Algal culturing techniques, 4, 35-63, 2005.

https://doi.org/10.5194/bg-2019-179
Preprint. Discussion started: 27 May 2019

Sunday, J. M., Bates, A. E., and Dulvy, N. K.: Thermal tolerance and the global redistribution of animals, Nature Climate Change, 2, 686, https://doi.org/ 10.1038/nclimate1539, 2012.

Tatters, A. O., Schnetzer, A., Xu, K., Walworth, N. G., Fu, F., Spackeen, J. L., Sipler, R. E., Bertrand, E. M., McQuaid, J. B., and Allen, A. E.: Interactive effects of temperature, CO_2 and nitrogen source on a coastal California diatom assemblage, J. Plankton Res., 40, 151-164, 2018.

Thomas, M. K., Kremer, C. T., Klausmeier, C. A., and Litchman, E.: A global pattern of thermal adaptation in marine phytoplankton, Science, 338, 1085-1088, https://doi.org/ 10.1126/science.1224836, 2012.

Vázquez, D. P., Gianoli, E., Morris, W. F., and Bozinovic, F.: Ecological and evolutionary impacts of changing climatic variability, Biological Reviews, 92, 22-42, https://doi.org/ 10.1111/brv.12216, 2017.

Vasseur, D. A., DeLong, J. P., Gilbert, B., Greig, H. S., Harley, C. D., McCann, K. S., Savage, V., Tunney, T. D., and O'Connor, M. I.: Increased temperature variation poses a greater risk to species than climate warming, Proceedings of the Royal Society of London B: Biological Sciences, 281, 20132612, https://doi.org/ 10.1098/rspb.2013.2612, 2014.

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

https://doi.org/10.5194/bg-2019-179
Preprint. Discussion started: 27 May 2019

Yvon-Durocher, G., Allen, A. P., Cellamare, M., Dossena, M., Gaston, K. J., Leitao, M., Montoya, J. M., Reuman, D. C., Woodward, G., and Trimmer, M.: Five years of experimental warming increases the biodiversity and productivity of phytoplankton, PLoS Biol., 13, e1002324, https://doi.org/10.1371/journal.pbio.10023242015.

Zander, A., Bersier, L. F., and Gray, S. M.: Effects of temperature variability on community structure in a natural microbial food web, Glob. Chang. Biol., 23, 56-67, https://doi.org/10.1111/gcb.13374, 2017.

Zhang, M., Qin, B., Yu, Y., Yang, Z., Shi, X., and Kong, F.: Effects of temperature fluctuation on the development of cyanobacterial dominance in spring: Implication of future climate change, Hydrobiologia, 763, 135-146, https://doi.org/10.1007/s10750-015-2368-0, 2016.

Zhu, Z., Qu, P., Fu, F., Tennenbaum, N., Tatters, A. O., and Hutchins, D. A.: Understanding the blob bloom: Warming increases toxicity and abundance of the harmful bloom diatom *Pseudo-nitzschia* in California coastal waters, Harmful Algae, 67, 36-43, https://doi.org/10.1016/j.hal.2017.06.004, 2017.

https://doi.org/10.5194/bg-2019-179
Preprint. Discussion started: 27 May 2019

Figure legends:

Fig. 1 Thermal performance curves (TPCs) showing cell-specific growth rates (d^{-1}) of *Emiliania huxleyi* CCMP371 across a temperature range from 8.5 to 28.6 °C. Symbols represent means and error bars are the standard deviations of three replicates at each temperature, but in many cases the errors bars are smaller than the symbols.

Fig. 2 Changes in *Emiliania huxleyi* TPC/PON ratios, POC/PON ratios, PIC/POC ratios and Cha/POC ratios across a temperatures range from 8.5 to 28.6 °C. Dashed lines represent the average ratios for the entire temperature range. Bars represent means and error bars are the standard deviations of three replicates at each temperature. Symbols * represent the significant difference ($p<0.05$) between average ratios and the ratio at each temperature.

Fig. 3 *Emiliania huxleyi* growth rate responses to constant temperatures, and during the warm and cool phases of the two thermal variation frequencies (one-day and two-day), under low (**A**) and high (**B**) mean temperatures. The thick black line in the boxplots represent median values for each experimental treatment; whiskers on boxplots indicate 1.5 × interquartile range. Listed p-values with their respective brackets are the statistical significance between two treatments.

Fig. 4 Responses of *Emiliania huxleyi* PIC/POC ratios to constant temperatures, and during the warm and cool phases of two thermal variation frequencies (one-day and two-day), under low (**A**) and high (**B**) mean temperatures. LT: Low temperature; HT: High temperature. The thick black line in the boxplots represent median values for each experimental treatment; whiskers on boxplots indicate 1.5 × interquartile range. Listed

https://doi.org/10.5194/bg-2019-179
Preprint. Discussion started: 27 May 2019

p-values with their respective brackets denote the statistical significance between two treatments.

Fig. 5 Responses of *Emiliania huxleyi* total carbon fixation (photosynthesis + calcification), photosynthetic and calcification rates to constant temperatures, and during the warm and cool phases of two thermal variation frequencies (one-day and two-day), under low (**A**, **C**, **E**) and high (**B**, **D**, **F**) mean temperatures. LT: Low temperature; HT: High temperature. The thick black line in the boxplots represent median values for each experimental treatment; whiskers on boxplots indicate 1.5 × interquartile range. Listed p-values with their respective brackets denote the statistical significance between two treatments.

Fig. 6 Responses of *Emiliania huxleyi* calcification to photosynthesis ratios (cal/photo) to constant temperatures, and during the warm and cool phases of two thermal variation frequencies (1 day and 2 day), under low (**A**) and high (**B**) mean temperatures. LT: Low temperature; HT: High temperature. The thick black line in the boxplots represent median values for each experimental treatment; whiskers on boxplots indicate 1.5 × interquartile range. Listed p-values with their respective brackets denote the statistical significance between two treatments.

Fig. 7 Responses of *Emiliania huxleyi* elemental ratios in two thermal variation frequency treatments (1 day and 2 day) compared to constant temperatures, for: TPC/PON (**A**, cool phase and **B**, warm phase), PON/POP (**C**, cool phase and **D**, warm phase) and Chl *a*/POC ratios (**E**, cool phase and **F**, warm phase). LT: Low temperature; HT: High temperature. The thick black line in the boxplots represent median values for

https://doi.org/10.5194/bg-2019-179
Preprint. Discussion started: 27 May 2019

each experimental treatment; whiskers on boxplots indicate 1.5 × interquartile range. Listed p-values with their respective brackets denote the statistical significance between two treatments.

Fig. 8 Thermal performance curves (TPCs) based on specific growth rates (d^{-1}) of *Emiliania huxleyi*, including our experimentally determined constant temperature TPC (black symbols and solid line) and an Eppley model-predicted fluctuating temperature TPC (dashed line). Measured growth rates from the two low and high temperature experiments are shown for constant thermal conditions (red symbols), one-day (green symbols) and two-day (blue symbols) variation treatments.

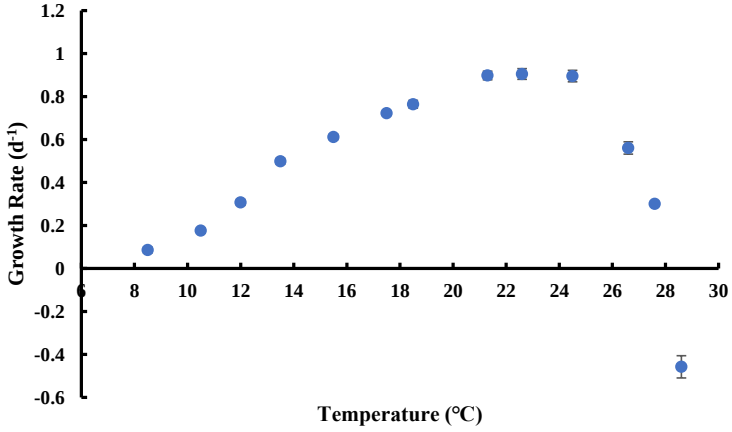
https://doi.org/10.5194/bg-2019-179
Preprint. Discussion started: 27 May 2019

Table 1 The effect of temperature variation under low and high temperature on total Carbon (pg/cell), cellular POC (pg/cell), cellular PIC (pg/cell), cellular PON (pg/cell), cellular POP (pg/cell) and cellular Chl *a* (pg/cell) of *Emiliania huxleyi*.

Treatment		**Total Carbon**	**Cellular PON**	**Cellular POP**	**Cellular POC**	**Cellular PIC**	**Cellar Chl *a***
low temperature	18.5 °C	11.5±0.4	1.8±0.2	0.17±0.00	8.0±0.6	3.5±0.3	0.14±0.00
	One-day cool point (16)	13.0±0.5	2.2±0.3	0.18±0.00	8.9±0.3	4.1±0.3	0.15±0.01
	One-day warm point (21)	12.0±0.7	2.1±0.3	0.19±0.00	9.3±0.9	2.7±0.9	0.19±0.00
	Two-day cool point (16)	10.1±0.7	1.3±0.2	0.16±0.01	6.0±0.9	4.0±0.3	0.12±0.01
	Two-day warm point (21)	10.4±0.5	1.5±0.2	0.17±0.01	6.6±0.5	3.8±0.3	0.15±0.01
high temperature	25.5 °C	15.0±0.7	2.0±0.1	0.21±0.01	9.5±0.3	5.5±0.7	0.18±0.02
	One-day cool point (23)	16.1±1.4	3.0±0.2	0.21±0.00	12.9±1.5	3.2±0.2	0.15±0.01
	One-day warm point (28)	19.1±0.8	4.4±0.3	0.24±0.01	17.0±0.6	2.1±0.2	0.20±0.02
	Two-day cool point (23)	12.4±1.0	1.9±0.2	0.19±0.01	7.5±1.0	4.8±0.3	0.13±0.01
	Two-day warm point (28)	19.4±2.0	3.9±0.8	0.25±0.03	18.3±3.7	2.1±0.9	0.25±0.02

https://doi.org/10.5194/bg-2019-179
Preprint. Discussion started: 27 May 2019

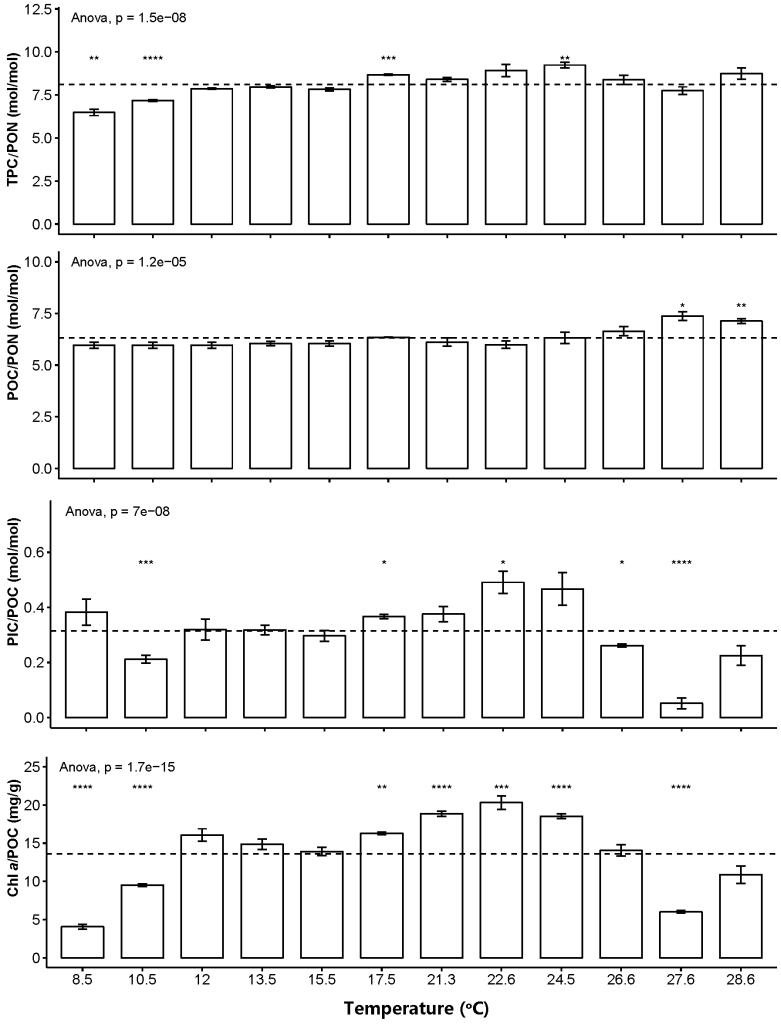
Fig. 1



https://doi.org/10.5194/bg-2019-179
Preprint. Discussion started: 27 May 2019

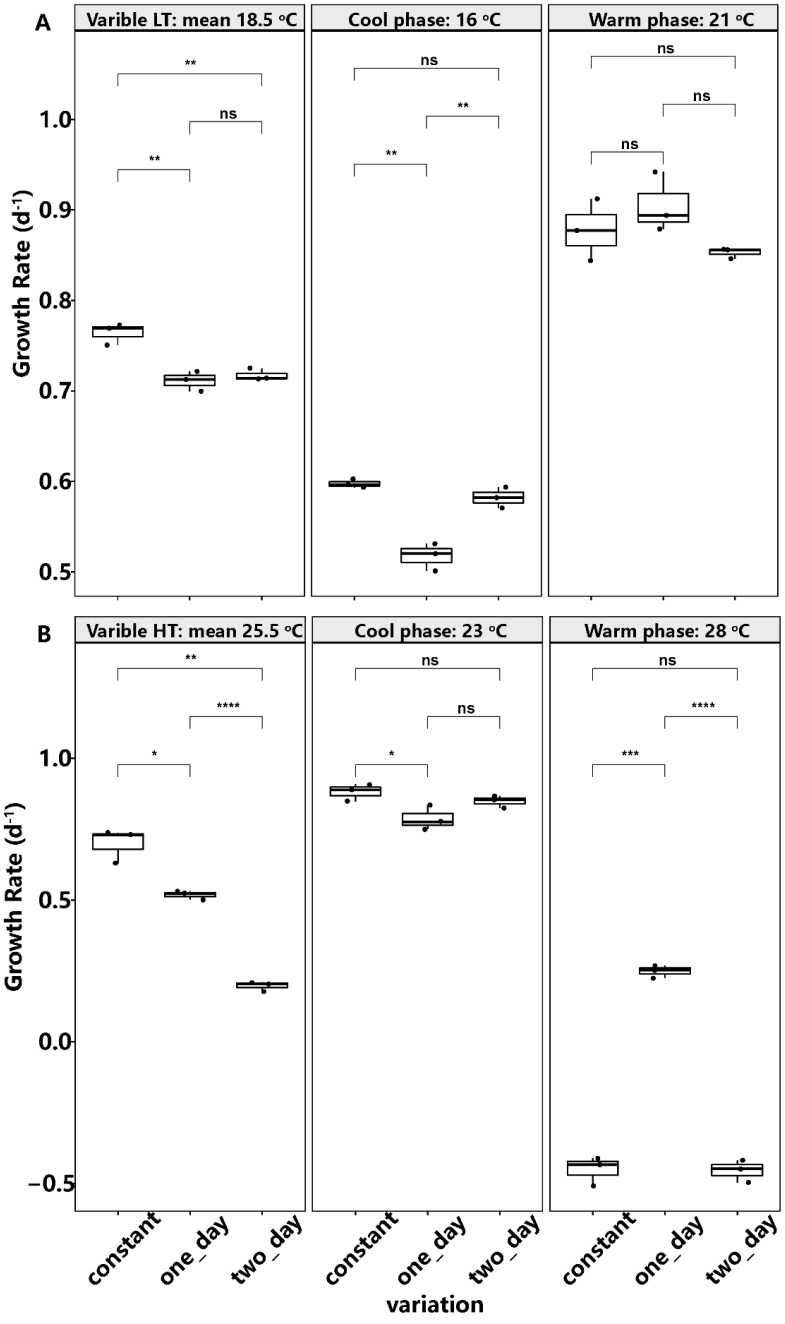
Biogeosciences Discussions
Open Access

Fig. 2



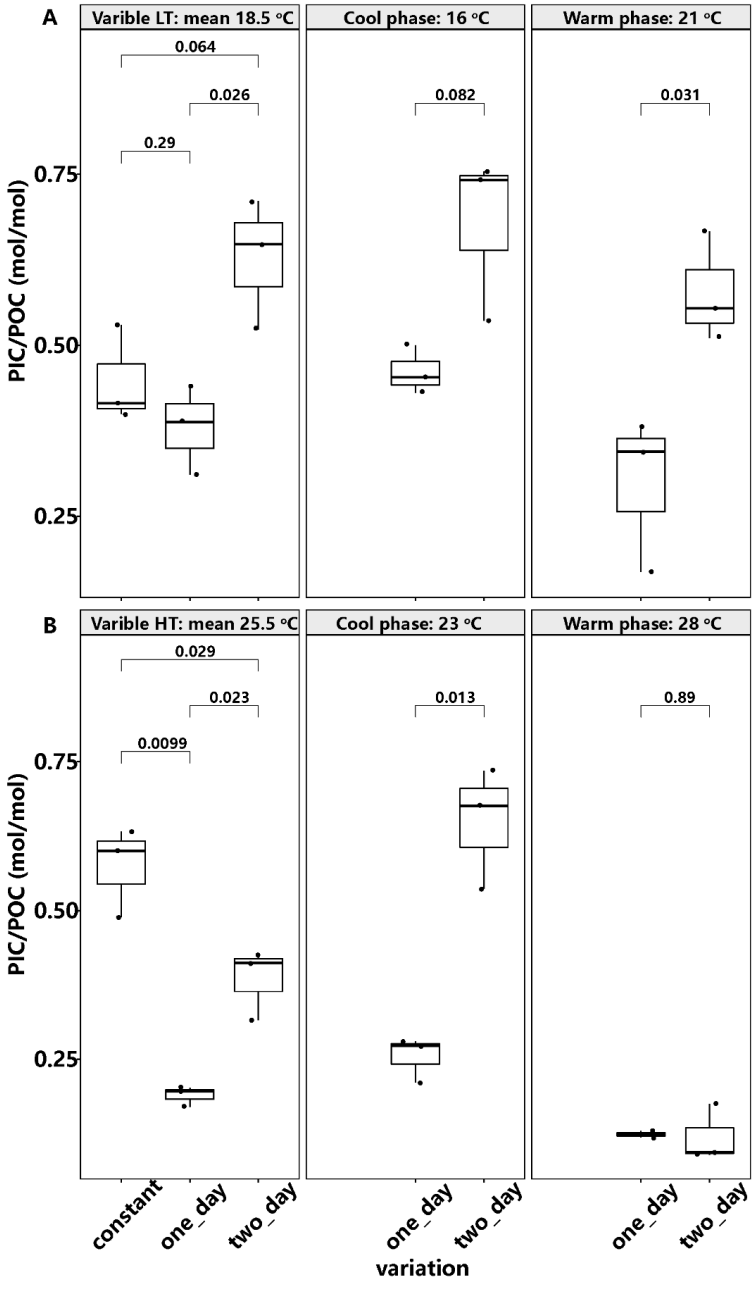
https://doi.org/10.5194/bg-2019-179
Preprint. Discussion started: 27 May 2019

Fig. 3



https://doi.org/10.5194/bg-2019-179
Preprint. Discussion started: 27 May 2019

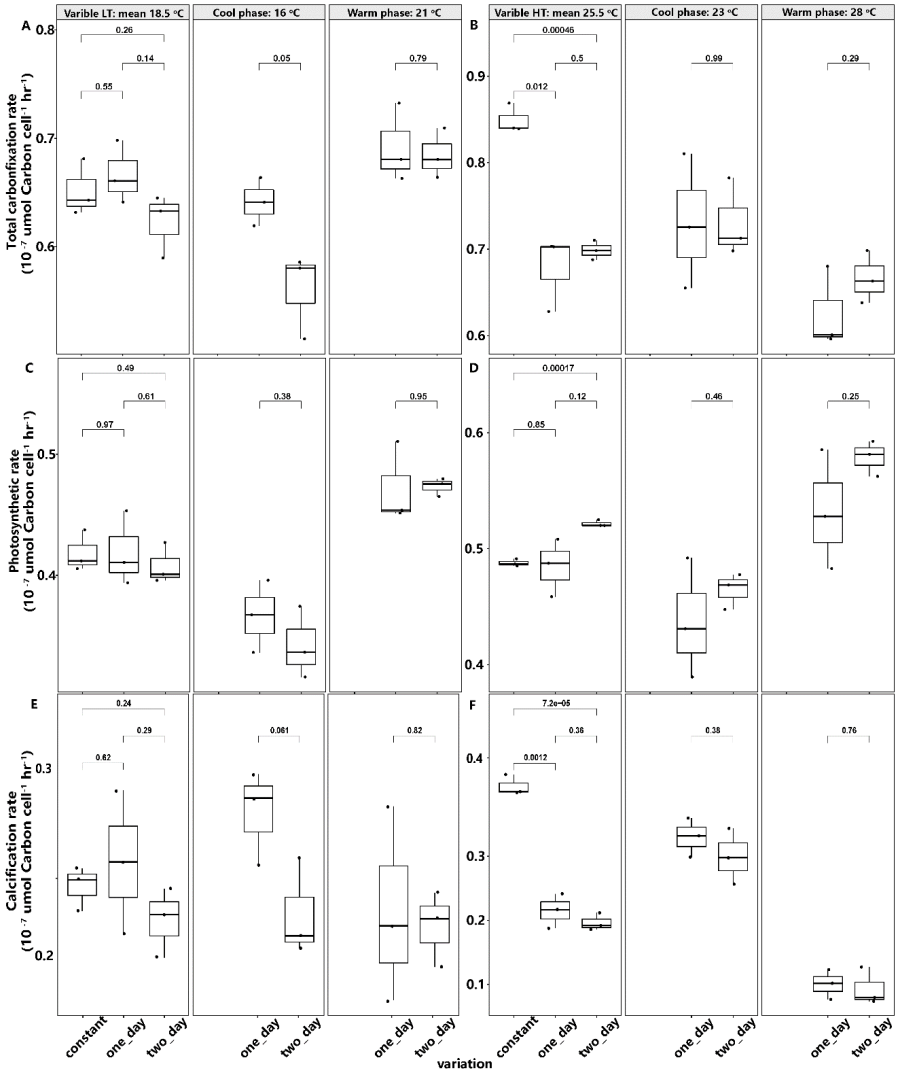
Fig. 4



https://doi.org/10.5194/bg-2019-179
Preprint. Discussion started: 27 May 2019

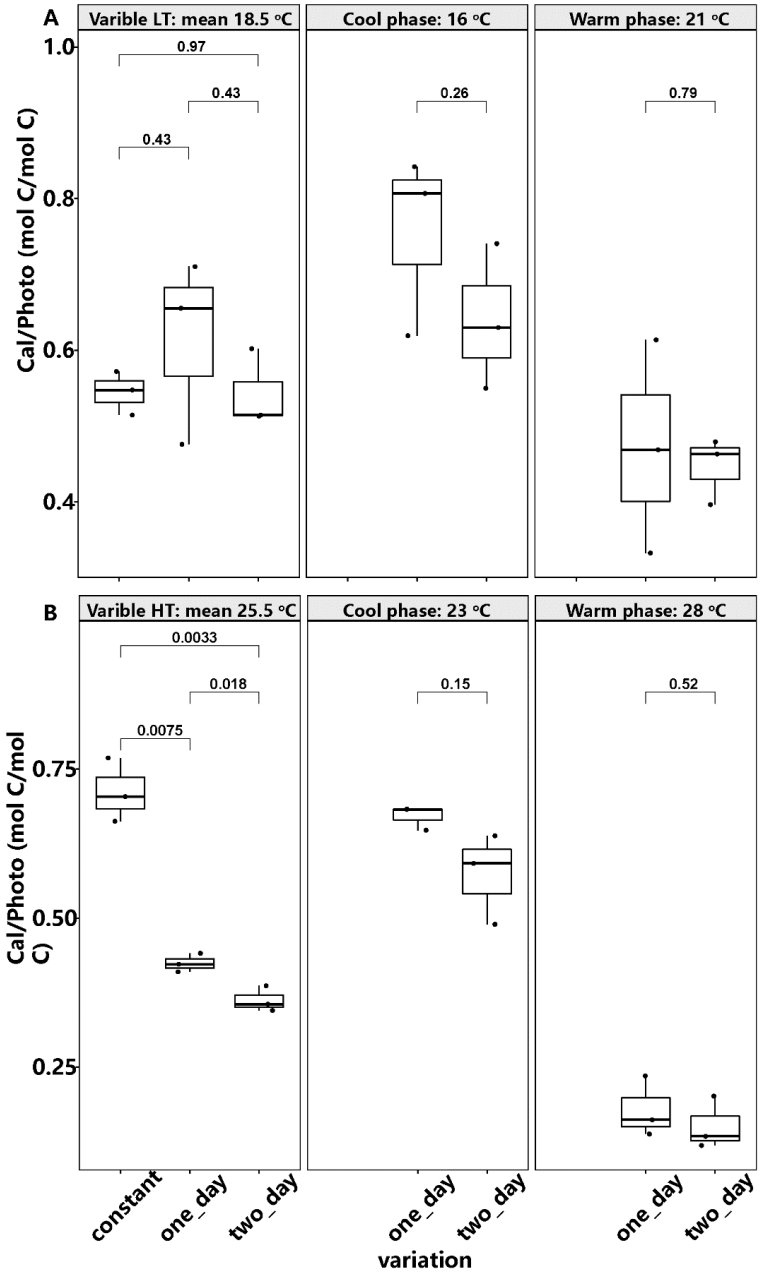
Biogeosciences Discussions
Open Access
EGU

Fig. 5



https://doi.org/10.5194/bg-2019-179
Preprint. Discussion started: 27 May 2019

963 **Fig. 6**



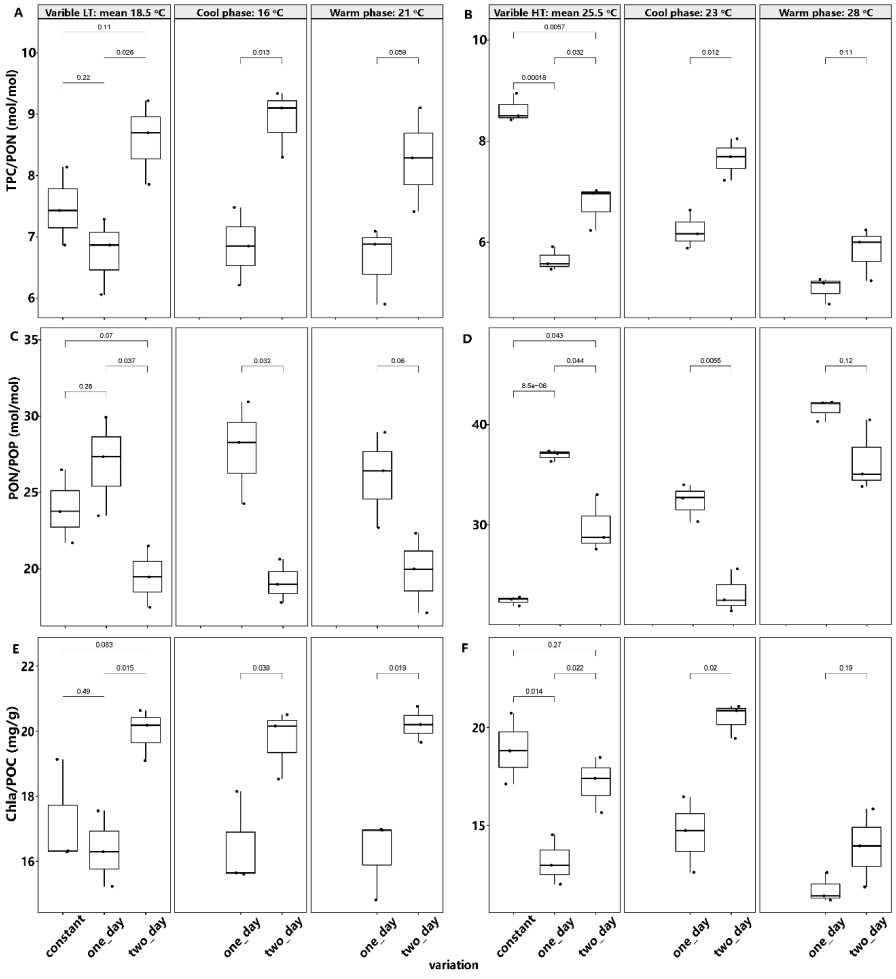
964

965

https://doi.org/10.5194/bg-2019-179
Preprint. Discussion started: 27 May 2019

Biogeosciences
Discussions
Open Access
EGU

Fig. 7



https://doi.org/10.5194/bg-2019-179
Preprint. Discussion started: 27 May 2019

Fig. 8