



Environmental controls on the elemental composition of a Southern Hemisphere strain of the coccolithophore *Emiliana huxleyi*

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Abstract. A series of semi-continuous incubation experiments were conducted with the coccolithophore *Emiliana huxleyi* strain NIWA1108 (Southern Ocean isolate) to examine the effects of five environmental drivers (nitrate concentration, phosphate concentration, irradiance, temperature and
20 $p\text{CO}_2$) on both the physiological rates and elemental composition. Here, we report the alteration of the elemental composition of *E. huxleyi* in response to the changes in these environmental drivers. A series of dose response curves for the cellular elemental composition of *E. huxleyi* were fitted for each of the five drivers across an environmentally-representative gradient. The importance of each driver in regulating the elemental composition of *E. huxleyi* was ranked using a semi-quantitative approach. The
25 percentage variation in elemental composition arising from the change in each driver between present day and model-projected conditions for the year 2100 were calculated. Temperature was the most important driver controlling both cellular particulate organic and inorganic carbon content, whereas nutrient concentrations were the most important regulator of cellular particulate nitrogen and phosphorus of *E. huxleyi*. In contrast, elevated $p\text{CO}_2$ had the greatest influence on cellular particulate
30 inorganic carbon to organic carbon ratio, resulting in a decrease in the ratio. Our results indicate that the



different environmental drivers each play specific roles in regulating the cellular elemental composition of *E. huxleyi* with wide-reaching implications for coccolithophore biogeochemistry, as a consequence of the regulation of *E. huxleyi* physiological processes.

1 Introduction

5 Climate change is causing a wide range of alterations to the marine environment including ocean acidification (OA), rising sea surface temperature (SST) and intensified stratification due to increased density gradients between surface and subsurface waters, with associated shifts in mean irradiance levels and nutrient availability in the upper water column (Boyd and Doney, 2002; Rost and Riebesell, 2004; Stocker, 2013). All these global changes in environmental variables will affect the physiology and
10 ecology of phytoplankton, both individually and interactively, in a complex way (Boyd and Hutchins, 2012; Boyd et al., 2010; Boyd et al., 2015; Feng et al., 2017).

Phytoplankton elemental composition is an important cellular property that reflects the metabolic rates of phytoplankton (Raven and Geider, 1988). Elemental composition is strongly influenced by environmental conditions and by phytoplankton adaptations to these conditions (Sterner and Elser,
15 2002) which in turn influences marine foodweb structure, particulate carbon export to the deep ocean, and ultimately marine biogeochemistry (Finkel et al., 2010 and references therein). The widely-recognized average molar elemental ratio of C:N:P is 106:16:1 for marine phytoplankton assemblages - the Redfield ratio (Redfield et al., 1963). However, individual phytoplankton species may have elemental ratios deviating, on short timescales (days to months) from Redfield depending on the
20 environmental conditions they encounter. Such deviations subsequently influence the accumulation of these elements in the upper food web and also marine biogeochemistry (Finkel et al., 2010; Ho et al., 2003; Sardans et al., 2012).

Different environmental drivers may play a range of roles in regulating the stoichiometry of marine phytoplankton. Nutrient availability (Hecky et al., 1993; Perry, 1976) has been proven to affect
25 phytoplankton stoichiometry directly. Irradiance provides the energy source for nutrient assimilation in the cells (Goldman, 1986). In addition, temperature changes, which mainly alter metabolic rates, can also influence the diffusive uptake of nutrients into cells (Raven and Geider, 1988; Roleda et al., 2013).



Increased levels of dissolved CO₂ during cell growth may result in higher cellular C:N and C:P ratios, due to increased CO₂ availability as a substrate for photosynthesis (Beardall et al., 2009; Feng et al., 2008; Fu et al., 2007; Fu et al., 2008). However, the dependency of C:N or C:P ratios on CO₂ availability can be species-specific (Burkhardt and Riebesell, 1997; Burkhardt et al., 1999). The effect
5 of rising *p*CO₂ on the N:P ratio is still unclear, due to large variations observed in previous environmental manipulation studies (Sardans et al., 2012). For example, the N:P ratio of *Synechococcus* increased with elevated CO₂ concentration, but remained unchanged for *Prochlorococcus* (Fu et al., 2007) and *Emiliana huxleyi* (Feng et al., 2008).

Marine coccolithophores are responsible for almost half of the global marine calcium carbonate
10 production, and are important in the marine carbon cycle through both the organic carbon pump and the inorganic carbon counter pump (Rost and Riebesell, 2004). *Emiliana huxleyi* is the most widely distributed coccolithophore species (Balch et al., 1991; Holligan et al., 1993; Holligan et al., 1983), and has been selected as a model phytoplankton species in the context of the marine carbon cycle (Westbroek et al., 1993). A wide range of environmental drivers, such as CO₂ concentration, nutrient
15 concentration, irradiance and temperature influence the growth, photosynthesis and calcification of *E. huxleyi* both individually and interactively (Feng et al., 2017; Raven and Crawford, 2012; Zondervan, 2007). Changes in these physiological processes may in turn alter the elemental stoichiometry and composition of coccolithophores. Knowledge of how different environmental drivers will affect the elemental composition of *E. huxleyi* is important for a more complete understanding of the
20 physiological responses of this species to the changing environment and the consequent effects on biogeochemical cycles. In addition, the magnitude of change of each environmental driver will be different with the future climate change, depending on location and scenario; hence a systematic study across a gradient of each driver is required.

This study advances previous findings by relating the change in elemental composition of *E. huxleyi*
25 cells, in response to environmental forcing, to the physiological rate responses presented in the study of Feng et al. (2017). The major objective of the present study is to investigate and rank the importance of the environmental drivers, nitrate and phosphate concentrations, irradiance, temperature and *p*CO₂, on setting the elemental composition of a Southern Ocean strain of *E. huxleyi*. The combined results of



this study and Feng et al. (2017) provide new insights into how changing environmental drivers changes will impact the marine biogeochemical cycles related to *E. huxleyi*.

2 Materials and methods

2.1 Experimental setup

5 The marine coccolithophore *Emiliania huxleyi* (morphotype A, strain NIWA1108) was isolated from the Chatham Rise, east of New Zealand by Dr. H. Chang in 2009, as detailed in Feng et al. (2017). The stock culture was maintained in the laboratory at 14°C and an irradiance of $\sim 140 \mu\text{mol m}^{-2} \text{s}^{-1}$, under a light/dark cycle of 12 h/12 h. The medium used for maintaining the stock culture was seawater obtained from Otago Harbour, New Zealand (nutrient concentrations: phosphate 0.3-0.6 μM , nitrate 3-6 μM),
10 filtered using 0.2 μm pore size filtration cartridge (WhatmanTM) and supplemented with nutrient stock solutions to give final concentrations of nitrate 96 μM and phosphate 6 μM , without silicate addition. Trace metal and vitamin stock solutions were added according to the f/20 recipe for both stock culture and the manipulation experiments (10 times dilution of f/2 level; Guillard and Ryther, 1962).

For the manipulation experiments, *E. huxleyi* cells, in exponential growth phase, were transferred into
15 acid-cleaned 500-mL polycarbonate bottles with screw caps and subjected to a series of semi-continuous incubation experiments under different nutrient, irradiance, temperature and $p\text{CO}_2$ conditions (Feng et al., 2017). Only one environmental driver was manipulated at a time for each incubation experiment, with the other environmental drivers remaining the same as the stock culture conditions. The manipulation of each of the different drivers was carefully selected to cover a broad
20 range of conditions observed in the natural environment and those commonly employed for laboratory incubations (Feng et al., 2017). Initial cell abundances were $\sim 10^4 \text{ cell mL}^{-1}$ and *in vivo* chlorophyll *a* (Chl-*a*) fluorescence readings were monitored daily as indicators of Chl-*a* biomass and cell growth. After 2-3 days of acclimation under the experimental conditions daily dilution was started by adding freshly made seawater medium into each incubation bottle to adjust the biomass to the level of the
25 previous day. All the incubation experiments were carried out in walk-in growth chambers (Model 650, Contherm, New Zealand), with metal halide lamps (full spectrum) as the light source, under a light/dark



cycle of 12 h/12 h. Irradiance levels inside the incubation bottles were measured using a quantum PAR sensor (2100 series, Biospherical Instruments Inc., USA). The temperature in each incubation experiment was monitored continuously using a HOBO Pendant[®] temperature/light data logger (Onset, Australia), with variation no more than $\pm 0.5^{\circ}\text{C}$.

- 5 The experimental conditions for each environmental driver used for the manipulation experiments are presented in Table 1, and described in Feng et al. (2017). All the treatments were conducted in triplicate. For each culture, the final sampling was performed after the daily monitored growth rate remained relatively constant (daily variations $< 10\%$) for more than seven generations (Feng et al., 2008). This yielded total acclimation of the cultures to the experimental conditions for ~ 20 days. Samples were
- 10 collected for cell counts, Chl-*a* biomass and elemental components, including particulate organic carbon (POC), particulate inorganic carbon (PIC), particulate organic nitrogen (PON) and particulate organic phosphorus (POP).

2.2 Sample analyses

2.2.1 Cell counts and Chl-*a*

- 15 One mL subsamples for cell counts were taken from each incubation bottle, preserved by adding 6 μL modified Lugol's solution, prepared by dissolving 10 g KI and 5 g iodine crystals in 20 mL Milli-Q water, then adding 50 mL of 5% anhydrous sodium acetate solution, and stored in dark at 4°C . Cell abundance was determined with a nanoplankton counting chamber (PhycoTech, USA) using a Zeiss microscope (Axiostar plus, Germany). *In vitro* Chl-*a* concentration was analysed using a Turner 10-AU
- 20 fluorometer (Turner design, USA) with 90% acetone extracted samples, as detailed in Welschmeyer (1994).

2.2.2 Elemental composition

- Subsamples from each incubation bottle for PON, total particulate carbon (TPC) and POC measurement were filtered onto pre-combusted GF/F glass fibre filters (WhatmanTM), and analysed using an
- 25 elemental combustion system (Elementar vario EL III, Germany). Filters for POC analysis, were fumed with saturated HCl for 3 hours in order to remove all the inorganic carbon (Zondervan et al., 2002). The



PIC content was calculated by subtracting POC from TPC values. Subsamples for particulate organic phosphate (POP) measurements were filtered onto pre-combusted GF/F filters (WhatmanTM), and analysed following the molybdate colorimetric method of Solórzano and Sharp (1980). The particulate inorganic nitrogen (PIN) and particulate inorganic phosphorus (PIP) contents were both neglected for
5 the calculation, due to the relative low values for laboratory cultures.

2.2.3 Data analyses

The effects of different environmental drivers on the elemental composition of *E. huxleyi* and their stoichiometric ratios were identified with one-way analysis of variance (ANOVA) using the commercial statistical software package SigmaStat (Version 3.5; Jandel Scientific, San Rafael, CA, US). Differences
10 between treatments were considered significant when $p < 0.05$. Post-hoc comparisons using the Student-Neuman-Keuls (SNK) test were conducted to determine any differences between particular treatments.

For the environmental drivers that had significant effects on the elemental composition and ratios of *E. huxleyi* within the examined range, the response curves to the drivers were fitted using the models listed in Table S1. All of the curve-fitting was performed using least square fit with Prism software (version
15 5.0; GraphPad Prism Software, US) with all the triplicate data for each of the experimental treatments.

The same approach as used in Feng et al. (2017) was performed to rank the relative importance of each environmental driver, that was found to have significant effects using the one-way ANOVA analyses, on the elemental composition of *E. huxleyi*. Firstly, the two values for the elemental composition at the average present-day conditions and the projected conditions for the year 2100 were derived from each
20 fitted dose-response curve for the environmental driver that had significant effects. The environmental conditions were projected using Coupled Model Intercomparison Project phase 5 (CMIP5) models (Boyd and Law, 2011; Law et al., 2016; Rickard et al., 2016), which suggested 33% decreases in both nitrate and phosphate concentrations, 2°C warming, a 25% increase in irradiance and an increase in $p\text{CO}_2$ from 39 to 75 Pa in the Chatham Rise area for the year 2100 compared to present day conditions
25 (Feng et al., 2017). The magnitude and direction of percentage change in the elemental composition under projected future conditions relative to present day condition for each environmental driver was then calculated. The ranking was finally determined by comparing the absolute values of the calculated



percentage changes of the physiological metrics caused by each driver. The driver that caused the largest percentage change was selected as the most important controlling driver.

3 Results

3.1 Changes in cellular POC content in response to environmental drivers

5 Cellular POC content was significantly affected by alteration of irradiance, temperature and $p\text{CO}_2$ (Fig. 1). Increasing irradiance from 14 to 80 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ increased the cellular POC content by around two-fold from 8.20 ± 2.39 to $14.07 \pm 1.17 \text{ pg cell}^{-1}$ ($p < 0.05$). POC content then decreased at the two highest irradiance levels (350 and 650 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$, Fig. 1c). A trend of decreased *E. huxleyi* cellular POC content with elevated temperature was evident from the temperature manipulation
10 experiment (Fig. 1d). The cellular POC content ($28.85 \pm 6.98 \text{ pg cell}^{-1}$) was significantly higher than all the other treatments ($p < 0.05$) at the lowest temperature of 4°C and significantly reduced by ~ 70% at both 20°C and 25°C ($p < 0.05$). Raising $p\text{CO}_2$ from 8 to 15 Pa significantly increased the cellular POC content from 9.63 ± 1.67 to $12.93 \pm 1.84 \text{ pg cell}^{-1}$ (Fig. 1e), with cellular POC content being relatively uniform from 15 to 109 Pa.

15 3.2 Alteration of cellular PIC content in response to environmental drivers

Temperature was the only driver that significantly altered the cellular PIC content (Fig. 2). There was a general trend of decreased cellular PIC content of *E. huxleyi* with warming from 11°C to 20°C (Fig. 2d). The cellular PIC content was significantly lower at 20°C and 25°C compared to the other four temperature treatments ($p < 0.05$). More than a 50% decrease in cellular PIC content was observed at the
20 two highest temperature conditions, relative to the 7°C treatment. However, there were no significant differences in cellular PIC content between the other temperature treatments. The fitted Q_c value (the plateau for one phase decay) was $6.94 \pm 0.93 \text{ pg cell}^{-1}$, close to the average value at the two highest temperatures (Table S1).



3.3 Changes in the cellular PIC:POC ratio in response to environmental drivers

As for cellular POC, the cellular ratio of PIC:POC was mainly affected by changes in irradiance, temperature and $p\text{CO}_2$ (Fig. 3). The highest cellular PIC:POC ratio of 1.20 ± 0.09 was observed at the lowest irradiance ($19 \mu\text{mol photons m}^{-2} \text{s}^{-1}$; $p < 0.05$, compared to all other irradiance treatments). The ratio then decreased with increasing irradiance to 0.72 ± 0.10 at $190 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ and slightly increased again at the two highest irradiances ($p < 0.05$ between 190 and $650 \mu\text{mol photons m}^{-2} \text{s}^{-1}$, Fig. 3c). In the temperature manipulation experiment, the PIC:POC ratio was significantly lower ($p < 0.05$) at the lowest temperature (4°C) than any other treatment, with a value of $0.45 \pm 0.03 \text{ pg cell}^{-1}$. The PIC:POC value then leveled off between the range of 7°C to 25°C , with the average value more than double that at 4°C (Fig. 3d). With the variation of $p\text{CO}_2$ levels, the cellular PIC:POC ratio decreased by more than 40% from $1.46 \pm 0.02 \text{ pg cell}^{-1}$ at 8 Pa to 0.90 ± 0.15 at 39 Pa and stayed similar between the range of 39 and 109 Pa ($p < 0.05$) (Fig. 3e), mainly due to the increased cellular POC quota with rising $p\text{CO}_2$.

3.4 Alteration of cellular PON content in response to environmental drivers

The cellular PON content increased with increasing nitrate concentration. The content at the two lowest nitrate concentrations of 3.7 and $6.0 \mu\text{M}$ was less than half of the average value ($2.06 \pm 0.36 \text{ pg cell}^{-1}$) of the three highest nitrate treatments (Fig. 4a). Warming from 4°C to 25°C decreased the cellular PON content ($p < 0.05$). The value of $4.07 \pm 0.00 \text{ pg cell}^{-1}$ at 4°C was double that at 15°C ($1.93 \pm 0.10 \text{ pg cell}^{-1}$), and three-fold greater than the PON content of $1.31 \pm 0.24 \text{ pg cell}^{-1}$ at 25°C (Fig. 4d).

3.5 Changes in cellular POP content in response to environmental drivers

The cellular POP content of *E. huxleyi* was significantly altered by nitrate, phosphate, temperature, and $p\text{CO}_2$. POP content was slightly less at the three low nitrate concentrations (3.7, 6.0 and $12 \mu\text{M}$), compared to those at 96 and $200 \mu\text{M}$ ($p < 0.05$; Fig. 5a). Cellular POP content significantly increased with rising phosphate concentration (Fig. 5b), with the highest POP content observed at $20 \mu\text{M}$ phosphate. As observed for cellular POC and cellular PON contents, warming greatly decreased the cellular POP content (Fig. 5d), with a reduction of 65% from $1.08 \pm 0.14 \text{ pg cell}^{-1}$ at 4°C to 0.38 ± 0.04



pg cell⁻¹ at 11°C, but then only a further decrease of ~0.1 pg cell⁻¹ from 15°C to 25°C. Significant differences in POP content were detected between the two lowest temperature treatments compared to all others. Conversely, with rising *p*CO₂ level there was a trend of increased cellular POP content (Fig. 5e), which almost doubled from 0.20 ± 0.04 pg cell⁻¹ at 8 Pa to 0.38 ± 0.02 pg cell⁻¹ at 109 Pa (*p*<0.05).

5 3.6 Alteration of cellular C to Chl-*a* ratio in response to environmental drivers

Alteration of all the five environmental drivers greatly affected the cellular ratio of POC to Chl-*a* content (C:Chl-*a*, g:g) (*p*<0.05) (Fig. 6). C:Chl-*a* decreased exponentially with increased nitrate concentration up to 50 μM, but stabilised between 50 and 200 μM (Fig. 6a). The highest ratio of 422.36 ± 74.28 was observed at the lowest nitrate concentration of 3.7 μM, significantly higher than all other treatments (*p*<0.05). The ratio then decreased by 87% at 200 μM. An increase in phosphate concentration, however, only slightly decreased the C:Chl-*a* ratio (Fig. 6b). Compared to the ratios at the two lowest concentrations, a significant decrease (*p*<0.05) at 6.0 μM and 20 μM was observed (by ~20% each). Increased irradiance increased the C:Chl-*a* ratio linearly, with more than a doubling at 650 μmol photons m⁻² s⁻¹ compared to the ratio of 47.45 ± 12.58 at 14 μmol photons m⁻² s⁻¹ (Fig. 6c). The C:Chl-*a* ratio dramatically decreased with warming, with a decline of 64% from 4°C to 25°C (Fig. 6d). The ratio of 131.26 ± 42.96 observed at 4°C was significantly higher than all the other temperatures (*p*<0.05). Significantly lower C:Chl-*a* ratios were observed at the two lowest *p*CO₂ levels of 8 and 15 Pa compared with the other treatments (*p*<0.05, Fig. 6e), with the ratio increasing by 42% from low to high *p*CO₂.

20 3.7 Shifts in cellular elemental molar ratios in response to environmental drivers

The PON to POP (N:P) ratio was significantly lower (*p*<0.05) at the two lowest nitrate treatments compared to the others (bold values in Table 2). In contrast, the POC to PON (C:N) ratio was significantly higher at the two lowest nitrate concentrations. There was no significant difference in C:N ratios across the other four nitrate treatments (*p*>0.05). Changes in nitrate concentration did not significantly affect the POC to POP (C:P) ratio.



The N:P ratio of *E. huxleyi* increased at low phosphate concentrations (0.4 and 2 μM), with highest value in the 0.4 μM phosphate treatment ($p < 0.05$). There was a significant increase in the C:P ratio ($p < 0.05$) at the two lowest phosphate concentrations compared to the others. The highest C:P ratio, recorded at the lowest phosphate concentration (0.4 μM), was almost double the value at 2 μM , and 5 more than three times the average ratio of the other treatments (Table 2). In contrast, there were no significant differences in the calculated C:N ratio across the phosphate treatments ($p > 0.05$).

Decreased C:N ratios were observed for low irradiances; the value at 14 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ being significantly lower than the three highest irradiances ($p < 0.05$). Similarly, a decreased C:P ratio was found at low irradiance, with a significantly lower value at 14 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ compared to the 10 three highest irradiances. Warming significantly increased the N:P ratio from 4°C to 20°C (Table 2).

3.8 Ranking the importance of environmental drivers in altering *Emiliania huxleyi* elemental composition

Ranking the response of the Southern Ocean *E. huxleyi* isolated to projected future changes in oceanic properties revealed differential responses between drivers and processes (Table 3, Fig. 7). Cellular POC and cellular PIC:POC ratio were both significantly influenced by CO₂ and temperature, with temperature affecting cellular POC content the most, while CO₂ was the most important factor regulating PIC:POC. The cellular PON content was significantly affected by future nitrate concentration and temperature, with nitrate ranking the most important. However, only one driver (temperature) significantly regulated cellular PIC, with a 4°C warming causing a 14.2 % decrease. Four (phosphate, 20 temperature, CO₂ and nitrate) out the five environmental drivers, under end of the century conditions, significantly affected cellular POP content: with future phosphate concentration playing the most important role. The rankings associated with statistically non-significant differences among the treatment intervals, as marked in Table 3 and Fig. 7, need to be considered with caution (see Feng et al. (2017)).



4 Discussion

This is the first detailed study of the individual effect of five environmental drivers (nitrate concentration, phosphate concentration, irradiance, temperature and $p\text{CO}_2$) on the cellular elemental composition of the coccolithophore *E. huxleyi*. Moreover, it is the first to rank the importance of the predicted changes in these environmental drivers on *E. huxleyi* elemental stoichiometry for the year 2100 relative to the present-day conditions. Relating changes in elemental composition is an important addition to the responses of growth, photosynthesis and calcification rates (Feng et al., 2017), providing insights into the biogeochemical consequences of the physiological effects induced by change in the five essential environmental drivers.

4.1 Effects of nutrient concentration on the elemental stoichiometry of *Emiliania huxleyi*

The PON and POP cell quotas of *E. huxleyi* in the present study were mainly controlled by nitrate and phosphate concentrations, respectively, as phytoplankton relies on seawater nutrient availability as the external elemental source (Hecky et al., 1993; Price, 2005; Sakshaug and Holmhansen, 1977). Nitrate concentration plays an important role in regulating the growth, photosynthetic and calcification rates of *E. huxleyi* (Feng et al., 2017); however, the three lowest nitrate concentrations only resulted in slightly decreased cellular POP contents and had no significant effect on cellular POC or PIC content. This indicates that the regulation of the nitrate concentration on the POC and PIC productivity in our study was mainly a consequence of decreased growth rate of the cells under nitrate limitation, as shown by Feng et al. (2017). This finding is in contrast to Paasche (1998) who observed higher *E. huxleyi* cellular PIC:POC ratios under nitrate limitation as a result of decreased cellular POC and increased coccolith abundance per cell in *E. huxleyi* strain BOF 92 isolated from the North Atlantic. Higher PIC:POC ratios under nitrate limitation was alternatively attributed to increased calcite mass per lith of *E. huxleyi* strain CCMP 378 isolated from the Gulf of Maine (Fritz, 1999). In addition, phosphate concentration did not significantly affect *E. huxleyi* cellular carbon content nor the PIC:POC ratio of cells in the present study. However, Paasche (1998) observed greatly increased PIC content of *E. huxleyi* (strain BOF 92) under phosphate limiting conditions, and Riegman et al. (2000) observed that a greater increase PIC quotas under phosphate limitation than nitrate limitation for *E. huxleyi* (strain L).



These discrepancies between studies in the nitrate or phosphate effects on cellular PIC:POC ratio are mainly due to the different nutrient concentrations in the culturing media. Paasche (1998) observed an increase in *E. huxleyi* PIC cell quota under the stationary phase of batch incubation, i.e. when cell division ceased as nitrate dropped to $\leq 0.2 \mu\text{M}$ and phosphate dropped to $\leq 0.03 \mu\text{M}$. This supports the findings of both Riegman et al. (2000) and Fritz (1999) who conducted continuous incubations with high cell densities of *E. huxleyi*. These studies observed an increased cellular PIC content when phosphate concentration fell below 0.4 nM (Riegman et al., 2000) or nitrate concentration was below the detection limit (Fritz, 1999). However, the present study used a semi-continuous incubation method with higher and relatively steady nutrient concentrations (with lowest nitrate and phosphate concentrations of 3.6 and $0.4 \mu\text{M}$, respectively) and the cells were grown and sampled at a healthy exponential growth phase. Similarly, Müller et al. (2008) only found higher *E. huxleyi* (strain CCMP371) cellular calcite content during the stationary but not the exponential growth phase under both nitrate and phosphate limitation, due to the different cell cycle phases during which the calcification and cell division occurred. The authors explained that calcification continued during the G1 phase of cell assimilation when cell division was restricted under nutrient limitation, and thus the cellular PIC content was increased (Müller et al., 2008). Further studies at extremely low nutrient concentrations ($< 0.1 \mu\text{M}$) in a steady-state growth phase are still needed to understand the potential connection between carbon production and extreme nutrient limitation, given reports of areal expansion of oligotrophic waters in the world oceans with global climate change (Polovina et al., 2008).

20 **4.2 Irradiance effects on the elemental stoichiometry of *Emiliana huxleyi***

In the present study, irradiance was the main environmental factor affecting cellular POC content which in turn altered the PIC:POC ratio. The increased PIC:POC cellular ratio at low irradiance indicates that calcification is less dependent on irradiance than organic carbon fixation, as discussed in Feng et al. (2017). Although both processes require light as an energy source, calcification requires less energy (Anning et al. 1996) than photosynthesis (Paasche, 1965; Balch et al., 1992). Therefore, the calcification rate is generally saturated at lower irradiance levels than photosynthesis (Paasche, 1964; Zondervan, 2007). Feng et al. (2017) reported greatly reduced photosynthetic rates under the two lowest irradiance



levels, while observing that this trend was less significant for the calcification rate. Hence, limiting irradiance will lead to less POC content in the cells compared to the cellular PIC quota, and thus a higher cellular PIC:POC ratio would be expected at low irradiance when growth and photosynthesis are light-limited (Raven and Crawford, 2012), as confirmed by the response of calcification:photosynthesis
5 in Feng et al. (2017).

Increasing irradiance also elevated the C:Chl-*a* ratio linearly in the present study, due to the increase in POC quota and a decrease in Chl-*a* quota, as also reported for diatoms and dinoflagellates (Geider, 1987). The reduced cellular pigment quota under high irradiance helps to reduce the energy required for light harvesting in phytoplankton cells, which is a strategy to balance the energy demands for growth
10 and POC production with photon harvesting (Kiefer, 1993). In addition, the present study revealed that the C:N and C:P ratios of *E. huxleyi* both increased at high light levels, as a consequence of increased cellular POC content driven by increased irradiance but no significant change in cellular PON or POP quota, further suggesting that organic carbon content is more light dependent than the accumulation of cellular N or P (Geider et al., 1998).

15 **4.3 Temperature effects on the elemental stoichiometry of *Emiliania huxleyi***

Temperature is important in regulating dissolved chemical diffusion and transport, non-enzymatic and enzymatic reactions, and the metabolic rates of phytoplankton (Raven and Geider, 1988). In our accompanying study, the growth, photosynthetic and calcification rates all increased with rising temperature until the optimal temperature was reached (Feng et al., 2007). Conversely, the cellular
20 POC, PON and POP content all reduced significantly as temperature increased in the present study. It has been proposed that reduced cell size is a universal strategy in response to increasing temperature for both terrestrial and aquatic organisms (Gardner et al., 2011), following a hypothesis suggested by Atkinson et al. (2003). A study on the coccolithophores *E. huxleyi* (strain EH2) and *Gephyrocapsa oceanica* (strain GO1) observed decreased cell size and thinner coccospheres upon raising temperature
25 from 10°C to 25°C, which was attributed to the relatively suppressed cell division at low temperature (Sorrosa et al., 2005). This decrease in cell volume could be the main cause of reduced cellular elemental components in the present study. Previous studies also reported that warming resulted in



reduced cell volume of *E. huxleyi* (strain AC481, De Bodt et al., 2010; strain L, Van Rijssel and Gieskes, 2002), and decreased cellular POC and PIC quotas of coccolithophore *Coccoliths pelagicus* when the temperature was raised from 10°C to 15°C (Gerecht et al., 2014). Similarly, warming significantly decreased the cellular elemental contents to their lowest levels measured in the present study over the range from 4°C to 25°C, with a decrease in cell size at higher temperatures (data not shown), as growth rate increased (Feng et al, 2017).

However, contrary to the observed changes in POC, PON and POP cell quota, the cellular PIC content of *E. huxleyi* only decreased when temperature was higher than 10°C in the present study, due to the strongly reduced calcification and malformation at low temperatures of 4°C and 7°C (Feng et al., 2017). The reduced cell division rate (i.e. enlarged cell volume) offset the reduced calcification rate at lower temperatures, and so there was no significant difference in PIC cell quota at temperatures below 10°C. Consequently, the cellular PIC:POC ratio was lower at 4°C and 7°C, consistent with the trend observed for the calcification: photosynthesis ratio (Feng et al., 2017), indicating suppression of PIC formation relative to POC production at low temperature (Watabe and Wilbur, 1966). The PIC:POC ratio then decreased with warming from 11°C to 15°C and remained relatively steady afterwards, mainly due to the lower optimal temperature for calcification (20°C) compared to photosynthesis (24°C) as suggested in Feng et al. (2017).

Furthermore, warming from 4°C to 20°C significantly increased the *E. huxleyi* cellular N:P ratio in the present study, in agreement with the recent model study on a natural phytoplankton community (Toseland et al., 2013). Toseland et al. (2013) found that with increasing temperature the rate of cellular protein synthesis in phytoplankton was higher, but with a lower number of phosphorus-rich ribosomes, thereby increasing the cellular N:P ratio. In the present study, the cellular N:P ratio of *E. huxleyi* at 20°C was almost double that at 4°C, in spite of both cellular PON and cellular POP decreasing with warming. Although this study presents results for a single strain of *E. huxleyi*, if the temperature dependency of cellular resource allocation is a universal trend for all the *E. huxleyi* genotypes, we can speculate that the diverse *E. huxleyi* strains growing in different temperature regions might have different requirements for nitrogen vs. phosphorus, and that the growth of *E. huxleyi* strains in the temperate to tropical regions might be more readily limited by nitrate than sub-polar strains. Moreover, this trend of



temperature-dependent N:P ratio also indicates that future warming might accentuate nitrate limitation in the oceans (Toseland et al., 2013).

4.4 Effects of CO₂ on the elemental stoichiometry of *Emiliania huxleyi*

The photosynthesis of *E. huxleyi* was saturated at a higher $p\text{CO}_2$ than that for growth rate (Feng et al., 2017). In the present study, CO₂ plays the most important role in regulating the cellular PIC:POC ratio. The PIC:POC ratio was significantly higher at the lowest $p\text{CO}_2$ level, as a consequence of the lower cellular POC and higher cellular PIC at 8 Pa. In general, cell growth of *E. huxleyi* is less limited by low CO₂ concentrations than in other phytoplankton groups (Clark and Flynn, 2000; Paasche et al., 1996; Riebesell et al., 2000a). Moreover, recent studies suggest that *E. huxleyi* operates an active carbon concentrating mechanism (CCM) to utilize HCO₃⁻ through the enzyme carbonic anhydrase (CA; Reinfelder, 2011), and may have high affinity for CO₂ in photosynthesis (Stojkovic et al., 2013). However, the efficiency of CCMs in *E. huxleyi* (strain B92/11) is considered to be low as a consequence of the leakage of CO₂ from the cell (Rost et al., 2006), and so coccolithophore photosynthesis is more dependent than cell growth on CO₂ concentration (Rost and Riebesell, 2004). This discrepancy between growth and organic carbon fixation can lead to a decrease in cellular POC at low $p\text{CO}_2$. This difference in CO₂ requirements between the two processes may also have resulted in the lower cellular POP content at 8 Pa compared to other $p\text{CO}_2$ treatments.

The increasing trend observed for cellular POC and POP was not apparent for cellular PIC quota, as calcification rates significantly decreased with increasing $p\text{CO}_2$ level >40 Pa (Feng et al., 2017). Hence the cellular PIC:POC ratio was significantly higher at the two lowest $p\text{CO}_2$ levels, consistent with previous findings for CO₂ manipulations at saturating irradiances on *E. huxleyi* (strain PML B92/11A; Zondervan et al., 2002; Zondervan et al., 2001). No further significant change in cellular carbon content or PIC:POC ratio occurred at higher $p\text{CO}_2$, in contrast to the linear decrease in the calcification:photosynthesis ratio with rising $p\text{CO}_2$ (Feng et al., 2017). This difference is noteworthy for both cellular PIC:POC and calcification:photosynthesis ratios are commonly used to examine the relative change of PIC and POC production in coccolithophores (Raven and Crawford, 2012). These changes both have ecological implications for the marine rain ratio in the carbon cycle (Klaas and



Archer, 2002; Rost and Riebesell, 2004), which is the export ratio of calcite to organic carbon into the deep ocean. The ^{14}C -labelling technique used in this study to measure carbon fixation (photosynthesis and calcification) rates was conducted during the light period, thus the measured rate is an indicator of net carbon fixation, that does not account for the energy-consuming respiratory process or CO_2 leakage out of the cells (Bach et al., 2015, 2013; Rost et al., 2006). Conversely, the cellular carbon content indicates the gross accumulated carbon in the cells over longer period of growth (Engel et al., 2010; Fabry and Balch, 2010). The most compelling reason for the relatively higher PIC:POC ratio (~ 1.5) than calcification: photosynthesis ratio (~ 1.0) in the lowest $p\text{CO}_2$ treatment (8 Pa, 79 ppm) in our study may then be attributed to diffusive CO_2 loss limiting inorganic carbon active uptake from the substrate (Bach et al., 2013), resulting in less POC fixation into the cells relative to the PIC fixation by the calcification process.

The C:Chl-*a* ratio of *E. huxleyi* was lowest at $p\text{CO}_2$ of 8 Pa across all the $p\text{CO}_2$ treatments in the present study, mainly due to the decreased cellular POC at low $p\text{CO}_2$, rather than any change in cellular Chl-*a* content. However, increasing $p\text{CO}_2$ did not have significant effects on the C:N, N:P or C:P ratios in the present study. This is in accordance with a recent study on *E. huxleyi* (strain PML B92/11A), which also exhibited constant C:N:P ratios across a $p\text{CO}_2$ range of 18 to 75 Pa for cultures at steady growth phase under phosphate-limited continuous incubation (Engel et al., 2014).

4.5 Biogeochemical implications and future directions

The comparisons between present day conditions and those projected for year 2100 for the Chatham Rise area are summarized in the conceptual model in Fig. 7. These results indicate that the 2°C warming will decrease both POC and PIC cellular quotas of *E. huxleyi*, but may slightly increase the PIC:POC ratio. Rising $p\text{CO}_2$ alone will result in decreased cellular PIC:POC ratio. Although the 33% decrease in nitrate concentration is the major factor controlling the growth, photosynthetic and calcification rates (Feng et al., 2017), change in nitrate concentration did not significantly affect the elemental stoichiometry except for the cellular PON contents of *E. huxleyi*. In addition, increasing temperature may increase the cellular N:P ratio; while rising $p\text{CO}_2$ will decrease the N:P and C:P ratios. These results provide a more detailed perspective that can improve our knowledge on how the model



coccolithophore species, *E. huxleyi*, may respond to future environmental changes. For example, our results suggest that rising $p\text{CO}_2$ in the future oceanic environment will decrease the *E. huxleyi* cellular PIC:POC ratio by 5.4%; however, the projected warming and increase in irradiance level may offset this decreased PIC:POC by 2.4% and 0.3%, respectively. The changes in PIC:POC have implications for the marine “rain ratio” and so alter the marine carbon cycle (Rost and Riebesell, 2004). Similarly, the cellular N:P ratio will be decreased by rising $p\text{CO}_2$, although this trend may be canceled out by warming. The altered C:N:P stoichiometry will in turn affect the nutrient cycle at higher trophic levels (Jones and Flynn, 2005) and marine biogeochemical cycles (Beardall and Raven, 2004).

It is noteworthy that the research presented here only examined the physiological response norms of *E. huxleyi* to a single environmental driver when other drivers were all kept at the stock culture growth condition (i.e. a set of single dimensional space experiments). However, these responses (such as the shape of the curves and the optimal conditions) may be different when the other background conditions are changed. For example, Sett et al. (2014) observed the dose-response curves of calcification of *E. huxleyi* PML B9/11 to CO_2 concentration was regulated by temperature. Therefore, in order to comprehensively understand how *E. huxleyi* physiology will respond to multiple environmental drivers and fill this knowledge gap, future research on a full environmental matrix is still necessary. In addition, the present study is only based on a single strain of southern hemisphere *E. huxleyi*. Due to the wide distribution of this species in the natural marine environment, *E. huxleyi* presents high variability in terms of genetic, morphological and physiological characteristics (Cook et al., 2011; Read et al., 2013; Young et al., 2014). Therefore, the physiology of different *E. huxleyi* strains isolated from different geographic locations might respond differently to changing environmental drivers. For example, within the context of OA research, extensive previous studies suggest a strain-specificity of *E. huxleyi* in response to changes in seawater carbonate chemistry (Langer et al., 2009; Raven and Crawford, 2012). It has also been observed that different *E. huxleyi* ecotypes/morphotypes responded differently to OA (Müller et al., 2015), which is likely a consequence of their genetic variation (Cook et al., 2011). The present study and Feng et al. (2017) demonstrate the important roles of different environmental drivers in controlling the physiology of *E. huxleyi* strain NIWA1108, and so further work is required to determine if the findings apply to other strains.



In summary, this study, in combination with Feng et al. (2017), have a number of implications for research into the response of *E. huxleyi* to ocean acidification and global climate change. In addition to seawater carbonate chemistry (Riebesell et al., 2010), it is necessary to report the experimental conditions of all the environmental drivers carefully. The predictions presented will provide useful
5 information for biogeochemical models, such as that of Bopp et al. (2001), of how the elemental stoichiometry of *E. huxleyi* will respond to the alteration of these environmental conditions individually, in order to predict the future changes in the marine biogeochemical cycles. In addition, multiple environmental drivers tend to change simultaneously in the future global climate change scenario (Boyd and Hutchins, 2012), and so future studies should also investigate the interactions between these
10 multiple drivers on phytoplankton physiology. The predicted future changes in marine physical properties (such as sea surface temperature (SST) and mixed layer depth) will vary from one oceanic region to another (Boyd and Doney, 2002). The dose response curves from our study suggest that the range of alteration in environmental drivers may control the outcome of the effects of environmental perturbation on *E. huxleyi* physiology and biogeochemistry. Therefore, for future multi-factorial
15 manipulation experiments, our results suggest that the magnitudes of change in each environmental driver need to be determined/decided cautiously and should have environmental relevance in order to make more accurate predictions.

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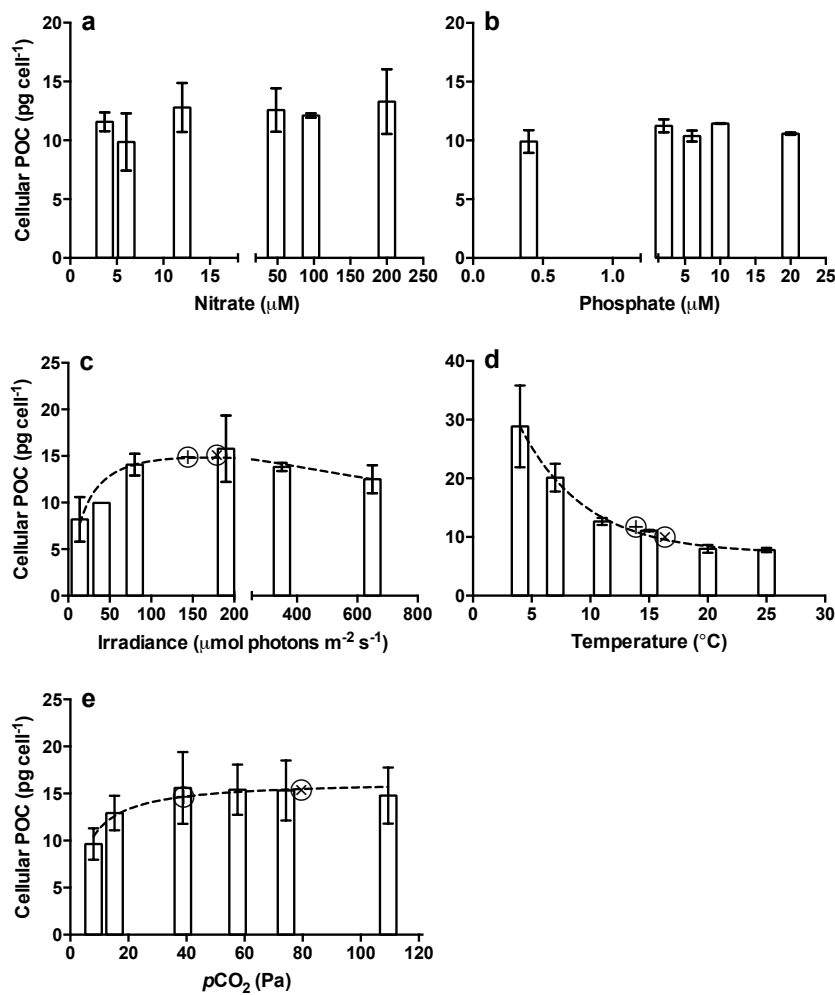


Figure 1. Changes in *Emiliania huxleyi* cellular particulate organic carbon (POC) content in response to different environmental drivers: a) cellular POC vs. nitrate concentration; b) cellular POC vs. phosphate concentration; c) cellular POC vs. irradiance; d) cellular POC vs. temperature; and e) cellular POC vs. $p\text{CO}_2$. Error bars represent standard deviations ($n=3$).

5 For Figures1-5: The dashed lines represent the fitted dose-response curves. “+” represent the fitted values for the present day conditions in the Chatham Rise area, and “X” represent the fitted values for the predicted future conditions (2100) in the Chatham Rise area. Error bars represent standard deviation ($n=3$).

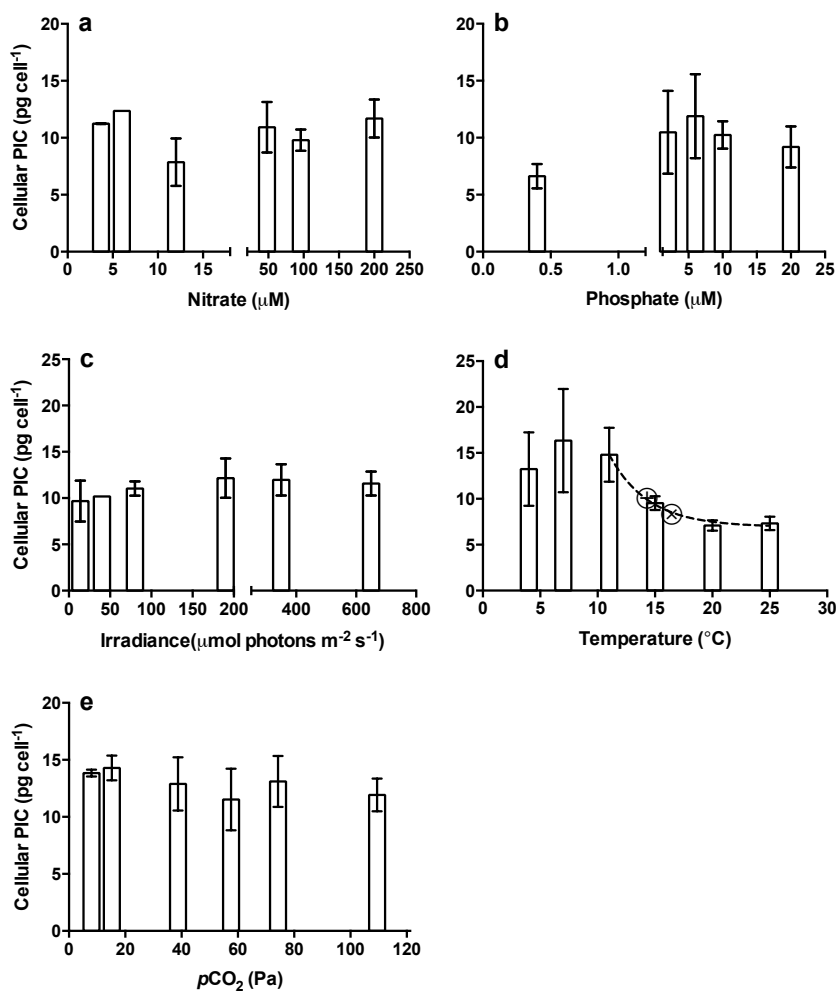


Figure 2. Changes in *Emiliana huxleyi* cellular particulate inorganic carbon (PIC) content in response to different environmental drivers: a) cellular PIC vs. nitrate concentration; b) cellular PIC vs. phosphate concentration; c) cellular PIC vs. irradiance; d) cellular PIC vs. temperature; and e) cellular PIC vs. $p\text{CO}_2$.

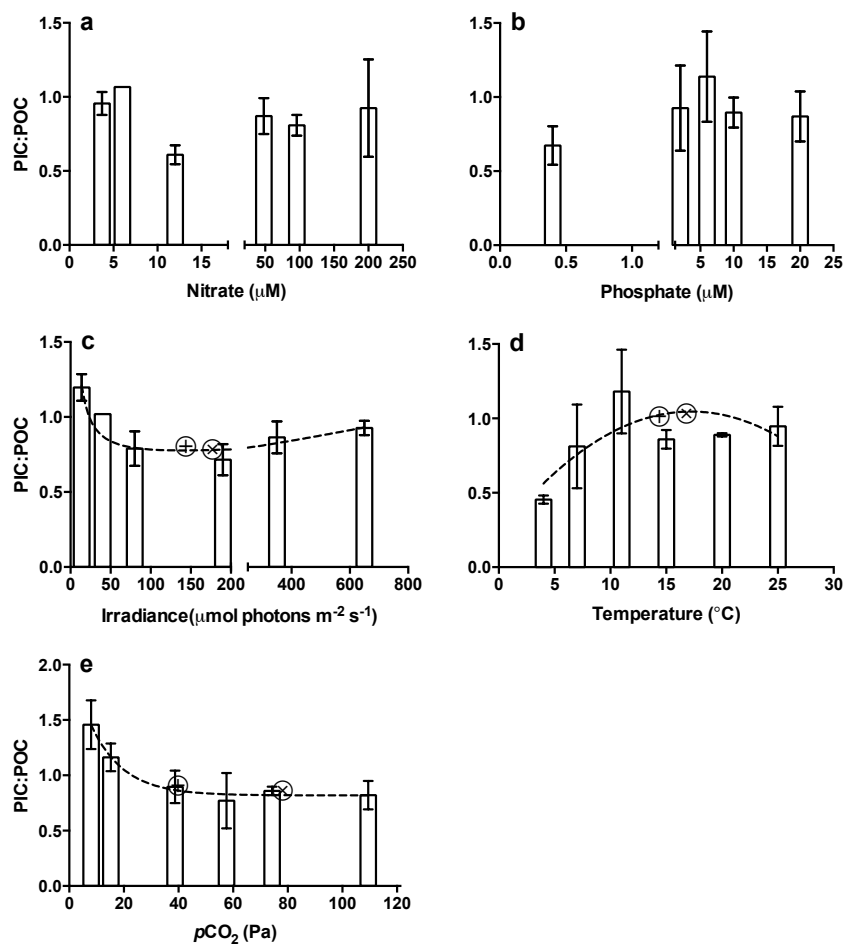


Figure 3. Changes in the ratio of *Emiliana huxleyi* cellular particulate inorganic carbon content to particulate organic carbon content (PIC:POC) in response to different environmental drivers: a) PIC:POC ratio vs. nitrate concentration; b) PIC:POC ratio vs. phosphate concentration; c) PIC:POC ratio vs. irradiance; d) PIC:POC ratio vs. temperature; and e) PIC:POC ratio vs. $p\text{CO}_2$.

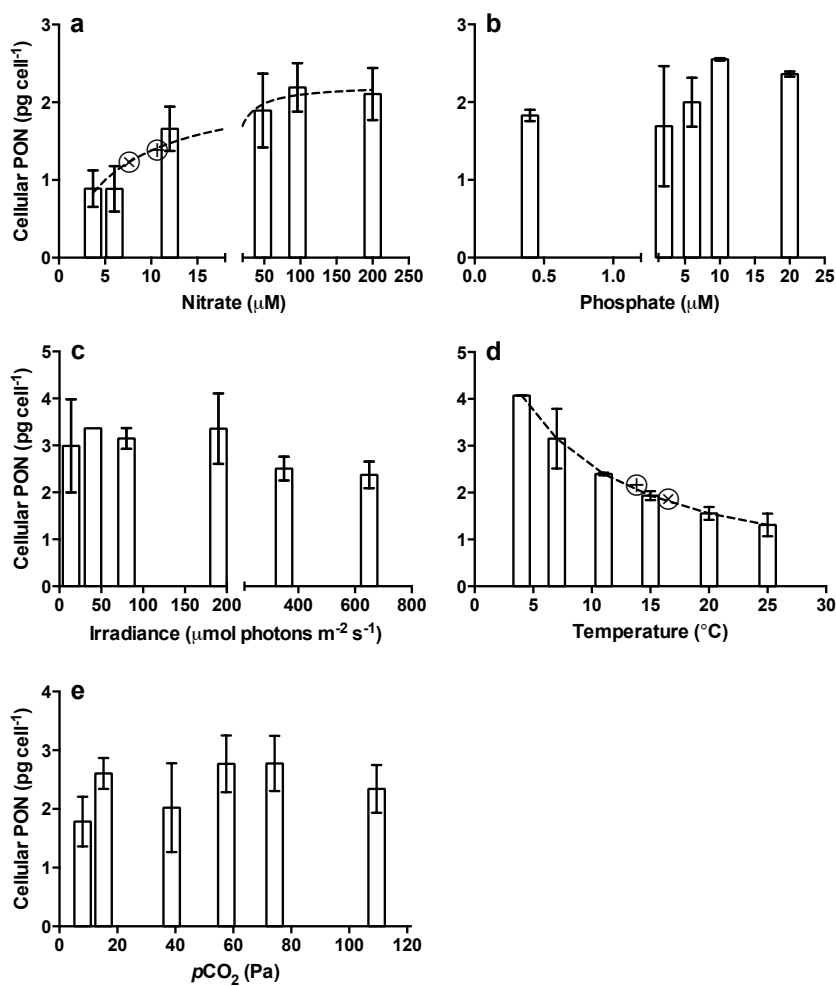


Figure 4. Changes in *Emiliana huxleyi* cellular particulate organic nitrogen (PON) content in response to different environmental drivers: a) cellular PON vs. nitrate concentration; b) cellular PON vs. phosphate concentration; c) cellular PON vs. irradiance; d) cellular PON vs. temperature; and e) cellular PON vs. pCO₂.

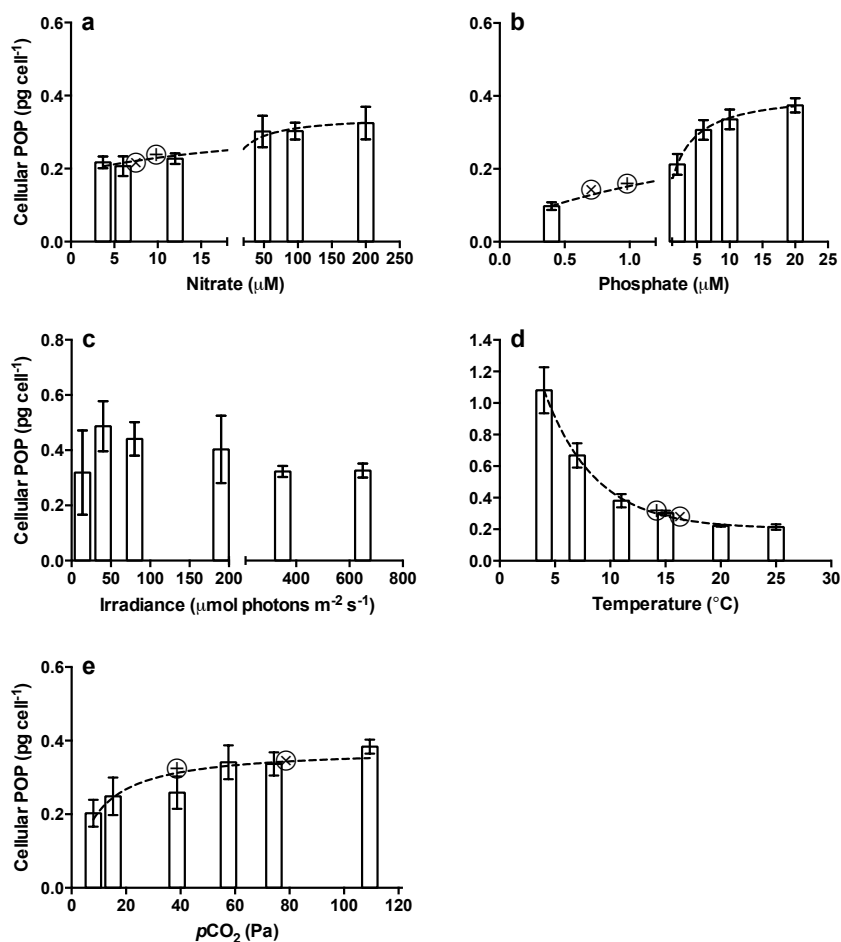


Figure 5. Changes in *Emiliana huxleyi* cellular particulate organic phosphorus (POP) content in response to different environmental drivers: a) cellular POP vs. nitrate concentration; b) cellular POP vs. phosphate concentration; c) cellular POP vs. irradiance; d) cellular POP vs. temperature; and e) cellular POP vs. pCO₂.

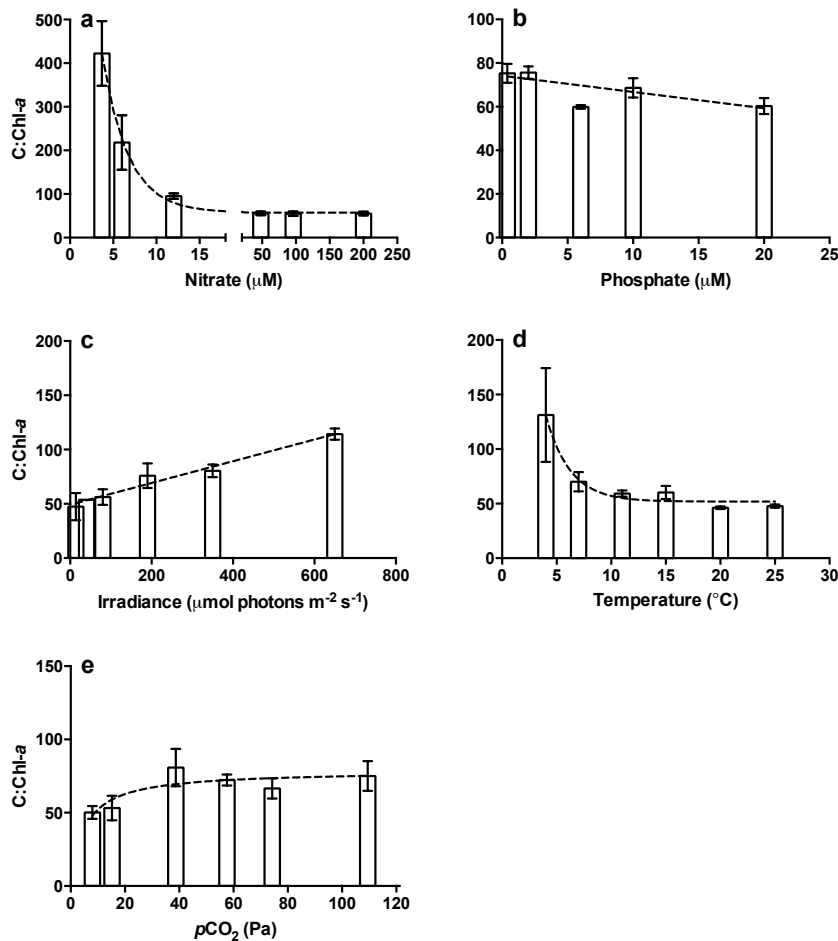


Figure 6. Changes in the ratio of *Emiliana huxleyi* cellular particulate organic carbon content to chlorophyll *a* content (C:Chl-*a*) in response to different environmental drivers: a) C:Chl-*a* ratio vs. nitrate concentration; b) C:Chl-*a* ratio vs. phosphate concentration; c) C:Chl-*a* ratio vs. irradiance; d) C:Chl-*a* ratio vs. temperature; and e) C:Chl-*a* ratio vs. $p\text{CO}_2$. Error bars represent standard deviations ($n=3$).

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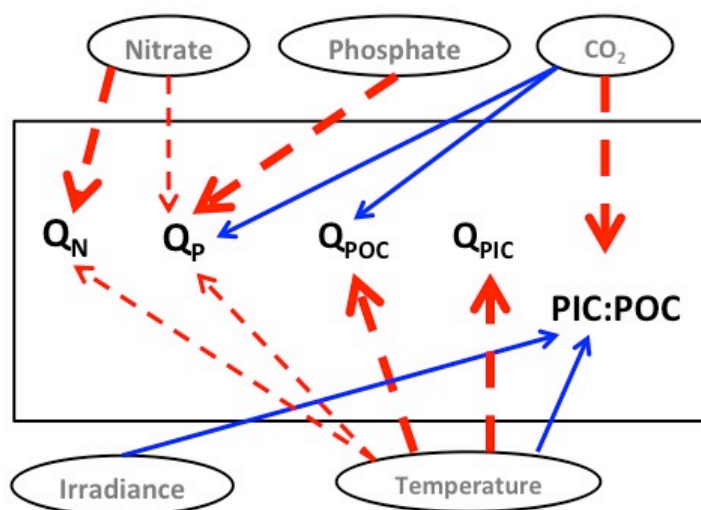


Figure 7. Conceptual model of the specific effects of each of the five environmental drivers, under the projected future conditions (year 2100), on the elemental composition of *Emiliana huxleyi*.

5 ***The box denotes the *E. huxleyi* cell. Solid blue arrows indicate positive effects of the future environmental changes; and dash red arrows indicate negative effects of the future environmental changes. Arrows in bold indicate the environmental drivers that play the most important role regulating the connected physiological metrics under the predicted environmental conditions for the year 2100.



Table 1. Treatment conditions for each environmental driver used in the manipulation experiments.

Environmental driver	Treatments
Nitrate (μM)	3.7; 6; 12; 48; 96; 200
Phosphate (μM)	0.4; 2; 6; 10; 20
Irradiance ($\mu\text{mol photons m}^{-2} \text{s}^{-1}$)	14; 40; 80; 190; 350; 650
Temperature ($^{\circ}\text{C}$)	4; 7; 11; 15; 20; 25
$p\text{CO}_2$ (Pa)	8; 15; 39; 58; 74; 109



Table 2. Elemental molar ratios of N:P, C:N and C:P of *Emiliana huxleyi* from the five single-factorial manipulation experiments. The errors are standard deviations around the mean (n=3).

Environmental driver	Treatment	N:P (mol:mol)	C:N (mol:mol)	C:P (mol:mol)
Nitrate (μM)	3.7	9.09±2.48	15.90±4.09	137.69±8.27
	6	9.07±1.55	13.16±1.13	118.45±10.06
	12	15.90±1.38	9.00±0.07	143.08±11.28
	48	14.46±1.39	7.01±0.64	107.76±11.93
	96	16.16±3.45	6.56±1.07	103.56±6.54
	200	14.31±0.40	7.32±0.47	104.82±8.34
Phosphate (μM)	0.4	41.57±4.14	6.32±0.42	261.67±17.30
	2	21.47±0.28	9.27±5.08	137.57±11.31
	6	14.17±3.59	6.16±1.24	85.00±4.51
	10	17.06±2.06	5.24±0.06	89.28±9.80
	20	13.99±0.89	5.22±0.08	73.04±3.48
Irradiance ($\mu\text{mol photons m}^{-2} \text{s}^{-1}$)	14	22.03±7.27	4.26±0.29	73.82±6.48
	40	14.27**	4.38**	62.50**
	80	16.03±2.90	5.24±0.69	83.96±17.05
	190	18.71±1.74	5.99±0.23	112.39±14.60
	350	17.15±0.83	6.47±0.54	110.63±3.98
	650	16.11±1.77	5.70±0.31	91.49±6.66
Temperature ($^{\circ}\text{C}$)	4	8.92±1.29	8.67±2.64	71.34±27.92
	7	10.46±2.05	7.60±1.32	78.70±14.78
	11	13.58±1.91	6.21±0.32	86.56±11.92
	15	14.12±0.66	6.70±0.31	94.47±3.84
	20	15.53±1.06	5.98±0.15	92.88±6.33
	25	13.67±2.99	7.08±1.39	93.96±4.87
$p\text{CO}_2$ (Pa)	8	19.39±2.41	6.81±1.09	122.61±9.97
	15	24.01±6.80	5.80±0.71	137.50±33.51
	39	16.96±3.62	7.41±0.06	155.64±31.42
	58	17.89±0.80	6.55±1.16	116.67±16.15
	74	18.22±2.45	6.42±0.43	116.93±17.60
	109	13.56±2.78	7.41±1.20	99.25±17.70

** Sample loss during analysis resulted in only single values at this irradiance.