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Day length as a key factor moderating the response of coccolithophore growth to elevated $p\mathrm{CO}_2$

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Abstract

The fate of coccolithophores in the future oceans remains uncertain, in part due to key factors having not been standardized across experiments. A potentially moderating role for differences in day length (photoperiod) remains largely unexplored. We therefore cultured four different geographical isolates of the species *Emiliania huxleyi*, as well as two additional species, *Gephyrocapsa oceanica* (tropical) and *Coccolithus braarudii* (temperate), to test for interactive effects of $p\mathrm{CO}_2$ with the light : dark (L : D) cycle. We confirmed a general regulatory effect of photoperiod on the $p\mathrm{CO}_2$ response, whereby growth and particulate inorganic carbon and particulate organic carbon (PIC : POC) ratios were reduced with elevated $p\mathrm{CO}_2$ under 14 : 10 h L : D, but these reductions were dampened under continuous (24 h) light. The dynamics underpinning this pattern generally differed for the temperate vs. tropical isolates. Reductions in PIC : POC with elevated $p\mathrm{CO}_2$ for tropical taxa were largely through reduced calcification and enhanced photosynthesis under 14 : 10 h L : D, with differences dampened under continuous light. In contrast, reduced PIC : POC for temperate strains reflected increases of photosynthesis that outpaced increases in calcification rates under 14 : 10 h L : D, with both responses again dampened under continuous light. A multivariate analysis of 35 past studies of *E. huxleyi* further demonstrated that differences in photoperiod account for as much as 40% (strain B11/92) to 55% (strain NZEH) of the variance in reported $p\mathrm{CO}_2$-induced reductions to growth but not PIC : POC. Our study thus highlights a critical role for day length in moderating the effect of ocean acidification on coccolithophore growth and consequently how this response may play out across latitudes and seasons in future oceans.

Primary productivity by oceanic phytoplankton drives the flux and eventual sequestration of carbon from the atmosphere into the deep ocean. Calcium carbonate (CaCO_3) biomineralizing nanoplankton (coccolithophores) are considered particularity critical to this role as their photosynthesis directly assimilates CO_2, while their calcification provides dense mineral for ballasting and facilitates export to the deep sea (Klaas and Archer 2002; Bach et al. 2016). Coccolithophores appear particularly susceptible to ocean acidification (OA), the process whereby rising atmospheric $p\mathrm{CO}_2$ concentrations are resulting in lower ocean pH and modifications to carbonate chemistry (see Meyer and Riebesell, 2015).

Experiments conducted on laboratory monocultures, as well as natural populations, generally observe that exposure to elevated $p\mathrm{CO}_2$ decreases coccolithophore calcification and/or increases photosynthetic rates (Riebesell 2004; Hoppe et al. 2011). Shifts in the balance of calcification to photosynthesis drive a decline in cellular ratios of particulate inorganic carbon (PIC) to particulate organic carbon (POC), implying significant biogeochemical implications (Ridgwell et al. 2009; Meyer and Riebesell

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2015). However, coccolithophore responses are moderated by several factors, which influence their productivity, other than ocean carbonate chemistry, including temperature (Sett et al. 2014; Feng et al. 2017), nutrient availability (Lefebvre et al. 2011; Rouco et al. 2013; Tong et al. 2016), life-cycle stage (Rokitta and Rost 2012; Kottmeier et al. 2014), and taxonomy (Langer et al. 2006; Rickaby et al. 2010). Coccolithophore responses may also be strain-specific (Langer et al. 2009) and dependent upon environmental history and local adaptation (Lohbeck et al. 2012).

Many OA studies have focused on the ubiquitous and abundant coccolithophore species *Emiliania huxleyi*. Several morphotypes and strains of *E. huxleyi* exist (Brand 1982; Young 1994), which demonstrate varying responses to elevated pCO_2 (e.g., Langer et al. 2009), potentially reflecting localized adaptations to specific oceanic regions (Findlay et al. 2011). Species other than *E. huxleyi* may also be regionally significant calcifiers (Daniels et al. 2014, 2016). For example, *Gephyrocapsa* spp. can contribute significantly to the coccolithophore community and suspended PIC pool in the southeast Pacific and Mediterranean Sea (Beaufort et al. 2007; Carmen Álvarez et al. 2010). However, only five coccolithophore species other than *E. huxleyi* have currently been studied in the context of OA: *Calcidiscus leptoporus* (Langer et al. 2006; Diner et al. 2015), *Calcidiscus quadriperforatus* (Diner et al. 2015), *Coccolithus braarudii* (Langer et al. 2006; Rickaby et al. 2010), *Gephyrocapsa oceanica* (Rickaby et al. 2010; Sett et al. 2014), and *Syrachosphaera pulchra* (Fiorini et al. 2011). Given that different species and strains represent populations adapted to specific environmental conditions, it is plausible to expect that the pCO_2 response of tropical species (and isolates) may differ from that for temperate species, for example, due to differences in the temperature, light, and nutrient climates, as well as carbonate chemistry buffering of their natural environment.

Light availability is a key environmental driver of coccolithophore productivity, with changes to dosage via intensity (Zondervan et al. 2001; Rost et al. 2002; McCarthy et al. 2012; Rokitta and Rost 2012; Jin et al. 2017), spectral quality (Gao et al. 2012; Jin et al. 2013), and frequency (Jin et al. 2013), all moderating how they respond to OA. Only three studies, on a single temperate isolate of *E. huxleyi* (PML B92/11; Rost et al. 2002, 2006; Zondervan et al. 2002), have tested for an interactive role of pCO_2 with differences in light dose (day length). Although these studies did not report any significant responses, this outcome is not directly consistent with physiological expectations.

First, both increased pCO_2 (Bach et al. 2013; Kottmeier et al. 2014) and day length (Rost et al. 2006) decrease the dependency of photosynthesis on HCO_3^- and hence on carbon concentrating mechanism (CCM) activity (Bach et al. 2013). Although photosynthetic rates are often higher under increased pCO_2, photosynthetic efficiency is lower with increased photoperiod (Nielsen 1997; Rost et al. 2002, 2006; Zondervan et al. 2002) as affinity for inorganic carbon (Ci) is reduced (Rost et al. 2006). Enhancement of photosynthesis by increased pCO_2 may then be canceled out when the photoperiod is increased. Second, calcification decreases with pCO_2 due to elevated H^+ concentration ($[H^+]$; Bach et al. 2013, 2015; Fukuda et al. 2014) but appears unchanged with photoperiod (Rost et al. 2002; Zondervan et al. 2002). As calcification directly competes with photosynthesis for HCO_3^- as a substrate (Rokitta and Rost 2012; Bach et al. 2013; Bolton and Stoll 2013), the decreasing dependency of photosynthesis on HCO_3^- (CCM activity), via increased pCO_2 and photoperiod, may then ultimately reduce the negative impact of elevated $[H^+]$ (Bach et al. 2013).

These two lines of evidence indicate that the decline in the PIC to POC ratio (PIC : POC) with increased pCO_2 (see Meyer and Riebesell 2015) should be lessened when the photoperiod is also increased. However, such an expectation may ultimately depend on the strength of the CCM (Rost et al. 2002, 2006) and the mechanism of Ci acquisition for photosynthesis and/or calcification (Rickaby et al. 2010; Meyer and Riebesell 2015; Taylor et al. 2017), and hence the coccolithophore species (or even isolate).

Photoperiod is a key factor regulating the timing and duration of seasonal productivity (Longhurst et al. 1995), which becomes increasingly seasonally extreme toward polar latitudes that are the least buffered against changes due to elevated pCO_2 and OA (Shadwick et al. 2013). While day length is not influenced by climate change, ocean warming and the associated stronger stratification will alter the light dose (and nutrient availability) for phytoplankton in the upper ocean. Differences in photoperiod have not been standardized across laboratory experiments (Meyer and Riebesell 2015) or field studies that span broad latitudinal gradients (Poulton et al. 2013; Richier et al. 2014) and therefore, represents a key untested source of variability in pCO_2 responses. We conducted a multifactorial experiment to examine interactions between photoperiod and pCO_2 upon geographically diverse coccolithophores. Specifically, we tested the hypothesis that OA-induced declines to PIC : POC ratios will be significantly lower under continuous 24 h light regimes compared to a 14 : 10 h light : dark (L : D) photoperiod. To consider our experimental results more broadly, we also constructed a database of responses from previous published studies to examine the potential interactive influence of photoperiod to elevated pCO_2.

Methods

Strain selection and culture conditions

Six coccolithophore isolates were selected to enable intercomparison of day length–CO_2 interactions within and between species: four isolates of *E. huxleyi* (PCC70-3, PCC124-3, RCC962, and NZEH) and one isolate each of *G. oceanica* (RCC1804) and *C. braarudii* (PLY182) (Table 1). These isolates were chosen to represent a cross section of geographical origin, isolation date,

Table 1. Strain information (*E. huxleyi* morphotype [B, A, R] is indicated where known). SST data from optimum interpolation sea surface temperature database and minimum and maximum temperatures for 2016 in°C. Light dose is calculated from day length and mean PAR values (derived using the "R" package "phytotools") and is in mol photons m^{-2} d^{-1}.

Species	Strain identifier(s)	Location	Isolation date	SST (min–max)	Day length (min–max)	Light dose (min–max)
E. huxleyi	RCC962/Biosope_32B_FL1-3	French polynesia, 8°19′S, 141°15′E	Oct 2004	26.5–30.6	11–13	21.2–30.5
E. huxleyi (B)	PCC70-3	Northern North Sea, 56°17′N, 3°21′E	Jul 2011	6.5–16.2	8–16	1.3–40.5
E. huxleyi (A)	PCC124-3	Bay of Biscay, 46°6′N, 7°8′W	Jun 2011	12.0–19.5	9–15	4.6–37.9
E. huxleyi (R)	NZEH/PLY M219/COWPO6	South Pacific (New Zealand), 47°41′S, 174°1′E	1992	7.8–13.1	9–15	4.0–40.3
G. oceanica	RCC1804/Sipadan DM2-4	Sipadan, Malaysia, 4°6′N, 118°37′ E	Dec 2008	28.4–30.0	11–12	25.7–25.8
C. braarudii	PLY 182G	English Channel, 50°10′N, 4°17′W	Nov 1990	9.8–17.2	8–16	2.8–40.7

morphotype (for *E. huxleyi*), and cellular inorganic content (PIC per cell; Supporting Information Table S2) under steady state ambient pCO_2. Isolates of *E. huxleyi* examined included those from temperate (PCC70-3, PCC124-3, and NZEH) and tropical (RCC962, French Polynesia, 8°19′S, 141°15′W) locations and encompassed morphotypes A (PCC124-3), B (PCC70-3), and R (NZEH; Young 1994). *C. braarudii* is a heavily calcifying coccolithophore commonly found in temperate coastal and upwelling regions (Daniels et al. 2014), whereas *G. oceanica* is found in temperate and subtropical open-ocean regions, with the isolate used in this study originating from the tropics (Sipadan, Malaysia, 4°6′N, 118°37′E).

All species were grown as semicontinuous cultures in climate-controlled growth cabinets (Sanyo Gallenkamp, Fitotron PG660), where temperate organisms (*E. huxleyi* stains PCC70-3, PCC124-3, and NZEH and *C. braarudii*) were maintained at 17°C and the tropical organisms (*E. huxleyi* stain RCC962 and *G. oceanica*) were maintained at 20°C. All cultures were grown at a light intensity of 150 μmol photons m^{-2} s^{-1}. Cultures were maintained in artificial seawater enriched with f/2 nutrients (Guillard 1975), plus selenium, and diluted where necessary to maintain cells in exponential growth. All media was filter sterilized via a 0.2-μm filter (Polycap 36AS, Whatman Filters) as autoclaving resulted in significant reductions to the total alkalinity. Triplicate 750 mL volumes for each isolate were simultaneously grown at ambient pCO_2 (present day, ~ 390 ppm) and under elevated pCO_2 representative of future representative concentration pathway 8.5 (IPCC 2014) for 2080 (~ 1000 ppm). Two different photoperiods were used: a 14 : 10 h L : D cycle vs. continuous 24 h light, with a total daily photon dose of 15.1 and 25.9 mol photons m^{-2} d^{-1}, respectively. Cultures were monitored daily via cell counts using a Neubauer hemocytometer. All cultures were harvested for physiological analyses once steady state growth rates (μ, d^{-1}) remained stable (< 5% difference according to a daily running average), which typically required 3 to 4 weeks (= 15–20 generations).

Controlling and monitoring the carbonate system

Carbonate chemistry within the culture vessels was maintained by bubbling with CO_2-enriched air via a series of mass flow controllers (MFCs, EL-FLOW F-201AV; Bronkhorst High-Tech), with a cylinder of 10% CO_2 (BOC) and an air compressor (Bambi, HT15 oil-free compressor). Each culture vessel was connected separately to an MFC via nylon tubing. Ambient air fed via the compressor was first passed through soda lime (Sigma 23,888) to remove CO_2 and thus standardize the CO_2 content. MFCs were connected to PC-software (Bronkhorst High-Tech) that both controlled and recorded the airflow and subsequently to a gas mixer equipped with four taps. One tap fed to an infrared gas analyzer (Li-Cor, LI-820 analyzer) connected to a laptop to verify the desired CO_2 concentration, whereas the other three taps ran into one set of triplicate cultures at a rate of 150 mL min^{-1}. All air-in and air-out ports on the culture vessels were fitted with 0.2 μm hydrophobic air filters (Millipore), and an air stone attached to the outflow of the air-in line within each culture vessel maximized diffusion of the gas into the media. Sterile f/2 media was bubbled with the target pCO_2 for 24 h before inoculation to allow for pre-equilibration of the carbonate chemistry. All culture vessels were continuously stirred via a magnetic stir plate to reduce formation of gas gradients and of cells settling and/or clumping within the cultures. To ensure that the MFCs provide full control over the biology, an initial experiment using batch cultures for each isolate was used to identify the cell concentration at which each isolate began to induce modifications to the carbonate chemistry. In all cases, cell concentrations of ~ 1 to 1.5 x 10^5 cells mL^{-1} began to induce significant drawdown of total alkalinity. Periodic dilutions of the main experimental cultures to maintain cells in exponential growth were then used to maintain cell concentrations below this threshold.

Several parameters of the carbonate chemistry were monitored daily from all culture vessels: total alkalinity (A_T) was measured using a Titrino auto-titrator (Metrohm; 0.024 mol L^{-1} HCl) on 20 mL aliquots gently gravity-filtered through a 0.2-μm syringe filter (Minisart filter, Sigma-Aldrich). Dissolved inorganic carbon (C_T) was determined on a separate 15 mL aliquot, also through a 0.2-μm syringe filter, whereby the filtrate was transferred to ashed glass vials (500°C, 3 h). Samples were then analyzed using a total carbon analyzer (Shimadzu TOC-VCSH Total Organic Carbon Analyzer with ASV-I autosampler) calibrated with bicarbonate standards. Both A_T and C_T were then used to determine the entire carbonate system via CO2SYS software (ver. 14) (Pierrot et al. 2006). Carbonate chemistry recorded throughout the experiment in this way demonstrated that our approach consistently maintained $p\mathrm{CO}_2$ at the desired concentrations (see Supporting Information Table S1); it is important to note that even with regular dilution and the protocols employed above, some drawdown in A_T and C_T was still evident, particularly in the high CO_2 treatments.

All cultures were finally sampled for the following:

PIC and POC Analysis—Two aliquots of 150 mL were each filtered down onto ashed (500°C for 3 h) 25 -m MF300 glass fiber filters (Fisher Scientific) and then placed in a desiccator to dry for 24 h. Samples were stored in sterile cryotubes for subsequent analysis. One of the paired filters was acidified with ~ 2 mol L^{-1} HCl to drive off inorganic carbon and further dried for 24 h. Total carbon on both filters was then determined using a carbon analyzer (Shimadzu TOC-VCSH Total Organic Carbon Analyzer with ASV-I autosampler) calibrated using a glucose standard. POC was quantified on the acidified filters, and PIC was calculated by subtracting POC from the total carbon from the nonacidified filters. PIC and POC content was normalized to corresponding measurements of cell concentration.

Growth rates and cell size—Cell concentrations were quantified daily from each culture using a Neubauer hemocytometer (Fisher Scientific). Doubling rates (μ) were calculated as μ (d^{-1}) = ($\ln c1 - \ln c0$) $\cdot$ Δt^{-1}, where $c0$ is the initial count (cells mL^{-1}), $c1$ is the final count (cells mL^{-1}), and Δt is the time between the two counts (d). Mean cell size was measured from a separate 15 mL aliquot from each culture using a Z1 Coulter Particle Counter (Beckman Coulter). Coccosphere thickness was estimated by adding 5 μL of 0.25 mol L^{-1} HCl to the sample to dissolve the coccoliths before rerunning each sample through the particle counter, after Franklin et al. (2010). Difference in mean cell diameter before and after acidification was taken as an estimate of the thickness of the coccosphere.

Data treatment and analysis

To directly examine the potential influence of day length and species upon any OA response, absolute values for each core variable (μ, cellular PIC content, cellular POC content, PIC : POC, cell size; see Supporting Information Table S2) were used to calculate the % change with increasing $p\mathrm{CO}_2$ (Suggett et al. 2013). For this, values from replicate treatment vessels V_{t1}, V_{t2}, V_{t2} were always expressed as a % change from its respective replicate control vessel, V_{c1}, V_{c2}, V_{c2} since vessels V_{t1}–V_{c1}, V_{t2}–V_{c2}, and V_{t3}–V_{c3} were located pairwise in the same area of the incubator. Periodic light measurements (QSL-101 scalar quantum irradiance sensor, Biospherical Instruments) demonstrated that the light fields for the pairwise vessel arrangement in the incubators were the same for control and respective treatment. Percentage data were tested for normality (MATLAB) and divided by 100 prior to arcsin transformation. The interactive influence of day length and species on the percentage change for each response variable to elevated $p\mathrm{CO}_2$ was then tested via two-way analysis of variance (ANOVA; MATLAB). The interactive effect of CO_2 and L : D cycle was also tested on the absolute values for μ, cellular PIC content, cellular POC content, and PIC : POC for each species via two-way ANOVA (see Supporting Information Table S3).

E. huxleyi database

A database was constructed from past OA studies on coccolithophores to more broadly consider our experimental results of the potential influence of photoperiod upon growth and cellular (sin)organic carbon content (Supporting Information Table S4). Values for growth rate (μ) and PIC : POC were extracted along with corresponding growth conditions (temperature, T; salinity, S; photoperiod, L : D; nitrogen-to-phosphate ratio, N : P; light intensity, E; total alkalinity, A_T; and $p\mathrm{CO}_2$) and strain identification. CO2SYS was used to calculate A_T and/or $p\mathrm{CO}_2$ where other carbonate chemistry parameters were reported; however, only A_T and $p\mathrm{CO}_2$ were included in subsequent multivariate analysis to minimize potential autocorrelation.

To be consistent with our experimental approach, data were only selected from studies where $p\mathrm{CO}_2$ was manipulated via the C_T pool as opposed to A_T or Ca^{2+} availability (see Meyer and Riebesell 2015). Noncalcifying isolates were not included in the analysis, or instances where PIC per cell was not measured alongside POC per cell and μ. The method used to manipulate C_T (i.e., CO_2 bubbling vs. acid–base additions with bicarbonate) was not considered a variable in our analysis since these two alternative C_T manipulation approaches have been shown to perturb the carbonate system in a similar way (Gattuso and Lavigne 2009). Values for μ, PIC : POC, and $p\mathrm{CO}_2$ for the "OA treatments" (T) were normalized relative to those for the corresponding present-day ambient (A) control ($\mu_{\mathrm{T:A}}$, PIC : $\mathrm{POC}_{\mathrm{T:A}}$, and $p\mathrm{CO}_{2\mathrm{T:A}}$). This ensured that any residual variability of $\mu_{\mathrm{T:A}}$ or PIC : $\mathrm{POC}_{\mathrm{T:A}}$ not explained by $\mathrm{CO}_{2\mathrm{T:A}}$ must be from other experimental factors and/or isolate (as per Suggett et al. 2013); this approach also accounts for potential discrepancies in the measurements (e.g., analytical accuracy) of the independent variables across studies. Normalized values for the control (i.e., $\mu_{\mathrm{T:A}}$, PIC : $\mathrm{POC}_{\mathrm{T:A}}$, and $\mathrm{CO}_{2\mathrm{T:A}} = 1$) were then discarded from the data set. Following these criteria, only a few data sets were available for coccolithophore species other than *E. huxleyi* and therefore we finally restricted our wider analysis to isolates of only this species, to yield 159 data points from across 35 studies (Supporting Information Table S4).

Positive (irradiance [E], $\mathrm{CO}_{2\mathrm{T:A}}$) or negative (PIC : $\mathrm{POC}_{\mathrm{T:A}}$) variables were initially identified (MATLAB) and then square root or square transformed, respectively, to stabilize the variance. The PRIMER-BEST match permutation (PRIMER v6, PRIMER-E Ltd.) was then used to identify variables that best explained variance of $\mu_{\mathrm{T:A}}$ or PIC : $\mathrm{POC}_{\mathrm{T:A}}$ via repeated (99) permutation testing. Data were standardized within each variable category to ensure comparable measurement scaling and Euclidian distance used to produce the corresponding resemblance matrix. Significant variables identified from the PRIMER-BEST match were then entered into a multiple stepwise regression (MSR; MATLAB).

Results

Experimental conditions

It was important to ensure the carbonate system was as tightly controlled as possible. Final $p\text{CO}_2$ tended to be slightly higher than the target in the ambient cultures but overall by only ± 15% in the 1000 ppm cultures. A_T was typically reduced in cultures via calcification. Media in ambient CO_2 cultures was more stable, with A_T only drifting by 2% to 7% below that expected (~ 2400 μmol kg^{-1}), whereas the high CO_2 cultures typically fell below 2000 μmol kg^{-1}. The total C_T pool ranged from 1851 to 2428 μmol kg^{-1} and was consistently lower in high CO_2 cultures. Overall, the different L : D cycles did not affect changes in carbonate chemistry.

Growth and carbon assimilation

Growth rates (μ, d^{-1}) for the 14 : 10 h photoperiod typically declined with elevated $p\text{CO}_2$ (Supporting Information Table S2), whereby the percentage change in growth rate (μ) from ambient to elevated $p\text{CO}_2$ was −50% to −60% (*E. huxleyi* strain 962, *G. oceanica*) or −15% (*E. huxleyi* strains NZEH, 70-3, and 124-3; Fig. 1A). Only *C. braarudii* exhibited a slight increase in μ (4%) with elevated $p\text{CO}_2$. In contrast, under constant light, the decrease in μ from ambient to elevated $p\text{CO}_2$ was only −50% (*E. huxleyi* strain 962) or −10% to −20% (*E. huxleyi* strain NZEH and *G. oceanica*), and for all other taxa μ increased by 3% to 5%. Thus, the longer photoperiod generally dampened or fully reversed the extent with which elevated $p\text{CO}_2$ lowered the growth rate (see also Table 2).

A similar response to photoperiod across taxa was observed for the percentage change in PIC : POC from ambient to elevated $p\text{CO}_2$ (Fig. 1B). Specifically, PIC : POC was decreased with elevated $p\text{CO}_2$ by −70% (*E. huxleyi* 962, *G. oceanica*) or −5% to −30% (all other taxa) under the 14 : 10 h photoperiod compared to only −40% to −50% (*E. huxleyi* stain 962 and *G. oceanica*) or −5% to 10% (all other taxa) under continuous light. While small differences in the extent of $p\text{CO}_2$ increase between control and treatments were evident between isolates (Supporting Information Table S1), neither the percentage change in μ nor in PIC : POC for either photoperiod significantly correlated with the corresponding percentage change in $p\text{CO}_2$ across taxa (Fig. 1C,D) and thus are not considered a major source of variance.

Greatest reductions of μ and PIC : POC with increased $p\text{CO}_2$ under both photoperiods were observed for the two tropical isolates, *E. huxleyi* stain RCC962 and *G. oceanica* and reflected PIC per cell and POC per cell responses that were very different compared to all other taxa (Fig. 1E,F). First, both *E. huxleyi* stain RCC962 and *G. oceanica* were the only two taxa to exhibit decreased PIC per cell with elevated $p\text{CO}_2$; this effect was greater under the 14 : 10 h photoperiod (−60% to −70%) than under continuous light (−20% to 2%) (Fig. 1F). All other taxa exhibited an increase in PIC per cell with increased $p\text{CO}_2$ by 10% to 30% for the 14 : 10 h photoperiod, and to a lesser extent (10–20%, and in the case of *C. braarudii* – 2%) under continuous light.

Second, all taxa exhibited an increase in POC per cell with increased $p\text{CO}_2$ under the 14 : 10 h photoperiod (25–55% *E. huxleyi* stain 962 and *G. oceanica*; 30–80% all other taxa; Fig. 1E). However, the percentage change of POC per cell with increased $p\text{CO}_2$ was higher for *E. huxleyi* stain RCC962 and *G. oceanica* under continuous light (65–75%), whereas it was lower for all other taxa (0–15%) compared to the 14 : 10 h photoperiod. Together, these trends indicate two contrasting functional responses among the taxa examined: (i) $p\text{CO}_2$-driven increases in photosynthesis and decreases in calcification were enhanced and dampened, respectively, with the longer photoperiod for *E. huxleyi* stain RCC962 and *G. oceanica* and (ii) $p\text{CO}_2$-driven increases in both photosynthesis and calcification were both dampened (but with a greater dampening of photosynthesis over calcification) with the longer photoperiod for all other taxa.

General, but subtle, differences were also evident among these two functional response groupings in cellular allocation of PIC and POC for the different $p\text{CO}_2$ and photoperiod treatments (Figs. 1 and 2). Under the 14 : 10 h photoperiod, all taxa generally exhibited reduced (or slightly elevated; *C. braarudii*) μ but larger cells with elevated $p\text{CO}_2$ (Fig. 2A); these larger cells were generally accompanied by substantially thinner (*E. huxleyi* stain 962 and *G. oceanica*) or thicker (all other taxa except *C. braarudii*) coccospheres (Fig. 2B; Supporting Information Table S2). No consistent trends in cell size or coccosphere thickness under elevated $p\text{CO}_2$ were evident under continuous light between these two groups, but all taxa still generally exhibited reduced growth rates (and lower PIC : POC).

A two-way ANOVA (see Supporting Information Table S3) to test for possible interactive effects of $p\text{CO}_2$ and day length on the absolute values for growth and carbon allocation further showed that L : D cycle was more important in driving the changes observed across μ, PIC, POC, and PIC : POC for the temperate isolates (*E. huxleyi* stains NZEH, 70-3, and 124-3) with interactive effects of L : D with $p\text{CO}_2$ for μ and POC. In contrast, CO_2 was the more important variable for the two tropical isolates (*E. huxleyi* stain RCC962 and *G. oceanica*).

Broader data analysis for *E. huxleyi*

Collation of data from across past studies did not result in any clear trends between elevated $p\text{CO}_2$ ($[\text{CO}_2]_{\text{T:A}}$) and the relative change in growth rate ($\mu_{\text{T:A}}$) when considering data from previous studies (Fig. 3; Table 3). However, the BEST test analysis (and MSR) identified that photoperiod (L : D), along with nutrient availability (N : P), was a significant variable in

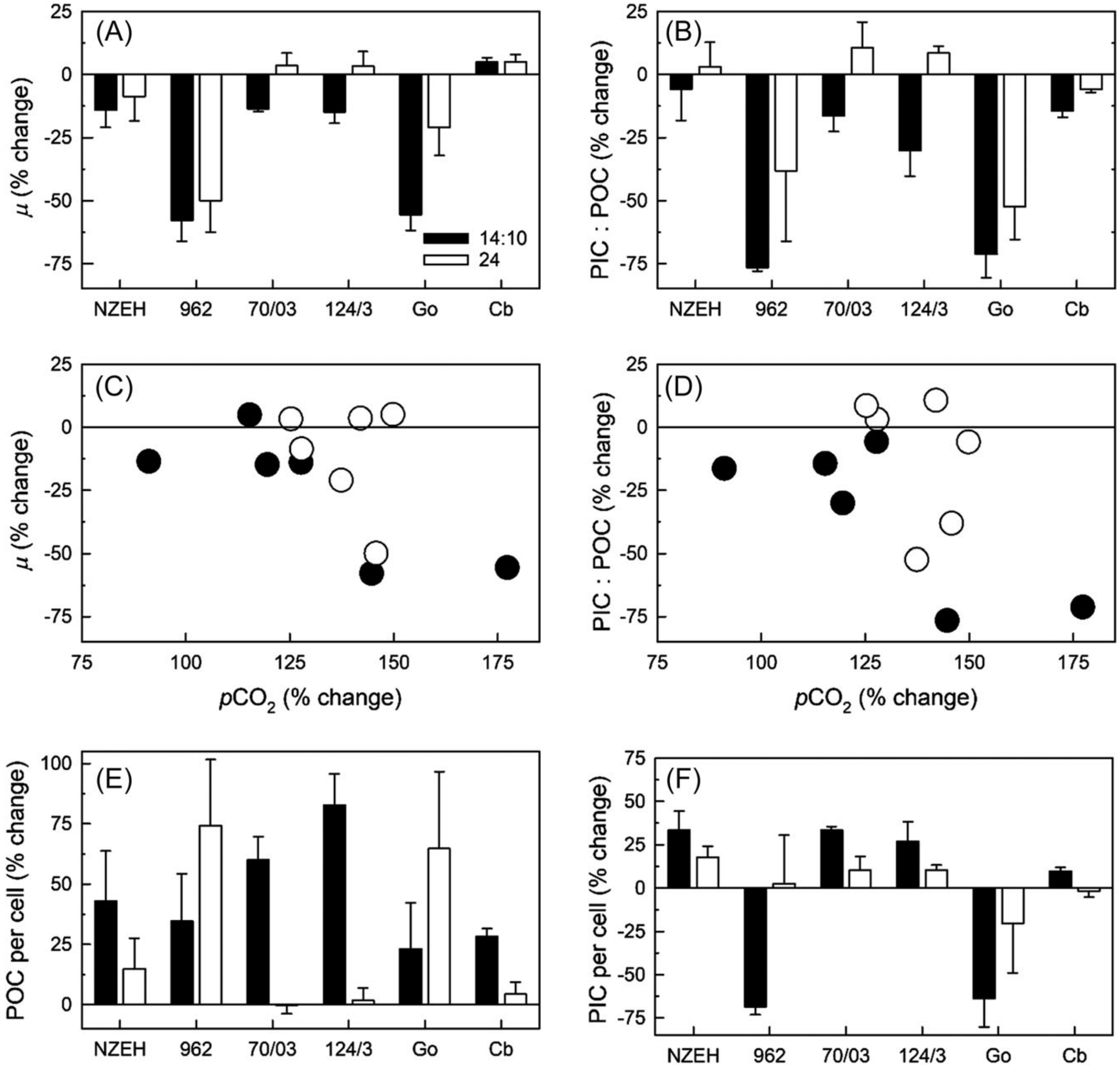

Fig. 1. The combined effect of elevated pCO_2 and light : dark cycle on growth rate (μ) (A **and C)**, calcification (PIC : POC) **(B and D)**, and cellular POC **(E)** and PIC **(F)** quotas on six coccolithophores: four strains of *E. huxleyi* (NZEH, 962, 70-3, and 124-3), *G. oceanica* (Go), and *C. braarudii* (Cb). The data are presented as percent change between low (400 μatm) and high (1000 μatm) CO_2.

Table 2. Summary of two-way ANOVA examining the influence of genotype (coccolithophore species, *E. huxleyi* isolate, $n = 6$; Table 1) and photoperiod (14 : 10 h vs. 24 h) upon the percentage change in response variable to elevated pCO_2 (see Methods section for procedures describing pretest normalization). Significant outcomes and interactions are highlighted in bold.

		Growth		PIC per cell		POC per cell		PIC : POC		Cell size	
Source of variation	**df**	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*
Genotype	5	55.7	**<0.001**	27.1	**<0.001**	3.16	**0.025**	37.0	**<0.001**	2.67	**0.047**
Photoperiod	1	30.6	**<0.001**	3.81	0.060	5.35	**0.030**	38.8	**<0.001**	8.30	**0.008**
Genotype x photoperiod	5	4.19	**0.007**	12.6	**<0.001**	10.4	**<0.001**	2.35	0.072	2.70	**0.045**

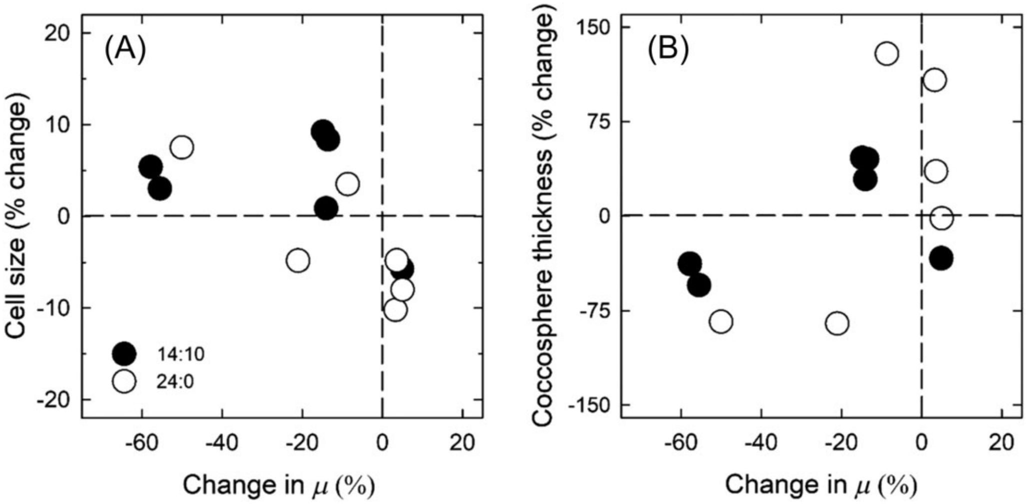

Fig. 2. Relationship between changes in cell size **(A)** and coccosphere thickness **(B)** with changes in growth rate (μ) at elevated $p\text{CO}_2$ under two different light : dark cycles for six coccolithophores: four strains of *E. huxleyi* (NZEH, 962, 70-3, and 124-3), *G. oceanica* (Go), and *C. braarudii* (Cb). The data are presented as percent change between low (400 μatm) and high (1000 μatm) CO_2.

controlling the trends in $\mu_{\text{T:A}}$ across all the studies included in the analysis, though they only explained < 10% of the variance. Our experimental data strongly indicated that *E. huxleyi* isolate was a key source of variance in the relative response of μ to elevated $p\text{CO}_2$ (Fig. 1, above); therefore, we repeated this analysis for individual *E. huxleyi* isolates where relatively large data sets were available (B92/11 and NZEH; see Supporting Information Table S4). The BEST test analysis identified that both temperature (*T*) and L : D cycle were significant variables in explaining the variance of $\mu_{\text{T:A}}$ for B92/11 and NZEH. However, the MSR was not able to incorporate all variables into a single model; only *T* for B92/11 and L : D cycle for NZEH were successfully incorporated, explaining 38% and 54% of the observed variance, respectively.

In contrast to $\mu_{\text{T:A}}$, PIC : $\text{POC}_{\text{T:A}}$ was consistently related to elevated $p\text{CO}_2$ ($[\text{CO}_2]_{\text{T:A}}$) when considering all data (25.9%) but also when considering data for only B92/11 (58%) or NZEH (48%) (Table 3). Additionally, *T* was consistently identified as another important variable in explaining the variance in PIC : $\text{POC}_{\text{T:A}}$. While the decline in PIC : POC with elevated $p\text{CO}_2$ from our experiments varied substantially between *E. huxleyi* isolates (Fig. 3), the regression slope of PIC : $\text{POC}_{\text{T:A}}$ vs. $[\text{CO}_2]_{\text{T:A}}$ for B11/92 was not significantly different from that for NZEH (see Fig. 3 legend). However, overall, and in contrast to our experimental data, photoperiod was not identified as a significant moderating variable in the response of PIC : POC to $p\text{CO}_2$ in this wider data set.

Discussion

Both photoperiod and Ci availability are well recognized to influence the productivity and growth of microalgae, including coccolithophores (Nielsen 1997; Rost et al. 2006). However, previous studies investigating an interactive role of photoperiod with $p\text{CO}_2$ on a single isolate (*E. huxleyi* stain B92/11) did not report any significant responses in terms of productivity or growth (Rost et al. 2002, 2006; Zondervan et al. 2002). In contrast, our multifactorial experiment demonstrated a clear moderating role for photoperiod upon elevated $p\text{CO}_2$ exposure for several isolates/species. However, while some patterns were evident (i.e., decreased growth rates with elevated $p\text{CO}_2$ under the shorter photoperiod), the interaction of $p\text{CO}_2$ and photoperiod ultimately yielded a more complex set of responses among the isolates. For *E. huxleyi*, such a trend is consistent with previous evidence for substantial intraspecific variation (Langer et al. 2006, 2009), whereas few studies have considered the isolates included in our study. Together, our data indicated general functional responses for Ci assimilation among isolates:

i. *E. huxleyi* stain RCC962 and *G. oceanica*: POC per cell (photosynthesis) increased and cells became larger, whereas PIC per cell (calcification) decreased and coccospheres thinned under elevated $p\text{CO}_2$ for the 14 : 10 h photoperiod. $p\text{CO}_2$-driven increases of photosynthesis and reductions of calcification were exacerbated and dampened, respectively, under continuous light. This was accompanied by larger cells and thicker coccospheres for *E. huxleyi* stain RCC962 relative to smaller cells and thinner coccospheres for *G. oceanica*.
ii. *E. huxleyi* stains 70-3, 124-3, and NZEH: photosynthesis and calcification increased, cells became larger, and coccospheres thickened with elevated $p\text{CO}_2$ for the 14 : 10 photoperiod. $p\text{CO}_2$-driven increases to photosynthesis and calcification were lessened under continuous light, with cells becoming smaller while coccospheres remained thickened.
iii. *C. braarudii*: photosynthesis and calcification increased but cells became smaller with a thinner coccospheres under elevated $p\text{CO}_2$ for the 14 : 10 h photoperiod. The same response, except for unchanged calcification (PIC per cell), was observed under elevated $p\text{CO}_2$ with continuous light.

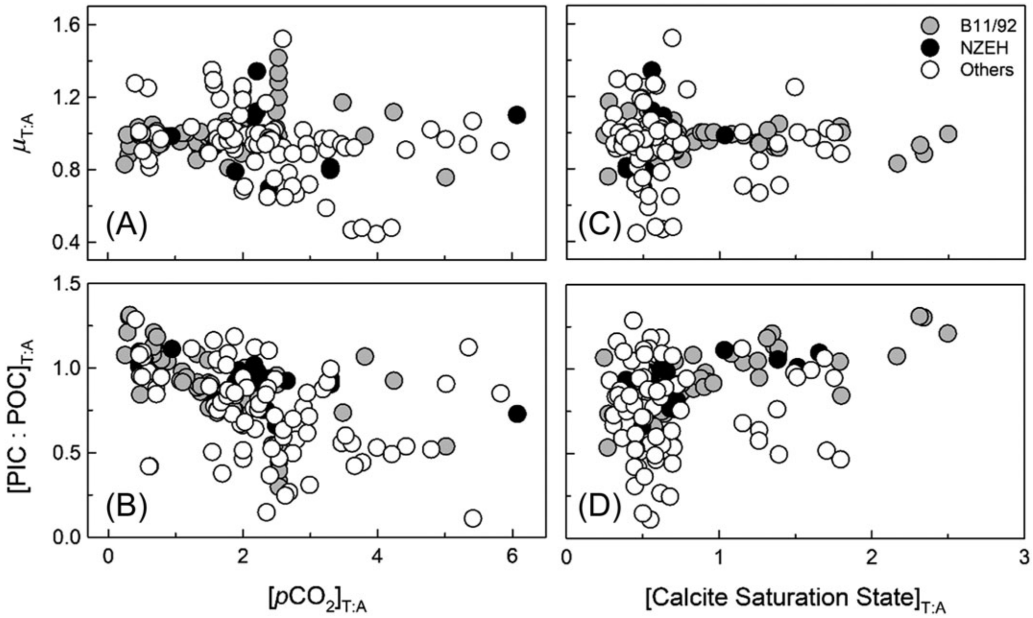

Fig. 3. Changes in growth rate (μ) and calcification (PIC : POC) of strains of *E. huxleyi* (B11/92, NZEH, and all other strains) with changing pCO$_2$ **(A and B)** and calcite saturation state **(C and D)**. Data are from previously published works (see Supporting Information Table S3) and normalized to control values (T = "treatment" values, A = "ambient" values).

In agreement with our original hypothesis, these functional responses always led to an overall decrease in PIC : POC with elevated pCO$_2$, which was notably lessened when day length was longer. We observed differences in the extent of this response between isolates/taxa, which presumably reflects differences in the strength of the CCM (Rost et al. 2002, 2006) and/or mechanism of Ci acquisition for photosynthesis and/or calcification (Rickaby et al. 2010; Meyer and Riebesell 2015). It is typically accepted that coccolithophores have an inefficient Ci pump (Rost et al. 2003), but the vast majority of this data comes from only examining *E. huxleyi*. This species has a low affinity for external CO$_2$ and has been shown to utilize bicarbonate (Zondervan et al. 2001; Rost et al. 2002; Trimborn et al. 2007). *E. huxleyi* also has low or undetectable carbonic anhydrase (CA) activity (Nimer et al. 1997). As such, calcification has previously been proposed as a means of driving photosynthesis by directly providing CO$_2$ (Nimer and Merrett 1996; Sikes et al. 2009).

Previous work has demonstrated that *G. oceanica* relies mostly on simple diffusion of CO$_2$, but sometimes utilizes external CAs in a manner similar to *E. huxleyi* (see Rickaby et al. 2010). Our observations of *G. oceanica* showing a generally (but not exclusively) similar response to *E. huxleyi* stain RCC962 vs. the other *E. huxleyi* isolates could indicate that these taxa fall across a spectrum of reliance upon external CAs and/or simple diffusion. In contrast, carbon for both photosynthesis and calcification by

Table 3. Multivariate output between environmental variables and (i) growth rate or (ii) PIC : POC. Environmental variables were temperature (*T*), salinity (*S*), light intensity (*E*), photoperiod (L : D), nitrogen-to-phosphate ratio (N : P), total alkalinity (A_T), as well as *E. huxleyi* isolate (see Supporting Information Table S3). Values for pCO$_2$, as well as the independent variables, were values normalized as the pCO$_2$ treatment relative to pCO$_2$ control (ambient, T : A; see main text) and with all values = 1 removed to avoid weighting by ambient data. The BEST match permutation test shown is that resulting in the highest correlation coefficient (ρ) from the resemblance matrices of the environmental data; the variables identified were subsequently included in the MSR.

		BEST			MSR			
Independent variable	**Taxa (*n*=)**	***ρ***	***p***	**Variables**	**Model**	***F***	***r*²**	***p***
$\mu_{T:A}$	All (155)	0.182	<0.01	L : D, N : P	(−0.0113 · L : D)+1.177	15.8	0.095	<0.001
	B92 (55)	0.342	<0.05	L : D, T, N : P	(0.0394 · T) + 0.389	32.2	0.378	<0.001
	NZEH (14)	0.425	0.103	T, E, L : D	(0.0259 · L : D)+0.557	14.0	0.539	0.003
PIC : POC$_{T:A}$	All (159)	0.200	<0.01	S, T, [CO$_2$]$_{T:A}$	(−0.107 · [CO$_2$]$_{T:A}$)+(0.017 · T)+0.768	27.1	0.259	<0.001
	B92 (55)	0.311	<0.05	T, [CO$_2$]$_{T:A}$	(−0.106 · [CO$_2$]$_{T:A}$)+(0.0595 · T)+1.991	35.4	0.576	<0.001
	NZEH (20)	0.505	<0.01	T, [CO$_2$]$_{T:A}$	(−0.041 · [CO$_2$]$_{T:A}$)+(0.0301 · T)+0.454	7.92	0.482	0.004

C. braarudii is derived from one shared internal pool of DIC actively transported into the cell, unless C_T concentrations are high enough in the surrounding seawater for cells to rely on simple CO_2 diffusion (Rickaby et al. 2010). This perhaps explains why this species responded differently to the other taxa tested here. Unfortunately, without a full mechanistic analysis of the carbon assimilation pathways amongst the different isolates, resolving the underlying basis for these differences is not currently possible.

Light availability is known to affect Ci acquisition in *E. huxleyi*, with both continuous light (Zondervan et al. 2001) and high irradiances (Kottmeier et al. 2014; Zhang et al. 2015) inhibiting HCO_3^- uptake and resulting in a greater dependence on CO_2 diffusion. Both CO_2 and high light have been reported to interactively cause a H^+ driven inhibition of HCO_3^- uptake (Kottmeier et al. 2016), which may explain why continuous light appears to dampen the CO_2-driven enhancement of POC per cell (at least for the temperate isolates). Cells generally grew more rapidly under continuous light (Supporting Information Table S2), leading to a higher Ci requirement. However, the reliance on CO_2 diffusion imposed by both continuous light and elevated pCO_2 presumably does not allow an adequate Ci supply to be maintained, since photosynthesis is not as enhanced despite external Ci concentrations being high. At the same time, PIC per cell either increased with CO_2 (*E. huxleyi* stains NZEH, 70-3, and 124-3) or was at least not as impaired by CO_2 as it was under the 14 : 10 h photoperiod (*E. huxleyi* stain RCC962 and *G. oceanica*). This is possibly in order to use H^+ generated by calcification to drive external CAs, a mechanism previously observed in *G. oceanica* (Rickaby et al. 2010), as a means of "overcoming" the imposed reliance on CO_2 diffusion and keeping up with Ci demand.

The functional groupings we observed for the interactive photoperiod-pCO_2 responses at face value appear to correspond with biogeographic origin, i.e., "temperate" (*E. huxleyi* stains NZEH, 70-3, and 124-3 and *C. braarudii*) vs. "tropical" (*E. huxleyi* stain RCC962 and *G. oceanica*) isolated species (see Table 1). Considerable phenotypic variability is known to exist between isolates of *E. huxleyi* (Iglesias-Rodriguez et al. 2006; Müller et al. 2015), although past efforts have failed to fully reconcile differences in isolate environmental history with OA responses (Langer et al. 2009; Findlay et al. 2011; Blanco-Ameijeiras et al. 2016). Furthermore, the previous pCO_2 responses under both 14 : 10 h and continuous light regimes for the temperate *E. huxleyi* strain B92/11 (Rost et al. 2002, 2006; Zondervan et al. 2002) are arguably more similar to those for our "tropical" then "temperate" isolates.

A major potential source of variability underpinning our observations is whether the experimental conditions (e.g., growth temperature and instantaneous irradiance) we examined confound direct comparisons of our observations across taxa. While we see consistencies in trends across taxa with two apparent functional groupings, we cannot determine whether these reflect exposure to suboptimum or optimum growth conditions equally across all isolates. For example, 20°C is below the growth optimum (25°C) for *G. oceanica* (Rhodes et al. 1995; Buitenhuis et al. 2008). Elevated temperatures have also been reported to decrease PIC per cell in coccolithophores (Langer et al. 2007; Feng et al. 2009) and sometimes cause malformation in coccoliths (Gerecht et al. 2018), although this is not consistent across studies (Feng et al. 2008; Sett et al. 2014). High irradiances may cause greater sensitivity to CO_2 (e.g., *G. oceanica*), and can shift the CO_2 optima for growth, calcification, and photosynthesis to lower concentrations (Zhang et al. 2015). CO_2 sensitivity in *E. huxleyi* has been shown to be dependent on photon flux density, with CO_2 effects observable above 150 μmol m^{-2} s^{-1} (Rost et al. 2002; Zondervan et al. 2002). In the current study, cultures were maintained in ~ 150 μmol m^{-2} s^{-1}, and while this is within the limits of typical saturating light levels for *E. huxleyi* under ambient CO_2 conditions (Nimer and Merrett 1993), growth does not become inhibited until much higher irradiances under high CO_2 conditions (Zhang et al. 2015). Tong et al. (2016) demonstrated that *G. oceanica* PIC per cell is highest in low light conditions (50 μmol m^{-2} s^{-1}), but daily PIC production between 50 and 190 μmol m^{-2} s^{-1} is not significantly different. As such, the distinct response observed in our two tropical isolates may in fact be driven by very different suboptimal growth conditions compared to the temperate isolates; i.e., the low growth temperature used generally inducing higher sensitivity to CO_2, which is then coupled with a high light dose to induce the more inhibited growth observed under continuous light.

Although we observed a significant interactive effect of photoperiod upon the pCO_2 response of μ and PIC : POC (Fig. 1; Table 2) across all isolates we examined, a strong moderating role for photoperiod across our broader analysis of past studies was not observed (Table 3; with the exception of NZEH growth rate). Consistent with previous analyses, we also observed a general negative trend of CO_2 on PIC : POC (Hendriks et al. 2010; Findlay et al. 2011), although with much heterogeneity between studies (Meyer and Riebesell 2015). This may reflect differences in carbonate manipulation method across studies, which can significant impact on POC per cell (and hence PIC : POC) but not PIC per cell (Meyer and Riebesell 2015), as well as differences in the way pCO_2 is calculated (Hoppe et al. 2012). However, the lack of covariance most likely highlights that different aspects of coccolithophore physiology are sensitive to different characteristics of the carbonate system (Bach et al. 2011, 2015; Meyer and Riebesell 2015), as observed in our controlled experiments across isolates. The lack of evidence for a moderating role of photoperiod presumably reflects the extent with which it has varied (compared to other factors regulating growth) across prior studies.

Our statistical models indicated that salinity and temperature appear to be significant factors driving the sensitivity of PIC : POC (and light influencing growth rate) to elevated pCO_2 (Table 3) across past studies. This could further support the notion that the "tropical" vs. "temperate" responses observed during the culture experiments reflect different proximities to environmental optima. Previous meta-analyses showed that differences in nutrient regime and A_T (Findlay et al. 2011), as well as the method of carbonate manipulation (Meyer and Riebesell 2015), have a strong regulatory effect on PIC : POC response to elevated CO_2. These studies used smaller data sets than the analysis presented here, which means the range of variance for the factors included will be different between studies. Photoperiod may not return as a significant variable simply because it is modified to a much smaller extent across experiments. Additionally, these analyses are likely further confounded by differences in achieved growth rates across studies, which can have a significant effect on PIC : POC (Daniels et al. 2014).

In summary, our results demonstrate how differences in photoperiod can alter the observed physiological responses to $p\mathrm{CO_2}$ in coccolithophores. Differences in functional response between the tropical and temperate isolates may reflect suboptimal growth conditions for *E. huxleyi* stain RCC962 and *G. oceanica*, and thus further highlight how light and $p\mathrm{CO_2}$ availability interact to influence growth and productivity depending on physiological optima to which cells are poised to utilize resources. This underlines the need to further resolve the complex nature of environmental optima such as light (including L : D cycle; Zondervan et al. 2001), temperature (Sett et al. 2014) and nutrient availability (Lefebvre et al. 2011; Müller et al. 2017) that different species and isolates are acclimated to and how these interact with CO_2 availability.

Our results have significant implications for predictions of the response of coccolithophores to OA in the global ocean. Although we have shown that coccolithophores will continue to grow in future oceans, the way in which OA changes this growth will be dependent on location, since natural L : D cycles are dictated by latitude. The nature in which elevated CO_2 changed the cellular organic and inorganic carbon content was dependent on L : D cycle in our culture experiments, thus changes to biogeochemical cycles (and in particular carbon cycling) as climate change and OA progress will possibly be dependent on latitude. Longer photoperiods dampened the overall impacts of elevated CO_2, which means that OA could have more negative impacts closer to the equator, where L : D cycles do not fluctuate much around 12 : 12 h. However, as sea surface temperatures increase, phytoplankton have been migrating to higher latitudes (Thomas et al. 2012), and tropical plankton species have been reported as far north as the North Sea (Barnard et al. 2004) and the Arctic Circle (Bjørklund et al. 2012). Changes in day length as these populations move away from the equator may also provide some refuge from the impacts of increasing $p\mathrm{CO_2}$, but it is important to note that OA will progress much more rapidly at higher latitudes (McNeil and Matear 2008). Our results further demonstrate that a better understanding how other environmental variables interact with elevated $p\mathrm{CO_2}$ is needed to better predict how different areas of the global ocean will change over the coming century.

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Conflict of Interest

None declared.

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