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Interactive Effects of CO_2, Temperature, and Nutrient Limitation on the Growth and Physiology of the Marine Coccolithophore *Emiliania huxleyi* (Prymnesiophyceae)

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Abstract: The marine coccolithophore *Emiliania huxleyi* (strain PnB 272 B10) was grown in a continuous culture system on a 12:12 light:dark cycle at temperatures from 10 °C to 28 °C under either nutrient-replete or nitrate-limited conditions and at either 493 ppmv (low) or 1003 ppmv (high) partial pressures of CO_2 (pCO_2). The maximum and minimum nutrient-replete growth rates were 0.751 d^{-1} at 20 °C and 0.365 d^{-1} at 10 °C, respectively. Ratios of particulate organic carbon (POC) to particulate inorganic carbon (PIC) were independent of pCO_2 and of the degree of nitrate limitation but were temperature dependent and lower at 10 °C and 28 °C than at intermediate temperatures. Nutrient-replete growth rates were higher at high versus low pCO_2 at 25 °C but did not differ significantly at other temperatures. Ratios of POC to particulate nitrogen (PN) were independent of temperature under nutrient-replete conditions (mean ± standard deviation = 5.07 ± 0.48 g g^{-1}) and under nitrate-limited conditions at half the nutrient-replete growth rates at the same temperature and pCO_2 (5.52 ± 0.60 g g^{-1}), but POC/PN ratios were significantly higher at low pCO_2 (7.26 ± 0.88 g g^{-1}) versus high pCO_2 (5.52 ± 0.59 g g^{-1}). Ratios of POC to chlorophyll *a* were positively correlated with temperature under nitrate-limited conditions and negatively correlated with temperature under nutrient-replete conditions. The ratio of productivity indices under nitrate-limited and nutrient-replete conditions was positively correlated with temperature and exceeded 1.0 at temperatures of 15 °C or higher. Growth efficiencies were significantly reduced at suboptimal and supraoptimal temperatures and by a transition from nutrient-replete to nitrate-limited conditions, but they were not significantly different under high and low pCO_2 conditions. Calcification by this strain of *E. huxleyi* appeared to be very insensitive to environmental conditions over the range of conditions that we examined.

Keywords: climate change; calcification; photosynthesis; respiration; phytoplankton

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1. Introduction

Ocean acidification is expected to adversely affect calcifying organisms because increases of [CO_2] are accompanied by a decrease in the concentration of carbonate ions and hence a decrease in the saturation state of $CaCO_3$ (Ω). Coccolithophores are the dominant pelagic calcifiers in the ocean, and *Emiliania huxleyi* is the most numerous and widely distributed of the coccolithophores [1]. Caldeira and Wickett [2] have estimated that the pH of surface seawater will fall by 0.7 by the year 2250 and will remain at that level for another 500 years if all known fossil fuel reserves are burned. Because of the associated reduction in the concentration of carbonate ions, calcification is very sensitive to pH and "essentially does not occur below *c*. pH 7.6" [3] (p. 747). However, controlled experiments have shown that not all marine calcifiers respond as expected to reductions of Ω, and the rate of calcification by a few species is negatively correlated with Ω in the range 0.5–2.0 [4]. It is now apparent that there is considerable variation among coccolithophores with respect to their sensitivity to ocean acidification [5–9] and that even within the species *E. huxleyi*

there are substantial differences between strains in responses to changes of pCO_2 that probably have a genetic basis [10,11].

The present study was motivated in part by the results of the study of Feng et al. [12], who have concluded, "Other environmental drivers may be equally or more influential than CO_2 in regulating the physiological responses of *E. huxleyi* [to climate change]". We therefore undertook a comprehensive assessment of the likely impact of changes in abiotic conditions on the growth and calcification of a strain of *E. huxleyi*. We examined the effects of changes in temperature from suboptimal to supraoptimal temperatures, nitrate limitation of growth rate, and an increase in pCO_2 from ~500 to ~1000 ppmv. The null hypotheses were (1) increasing the pCO_2 from ~500 to ~1000 ppmv and varying the temperature from suboptimal to supraoptimal temperatures would have no effect on the growth rate and/or calcification of *E. huxleyi*, (2) there would be no interactive effects of pCO_2, temperature, and degree of nitrate limitation on calcification by *E. huxleyi*, and (3) stresses associated with reductions of pH, suboptimal or supraoptimal temperatures, and nitrate-limited versus nutrient-replete growth conditions would not be reflected by reductions of growth efficiency. We also tested the hypothesis of Goldman [13] that the organic carbon-to-nitrogen ratio of the cells would be a unique function of their relative growth rate.

2. Materials and Methods

2.1. Culturing Conditions

The coccolithophore *E. huxleyi* was obtained from the laboratory of Dr. Iglesias-Rodriguez at the University of California, Santa Barbara (strain PnB 272 B10). The culture was collected on 15 September 2015 from a water sample taken from the California Current near Santa Barbara. *E. huxleyi* was isolated on 16 September 2015 and has been maintained as a unialgal culture in the laboratory of Dr. Iglesias-Rodriguez since then. We acquired the culture from Dr. Iglesias-Rodriguez in January 2021 and began maintaining it at a temperature of ~22 °C on a 12:12 light:dark cycle of illumination. The light (50 mmol quanta m^{-2} s^{-1} of 400–700 nm radiation) was provided by a bank of light-emitting diodes (LEDs). The growth medium was f/2 medium [14,15] that was sterile-filtered (0.2 μm) into autoclaved, 125-mL glass flasks.

Continuous culture studies were carried out in a system identical to that described by Laws et al. [16], except that the growth medium contained no added silicate. The experiments were conducted from February 2021 through February 2023 and included a total of 20 steady states at five temperatures (10, 15, 20, 25, and 28 °C), low and high pCO_2 (~500 and ~1000 ppmv, respectively), either nitrate-limited or nutrient-replete growth conditions, and an irradiance of 50 mmol quanta m^{-2} s^{-1} of 400–700 nm radiation provided by a bank of LEDs. The growth chamber was a double-walled, glass reaction flask (Ace Glass, Inc., Vineland, NJ, USA) with a working volume of 2130 mL. Temperature was controlled to within 0.1 °C by circulating water from a thermoregulated water bath (model DC10, Haake Mfg. Co., De Soto, MO, USA) through the space between the inner and outer walls of the growth chamber. An initial set of experiments was carried out under nutrient-replete conditions to determine, inter alia, the nutrient-replete growth rate as a function of temperature and pCO_2. The irradiance was measured at the center of the empty growth chamber with a quantum scalar light meter (Biospherical Instruments Model QSL 2100, San Diego, CA, USA). The growth medium consisted of artificial seawater enriched with vitamins and inorganic nutrients sufficient to produce a salinity of 35 and a total alkalinity (TA) of 2.4 meq kg^{-1}. Trace metals were added in an ethylenediaminetetraacetic acid-buffered solution as specified by Sunda and Hardison [17]. The nitrate concentrations in the growth medium used for the nitrate-limited and nutrient-replete studies were 20 μM and 882 μM, respectively. Concentrations of phosphate and vitamins were identical to those specified for f/2 medium; silicate was omitted. The growth chamber and 40-L medium reservoir were sterilized by autoclaving, and the growth medium was sterile-filtered (0.2 μm) into the reservoir. Fresh medium was introduced into the growth chamber at a controlled rate via a peristaltic pump. The culture was stirred by a Teflon-coated

magnetic stir bar and via bubbling with sterile air (0.2-µm sterile filter), which also served to drive the overflow by pressurizing the headspace above the culture. The pCO_2 in the growth chamber was controlled by bubbling sterile air admixed with regulated amounts of pure CO_2 (Bone Dry 3.0 Grade) through the medium in the nutrient reservoir and from there into the growth chamber. The pCO_2 of the inflow air was monitored continuously by an infrared absorption-based pCO_2 m (AZ-0004; https://www.CO2meter.com, Ormond Beach, FL, USA). The low pCO_2 treatment involved bubbling with sterile laboratory air, and the low pCO_2 of that air was ~500 ppmv.

2.2. Inorganic Carbon System

The pH and total alkalinity of the growth chamber were monitored shortly after the start of the photoperiod, the middle of the photoperiod, and shortly before the end of the photoperiod. The pH was measured on the total hydrogen ion scale based on the spectrophotometric method described in SOP 6b by Dickson et al. [18]. Absorbances for pH determination were measured in a 5-cm glass cuvette on a Spectronic Helios Delta UV–Visible spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Total alkalinity was determined based on the single-point titration described by Breland and Byrne [19]. Briefly, 100 mL of culture was brought to 25 °C and titrated to an approximate pH of 4.0–4.3 with standardized HCl (Thermo Fisher Scientific) diluted to 0.10 N in 0.6 M NaCl. The solution was stirred vigorously during the titration and for 5 min continuously. Approximately 0.5 mL of 2 mM bromocresol green (pH 4.2) was then added to the titrated solution. The precise endpoint pH of the resultant solution was then determined based on its absorbance at 750, 616, and 444 nm using the same configuration used for the pH measurements (vide supra). The dissolved inorganic carbon (DIC) concentration was calculated based on pH and total alkalinity using the equations and equilibrium constants in Zeebe and Wolf-Gladrow [20]. The pCO_2 in the growth chamber was calculated based on the DIC concentration and total alkalinity in accordance with EU [21] using the CO2calc software package [22] version 1.1. Table S1 in the Supporting Information summarizes the characteristics of the growth medium as a function of temperature and pCO_2.

2.3. Sampling and Analysis

The optical density of the culture was recorded at the same time of day each day during the light period. A sample of the culture was placed in a 5-cm cuvette, and the extinction coefficient at 750 nm was measured with a Spectronic Helios model Delta spectrophotometer (Thermo Fisher Scientific). Dilution rates of the growth chamber (volume of overflow during an interval of time) were recorded at the same time. Sampling of the growth chamber for characteristics other than optical density did not begin until the optical density had stabilized (coefficient of variation no more than 2%) and four doubling times (i.e., generation times) had elapsed at each dilution rate. Growth rates in continuous cultures are equal to the dilution rates once optical densities have stabilized. Once begun, sampling for chlorophyll a (Chl *a*) and for particulate carbon (PC) and particulate nitrogen (PN) was completed immediately after the start of the photoperiod and just before the end of the photoperiod.

The experimental design was expected to reveal effects of acclimation but not of adaptation, e.g., [23]. Samples were collected via a three-way valve; they were not collected from the growth chamber overflow. The growth chamber was always overflowing when a sample was withdrawn (i.e., the interval between two consecutive samples was always long enough to allow the volume removed for one sample to be replaced before the next sample was withdrawn). The lowest dilution rate was 0.152 d^{-1} under nitrate-limited conditions, high pCO_2, and a temperature of 10 °C. The medium pumping rate was therefore (0.152 d^{-1}) (2130 mL) = 324 mL d^{-1} or 162 mL every 12 h. Under these conditions, we collected only two 50-mL samples at lights-on and another two 50-mL samples at lights-off from the growth chamber each day after the optical density had been constant for four doubling times = 18 days. Because we wanted a total of five replicate samples for both

PN and Chl *a* and 10 replicate samples for PC (vide infra) at both lights-off and lights-on, sampling at this growth rate required 18 + (5 + 5 + 10)/2 = 28 days. Sampling at higher growth rates obviously required less time.

Samples for determination of PC, PN, and Chl *a* were collected on glass fiber filters (GF/F; Whatman, UK). Concentrations of Chl *a* were measured in methanol extracts on a Cary model 50 UV–visible spectrophotometer (Agilent Technologies, Santa Clara, CA, USA) using the protocol of Holm-Hansen and Riemann [24]. PC and PN were measured on an elemental analyzer (model CE 440; Exeter Analytical, Chelmsford, MA, USA).

Concentrations of particulate inorganic carbon (PIC) were distinguished from particulate organic carbon (POC) by fuming the filters collected for PC analysis with concentrated HCl for 12 h to drive off PIC [25,26]. The PIC was then equated to the difference between the PC on replicate filters that were not acid-fumed and the POC that remained on the filters after acid-fuming.

Rates of POC production in the growth chamber during the photoperiod were calculated by integrating the differential equation:

$$\frac{\mathrm{d(POC)}}{\mathrm{dt}} = \pi - \mu \cdot \mathrm{POC} \tag{1}$$

where π is the rate of POC production (mg C h^{-1}), and μ is the dilution rate per hour. If π is constant during the photoperiod, the solution to Equation (1) is

$$\mathrm{POC}_{12} = \mathrm{POC}_0 e^{-12\mu} + \frac{\pi}{\mu}\left(1 - e^{-12\mu}\right) \tag{2}$$

where POC_0 and POC_{12} are the POC concentrations at the beginning and end of the 12-h photoperiod, respectively. The rate of POC production during the photoperiod was then estimated from Equation (3):

$$\pi = \mu\left(\frac{\mathrm{POC}_{12} - \mathrm{POC}_0 e^{-12\mu}}{1 - e^{-12\mu}}\right) \tag{3}$$

Production rates calculated with Equation (3) were divided by the average of the Chl *a* concentrations at the beginning and end of the photoperiod to estimate the productivity index during the photoperiod in units of g C·(g Chl *a*)$^{-1}$·h^{-1}.

The rate of change of the POC concentration in the growth chamber during the 12-h dark period was assumed to be described by Equation (4):

$$\frac{\mathrm{d(POC)}}{\mathrm{dt}} = -(\mu + \mu_r) \cdot \mathrm{POC} \tag{4}$$

where μ_r is the dark respiration rate. Integrating Equation (4) over the one-half day of darkness and rearranging gives

$$\mu_r = 2\ln\left(\frac{\mathrm{POC}_{12}}{\mathrm{POC}_0}\right) - \mu \tag{5}$$

where POC_0 and POC_{12} are the POC concentrations at the beginning and end of the photoperiod, respectively, and μ_r and μ have units of d^{-1}. Muscarella et al. [27] have defined growth efficiency as the proportion of assimilated carbon converted into biomass. For a photosynthesis organism growing on a 12:12 L:D cycle, we defined the analogous efficiency to be the proportion of carbon fixed during the photoperiod that was retained in biomass (i.e., not respired at night). We calculated that efficiency with Equation (6).

$$\text{efficiency} = \frac{2\mu}{2\mu + \mu_r} \tag{6}$$

2.4. Statistical Analysis

All statistical tests were carried out using Matlab version R2024a software. Tests for normality and homoscedasticity were made using a Kolmogorov–Smirnov test (kstest) and Bartlett's test (vartestn), respectively. When the assumptions of normality and homoscedasticity could be accepted, tests for treatment effects were carried out using paired *t*-tests (ttest) or one-way analysis of variance (anova1). Pearson's correlation coefficient (corr) was used to judge the significance of correlations between pairs of variables. Linear regression analyses were carried out using model I linear regression (polyfit).

3. Results

Measurements of pH and TA in the growth chamber indicated that the pCO_2 in the growth chamber was 493 ± 29 ppmv in the low pCO_2 treatment and 1003 ± 83 in the high pCO_2 treatment. The pH averaged 7.8 ± 0.03 and 7.6 ± 0.02 under low and high pCO_2 conditions, respectively.

Under nutrient-replete conditions, *E. huxleyi* was able to grow at temperatures of 10–28 °C (Figure 1). At both high and low pCO_2, growth was most rapid at 20 °C. The effect of pCO_2 on growth rates depended on the temperature. There was virtually no effect of pCO_2 at temperatures of 10 and 28 °C. At temperatures of 15, 20, and 25 °C, growth rates were higher at high pCO_2 than at low pCO_2, but the difference was statistically significant only at 25 °C (analysis of variance, $p = 1.2 \times 10^{-5}$).

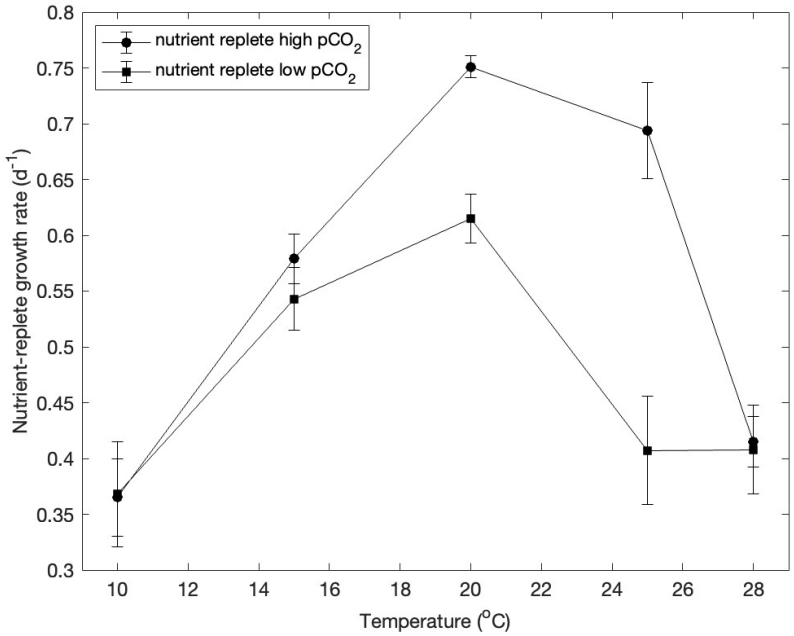


Figure 1. Nutrient-replete growth rates of *E. huxleyi* as a function of temperature and pCO_2. Values are means of 7–9 measurements made on consecutive days after optical densities had stabilized and four doubling times had elapsed. Error bars are standard deviations of the mean values (i.e., standard errors).

The saturation states of calcium carbonate (Ω) were higher at low pCO_2 than at high pCO_2 (paired *t*-test, $p = 0.002$), were higher under nutrient-replete conditions than under nitrate-limited conditions (paired *t*-test, $p = 0.0002$), and were positively correlated with temperature (Pearson's $r = 0.50$, $p = 0.02$) (Figure 2).

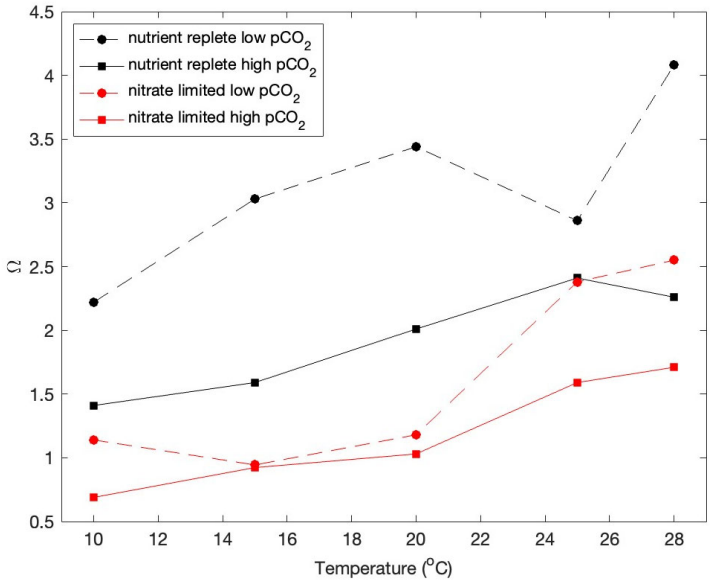


Figure 2. Saturation state (Ω) of calcium carbonate *E. huxleyi* growth chamber as a function of temperature for high and low pCO_2 and for nutrient-replete and nitrate-limited growth conditions. Values of carbonate concentrations were estimated from measurements of total alkalinity and pH at the beginning, middle, and end of the photoperiod and equations in Zeebe and Wolf-Gladrow [20]. Calcium ion concentrations were equated to the concentration of calcium ions in seawater with a salinity of 35.

Ratios of POC to PIC (Figure 3) did not differ significantly between nutrient-replete and nitrate-limited conditions (paired *t*-test, $p = 0.75$), and they did not differ significantly between high pCO_2 and low pCO_2 treatments (paired *t*-test, $p = 0.87$). However, they varied significantly as a function of temperature (one-way analysis of variance of log-transformed data, $p = 0.058$).

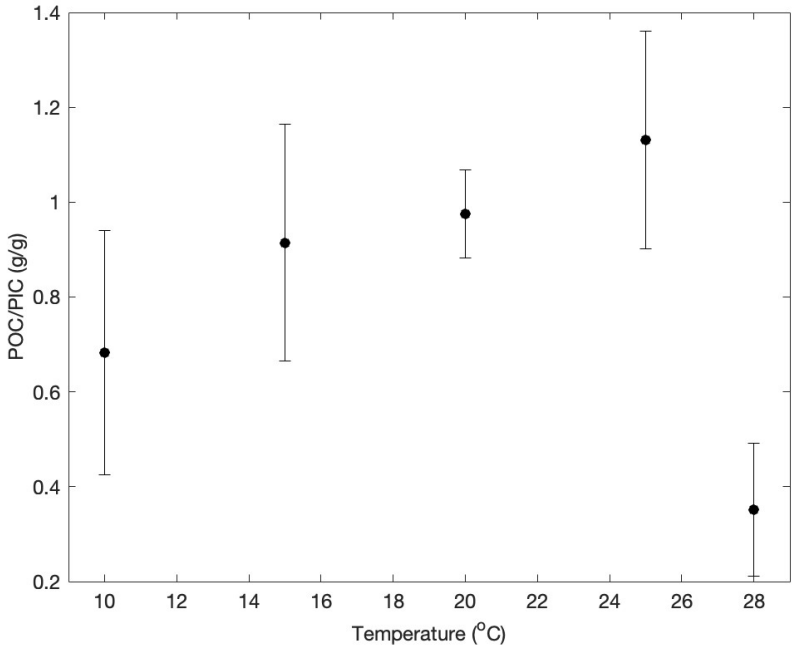


Figure 3. Ratios of POC to PIC versus temperature. Values are means of ratios at high and low pCO_2 and nutrient-replete and nitrate-limited conditions. Error bars are standard deviations of the mean values.

POC/PN ratios (Figure 4) were independent of temperature (Pearson's r = 0.002, p = 0.99), but they were higher under nitrate-limited than nutrient-replete conditions (paired t-test, p = 0.023), and they were higher under low pCO_2 than high pCO_2 conditions (paired t-test, p = 0.048).

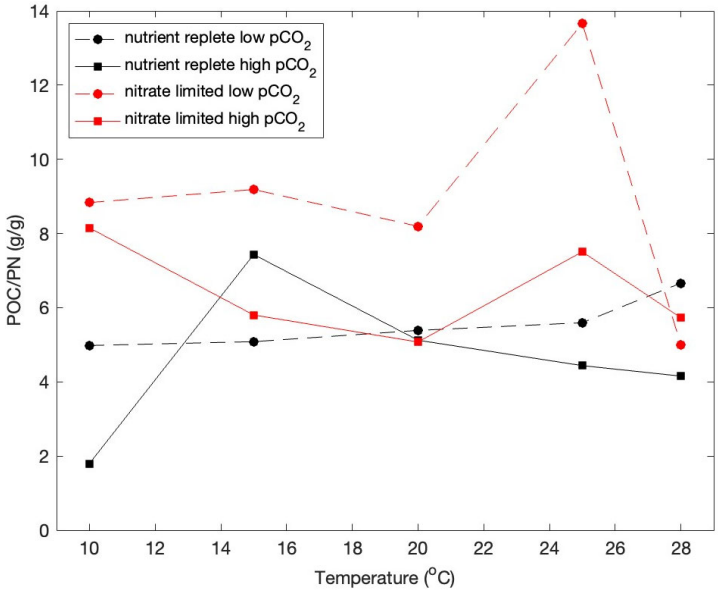


Figure 4. Molar POC/PN ratios of *E. huxleyi* as a function of temperature under nitrate-limited and nutrient-replete conditions and high pCO_2 and low pCO_2.

Ratios of POC to Chl *a* did not differ significantly between high pCO_2 and low pCO_2 treatments (paired t-test, $p > 0.05$), but their dependence on temperature was very different under nitrate-limited and nutrient-replete conditions (Figure 5). POC/Chl *a* ratios were positively correlated with temperature under nitrate-limited conditions (Pearson r = 0.96, p = 0.01), negatively correlated with temperature under nutrient-replete conditions (Pearson $r = -0.91$, p = 0.033), and they were higher under nitrate-limited than nutrient-replete conditions (sign test, p = 0.02).

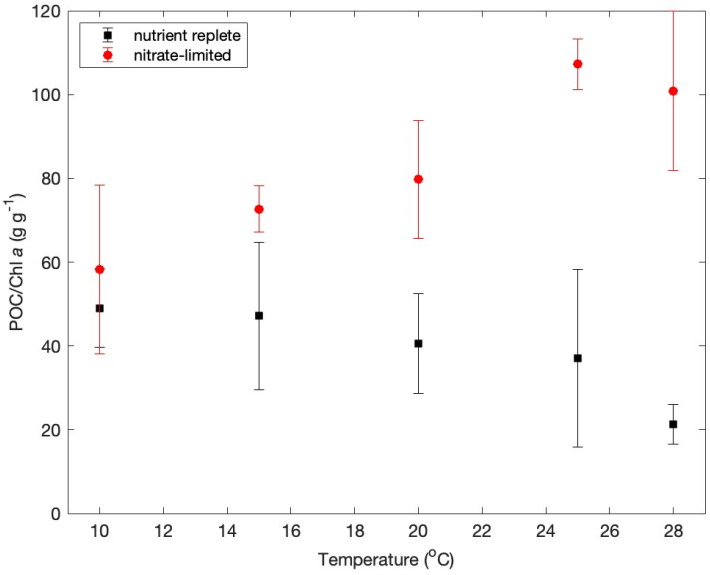


Figure 5. POC/Chl *a* ratios as a function of temperature under nutrient-replete and nitrate-limited conditions. Error bars show ranges of ratios under high pCO_2 and low pCO_2.

Productivity indices (Figure 6) did not differ significantly between high pCO_2 and low pCO_2 treatments (paired *t*-test, $p = 0.14$), but they were significantly higher under nitrate-limited conditions than nutrient-replete conditions at temperatures of 15 °C or higher (paired *t*-test, $p = 0.014$).

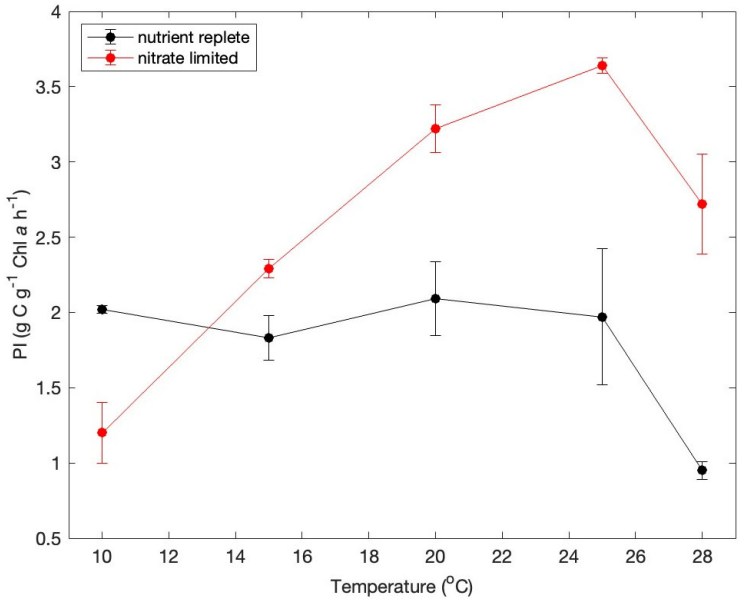


Figure 6. Productivity indices as a function of temperature under nitrate-limited and nutrient-replete conditions. Error bars are standard deviations of mean values under both high and low pCO_2 conditions.

Growth efficiencies were higher at high pCO_2 (mean ± standard deviation = 0.86 ± 0.08) than at low pCO_2 (mean ± standard deviation = 0.76 ± 0.03), but the difference was not significant (paired *t*-test, $p = 0.20$). However, growth efficiencies differed significantly between intermediate temperatures of 15–25 °C versus stressful temperatures of 10 and 28 °C (one-way anova, $p = 0.015$). Mean efficiencies were 0.94 and 0.36 at intermediate and stressful temperatures, respectively. Growth efficiencies also differed significantly between nutrient-replete and nitrate-limited conditions (paired *t*-test, $p = 0.05$). Mean efficiencies were 0.89 and 0.73 under nutrient-replete and nitrate-limited conditions, respectively.

4. Discussion

Raising the partial pressure of CO_2 in the atmosphere from its current level of ~423 parts per million (ppm) to ~1000 ppm is expected to have little positive effect on the growth rates of phytoplankton in the ocean for several reasons. Cell surface-to-volume considerations suggest that any positive effect would be most apparent for large cells, but previous experiments by Riebesell et al. [28] with large diatoms (*Ditylum brightwellii*, *Thalassiosira punctigera*, and *Rhizosolenia alata*) revealed a positive correlation between the concentration of dissolved CO_2 [CO_2] and growth rate only at [CO_2] less than 10–15 mM. Equilibrium concentrations of [CO_2] in the ocean are negatively correlated with temperature [20], and at a salinity of 35 and pCO_2 of 423 ppmv, they equal 26.6 mM and 11.2 mM at temperatures of 0 °C and 28 °C, respectively. Hence, there would seem to be no reason to think that higher partial pressures of pCO_2 would increase photosynthetic rates in the ocean. Hopkinson et al. [29] have estimated that the energy savings associated with down-regulation of the carbon concentrating mechanism in diatoms if [CO_2] were doubled could increase primary production by only a few percent. Subsequent experiments with the diatom *Thalassiosira weissflogii* by Goldman et al. [30] revealed no effect of varying pH and pCO_2 independently or in concert with the rates of growth, photosynthesis, or respiration of *T. weissflogii*. They concluded that the likely effects of ocean acidification on

the growth, photosynthesis, and respiration of diatoms and many other eukaryotic marine phytoplankton would be very small over the range of pCO_2 predicted for the 21st century.

The concentrations of [CO_2] are naturally higher in cold water than in warm water because of the temperature dependence of the Henry's Law constant [20]. In the Southern Ocean, for example, the high [CO_2] might therefore be expected to lead to enhanced use of CO_2 as opposed to bicarbonate as a source of inorganic carbon for photosynthesis. However, isotope disequilibrium studies carried out by Cassar et al. [31] and later by Tortell et al. [32] have revealed that bicarbonate accounts for 50% (56° S and 66° S, 172° W) to 65–90% (Ross Sea) of the inorganic carbon fixed by phytoplankton in the Southern Ocean. The implication of these laboratory and field studies has been that the increase of pCO_2 to ~2000 ppmv by the year 2250 as a result of fossil fuel burning [2] is unlikely to stimulate the photosynthetic uptake of inorganic carbon by more than a few percent.

For small phytoplankton such as *Prochlorococcus* and *Synechococcus*, surface area–to–volume considerations suggest that any increase in growth rates associated with an increase in pCO_2 would be negligible [28], and studies of *Synechococcus* CCMP 1629 by Laws and McClellan [33] have revealed that the growth rates of this isolate are in fact higher at a pCO_2 of 400 ppmv than at 1000 ppmv under almost all conditions of temperature from 20 to 45 °C and both high and low irradiance (300 and 50 mmol photons m^{-2} s^{-1}, respectively). The stress associated with lowering the pH of the growth medium is presumably the cause of the lower growth rates of *Synechococcus* at 1000 ppmv. The single exception to this pattern was the growth rate at 45 °C and high irradiance. Under those conditions, the nutrient-replete growth rate was 45% higher at 1000 ppmv than at 400 ppmv. Laws and McClellan [33] postulated that at 45 °C and high irradiance, losses associated with photorespiration may have been substantial and that, "The beneficial effect of increasing the pCO_2 may therefore have been the result of shifting the balance of competition between CO_2 and O_2 at the active sites of Rubisco".

Our results (Figure 1) clearly indicated that there was an interaction between the effects of temperature and pCO_2 on the growth rate of *E. huxleyi*. At clearly suboptimal and supraoptimal temperatures (10 and 28 °C, respectively), there was no effect of pCO_2 on growth rate. Increasing the pCO_2 enhanced growth rates at intermediate temperatures, but the effect was statistically significant only at 25 °C. At that temperature growth rates at high pCO_2 were close to optimal, but effects of photorespiration may have been significant at 493 ppmv pCO_2 because of the high temperature and shift of the balance of competition between the carboxylase and oxygenase roles of Rubisco. Because of the positive correlation between temperature and respiration rates [34], photorespiratory effects were presumably smaller at lower temperatures, and there was no significant difference between growth rates at high and low pCO_2 (vide supra). A temperature of 28 °C may have been sufficiently stressful that any benefit from lower rates of photorespiration was overwhelmed by other factors. Sett et al. [35] have also reported an interactive effect between temperature and pCO_2 on the growth rates of strains of *E. huxleyi* and *Gephyrocapsa oceanica*, but in their studies, the effect of the interaction was to shift the pCO_2 for optimum growth to higher values as the temperature was increased from low-to-intermediate values (10–20 °C) to high values (20–25 °C).

The dependence of Ω on temperature and pCO_2 is easily explained by the negative correlation between temperature and the solubility of CO_2 in seawater [20]. Simplistically, increasing the concentration of DIC at a fixed TA requires that some [CO_2] react with carbonate ions to form bicarbonate ions as follows:

$$CO_2 + H_2O + {CO_3}^{2-} \rightarrow 2{HCO_3}^{-} \quad (7)$$

The result is a reduction in the concentration of carbonate ions and hence of Ω. The lower values of Ω under nitrate-limited conditions reflect the fact that the residence time of cells in the growth chamber was twice as long under nitrate-limited versus nutrient-replete conditions because the nitrate-limited growth rates were half the nutrient-replete growth rates at the same temperature and pCO_2. The longer residence time in the growth chamber

allowed for more precipitation of $CaCO_3$ as well as more fixation of POC. The former resulted in a reduction in the TA in the growth chamber by an average of 0.55 ± 0.28 meq L^{-1}.

The effect of high pCO_2 on calcification has been a focus of several investigations [6,7,9]. In our experiments, Ω changed significantly between nutrient-replete and nitrate-limited conditions, between high and low pCO_2, and as a function of temperature, but only temperature significantly affected POC/PIC ratios (Figure 3). The effect of suboptimal and supraoptimal temperatures was clearly to suppress photosynthesis relative to calcification (Figures 1 and 3). Calcification in this case was relatively insensitive to temperature compared to photosynthesis.

Goldman [13] has hypothesized that the POC:PN ratios of phytoplankton are unique functions of their relative growth rates, where the relative growth rate is defined to be the dimensionless ratio of the growth rate to the nutrient-replete growth rate under otherwise identical conditions. The data available to test that hypothesis in 1980 came from multiple species grown under laboratory conditions, but all studies for a given species had been carried out at a fixed temperature and the same pCO_2. Our results with *E. huxleyi* provided an opportunity to test Goldman's [13] hypothesis over a range of temperatures from suboptimal to supraoptimal and at both high and low pCO_2. We found that the POC:PN ratios of *E. huxleyi* were remarkably constant at a relative growth rate of 1.0 (mean = 5.0 ± 0.5 g/g) and a relative growth rate of 0.5 (mean = 7.75 ± 0.8 g/g). There was no evidence of a temperature effect on the POC:PN ratios, but POC:PN ratios were significantly higher under low pCO_2 than high pCO_2 conditions at the same relative growth rate (vide supra). The mean difference was 1.7 ± 0.76 g/g. Our results were thus consistent with Goldman's [13] relative growth rate hypothesis with respect to the effects of nitrate limitation and temperature. The effect of pCO_2 on the POC:PN ratios at a fixed relative growth rate suggests that the pCO_2 can affect the composition of the cells in ways not anticipated in the original relative growth rate hypothesis [13]. The difference between the POC/PN ratios under high and low pCO_2 conditions presumably reflected acclimation of the cells in ways that tended to minimize effects on growth rates, which were not significantly different, except at 25 °C.

Balanced growth requires that the rates of the light and dark reactions of photosynthesis be equal [36–38]. Because light absorption by photosynthetic pigments is independent of temperature, whereas the rates of enzyme-mediated reactions are temperature-dependent, it is reasonable to expect that phytoplankton would allocate relatively more resources to the light reactions and fewer to the dark reactions with increasing temperature at a fixed irradiance. This rationale presumably explains the negative correlation between POC/Chl *a* ratios and temperature under nutrient-replete conditions (Figure 5). Under nitrate-limited conditions, a similar balance between the light and dark reactions can be achieved by diverting carbon from the dark reactions to storage products [36,37]. This line of reasoning presumably explains the positive correlation between POC/Chl *a* ratios and temperature under nitrate-limited conditions and of course the higher POC:PN ratios under nitrate-limited versus nutrient-replete conditions (Figure 4).

Productivity indices are the product of the POC/Chl *a* ratios and the growth rates during the photoperiod. Nitrate-limited growth rates were half the nutrient-replete growth rates at the same temperature and pCO_2, but POC/Chl *a* ratios were higher under nitrate-limited conditions, and the ratio of nitrate-limited POC/Chl *a* ratios to nutrient-replete POC/Chl *a* ratios steadily increased with increasing temperature. The result was that the ratio of nitrate-limited PIs to nutrient-replete PIs steadily increased with increasing temperature and exceeded 1.0 at temperatures of 15 °C and higher. The results in Figure 6 at temperatures of 15 °C and higher are contrary to the inference from fieldwork that PIs are positively correlated with nutrient sufficiency [39–41]. Laws and Bannister [42] have pointed out, however, that the positive correlation between Chl *a*/POC ratios and nutrient-limited growth rates confounds the use of PIs as metrics of nutrient limitation. This problem is clearly apparent in Figure 6, where the nutrient-replete PIs exceeded the nitrate-limited PIs only at 10 °C.

We expected that the stress associated with the reduction in pH from 7.8 at low pCO_2 to 7.6 at high pCO_2 might be reflected by a reduction in growth efficiency at high pCO_2, but the growth efficiencies were not significantly different. The reduction in growth efficiency from 0.89 under nutrient-replete conditions to 0.73 under nitrate-limited conditions is noteworthy because the warming of ocean surface waters is expected to increase thermal stratification and hence reduce inputs of nitrate from subsurface waters. Because growth rates in our nitrate-limited studies were half the corresponding nutrient-replete growth rates, the relative contribution of basal respiration would have been higher under nitrate-limited versus nutrient-replete conditions. This consideration likely accounts for the lower growth efficiency under nitrate-limited conditions. Temperature, however, exerted the most dramatic effect on growth efficiency. The losses to dark respiration were less than 10% of production during the photoperiod at intermediate temperatures, but those losses exceeded 60% at 10 and 28 °C. As phytoplankton become stressed by supraoptimal temperatures, they will likely be replaced by more thermotolerant species/strains.

Based on the results of this study, we rejected the null hypothesis that increasing the pCO_2 from ~500 to ~1000 ppm would have no effect on the growth rate of *E. huxleyi* strain PnB 272 B10. Increasing the pCO_2 increased the growth rate at the supraoptimal temperature of 25 °C. We accepted the null hypothesis that increasing the pCO_2 would have no effect on the calcification of *E. huxleyi* strain PnB 272 B10, and we accepted the null hypothesis that varying the temperature from suboptimal to supraoptimal temperatures would have no effect on calcification. The dependence of POC/PIC ratios on temperature (Figure 3) was due to temperature effects on photosynthesis, not calcification. We accepted the null hypothesis that there were no interactive effects of pCO_2, temperature, and degree of nitrate limitation on calcification by *E. huxleyi* strain PnB 272 B10. Calcification by this strain of *E. huxleyi* appeared to be remarkably robust over the range of conditions that we examined. We accepted the null hypothesis that the reduction in pH from 7.8 to 7.6 would have no effect on growth efficiency, but we rejected the null hypothesis that nitrate limitation and suboptimal or supraoptimal temperatures would have no effect on growth efficiency. Nitrate limitation and both suboptimal or supraoptimal temperatures clearly reduced growth efficiencies. With one caveat, we accepted Goldman's [13] relative growth rate hypothesis. The caveat concerned the effect of pCO_2 on POC/PN ratios, which were significantly higher at low pCO_2 than at high pCO_2 under otherwise identical conditions. The difference in POC/PN ratios presumably reflected acclimation by the cells to the doubling of pCO_2.

5. Conclusions

Several important conclusions emerge from this study. First, there was an interaction between the effects of temperature and pCO_2 on growth rates. The answer to what effect increasing the pCO_2 had on the growth rate depended on the temperature. When temperature became sufficiently stressful (either too high or too low), the pCO_2 had essentially no effect on the growth rate. At intermediate temperatures, raising the pCO_2 increased the growth rate if the temperature was sufficiently high, probably because the effects of photorespiration were significant, and raising the pCO_2 shifted the balance of competition between the roles of Rubisco as an oxygenase or carboxylase. Second, the degree of calcification of the cells appeared to be determined mainly by the time interval between cell divisions. Cells growing slowly accumulated more coccoliths than cells growing rapidly. Third, an important implication of our results for pelagic ecosystem functioning was that we did not see a cessation of calcification in the nitrate-limited, high-pCO_2 experiments. In all of those experiments, the pH was less than 7.6. This result contradicts the statement by Raven and Falkowski [3] (p. 747), that calcification "essentially does not occur below *c.* pH 7.6". Our high pCO_2 experiments, however, were carried out at a pCO_2 of ~1000 ppmv, and Caldeira and Wickett [2] have estimated that the pCO_2 of the atmosphere will rise to ~2000 ppmv by roughly the year 2250 if all known fossil fuels are burned. Furthermore, other strains of *E. huxleyi* may prove to be less tolerant to a reduction in Ω than PnB 272

B10. Future studies should therefore explore the effects of climate change—an increase of temperature, greater nutrient limitation, and increase in the pCO_2 to 2000 ppmv—on other strains of *E. huxleyi* to enable an informed assessment of the impact of climate change on this important species.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/w16223184/s1, Table S1: Summary of growth conditions and characteristics of *E. huxleyi* strain PnB 272 B10 grown on a 12:12 L:D cycle with irradiance of 50 mmol photons m^{-2} s^{-1}.

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