Interactive Effects of Ocean Acidification and Nitrogen-Limitation on the Diatom *Phaeodactylum tricornutum*

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**Abstract**

Climate change is expected to bring about alterations in the marine physical and chemical environment that will induce changes in the concentration of dissolved CO₂ and in nutrient availability. These in turn are expected to affect the physiological performance of phytoplankton. In order to learn how phytoplankton respond to the predicted scenario of increased CO₂ and decreased nitrogen in the surface mixed layer, we investigated the diatom *Phaeodactylum tricornutum* as a model organism. The cells were cultured in both low CO₂ (390 µatm) and high CO₂ (1000 µatm) conditions at limiting (10 µmol L⁻¹) or enriched (110 µmol L⁻¹) nitrogen concentrations. Our study shows that nitrogen limitation resulted in significant decreases in cell size, pigmentation, growth rate and effective quantum yield of *Phaeodactylum tricornutum*, but these parameters were not affected by enhanced dissolved CO₂ and lowered pH. However, increased CO₂ concentration induced higher rETR_{max} and higher dark respiration rates and decreased the CO₂ or dissolved inorganic carbon (DIC) affinity for electron transfer (shown by higher values for K_{1/2} DIC or K_{1/2} CO₂). Furthermore, the elemental stoichiometry (carbon to nitrogen ratio) was raised under high CO₂ conditions in both nitrogen limited and nitrogen replete conditions, with the ratio in the high CO₂ and low nitrate grown cells being higher by 45% compared to that in the low CO₂ and nitrate replete grown ones. Our results suggest that while nitrogen limitation had a greater effect than ocean acidification, the combined effects of both factors could act synergistically to affect marine diatoms and related biogeochemical cycles in future oceans.

**Introduction**

Rising atmospheric CO₂ concentrations enhance its absorption into the world’s oceans, which currently accounts for removal of nearly one third of anthropogenic CO₂ emissions from the atmosphere [1]. Atmospheric CO₂ concentrations are expected to reach 800–1000 ppmv by the end of this century according to the “business as usual” CO₂ emission scenario [2]. Dissolution of CO₂ into seawater has already induced a global drop in pH of 0.1 units since the end of the Industrial Revolution, and values are expected to drop another 0.3–0.4 units by the end of this century. This decline in pH driven by increased CO₂ is termed ocean acidification (OA) [3]. The decrease in seawater pH is a consequence of changes in marine chemistry, where increased dissolved CO₂ leads to increases in H₂CO₃ and hence to increases in H⁺ and HCO₃⁻ concentrations and decreased CO₃²⁻ concentration and CaCO₃ saturation state. Changes in pH also affect biogeochemical processes such as alterations to trace metal speciation, which can have significant biological effects [4], [3].

If the photosynthesis of marine eukaryotic phytoplankton were supported solely by the diffusional supply of CO₂ to the active site of the CO₂ fixing enzyme Rubisco, then this process would be severely limited at the concentrations of CO₂ currently found in seawater (<10–30 µmol) [6]. However, most algae have been shown to be able to make extremely efficient use of low levels of dissolved inorganic carbon (DIC) by virtue of inducible carbon concentrating mechanisms (CCMs) [7]. The CCMs act to maintain internal CO₂ concentrations higher than can be accounted for by diffusion-mediated entry of inorganic carbon.

CCM activity is down-regulated under high CO₂; enhanced CO₂ availability could thus reduce the energy cost for CO₂ transport [8], and the re-allocation of energy may play a critical role in modulating primary production as well as elemental stoichiometry and species composition [6]. This, however, also may depend on other environmental factors. The effects of ocean acidification can have positive, neutral or negative aspects depending on the physiological processes involved, and may be species-specific [9]. While increased primary production under high CO₂ has been found in many studies [10], energy loss due to enhanced respiration has also been reported under high CO₂/low pH conditions [11], possibly due to enhanced energy demand associated with the need to maintain intracellular acid-base stability [12]. The effects of ocean acidification are controversial with contradictory trends reported in the literature. While some of this could be due to species-specific responses it could also result from interactive effects with other environmental factors [13], [14].

Nutrient availability is well known to affect algal growth and production. This is especially so for nitrogen availability, which is seen in many cases as a major limiting factor for algal growth in the oceans [15]. Marine phytoplankton may experience increased nutrient limitation in the euphotic layer in the future due to
intensified stratification in a warming ocean [16], [17]. Ocean acidification, at the same time, may affect ion and nutrient assimilation of algae either directly by altering proton or ion channels or indirectly by changes in chemical speciation and nutrient availability [18], [19]. Thus, ongoing ocean acidification together with intensified stratification could further decrease marine nutrient availability and uptake rates. Decreased nitrogen availability is expected to lead to decreased synthesis of chlorophyll and proteins in algae, which would have a strong influence on photosynthesis and physiological performance. Nitrogen limitation is known to affect carbon fixation because of potential impacts on levels of RuBisco and other proteins and also because nitrate assimilation is energy dependent and will compete with carbon fixation for ATP and reductant [20]. Thus changes in C and N acquisition may be reflected in altered cell carbon and nitrogen contents [21].

While the impacts of nutrient limitation under present day CO₂ are well understood and there is an increasing literature on the effects of elevated CO₂ on phytoplankton physiology and ecology (see reviews by Beardall et al., Riebesell and Tortell and references therein) [10], [22] and elemental ratios [23], most studies on the effects of ocean acidification have been carried out under nutrient replete conditions [11], [24] and there is very little information on interactive effects between nutrient limitation and elevated CO₂ [10]. Since elevated CO₂ and ocean acidification in a future world is likely to go hand-in-hand with a more restricted nutrient supply that needs addressing.

Thus, this paper considers how phytoplankton responses to ocean acidification may be affected by nitrogen limitation. Specifically, we have measured the cell size, growth, pigmentation, quantum yield, respiration, and CCM activity as well as cell carbon and nitrogen contents of Phaeodactylum tricornutum grown under high CO₂ and low nitrogen conditions, to determine the possible interactive effects of ocean acidification and nitrogen limitation on this model diatom species.

Materials and Methods

Statement of ethics

The strain of the diatom Phaeodactylum tricornutum Bohlin (strain CCMA 106), originally isolated from the oligotrophic waters of the South China Sea in 2004, was obtained from the Center for Collections of Marine Bacteria and Phyttoplankton (CCMBP) of the State Key Laboratory of Marine Environmental Sciences (Xiamen University). No specific permits were required for using this species.

Algal culture conditions

The diatom Phaeodactylum tricornutum Bohlin (strain CCMA 106) were grown in artificial seawater with Aquil medium enrichment [25] except that the NO₃⁻ concentration was adjusted to 110 μmol L⁻¹ NO₃⁻ (HN) or 10 μmol L⁻¹ NO₃⁻ (LN). The nitrogen-limiting level of 10 μM was based on the surface inorganic nitrogen concentrations (unpublished) obtained from the oligotrophic South China Sea, ranging from 0 (undetectable) to 20 μM. Cultures were continuously aerated with ambient air of 390 μmol of CO₂ (LC) or with high CO₂ of 1000 μmol (HC) within plant CO₂ chambers (HP10000G-D, Ruhau Instrument & Equipment Co. Ltd, China) and bubbled at a constant flow rate of 300 ml min⁻¹. This allowed the following treatments, combining different N and CO₂ levels, to be performed: LC-HN, LC-LN, HC-HN, HC-LN. The cells were grown semi-continuously at 20 C under 70 μmol photons m⁻² s⁻¹ illumination with a 12L:12D photoperiod. Dilutions were carried out every 24 h to ensure cell concentrations did not exceed 3×10⁷ cells ml⁻¹ at their exponential growth phase so that pH change during growth at each CO₂ level was less than 0.02 (Table 1). Cells were acclimated to each NO₃⁻ and CO₂ combination for more than 10 generations before being used in the experiments described below.

The pH of cultures was measured daily, prior to dilution, with a pH probe (Mettler Toledo DL15 Titratior, Sweden), which was calibrated with standard NBS (National Bureau of Standards) buffer solutions (Hanna) at three pH points (pH 10.01, pH 7.01 and pH 4.01). Measurement of dissolved inorganic carbon (DIC) was carried out using an automated system (AS-C3, Apollo Scitech), which was connected to an infrared gas detector (Li-Cor 7000, Li-Cor). Calculation of the carbonate system components (HCO₃⁻, CO₂, CO₂ and TA) was carried out using known values of DIC, pH, nutrient concentration, salinity and temperature with a CO₂ system analysis software (CO2SYS) [26] (Table 1). Carbonic acid dissociation constants (K₁ and K₂) were according to Roy et al. [27], and that for boric acid (KB) was taken from Dickson [28].

Growth rate and cell size measurements

Cell numbers, mean cell volumes and size distributions were acquired with a Z2¹M¹ Coulter Counter (Beckman, Buckinghamshire, UK). Determinations of growth rates were based on the cell number changes every 24 h and were calculated according to the equation: μ = ln(N₁-ln(N₀))/t₁-d₀, where N₁ and N₀ are the cell concentrations before dilution (t₁) and after the previous dilution (d₀) respectively. Growth rates were calculated based on measurements of 11–12 replicates for triplicate cultures under each CO₂ level.

Carotenoid and Chlorophyll measurements

To determine the carotenoid and chlorophyll a and ε contents of cells cultured in the different CO₂ and NO₃⁻ conditions, cells were collected by filtration on to Whatman GF/F filters (pore size, 0.22 μm) and extracted overnight with absolute methanol at 4°C. The extracts were then centrifuged for 10 min at 5000 x g and the absorbance of the supernatant was scanned with a spectrophotometer (DU800, Beckman, Fullerton, California, USA). Calculation of chlorophyll a from the absorbance spectra followed the equation of Porra [29], chlorophyll ε was after Ritchie [30] and carotenoid was calculated according to Strickland and Parsons [31].

Quantum yield measurements

The quantum yield of cells grown in the different CO₂ and NO₃⁻ conditions was measured with a XE-PAM (Walz, Germany) at both mid-light phase (Fᵥ/Fm') and at the end of the dark phase (Fᵥ/Fm). The saturation light was set at 5000 μmol photons m⁻² s⁻¹ for 0.8 s.

Determination of CCM activity from rETR vs DIC curves

To estimate the affinity of cells for DIC (used as a proxy for CCM activity), cells cultured in different CO₂ and NO₃⁻ conditions were collected, washed with, and re-suspended into, DIC-free seawater with a pH of 8.20. Cell densities after re-suspension were between 3 and 4×10⁷ cells ml⁻¹. The DIC-free seawater was prepared by adding 1 mol L⁻¹ HCl to drop the pH below 3 and then bubbling with pure N₂ for 1 h. Tris-buffer was added to 20 mmol L⁻¹ to adjust the pH back to 8.20. Cells suspended in the DIC- free seawater were incubated at 150 μmol m⁻² s⁻¹ for 15 min to exhaust any intracellular DIC, and
NaHCO₃ solution was then added into each vial of algal suspension to obtain different DIC concentrations. After further incubation under a photon flux of 70 µmol m⁻² s⁻¹ for 10 min (less than 0.1% DIC was consumed), a rapid light curve was determined with the XE-PAM, and the resulting data fitted with the equation of Eilers and Peeters [32]: \( y = \frac{x}{ax^2 + bx + c} \), where \( a, b, c \) are estimated parameters, \( x \) photon flux density, and \( y \) the \( rETR \) value. Light saturated rates of electron transport \( (rETR_{max}) \) at the different DIC concentrations can be calculated from the fitted rapid light curve: \( rETR_{max} = \frac{1}{bx} \), and the light harvesting efficiency (\( \phi \)) was calculated with the equation: \( \phi = \frac{y}{ax^2 + bx + c} \). To quantify the relationship between \( rETR_{max} \) and DIC, we fitted the two parameters using the Michaelis-Menten equation to determine light- and DIC-saturated rates of photosynthesis and the half-saturation constant \( K_{1/2}^{DIC} \) and \( K_{1/2}^{CO_2} \) for DIC-dependent electron transport.

### Dark respiration measurements

Cells were gently filtered on to polycarbonate membrane filters (0.22 µm, Q/YX-8-48, Xinya, China) with a vacuum pump at a pressure of less than 0.02 Pa to ensure cells were intact, based on a microscopic checkup, and were then re-suspended into 20 mmol L⁻¹ Tris-buffered media of the respective composition (LC-HN, LC-LN, HC-HN and HC-LN). Each treatment had a known cell concentration of around 1 x 10⁶ cells ml⁻¹. Dark respiration rates were determined with a Clark type oxygen electrode (5300A, YSI) from changes in oxygen concentration over time at 20°C. A two-point calibration (seawater bubbled with air until equilibrium saturation and O₂ deprivation with excess sodium sulfite as zero oxygen) was carried out before respiration measurements. The possible contribution of bacterial respiration was tested on the culture filtrate that passed through a filter pore size of 1 µm (mixed cellulose lipid membranes), which would not exclude the few bacteria present, and there was no detectable bacterial O₂ consumption.

### Measurement of carbon and nitrogen contents

To determine particulate organic carbon (POC) and nitrogen (PON) in *Phaeodactylum tricornutum* grown under different CO₂ and NO₃⁻ concentrations, cells were collected in the mid-light period (0.22 µm, Q/YX-8-48, Xinya, China) with a vacuum pump at a pressure of less than 0.02 Pa. To ensure cells were intact, based on a microscopic checkup, and were then re-suspended into 20 mmol L⁻¹ Tris-buffered media of the respective composition (LC-HN, LC-LN, HC-HN and HC-LN). Each treatment had a known cell concentration of around 1 x 10⁶ cells ml⁻¹. Dark respiration rates were determined with a Clark type oxygen electrode (5300A, YSI) from changes in oxygen concentration over time at 20°C. A two-point calibration (seawater bubbled with air until equilibrium saturation and O₂ deprivation with excess sodium sulfite as zero oxygen) was carried out before respiration measurements. The possible contribution of bacterial respiration was tested on the culture filtrate that passed through a filter pore size of 1 µm (mixed cellulose lipid membranes), which would not exclude the few bacteria present, and there was no detectable bacterial O₂ consumption.

### Statistical analysis

One-way analysis of variance (ANOVA) and Tukey’s test were used to establish differences among treatments at a confidence level of 95%. Interactive effects between CO₂ and NO₃⁻ were analyzed using a Tukey post hoc test.

### Results

#### Growth rate

Growth rates were inhibited under nitrogen limited conditions under both LC (30%, \( P<0.001 \)) and HC (38%, \( P<0.001 \)) conditions (Figure 1). No direct effects on growth rate were found between the CO₂ treatments (nitrogen replete, \( P=0.24 \); nitrogen limited, \( P=0.72 \)). No interactive effect was found between CO₂ and NO₃⁻ levels (\( P=0.3 \)).

#### Chlorophyll and carotenoid contents

The effects of nitrogen and CO₂ treatments on cellular chlorophyll and carotenoid concentrations showed the same trend as cell size and growth rate. Nitrogen limitation decreased the carotenoid, chlorophyll \( a \) and \( c \) contents by 50% and 62% (Figure 2A), 48% and 60% (Figure 2B), 45% and 63% (Figure 2C) in the LC and HC groups, respectively (\( P<0.05 \)). No direct effects

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**Table 1. Chemical parameters of seawater carbonate system.**

| pCO₂ (µatm) | pH_NBS | DIC (µmol Kg⁻¹) | HCO₃⁻ (µmol Kg⁻¹) | CO₂ (µmol Kg⁻¹) | CO₂ (µmol Kg⁻¹) | TA (µmol Kg⁻¹) |
|-----------|--------|----------------|------------------|----------------|----------------|----------------|
| LC-HN     | 416.6±15.4a | 8.17±0.02a | 2063.6±13.4b | 1851.1±12.0b | 199.1±6.7b | 13.1±0.1a | 2349.3±19.1a |
| LC-LN     | 404.0±11.9a | 8.18±0.01a | 2052.4±8.3a | 1837.2±5.8a | 202.2±5.8a | 13.1±0.4a | 2343.4±15.2a |
| HC-HN     | 980.4±52.6b | 7.85±0.02b | 2209.7±19.3b | 2072.0±20.8b | 106.1±3.7b | 30.9±1.0b | 2341.4±14.1b |
| HC-LN     | 952.8±27.5b | 7.86±0.01b | 2200.8±11.5b | 2062.1±9.9b | 108.0±3.4b | 30.8±0.9b | 2336.2±15.1b |

Parameters of the seawater carbonate system under the ambient (390 µatm, LC) and elevated (1000 µatm, HC) CO₂ concentrations as well as nitrogen-replete (HN) and limited (LN) conditions before the partial renewal of the medium for the semi-continuous cultures. Total inorganic carbon (DIC), pH, salinity, nutrient concentration and temperature were used to derive all other parameters using the CO₂ system analyzing software CO2SYS [34]. Data are the means ± SD of 4 measurements. Different letters indicate significant differences among the treatments at the \( P<0.05 \) level.

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**Figure 1. Specific growth rates of *P. tricornutum*.** The growth rates were measured after cells acclimated for 10 generations under nitrogen limited (LN) and replete (HN) levels in 390 (LC) and 1000 µatm (HC) CO₂ conditions. The different letters indicate significant differences among the treatments at the \( P<0.05 \) level. Vertical bars are means ± SD, \( n=11–12 \).
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on pigmentation were found between the CO2 treatments \((P>0.05)\). The ratio of chlorophyll \(a\) to carotenoids showed no significant difference among the treatments \((P>0.05)\) (Figure 2D). No interactive effects on chlorophyll \(a\) \((P=0.086)\), \(\epsilon\) \((P=0.139)\), carotenoid \((P=0.475)\) and the ratio of chlorophyll \(a\) to carotenoids \((P=0.657)\) were found between CO2 and NO\(_3^-\) levels.

Quantum yield

Maximum quantum yields \(\left(F_v/F_m\right)\) measured at the end of the dark period, showed no significant differences among treatments \((P>0.05)\) (Figure 3A). However, in the mid-light period, cells cultured under nitrogen limited conditions showed decreases in effective quantum yield \(\left(F_v'/F_m'\right)\) by 12% and 15% in the LC \((P=0.01)\) and HC \((P=0.002)\) treatments respectively, compared to those under nitrogen replete conditions (Figure 3B). No significant (nitrogen replete, \(P=0.45\); nitrogen limited, \(P=0.91\)) change in the yield was found between the low and high CO2 levels (Figure 3A, B). No interactive effect on yield was found between CO2 and NO\(_3^-\) levels \((F_v/F_m, P=0.24\); \(F_v'/F_m', P=0.50)\).

Cell size

Increased dissolved CO2 concentration did not affect the mean cell size (as Effective Spherical Diameter) and cell volume of *Phaeodactylum tricornutum* under nitrogen replete conditions. However, nitrogen limitation did cause significant \((P<0.001)\) decreases in cell size and cell volume (Figure 4, 5). Mean cell volumes were 65.3 and 67.6 \(\mu m^3\) in LC and HC treatments, and nitrogen limitation significantly decreased these values to 43.0 and 41.3 \(\mu m^3\) (by 34% and 39%), respectively \((P<0.001)\) (Figure 5A). Mean cell sizes (as Effective Spherical Diameter) in LC and HC were 4.91±0.08 and 4.95±0.00 \(\mu m\) respectively, under nitrogen replete conditions (Figure 5B). Under nitrogen limitation, cell size decreased by 13% and 15% \((P<0.001)\), to 4.25±0.02 \(\mu m\) and 4.19±0.01 \(\mu m\) in LC and HC cells, respectively (Figure 5B). No significant differences in both cell size \((P=0.56)\) and volume \((P=0.39)\) were found between low and high CO2 levels under the nitrogen replete conditions. However, high CO2 cells showed a small but significant decrease of both cell size \((P=0.02)\) and volume \((P=0.04)\) when nitrogen was limited. No interactive effects were found between CO2 and NO\(_3^-\) levels in both cell size \((P=0.16)\) and cell volume \((P=0.14)\).

P vs DIC characteristics

With increasing DIC concentration in the medium, high CO2, N-replete cultures had a significantly \((p=0.04)\) elevated \(rETR_{\text{max}}\) (light and DIC-saturated rate of electron transfer) compared to low CO2, N-replete cells \(\left(rETR_{\text{max}}\right)\) values were HC-HN 110.08±5.76, LC-HN 97.47±4.55). The \(rETR_{\text{max}}\) under N-limited conditions was not affected by the CO2 level (LC-LN \((n=2)\) 93.24±2.12, HC-LN \((n=2)\) 98.31±5.99; \(P=0.48\)) (Figure 6A, B). The calculated \(K_{1/2}\) DIC values indicate that CCM activity was down-regulated under HC conditions \((P=0.04)\), with \(K_{1/2}\) DIC values in the high N-grown cells increasing from 57.0 \(\mu L\) to 103.4 \(\mu L\) \(1\) (HC-HN). N-limitation also caused a rise in \(K_{1/2}\) DIC to 110.4 \(\mu L\) \(1\) even under low CO2, and this increased to 134.2 \(\mu L\) \(1\) in HC-LN cells (Figure 6C). The \(K_{1/2}\) DIC shows the same trend as \(K_{1/2}\) DIC and values were 0.34, 0.66, 0.82 and 0.81 \(\mu L\) \(1\) in the HC-HN, HC-LN, HC-HN and HC-LN treatments respectively. The light harvesting efficiency \((\varepsilon)\) of the cells, derived from RLC at different DIC levels, was not affected \((P=0.12)\) by CO2 levels when nitrogen was limited, but was significantly \((P=0.01)\) elevated by the CO2 enrichment in N-replete conditions at a DIC level of 138 \(\mu L\) \(1\). Such a trend was still observed at 275 \(\mu L\) \(1\), although with the differences being statistically insignificant (nitrogen replete, \(P=0.35\); nitrogen limited, \(P=0.52\)) (Figure 7). At the ambient DIC level of 2200 \(\mu L\) \(1\), no significant difference (nitrogen replete, \(P=0.22\); nitrogen limited, \(P=0.23\)) in light harvesting efficiency was found among the treatments (Figure 7).
Diatom’s Response to CO₂ and N-Limitation

Dark respiration rates

Cells grown under nitrogen limitation, irrespective of CO₂ level, showed approximately a doubling in respiration rate, compared to ambient CO₂, nitrogen replete cells. With N-replete cells, high CO₂ also resulted in an increase in cellular respiration rates (Figure 7A). When dark respiration was expressed on a per chlorophyll a basis, rates were enhanced by 298%, 110% and 305% in LC-LN, HC-HN, HC-LN treatments respectively, compared to the LC-HN conditions (Figure 7B). No interactive effects on dark respiration were found between CO₂ and NO₃ levels (P = 0.16).

Carbon and nitrogen contents

Nitrogen limitation significantly decreased the nitrogen content of cells cultured in both LC (by ca. 32%) and HC (by ca. 28%) conditions compared with the nitrogen-replete treatments (P < 0.001) (Table 2). Nitrogen limitation led to an increase in the C:N ratio by 21% in the LC condition, and this enhancement increased to 45% in the HC condition (P < 0.001). However, under the nitrogen replete treatment the CO₂ concentration did not affect the C:N ratio (P = 0.24), even though the cells at the high CO₂ level significantly increased their nitrogen content by 13% (P = 0.04). Turkey’s post hoc test showed that there were significant interactive effects on C:N found between CO₂ and NO₃⁻ levels (P < 0.001).

Discussion

While the elevated CO₂ concentration of 1000 μatm did not cause significant differences in growth, pigment contents, effective quantum yield and cell size, nitrogen limitation decreased all these parameters in the diatom Phaeodactylum tricornutum (Figures 1, 2, 3, 4). Both the elevation of CO₂ and N-limitation led to a down-

regulation of CO₂ concentrating mechanism (CCM) activity, as reflected in the increased/decreased K₁/₂ DIC or K₁/₂ CO₂. N-limitation and increased pCO₂/reduced pH led to the lowest light use efficiency under Ci-limited conditions (Figure 7), with this trend effect being minimized under elevated levels of DIC. Ocean acidification increased dark respiration under N-limited conditions.
Previous studies on a green alga *Ulva rigida* [37] showed that both Fv/Fm and Fv'/Fm' were significantly down-regulated by high CO2, consistent with previous data on diatoms [11], [36], [44]. Growth under N-limited conditions caused an increase in K1/2 DIC in low-CO2 grown cells.

### Basic cell parameters

Confirming previous studies on growth of diatoms, including *Phaeodactylum tricornutum*, under elevated CO2 [33], [34], growth at the elevated CO2 levels that are expected by the end of the century did not cause a significant increase in growth rate. This was the case regardless whether the organism was grown under N depletion or N replete conditions. Earlier studies in our laboratory on the same species showed an enhanced growth rate (ca. 5%) under elevated CO2 and a PAR of 120 µmol m–2 s–1 [11]. In the current study we did not find improved growth under a PAR level of 70 µmol m–2 s–1. Recently, changes in light levels have recently been shown to mediate diatoms’ responses to ocean acidification [14]. Even although both light levels are sub-saturating for photosynthesis, less photosynthetic carbon fixation under 70 µmol m–2 s–1 should have resulted in less or no growth stimulation due to the enhanced respiratory carbon loss (Figure 8).

Although growth rates were clearly limited by a decrease in N-supply and, as has commonly been reported [35], chlorophyll levels were significantly decreased in N-limited cells, there was no change in the chl:carotenoid ratio, sometimes used as an indicator for N-limitation [35], under our experimental conditions (Figure 2).

A lack of effect of CO2 levels on cellular pigment content is consistent with previous data on diatoms [11], [36] and other microalgae [37]. Furthermore, dark-adapted maximal quantum yield showed no decrease in N-limited cells, although the effective quantum yield show a small, but significant drop; neither parameter was affected by growth at elevated CO2 (Figure 3). Previous studies on a green alga *Ulva rigida* showed that both Fv/Fm and Fv'/Fm' were significantly down-regulated by high CO2 and that nitrogen limitation further decreased both parameters [38]. A decline in Fv/Fm is a general response to nitrogen limitation [39]. There is a possibility that the urea cycle found in *Phaeodactylum* functions to support the photosynthetic machinery by recycling N under the N-limited conditions [40].

### Photosynthesis vs DIC characteristics

Elevated CO2 caused a slight increase in photosynthetic capacity (measured as rETRmax under light and DIC-saturated conditions) under N-replete, but not under N-limited conditions (Figure 6). Small increases in the DIC-saturated photosynthetic capacity of diatoms grown under elevated CO2 have been reported previously [11], [36], [44]. Growth under N-limited conditions caused an increase in K1/2 DIC in low-CO2 grown cells.
This indicates a down-regulation of CCM activity by nitrogen limitation. This is in contrast to the reports of Young and Beardall for the green alga Dunaliella tertiolecta showing cells grown under low CO₂ exhibited increasing affinity (lower K₅₀) for DIC under N-limited growth and work on Chlorella emersonii in which cells under 5% CO₂ showed partial induction of CCM activity when N-limited [45], [46]. Such responses have been viewed as a way of improving N-use efficiency and maintaining Rubisco activity with less Rubisco protein when resources such as nitrogen are in short supply [45]. However, induction of CCM activity under N-limitation does not always occur, especially when cells are grown under low CO₂ or are not severely N-limited (see Table 1) [39], [47]. In P. tricornutum, nitrogen storage strategy using the urea cycle could mediate the diatom’s CCM to decrease the influence of elevated CO₂ levels [40].

The changes in rETR max under elevated CO₂ and N-replete conditions may be partially negated by the rise in dark respiration and contribute to the lack of effect of high CO₂ on growth rate. However, low CO₂ grown cells that were N-limited also exhibited higher respiration rates, contributing to the lower growth rates found in N-limited cells. Growth at elevated CO₂ exposes cells to a lower pH, which might impose additional energetic costs for acid-base regulation to sustain metabolic integrity [48]. Metabolic processes also influence the pH in the immediate proximity to the cell surface [49], and under low N levels (here supplied as nitrate), the near cell pH would become less alkaline (NO₃⁻ uptake leads to OH⁻ extrusion), again leading to increased energy demand to maintain pH gradients across the cell membrane.

Elemental composition

The elemental composition and macromolecular composition of phytoplankton is critically important for secondary producers such as copepods, fish and shrimp, and food nutritional quality influences energy flow through marine food chains [50]. Recently Rossell et al. found that the fatty acid composition of the diatom Thalassiothrix pseudonana cultured at elevated CO₂ was altered and that this significantly affected the growth and egg production of a copepod, Acartia tonsa [51]. Riebesell et al. reported an increased C:N ratio in a mesocosm study dominated by diatoms [52], whereas Burkhardt et al. reported both increases and decreases in C:N ratio with increasing CO₂ dependent on the species [23]. In our study, nitrogen limitation decreased both the carbon and nitrogen contents per cell but these changes were not parallel and led to an enhanced C:N ratio in both LG and HC conditions. Cells cultured under high CO₂ showed increased cell quotas for both carbon and nitrogen, irrespective of nitrogen supply (Table 2). The highest C:N was found in the high CO₂/low nitrogen conditions that are expected to dominate the open ocean in the near future, indicating that these synergistic effects of ocean acidification and nitrogen limitation could decrease the food quality of marine phytoplankton.

Although increased marine dissolved CO₂ may bring some benefits in terms of improved carbon supply to some phytoplankton [9], these organisms also face an extra cost associated with changed marine chemistry, especially pH stress, which could also lead to more energetic constraints on growth. Thus, the net benefit of higher CO₂ will be a balance between gains and losses determined by the various environmental factors associated with climate change.

In conclusion, ocean acidification together with ocean change can act in the marine environment synergistically or antagonistically to affect diatom performance, depending on the levels of sunlight [14]. Intensified stratification may push the marine phytoplankton into nitrogen-limited status, and will thereby influence the physiological or biochemical characteristics of the phytoplankton cells. Increases in respiratory metabolism may counteract any increase in the rate of C gain through photosynthesis (and hence affect net growth) [11], [14], [36]. Enhanced C:N ratios, induced by high CO₂ and low nitrogen, can influence secondary producers as well as predators at higher levels. Effects of ocean acidification on marine primary producers can be species-specific due to their physiological diversities and vary between different oceanic regions correlated with different physical, chemical or biological conditions.

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Author Contributions

Conceived and designed the experiments: KSG. Performed the experiments: WL. Analyzed the data: WL KSG JB. Contributed reagents/materials/analysis tools: WL. Wrote the paper: WL KSG JB.

References

1. Sabine CL, Feely RA, Gruber N, Key RM, Lee K, et al. (2004) The oceanic sink for anthropogenic CO₂. Science 305: 367–371.
2. IPCC (2001) Climate change 2001: the scientific basis. Contribution of Working Group I to the Third Assessment Report of the Intergovernmental Panel on Climate Change. Cambridge University Press, Cambridge, United Kingdom and New York, NY, USA. 881 p.
3. Doney SC, Fabry VJ, Feely RA, Kleypas JA (2009) Ocean acidification: The other CO₂ problem. Annu Rev Mar Sci 1: 169–192.
4. Caldeira K, Wickett ME (2003) Anthropogenic CO₂ and ocean pH. Nature 425: 365–365.
5. Millero FJ, Wooley R, Ditroilo B, Waters J (2009) Effect of ocean acidification on the speciation of metals in seawater. Oceanography 22: 72–85.
6. Knüttleder JR (2011) Carbon concentrating mechanisms in eukaryotic marine phytoplankton. Annu Rev Marine Sci 3: 291–315.
7. Raven JA, Giordano M, Beardall J, Maberly SC (2011) Algal and aquatic plant carbon concentrating mechanisms in relation to environmental change. Photosynth Res 108: 201–296.
8. Hopkinson RM, Dupont CL, Allen AE, Morel FFM (2011) Efficiency of the CO₂-concentrating mechanism of diatoms. Proc Nat Acad Sci 108: 3830–3837.
9. Gao K (2011) Positive and negative effects of ocean acidification: Physiological responses of algae. Journal of Xiamen University (Natural Science) 30: 411–417. (In Chinese).
10. Riebesell U, Tortell PD (2011) Effects of ocean acidification on pelagic organisms and ecosystems. In: Gattuso JP, Hansson I, editors. Ocean acidiﬁcation. New York: Oxford University Press. 99–116.
11. Wu Y, Gao K, Riebesell U (2010) CO₂-induced seawater acidification affects physiological performance of the marine diatom Phaeodactylum tricornutum. Biogeosciences 7: 2915–2923.
12. Portner HO, Farrell AP (2008) Physiology and climate change. Science 322: 690–692.
13. Veron JEN, Hoegh-Guldberg O, Lenton TM, Lough JM, Obura DO, et al. (2009) The coral reef crisis: The critical importance of <350ppm CO₂. Mar Pollut Bull 58: 1428–1436.
14. Gao K, Xu J, Gao G, Li Y, Hutchins DA, et al. (2012) Rising CO₂ and increased light exposure synergistically reduce marine primary productivity. Nature climate change 2: 519–523.
15. Caraco N, Cole J, Likens GE (1990) A comparison of phosphorus immobilization in sediments of freshwater and coastal marine systems. Biogeochemistry 9: 277–290.
16. Doney SC (2006) The dangers of ocean acidification. Sci Am 294: 58–65.
17. Cerniør P, Dutkiewicz S, Harris RP, Follows M, Schofield O, et al. (2008) The role of nutrient depth in regulating the ocean carbon cycle. Proc Natl Acad Sci 105: 20344–20349.
18. Shi D, Xu Y, Hopkinson BM, Morel FFM (2010) Effect of ocean acidification on iron availability to marine phytoplankton. Science 327: 676–679.
19. Beman JM, Chow CE, King AL, Feng Y, Fuhrman JA, et al. (2011) Global declines in oceanic nitrification rates as a consequence of ocean acidification. Proc Natl Acad Sci 108: 208–213.
20. Hipkin CR, Thomas RJ, Syrett PJ [1983] Effects of nitrogen deficiency on nitrate reductase, nitrite assimilation and photosynthesis in unicellular marine algae. Mar Biol 77: 101–105.

21. Kaffes A, Thoms S, Trimborn S, Rost B, Langer G, et al. (2010) Carbon and nitrogen fluxes in the marine coccolithophore Emiliania huxleyi grown under different nitrate concentrations. J Exp Mar Biol Ecol 393: 1–8.

22. Beardall J, Sobrino C, Stojkovic S (2009) Interactions between the impacts of ultraviolet radiation, elevated CO2 and nutrient limitation on marine primary producers. Photosch Photosib Sci 8: 1257–1265.

23. Burkhardt S, Zondervan I, Riebesell U (1999) Effect of CO2 concentration on C:N:P ratio in marine phytoplankton: A species comparison. Limnol Oceanogr 44: 683–690.

24. Sobrino C, Ward ML, Neale PJ (2008) Acclimation to elevated carbon dioxide Thalassiosira pseudonana. J Phycol 44: 683–690.

25. Morel FMM, Rüeter JG, Anderson DM, Guillard RRL (1979) Aquil: A chemically defined phytoplankton culture medium for trace metal studies. J Physcol 15: 135–141.

26. Lewis E, Wallace DW, Tjiputra J (1998) Program developed for CO2 system calculations. ORNL/CDIAC-105. Carbon Dioxide Information Analysis Center, Oak Ridge National Laboratory, US Department of Energy, Oak Ridge, Tennessee.

27. Roy RN, Roy LN, Vogel KM, Porter-Moore C, Pearson T, et al. (1993) The chemically defined phytoplankton culture medium for trace metal studies. J Phycol 15: 135–141.

28. Dickson AG (1990) Standard potential of the reaction: AgCl(s)+½ H2(g) = Ag(s)+HCl(aq), and the standard acidity constant of the ion HSO4− in synthetic seawater from 273.15 to 318.15 K. J Chem Thermodyn 22: 113–127.

29. Porra RJ (2002) The chequered history of the development and use of simultaneous equations for the accurate determination of chlorophylls a and b. Photosynth Res 73: 149–136.

30. Ritchie RJ (2006) Consistent sets of spectrophotometric chlorophyll equations for acetone, methanol and ethanol solvents. Photosynth Res 89: 27–41.

31. Strickland JDH, Parsons TR (1968) A practical handbook of seawater analysis. B Fish Res Board Can 167: 49–80.

32. Eilers PHC, Peeters JCH (1988) A model for the relationship between light intensity and the rate of photosynthesis in phytoplankton. Ecol Model 42: 199–215.

33. Burkhardt S, Riebesell U, Zondervan I (1999) Effects of growth rate, CO2 concentration, and cell size on the stable carbon isotope fractionation in marine phytoplankton. Geochem Cosmochim Acta 63: 3729–3741.

34. Trimborn S, Lundholm N, Thoms S, Richter KU, Krock B, et al. (2008) Inorganic carbon uptake in potentially toxic and non-toxic diatoms: the effect of pH-induced changes in seawater carbonate chemistry. Physiol Plantarum 133: 92–105.

35. Burkhardt S, Amoroso G, Riebesell U, Sültemeyer D (2001) CO2 and HCO3− uptake in marine diatoms acclimated to different CO2 concentrations. Limnol Oceanogr 46: 1378–1391.

36. Yang G, Gao K (2012) Physiological responses of the marine diatom Thalassiosira pseudonana to increased pCO2 and seawater acidity. Mar Environ Res 10.1016/j.marenvres.2012.06.002.