Rising CO₂ Interacts with Growth Light and Growth Rate to Alter Photosystem II Photoinactivation of the Coastal Diatom *Thalassiosira pseudonana*

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**Abstract**

We studied the interactive effects of pCO₂ and growth light on the coastal marine diatom *Thalassiosira pseudonana* CCMP 1335 growing under ambient and expected end-of-the-century pCO₂ (750 ppmv), and a range of growth light from 30 to 380 μmol photons m⁻² s⁻¹. Elevated pCO₂ significantly stimulated the growth of *T. pseudonana* under sub-saturating growth light, but not under saturating to super-saturating growth light. Under ambient pCO₂ susceptibility to photoinactivation of photosystem II (σ₀) increased with increasing growth rate, but cells growing under elevated pCO₂ showed no dependence between growth rate and σ₀, so under high growth light cells under elevated pCO₂ were less susceptible to photoinactivation of photosystem II, and thus incurred a lower running cost to maintain photosystem II function. Growth light altered the contents of RbcL (RUBISCO) and PsaC (PSI) protein subunits, and the ratios among the subunits, but there were only limited effects on these and other protein pools between cells grown under ambient and elevated pCO₂.

**Introduction**

Atmospheric carbon dioxide (CO₂) is expected to rise from current levels of ~390 parts per million (ppm) to 700–1000 ppm by the end of this century, beyond the levels of the past 800 kyr of glacial-interglacial periods [1]. The dissolution of additional atmospheric CO₂ into seawater alters the inorganic carbon buffer [1]. The dissolution of additional CO₂ by the end of this century, beyond the levels of the past 800 kyr of glacials and interglacials [1], would increase the pCO₂ and decrease the pH [1,2], elevating CO₂ complicate the implications of rising CO₂ for marine phytoplankton. Low light increases accessory pigmentation, which may increase stratification and decrease the upper-mixed-layer depth, thus exposing phytoplankton cells to higher mean light intensities [17]. Available light is a key factor for phytoplankton. Low light increases accessory pigmentation, which elevates the likelihood of photon capture [22]; it leads also to an increase in total proteins [23]. Concomitantly light drives photoinactivation of photosystem II reaction centers through damaging key protein subunits, particularly PsbA, when can lead to photo-inhibition or cell death if photoinactivation outruns counter-acting repair processes [24,25].

Diatoms are a biogeochemically important group of marine phytoplankton. They contribute up to 40% of marine primary production and show relatively high carbon sequestration into the deep oceans because their silica frustules enhance their sinking rates [26,27]. Diatoms often dominate in well-mixed coastal or estuarine waters where steep attenuation of light results in fast light fluctuations for cells mixing through the water column. Diatoms...
succeed under variable light through their high plasticity in photoacclimation capacity and quantum-to-biomass conversion rate [28] as well as their photoprotection mechanisms [29,30]. Diatoms as a group [31,32] have lower susceptibility to photoinactivation of PSII than do other phytoplankton [33–35]. This lowers the diatom cost-of-growth, particularly under fluctuating light, since they do not need to allocate as much protein metabolic capacity [25,36] to counter PSII photoinactivation. Irradiance and elevated CO$_2$ have interactive influences on diatom physiology which may contribute to controlling future community structures [15–19,37].

In this paper we determined the interactive effects of elevated pCO$_2$ and growth light on the photophysiology of the model diatom Thalassiosira pseudonana CCMP 1335 [38,39], which is derived from a coastal environment, where light [40] and pCO$_2$ [41] already vary widely.

**Materials and Methods**

**Culture protocol**

The coastal centric diatom, Thalassiosira pseudonana (Hustedt) Halse et Heimdal obtained from the Provasoli-Guillard National Center of Marine Phytoplankton (CCMP 1335) was cultured in 2 cm thick cuvettes (450 ml volume) of FMT-150 photobioreactors (Photon Systems Instruments, Drasov, Czech Republic) at 18°C in enriched artificial seawater (EASW) prepared according to [42], except with 54.5 µM Si and 0.82 µM Sr to limit precipitation during autoclaving. Cultures were gently mixed by a curtain of bubbles emitted from four apertures across the cuvette bottom with air at either ambient (~390 ppmv) or elevated (~750 ppmv) pCO$_2$. Outdoor air was used for the ambient CO$_2$ treatment whereas the elevated CO$_2$ treatment was achieved by mixing 99.99% CO$_2$ with zero-CO$_2$ air using mass flow controllers (16 series, Qubit Systems, Kingston, Canada). Before bubbling into the bioreactor culture cuvette, the air streams were filtered through a 0.2 µm micro-filter and bubbled through sterile distilled water for humidification. The growth of T. pseudonana was not notably disrupted by this bubbling [43] although we earlier noted disruption of growth and cell breakage of larger diatoms using similar bubbling streams in these bioreactors (A. McCarthy & D. Campbell, unpub.).

Continuous growth light was measured with a micropheral quantum sensor (US-SQS, Waltz, Germany). The light intensities in culture vessels filled with seawater were set to 30, 80, 160, 240 or 380 µmol photons·m$^{-2}$·s$^{-1}$, provided by a panel of blue LED that cover the entire rear face of the cuvette. These light levels approximate a range from near the bottom of the euphotic zone at 1.5% of surface sunlight, up to the upper 35% of the euphotic zone in marine ecosystems.

**Carbon system analyses**

During the growth period, we continuously monitored the pH in culture replicates in each of the three photobioreactors using an InPro 325 x glass electrode (Mettler-Toledo) that was calibrated before setting up the culture systems with NBS buffers of pH 7.0 and 10.0 (Sigma-Aldrich). At the end of growth of each culture replicate, we measured dissolved inorganic carbon (DIC) with a CO2 analyzer (S151, Qubit systems, Kingston, Canada) [44]. We calculated total alkalinity, bicarbonate (HCO$_3^-$), carbonate (CO$_3^{2-}$) and pCO$_2$ on the base of the measured temperature (18°C), salinity (35 g L$^{-1}$), pH, DIC and the phosphate (21 µmol L$^{-1}$) or silica (52.5 µmol L$^{-1}$) contents of the prepared EASW using the CO2SYS program [45], with NIST scale constants from [46] and K1, K2 from [47] to calculate total alkalinity.

**Growth rate**

We obtained a preliminary growth curve by operating the bioreactors in batch mode to determine a cell density set-point for turbidostat mode that maintained a stable pH, indicating stable DIC status, and a detectable FO$_3$ fluorescence signal from the diatoms, monitored continuously using the onboard sensor in the bioreactors. For each of the ten different combinations of light and pCO$_2$, we then grew 3 or 4 replicate cultures by re-inoculating cells after cleaning and autoclaving the photobioreactor cuvettes.

We grew each experimental replicate culture from initial inoculation for 3–4 generations without dilution until the culture density reached the set-point (Fig. 1A) of basal fluorescence value (FO$_3$ = 290; 62±30 ng Chl a mL$^{-1}$ in this study). We then entered turbidostat mode to maintain this cell density by activating a peristaltic pump when the FO$_3$ value reached the set-point (Fig. 1A) to dilute the 450 ml culture with a 10% volumetric addition of media delivered from a reservoir, which was continuously pre-bubbled with the air stream to pre-equilibrate the dissolved inorganic carbon system of the media before addition to the culture volume. 3.5 L of media in the reservoir supported about 9.5 cell generations of growth for each replicate culture under the set conditions of light and pCO$_2$, before we harvested for the light-shift experiments and biochemical analyses described below. The temporal duration of each turbidostat run varied between 160 to 200 h, depending upon the achieved growth rate of the culture, which in turn varied with the applied growth light and pCO$_2$. We calculated the specific growth rate for a given culture replicate (Fig. 1A; Fig. 2A) by using the onboard detector to monitor OD$_{680}$, and then fitting the increase with time with an exponential growth function for the interval between each turbidostat 10% dilution cycle. We then averaged the exponential rates from the final 10 cycles of dilution to estimate the growth rate for the culture replicate. There were small variations in the light sources among the three bioreactor units we used, so we plotted specific growth rates and other parameters versus the actual light level applied to a given culture replicate, resulting in small offsets in data points along the X axes of plots (ex. Fig. 2A). Across the 10 combinations of growth light and pCO$_2$ we grew a total of 33 separate turbidostat runs between December 2011 and August 2012, although experiment failures and volume limitations meant that not every analytical measurement was performed for every turbidostat run. At the end of the turbidostat growth period we measured the cell volume and cell suspension density with a Multisizer 3 Counter (Beckman Coulter Inc., USA).

**Frustule thickness**

Samples were harvested for scanning electron microscopy (SEM) by collecting cells from approximately 15 mL of culture using a Millipore vacuum filtration apparatus containing a 25 mm diameter, 1 µm pore size polytetrafluoroethylene membrane (Steriltech Corp., Kent, WA). The cells were washed with 250 mL distilled water, resuspended in 1 mL distilled water and cleaned in disposable 18 x 150 mm borosilicate culture tubes (Fisher Scientific, Ottawa, ON) by adding 10 mL of each concentrated sulfuric and nitric acid in a boiling water bath for 45 min. Samples were washed again with 250 mL distilled water in the filtration apparatus and resuspended in 5 mL distilled water. For SEM examination, 0.1–0.5 mL of the cleaned samples were deposited on 10 x 10 mm squares of phonograph record substrate and dried at 45°C. This substrate provided microscopic grooves with side walls tilted 45 degrees relative to normal and allowed viewing of fractured valves at this angle [48]. Substrates were attached to 32 mm diameter aluminum support stubs using colloidal graphite, coated with ca. 5 nm gold in a Hummer 6.2
sputtering unit (Anatech USA, Union City, CA) and examined using a JEOL JSM-5600 SEM (JEOL USA, Peabody, MA) operating at 10 kV and 8 mm working distance. Six images of fractured valves oriented so that the fracture was facing upslope and parallel to the wall of the groove were collected for each treatment. Valve thickness was measured on magnification calibrated digital image files using the program dmfMeasure [49]. Thicknesses were blind measured by stripping identifying information from the images, renaming the files with a randomly generated file name, and mixing them with images from at least three other treatments. Measurements were made in the centermost part of the fracture in a region where the break appeared to be as perpendicular through the cell wall as possible. Measured thicknesses were corrected for tilt and coating thickness. Once measurements for all treatments were complete, 30 images were randomly selected and measured again. Test-retest variability was less than 2.3% in all cases.

### Photoinactivation ($\sigma_i$) determination

At the end of the turbidostat growth period we took a 280 mL culture sample from each replicate for determination of the functional absorption cross-section for photons driving PSII photoinactivation ($\sigma_i A^2$ quanta$^{-1}$) [31,34,50]. We divided the culture sample into two flasks, and supplemented one with a final concentration of 500 $\mu$g mL$^{-1}$ lincomycin (Sigma-Aldrich) to
inhibit chloroplast ribosome function [8,31,51] to block PSII repair. We placed both the flasks in the dark for 10 min to allow the antibiotic (if present) to penetrate into cells and inhibit ribosome function. We then shifted the flasks to 450 μmol photons m⁻² s⁻¹ blue light (LEE #183, Panavision; peak transmission at 435–479 nm and 50% transmission at 406–529 nm, approximating the growth light quality in the bioreactors). In this way, we aimed to assess the responses of cells acclimated to a range of light levels, to a shift to higher light simulating an upward mixing event to the upper region of the euphotic zone. After 15, 30, 60 and 90 min exposure to 450 μmol photons m⁻² s⁻¹, we took samples from both flasks for chlorophyll fluorescence measurements (Fig. 1B). After 90 min we returned the cultures to their culture growth light level and tracked any recovery over 30 and 60 min. We estimated the susceptibility of PSII to photoinactivation (σPSII, A² quanta⁻¹) by fitting the exponential decrease of PSII photochemical yield (Fv/FM) of the lincomycin-treated sample versus cumulative incident photons per area (Fig. 1B). To obtain Fv/FM, a 2 mL sample from each culture replicate at each time point was dark adapted for 5 min in a temperature-controlled cuvette holder (18°C). A blue-green modulated measuring light (4 Hz; Xenon-PAM, Waltz, Effeltrich, Germany) was applied to measure Fv, followed by a saturating light pulse (4000 μmol photons m⁻² s⁻¹, 600 ms) to measure the dark-adapted maximal fluorescence (FM). Fv/FM was calculated as:

\[ Fv/FM = (FM - F0)/FM \]

During the growth light recovery period we continued to monitor Fv/FM and if there was any increase in Fv/FM in the lincomycin treated cells we attributed the increase to slow relaxation of non-photochemical quenching, and used the amplitude of any relaxation to correct for the influence of non-photochemical quenching on changes in the measured Fv/FM. Such corrections were small to negligible and had no substantive effect on the results.

σPSII measurement

We measured the functional absorption cross-section for PSII photochemistry (σPSII, A² quanta⁻¹) for blue light using a Fluorescence Induction and Relaxation fluorometer (FIREc, Satlantic, Halifax, Nova Scotia, Canada). Ahead of the light-shift experiment, we dark-acclimated a 2 mL sample from each culture replicate for 5 min. We then triggered an 80 μs single turnover flash (blue LED 455±2 nm) to saturate PSII photochemistry and drive fluorescence to a maximum. After this we exposed the sample to the relevant culture growth light (30, 80, 160, 240 or 380 μmol m⁻² s⁻¹) for 2 min. We then again triggered the single turnover saturating flash to obtain the absorption cross-section for PSII centers remaining open under the culture light level (σPSII). We then applied a high actinic light of 450 μmol m⁻² s⁻¹ to the samples for 2 min, followed by triggering the single turnover saturating flash again for σPSII measurements of the absorption cross section for the remaining PSII centers still open under the high light treatment. The values of σPSII were extracted from the fluorescence rise curves using the FIREcWORX software [52] with the flash irradiance calibration factors provided by Satlantic (Halifax, Canada).

Photochemical quenching to track the fraction of PSII centers open and ready for photochemistry under the growth light level for a given culture, was calculated following van Kooten and Snel (1990) [53] as:

\[ \sigma_{\text{PSII}} = \frac{Fv/FM - (FM - F0)}{FM} \]

\[ q_P = \frac{(F_{M}^- - F_{S})}{(F_{M}^- - F_{0})} \]

Protein, Chl a and malondialdehyde measurements

Just prior to the high light shift experiments (T0 samples) or after the 90 min of high light treatment, both with or without lincomycin (T90 samples) we vacuum-filtered 50 or 30 mL of culture onto a binder-free Whatman GF/F glass fiber filter (25 mm in diameter), which was immediately flash frozen in liquid nitrogen and stored at −80°C until later analyses of protein and chlorophyll (50 mL filter) or malondialdehyde (30 mL filter).

Total protein was extracted from the frozen filters using the MPBio FastPrep®-24 with bead lysing matrix D (SKU 116913050) and 400 μL of 1× denaturing extraction buffer (0.1375 mol L⁻¹ TRIS buffer, 0.075 mol L⁻¹ EDTA, 0.1 mg mL⁻¹ Pefabloc) [54] for three cycles of 60 seconds at 65 m s⁻¹. Total protein concentration in the extracts was then determined using the Bio-Rad DC protein assay kit (500-0116) with known BGG standards. Molar levels of RbcL, PsbA, PsbD, PetC and PsbA in 1 μg total protein were then determined with quantitative immunoblotting [34,51,54].

For chlorophyll a (Chl a) measurement, 30 μL of protein extract was added to 470 μL 90% acetone (v/v) saturated with magnesium carbonate; after 15 min extraction in the dark at 4°C and 2 min centrifugation (13,000 g), we measured the absorbance of the supernatant at 664, 630 and 750 nm using a UV/VIS spectrophotometer (UV-1800, Shimadzu, Japan). Chl a content was estimated following Jeffrey and Humphrey (1975) [55]:

\[ [\text{Chl a}] = 11.47 \times (A_{664} - A_{750}) - 0.4 \times (A_{630} - A_{750}) \]

Malondialdehyde (MDA) content, an index of cumulative ROS toxicity, was determined with the thiobarbituric acid-reactive substance method [56,57]. Cells on the filter were homogenized with 0.8 mL of 20% (v/v) trichloroacetic acid (TCA), followed by centrifugation at 13,000 g (10 min). After this, 0.35 mL of the supernatant and 0.35 mL of thiorbarbituric acid reagent (0.5% in 20% TCA) was mixed, heated at 90°C for 30 min and cooled on ice. The absorbance of the mixture was measured at 532 and 600 nm with the extraction solvent as blank. Based on the A₅₃₂–A₆₀₀, MDA content was calculated by multiplying by the extinction coefficient of 6.45 μM cm⁻¹.

Carbon and nitrogen analyses

Prior to the light-shift experiment, we filtered 20 mL of turbidostat culture onto a pre-combusted (5 h, 450°C) Whatman GF/F glass fiber filter (13 mm in diameter), rinsed with 10 mL of 50 mmol L⁻¹ HCl to remove inorganic carbon, dried at 55°C for 12 h and stored in a desiccator for later analyses. Contents of carbon and nitrogen were measured with a Vario EL III Elemental Analyzer (Elementar, Hanau, Germany).

Data analysis

The response of growth rate and MDA content to culture growth light was fitted with the equation of Platt et al. (1980) [58]:

\[ \mu = \mu_{\text{max}} \times (1 - e^{-z \times t/\mu_{\text{max}}}) \times e^{(t \times b)/\mu_{\text{max}}} \]
where: $\mu$ is growth rate (d$^{-1}$) or MDA content (attomoles-cell$^{-1}$) at the particular light level; $\mu_{max}$ is the maximum growth rate (d$^{-1}$) or MDA content (attomoles-cell$^{-1}$) derived from the curve fit; $e$ is the base of natural logarithm; $\alpha$ is the initial quantum yield of growth or MDA content; $I$ is the growth light (µmol photons-m$^{-2}$-s$^{-1}$) and $\beta$ is an inhibition parameter.

We used ANOVA with Bonferroni post-tests (Prism 5, Graphpad Software) and comparisons of linear and non-linear curve fits to detect significant differences among the CO$_2$ and culture-light treatments.

**Results**

**Carbonate chemistry system**

Carbonate chemistry parameters in our turbidostat cultures were representative of current ambient (~390 ppmv) and the end of this century (~750 ppmv), except that the estimated pCO$_2$ level for cultures under the lowest growth light and elevated pCO$_2$ treatment was significantly lower than in the cultures under higher growth lights (Table 1). As expected, the elevated pCO$_2$ treatment increased DIC by 7.8%, HCO$_3$ by 13.6% and free CO$_2$ in the medium by 117%, while pH decreased by 0.33 units and [CO$_3$$^2-$] decreased by 39.5%. The total alkalinity in the media remained unchanged between the ambient and elevated pCO$_2$ treatments. The steady total alkalinity also shows that the differing rates of assimilation of ionic nitrogen and sulphur sources across the cultures growing at different rates did not significantly alter the media composition, which was instead dominated by the cycles of dilution with fresh media.

**Growth, cell volume, Chl a and cellular protein**

Cell growth rate increased with culture growth light from low (30 µmol photons-m$^{-2}$-s$^{-1}$) to moderate levels (160 µmol photons-m$^{-2}$-s$^{-1}$), but then decreased under higher light (240, 380 µmol photons-m$^{-2}$-s$^{-1}$) (Fig. 2A). Under bubbling with ambient pCO$_2$, Thalassiosira psuedonana CCMP 1335 achieved a $\mu_{max}$ of 2.15 d$^{-1}$. Bubbling with elevated pCO$_2$ significantly stimulated the growth rate across low to moderate light level, allowing cells to achieve a $\mu_{max}$ of 3.04 d$^{-1}$. Note that these estimates for $\mu_{max}$ are from the curve fits, which include an inhibition term. The measured maximum achieved growth rates for the cultures were 1.8 d$^{-1}$ for ambient pCO$_2$ and 2.3 d$^{-1}$ for elevated pCO$_2$. Above the saturating light level elevated pCO$_2$ did not stimulate growth rate (Fig. 2A). There was an increase in cell volume from 19 to 20 nm$^3$ as the growth light increased, but there was significant scatter among replicates and no significant effect of pCO$_2$ on cell volume (Fig. 2B). Frustule thickness varied from 52 to 80 nm and showed significant scatter among replicates but no significant trend with growth light nor with pCO$_2$ (data not presented).

Chl a content decreased from about 0.22 to 0.08 pg·cell$^{-1}$ as the growth light increased from low to middle levels, with no significant effect of elevated CO$_2$ (Fig. 2C). There were no significant effects of growth light nor pCO$_2$ on total protein per cell, although cultures growing under low light showed more scatter among replicates (Fig. 2D). The mass ratio of Chl a: protein decreased from 0.036 to 0.016 with the increasing growth light at ambient pCO$_2$ (Fig. 2E) and from 0.025 to 0.013 under elevated pCO$_2$, so cultures growing under elevated pCO$_2$ showed a lower Chl a: protein, but only under low growth light (p<0.05). Note that the mass ratios for Chl a: protein were calculated for the same extract from each culture, and thus the patterns of the ratio versus light and pCO$_2$ can achieve statistical significance even though the
unpaired chlorophyll and protein plots do not, possibly because of experimental variation in extraction efficiencies.

**Photophysiological parameters**

The cultures showed differential growth-light and growth-rate dependent changes in photophysiology under ambient and elevated pCO2 (Fig. 3). The functional absorption cross-section that serves PSII photochemistry ($\sigma_{PSII}$) decreased markedly from about 322 to 210 A$^2$·quanta$^{-1}$ with increasing growth light, with no significant effect of pCO2 (Fig. 3A). There was no pattern of $\sigma_{PSII}$ with respect to culture growth rate (Fig. 3B), and so $\sigma_{PSII}$ shows a clear pattern of acclimation to increasing culture growth light.

In marked contrast the functional absorption cross-section for incident photons driving PSII photoinactivation ($\sigma_i$) increased from approximately 7.3×10$^{-5}$ to 9.3×10$^{-5}$ A$^2$·quanta$^{-1}$, as the growth light increased from 30 to 160 µmol photons·m$^{-2}$·s$^{-1}$ for cultures bubbled with ambient CO2, and then decreased back to 7.1×10$^{-5}$ A$^2$·quanta$^{-1}$ as growth light increased further to 380 µmol photons·m$^{-2}$·s$^{-1}$ (Fig. 3C). Under elevated CO2 this curvilinear response to growth light disappeared and $\sigma_i$ remained steady at ~7×10$^{-5}$ A$^2$·quanta$^{-1}$ (Fig. 3C). The unexpected curvilinear light response of $\sigma_i$ is resolved by a plot of $\sigma_i$ versus growth rate (Fig. 3D) which shows a positive, linear correlation for cultures growing under ambient pCO2 ($R^2 = 0.64$, $p < 0.0001$). Cultures grown under elevated pCO2 lose this correlation of susceptibility to photoinactivation with growth rate, and thus show a significantly lower $\sigma_i$ under conditions of high growth rate ($p < 0.01$) (Fig. 3D). Thus, under elevated pCO2 rapidly growing cells show a decrease in susceptibility to photoinactivation of PSII, compared to cells under ambient pCO2. In contrast, under slow growth elevated pCO2 can even provoke increased susceptibility to photoinactivation [8].

The ratio of $\sigma_{PSII}/\sigma_i$, a baseline estimate of photochemical return on investment per cycle of PSII repair, demonstrated both growth light and growth rate-dependent effects at both CO2 levels. $\sigma_{PSII}/\sigma_i$ in cultures bubbled with ambient pCO2 curvedly decreased from 4.5×10$^{-6}$ to 2.8×10$^{-6}$ as growth light increased from low to moderate levels. Cultures bubbled with elevated pCO2 showed a linear decrease in $\sigma_{PSII}/\sigma_i$ with growth light (Fig. 3E). At the moderate, growth-saturating light of 160 µmol photons·m$^{-2}$·s$^{-1}$, the $\sigma_{PSII}/\sigma_i$ in cultures bubbled with ambient pCO2 is about 70% of the value for cultures bubbled with elevated pCO2.

**Figure 3. Photophysiology of cells grown under ambient and elevated CO2 versus growth light or growth rate.**

A) Functional absorption cross-section for Photosystem II photochemistry ($\sigma_{PSII}$, A$^2$·quanta$^{-1}$) versus growth light. Solid line: Polynomial curve for pooled growth light response of $\sigma_{PSII}$ under both ambient and elevated pCO2. Thin dotted lines: 95% confidence intervals on the fitted curve. B) Functional absorption cross-section for Photosystem II ($\sigma_{PSII}$) versus growth rate. C) Functional cross section for photoinactivation of Photosystem II ($\sigma_i$, A$^2$·quanta$^{-1}$) versus growth light. Solid line: Polynomial curve for growth light response of $\sigma_i$ under ambient pCO2. Dashed line: Polynomial curve for growth light response of $\sigma_i$ under elevated pCO2. Thin dotted lines: 95% confidence intervals on the fitted curves. D) Functional cross section for photoinactivation of Photosystem II ($\sigma_i$) versus growth rate. Solid line: Linear regression for growth response of $\sigma_i$ under ambient pCO2. Dashed line: Linear regression for growth response of $\sigma_i$ under elevated pCO2. Thin dotted lines: 95% confidence intervals on the fitted curves. E) Ratio of $\sigma_{PSII}/\sigma_i$ versus growth light. Solid line: Polynomial curve for light response of $\sigma_{PSII}/\sigma_i$ under ambient pCO2. Dash line: Polynomial curve for growth light response of $\sigma_{PSII}/\sigma_i$ under elevated pCO2. Thin dotted lines: 95% confidence intervals on the fitted curves. F) Ratio of $\sigma_{PSII}/\sigma_i$ versus growth rate. Solid line: Linear regression for growth response of $\sigma_{PSII}/\sigma_i$ under ambient pCO2. Dash line: Linear regression for growth response of $\sigma_{PSII}/\sigma_i$ under elevated pCO2. Thin dotted lines: 95% confidence intervals on the fitted curves.

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pCO2, indicating fewer rounds of photochemical charge separation per round of PSII repair under ambient CO2, where cells suffer more frequent photoinactivations of PSII under moderate light. At the fastest growth rates (Fig. 3F) cells under elevated pCO2 enjoy more potential photochemical return per round of PSII repair.

The functional absorption cross-section for PSII photochemistry measured under growth light ($\sigma_{PSII}$) (Fig. 4A) shows a similar trend to $\sigma_{PSII}$ measured from dark-acclimated cells (Fig. 3A), decreasing from about 311 to 156 Å$^2$ quanta$^{-1}$ with the increasing growth light, with no significant effect of pCO2 except under the highest growth light of 380 μmol photons·m$^{-2}·$s$^{-1}$ (Fig. 4A). Photochemical quenching (qP) that tracks the fraction of PSII centers open and ready for photochemistry, decreased from 0.89 to 0.63 as the growth light increased from low to moderate levels, then remained steady under higher growth light levels, with no significant CO2 effects (Fig. 4B). When we plotted the growth light $\sigma_{PSII}$ versus $\sigma_{PSII}$, all the points fell close to the 1:1 dotted line (Fig. 4C), indicating limited net down-regulation of the antenna size serving PSII under culture growth light, compared to the antenna size from dark-acclimated cells. However, when we plotted the functional absorption cross-section ($\sigma'_{PSII}$) measured under the high shift light (450 μmol photons·m$^{-2}·$s$^{-1}$) to the $\sigma_{PSII}$, all the points fell far below the 1:1 line, especially in the cultures from low growth light (Fig. 4D), showing a significant down-regulation of the functional antenna size under the excess light conditions as non-photochemical quenching is induced [59].

Carbon and nitrogen

Mean contents of carbon or nitrogen were 0.65 and 0.11 pmol·cell$^{-1}$, with no significant patterns in response to growth light nor pCO2 (data not presented). Under ambient pCO2 the molar ratio of C:N varied from 5.1 to 7.4 and showed a curved response as the growth light increased from low to moderate levels, followed by a decrease under higher growth light levels (Fig. 5A); moreover, C:N shows a linear increase with increasing growth rate ($R^2$ = 0.37, p < 0.05) (Fig. 5B) across both ambient and elevated pCO2 cultures.

RbcL, PsbA, PsbD, PetC and PsaC

Content of RbcL, the RUBISCO large subunit, varied from 3.2 to 11 atomomoles·cell$^{-1}$ and showed a modest positive correlation...
with increasing growth light ($R^2 = 0.21$, $p < 0.05$) (Fig. 6A). There were no significant effects of growth light nor pCO$_2$ on the contents of PsbA (PSII subunit) (Fig. 6B), PsbD (PSII subunit) (Fig. 6C) nor PetC (Cytochrome b$_6$f complex subunit) (Fig. 6D). However, PsaC (PSI subunit) declined significantly as growth light increased from low to moderate levels (Fig. 6E). Increasing growth light provoked significant increases in the ratios of RbcL/PsbD and PsbA/PsbD (Fig. 6F,G), but a decrease in the PsaC/PsbD ratio (Fig. 6H). Overall we did not detect significant effects of pCO$_2$ on protein subunit contents (Fig. 6), except under the growth light of 30 μmol photons m$^{-2}$s$^{-1}$ where the RbcL:PsbD ratio was marginally higher in cultures bubbled with elevated pCO$_2$ ($p < 0.05$) (Fig. 6F).

**Malondialdehyde content**

In cultures under ambient pCO$_2$ malondialdehyde (MDA), a product of lipid peroxidation, curvilinearly increased from 10 to 23 attomoles cell$^{-1}$ as growth light increased from low to moderate levels, followed by a decrease to 14 attomoles cell$^{-1}$ under the highest growth light (Fig. 7A). Elevated pCO$_2$ significantly increased the MDA content under low growth light (30 μmol photons m$^{-2}$s$^{-1}$), but not under higher growth light (Fig. 7A). The content of MDA was also increased after 90 min exposure of low-light-cultured cells (30 or 80 μmol photons m$^{-2}$s$^{-1}$) to 450 μmol photons m$^{-2}$s$^{-1}$ under both CO$_2$ treatments (Fig. 7B). Furthermore, there was a positive correlation ($R^2 = 0.75$, $p < 0.001$) between the MDA content and growth rate under ambient pCO$_2$, but not under elevated pCO$_2$ (Fig. 7C), mirroring the correlation of $\sigma_i$ with growth rate under ambient pCO$_2$ (Fig. 3D,7D).

**Discussion**

**Elevated pCO$_2$ stimulates diatom growth under low to moderate light**

A seawater inorganic carbon system that approximates conditions expected for the end of this century promoted the growth rate of a marine coastal diatom under sub-saturating growth light, but not under saturating to super-saturating growth light. The significant fertilization of elevated pCO$_2$ on growth of the diatom under lower light (Fig. 2A) may be accounted for by an increased diffusive supply of free CO$_2$ and bicarbonate concentrations, that could allow the diatom to down-regulate carbon concentrating mechanisms (CCM) [5,6] and thereby save about 20% of the energy demanded by CCM under current ambient pCO$_2$ [5,60,61]. The savings from this down-regulation can be re-allocated to support the increased growth rate (Fig. 2A). We see a hint of these resource re-allocations in the lower Chl: Protein ratio under low light and elevated pCO$_2$ (Fig. 2E), but surprisingly not in our determinations of C:N molar ratio (Fig. 5A) nor in representative subunits of major photosynthetic complexes (Fig. 6). Under higher growth lights savings from CCM down-regulation may become less significant relative to the costs of coping with excess light [7,16,18]. Furthermore with rapid growth under high light the increased demand for carbon assimilation may outweigh the influence of increased pCO$_2$.

In our cultures the moderate light of 240 μmol photons m$^{-2}$s$^{-1}$ (Fig. 2A) was sufficient to provoke the onset of inhibition of growth, particularly under elevated pCO$_2$. Cullen and Lewis (1988) [62], in contrast, achieved yet higher growth rates with T. pseudonana under light up to 2200 μmol photons m$^{-2}$s$^{-1}$. We suspect the difference arose because we grew the cultures under continuous illumination from narrow-band blue light LED sources, for consistency between our growth light spectra and our determinations of $\sigma_{PSII}$ and $\sigma_i$ for blue light. Blue light has a higher quantum yield for photoactivation of PSII of diatoms [51] and plants [63] than do longer wavelengths, so our illumination provoked a higher rate of photoactivation at a given light level than did the white light source of Cullen and Lewis (1988) [62].

**Elevated pCO$_2$ alters the photophysiological properties of diatoms**

When growing under current ambient pCO$_2$ the coastal diatom showed a curvilinear relation between the susceptibility of their PSII to photoactivation ($\sigma_i$) and growth light (Fig. 3C). This surprising pattern resolved to a linear correlation between $\sigma_i$ and growth rate (Fig. 3D). For determination of $\sigma_i$ we elevated the treatment light to accelerate the rate of photoactivation, to improve the accuracy of curve fits over reasonably short treatment times. As reviewed in Campbell & Tyystjärvi [50] $\sigma_i$ is applicable across the range of growth lights, although at lower light the rate of photoactivation ($\sigma_i$ times incident photons m$^{-2}$s$^{-1}$) will be slower. The turbidostat cultures maintained a stable growth rate, with stable Photosystem II function, tracked with $F_{v}/F_{m}$, and stable PSII protein content (Fig. 6B,C). In this steady state situation each photoinactivation event is countered by the PSI repair cycle. Therefore, the susceptibility to photoactivation is a direct measure of the metabolic cost the diatoms incur to maintain PSII function under a given growth light. Under ambient pCO$_2$ the faster growing diatoms suffer a cost of increased susceptibility to photoactivation and thus a proportionate increase in their

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**Figure 5. Molar ratio of C:N versus growth light and growth rate.** A) Molar ratio of carbon to nitrogen (C:N) versus culture growth light. Solid line: Polynomial curve for pooled growth light response for cultures from ambient and elevated pCO$_2$. Thin dotted lines: 95% confidence intervals on the fitted curves. B) Molar ratio of C:N versus growth rate ($\mu$, d$^{-1}$). Solid line: Polynomial curve for pooled growth light response for cultures from ambient and elevated pCO$_2$. Thin dotted lines: 95% confidence intervals on the fitted curves.

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Figure 6. Representative protein subunits per cell for complexes mediating photosynthesis versus growth light. A) RbcL subunit from RUBISCO. Solid line: Linear regression for pooled growth light response for cultures from ambient and elevated pCO2. Thin dotted lines: 95% confidence intervals on the fitted curve. B) PsbA subunit from PSII versus growth light. C) PsbD subunit from PSII versus growth light. D) PetC subunit from cytochrome b6f complex versus growth light. E) PsaC subunit from PSI. Solid line: Polynomial curve for pooled growth light response for cultures from ambient and elevated pCO2. Thin dotted lines: 95% confidence intervals on the fitted curve. F) Ratio of RbcL: PsbD versus growth light, proxy for RUBISCO:PSII. Solid line: Polynomial curve for growth light response for cultures from ambient pCO2. Dash line: Polynomial curve for growth light response for cultures from elevated pCO2. Thin dotted lines: 95% confidence intervals on the fitted curves. G) Ratio of PsbA: PsbD versus growth light, proxy for PSII repair cycle. Solid line: Linear regression for pooled growth light response for cultures from ambient and elevated pCO2. Thin dotted lines: 95% confidence intervals on the fitted curves. H) Ratio of PsaC: PsbD versus growth light, proxy for PSI:PSII. Solid line: Polynomial curve for pooled growth light response for cultures from ambient and elevated pCO2. Thin dotted lines: 95% confidence intervals on the fitted curve.

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metabolic cost to maintain photosynthesis. Under elevated pCO₂ these patterns of σᵢ disappear, so that at the very lowest growth rates diatoms under elevated pCO₂ show similar (Fig. 3D) or even increased susceptibility to photoinactivation compared to current ambient pCO₂ [8]. In contrast, under moderate to high growth rates the diatoms under elevated pCO₂ enjoyed significantly lower susceptibility to photoinactivation. At the optimal growth light of 160 μmol photons m⁻² s⁻¹ diatoms under elevated pCO₂ show a ~30% lower σᵢ and thus incur a proportionately lower cost to maintain their PSII pool and photosynthetic capacity.

Note that these determinations of susceptibility to photoinactivation are for diatoms shifted to the moderately high light of 450 μmol photons m⁻² s⁻¹ and that upon shifts to yet higher light additional factors interact with photoinactivation of PSII, including the catalytic capacity for ROS detoxification [64] and dumping of photosynthetic electrons back to oxygen [65]. Furthermore, our current study is of coastal diatoms under exponential growth and nutrient repletion and in some phytoplankton nutrient depletion strongly alters photophysiological responses [32,66].

We tested multiple hypotheses as to the underlying mechanism(s) for the effects of growth rate and of elevated pCO₂ upon photoinactivation. Based upon findings of Y. Wu & Z. Finkel (unpub., data) that elevated pCO₂ lowers the silica content of diatoms, we measured the frustule thickness (data not presented) to determine if changes in frustule thickness might underlie the changes in susceptibility to photoinactivation, since the diatom frustule can screen harmful radiation [67]. We did not find any significant correlation of frustule thickness with growth light (data not presented), nor with growth rate, nor with pCO₂, and so we did not find support for our initial hypothesis that pCO₂ influenced cellular optics by altering frustule thickness. More subtle changes in frustule structure [68], density or pigment binding [67] could still potentially contribute to changes in diatom susceptibility to photoinactivation of PSII. We did capture whole cell spectra using an integrating cavity spectrophotometer but did not detect significant changes in cellular coloration under changing pCO₂ (data not presented). The buffering influence of silicate upon pH changes under rising CO₂ might also influence PSII susceptibility to photoinactivation of diatom by altering CCM operation [69], but we have no data on this topic. More generally we find a suggestive correlation between the diatom frustule and the low susceptibility of diatoms, as a group, to photoinactivation [31], when compared to other phytoplankton measured under comparable conditions including coccolithophores [8,32,33], picocyanobacteria [34], prasinophytes [35], Bolidomonas [70] and other taxa (Campbell et al. unpub.). Even here, however, specificities of diatom photosystem subunit composition [71] and metabolism [65] provide alternate explanations for the unusually low susceptibility of diatoms to photoinactivation of PSII.

σPSII showed a simple negative correlation with growth light, was not significantly altered by elevated pCO₂ (Fig. 3A) and did not correlate with growth rate (Fig. 3B), thus showing straight forward light regulation of functional antenna size. Elevated pCO₂ had no significant effects on the down regulation of σᵢ under excess light (Fig. 4D). Therefore the effects of light and pCO₂ on σᵢ (Fig. 3C,D) appear to arise outside and separately from acclimatory changes in the functional size and short-term regulation of the antenna serving PSII. Across a taxonomic panel of diatoms of widely differing size [31] we found negative correlations of both σPSII and σᵢ with increasing cell biovolume, which is consistent with pigment packaging or self-screening effects in the larger cells [31,72,73]. We did see some increase in cell biovolume with increasing light in these experiments (Fig. 2B), but
there was no significant influence of pCO2 on biovolume and thus the small changes in biovolume explained neither the effects of pCO2 nor growth light on σT.

The change from ambient to elevated pCO2 did not significantly alter the pattern of PSII closure, measured as qP with increasing growth light (Fig. 4B), nor was there an effect of pCO2 upon qP measured under the higher treatment light (data not presented). Therefore we cannot attribute the pCO2 effect on susceptibility to photoinactivation to a simple change in excitation pressure on PSII [74].

We hypothesized that large re-allocations of nitrogen resources (Fig. 5) among the major complexes mediating photosynthesis might underlie the interactive effects of growth light and pCO2 on σT. RbcL (RUBISCO, Fig. 6A) and PsaC (PSI, Fig. 6E) contents changed with growth light, but we found only modest effects of elevated pCO2 on the ratio between RbcL (RUBISCO) and PsbD (PSII subunit) and only in cells growing under low light (Fig. 6F). We did not detect any significant influence of pCO2 on the cellular contents of PSII subunits, so a bulk change in PSII content is unlikely to explain the patterns of σT with growth light and pCO2.

We did detect a moderate increase in the PsbA:PsbD ratio under lower growth light (Fig. 7A). Intriguingly in cells under increased light, which may reflect increasing engagement of the PSII repair cycle and accumulation of intermediates in the overall PSII repair cycle [51,59,75], but which does not explain the patterns of σT that we observe.

We analyzed the cellular content of MDA, an end product of lipid peroxidation produced when reactive oxygen stress damages biological membranes [56,57,76]. Increasing growth light and elevated pCO2 both altered MDA content of the diatoms (Fig. 7A). A shift from growth light to excess light of 450 μmol photons·m−2·s−1 provoked some additional accumulation of MDA, but only in cells growing under low light; cells growing under higher light showed no additional MDA accumulation (Fig. 7B) pointing to pre-induction of detoxification systems [64] sufficient to counter any additional ROS production during the high light shift. MDA also correlated with growth rate (Fig. 7C), particularly in cells growing under ambient pCO2. Carbon limitation has been observed to induce increased expression of ROS-removing enzymes [77], which can explain the lower MDA in cells growing under ambient than elevated pCO2, particularly under lower growth light (Fig. 7A). Intriguingly in cells under ambient pCO2 increasing susceptibility to photoinactivation correlates with increasing cellular content of MDA (Fig. 7D), suggesting that in cells suffering significant photoinactivation, lipid peroxidation occurs in parallel, possibly driven by common or over-lapping mechanisms [78–80]. In contrast, under elevated pCO2 cellular content of MDA varies widely depending upon growth conditions but σT does not, so growth under elevated pCO2 breaks the correlation between an index of ROS toxicity and cellular susceptibility to photoinactivation (Fig. 7D). In summary, our analyses to date have not uncovered a mechanism for the interactive effects pCO2, light and growth rate on σT, but the effects are significant and represent biologically meaningful changes in the cost of maintaining the photosynthetic apparatus in this coastal diatom.

To maintain PSII function, every photoinactivation event must be countered by an expensive PSII repair process [25,59,75]. Assuming the similar cellular contents of PSII protein subunits under ambient and elevated pCO2 are proxies for similar overall pools of PSII, the lower σT under elevated pCO2 (Fig. 3D) implies a decreased metabolic burden to maintain a comparable PSII pool.

The ratio σPSII/σT ratio tracks the delivery of excitons to drive PSII photochemistry relative to photoinactivation events and sets an upper boundary on the return-on-investment for maintaining PSII under a given growth light condition. Elevated pCO2 markedly alters this relation (Fig. 3E,F) so fast-growing diatoms under elevated pCO2 enjoy a significantly higher metabolic return from their investments into PSII.

The coastal strains of T. pseudonana that can dominate in the well mixed coastal or estuarine waters [27,81] are well adapted to the sharply fluctuating light therein. Simultaneously, pH in these waters also varies widely, for example, from 7.0 to 8.4 in the Pearl River estuary of the South China Sea, causing a drastic change in pCO2 of 380 to 4800 ppmv [41]. Thus coastal phytoplankton including strains of T. pseudonana must be well adapted to changing CO2 levels. T. pseudonana is also a bloom forming species whose own blooms can occasionally considerably change the seawater CO2 and light regimes.

Elevated pCO2 and growth light interactively affect the growth rates, photophysiological parameters and key protein levels of the coastal diatom T. pseudonana CCMP 1335. The elevated CO2 provokes a decreased susceptibility to photoinactivation of PSII when the growth rates are high (Fig. 3D). In nutrient-rich coastal or estuarine waters, the growth rate of natural phytoplankton assemblages including diatoms can reach as high as 2.5 d−1 [82]. Moreover, the diatoms in these waters must exploit variable light environments due to sharp attenuation of the light gradient and fast vertical mixing rates [40]. Decreased susceptibility to photoinactivation due to rising CO2 thus supplies T. pseudonana with a potential competitive advantage over other phytoplankton species in coastal or estuarine waters.

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Author Contributions

Conceived and designed the experiments: GL DAC. Performed the experiments: GL. Analyzed the data: GL DAC. Contributed reagents/materials/analysis tools: DAC. Wrote the paper: GL DAC.

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