

Rubisco is a small fraction of total protein in marine phytoplankton

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Summary

- Ribulose 1,5 biphosphate carboxylase oxygenase (Rubisco) concentrations were quantified as a proportion of total protein in eight species of microalgae. This enzyme has been assumed to be a major fraction of total protein in phytoplankton, as has been demonstrated in plants, potentially constituting a large sink for cellular nitrogen.
- Representative microalgae were grown in batch and continuous cultures under nutrient-replete, nitrogen (N)-limited, or phosphorus (P)-limited conditions with varying CO₂. Quantitative Western blots were performed using commercially available global antibodies and protein standards. Field incubations with natural populations of organisms from the coast of California were conducted under both nutrient-replete and N-limited conditions with varying CO₂.
- In all experiments, Rubisco represented < 6% of total protein. In nutrient-replete exponentially growing batch cultures, concentrations ranged from 2% to 6%, while in nutrient-limited laboratory and field cultures, concentrations were < 2.5%. Rubisco generally decreased with increasing CO₂ and with decreasing growth rates.
- Based on a calculation of maximum Rubisco activity, these results suggest that phytoplankton contain the minimum concentration of enzyme necessary to support observed growth rates. Unlike in plants, Rubisco does not account for a major fraction of cellular N in phytoplankton.

Introduction

The carboxylating enzyme ribulose 1,5 biphosphate carboxylase oxygenase (Rubisco), which is responsible for catalyzing the bulk of the conversion of inorganic carbon to organic matter, is arguably one of the most important enzymes on the planet. Nonetheless, this enzyme has a relatively slow catalytic efficiency (*c.* 3 carbons active site⁻¹ s⁻¹), has difficulty discriminating between CO₂ and O₂ as a substrate, and in the surface ocean, is undersaturated for CO₂ (Badger *et al.*, 1998). Rubisco compensates for these catalytic inefficiencies by being present in high abundances. It is commonplace in the literature to claim that Rubisco is not only one of the most important enzymes, but also the most abundant protein on the planet (Ellis, 1979), accounting for up to half of total cellular protein. While this assertion is commonplace in the literature, it is based on relatively few studies (Dorner *et al.*, 1957; Pickersgill, 1986). The vast majority of measurements reveal much lower, but still high, concentrations in plants ranging from 5% to 40% of total protein. This variation is found among different species, including rice, tobacco, spinach and various grasses (Joseph *et al.*, 1981; Pickersgill, 1986; Rowland-Bamford *et al.*, 1991) and varies widely even within a single plant depending on the quantification method, growth stage or which leaf is sampled (Goldthwaithe & Bogorad, 1971; Seemann &

Berry, 1982; Sugiyama *et al.*, 1984; Campbell *et al.*, 1988; Evans, 1989; Makino *et al.*, 1992; Metodiev & Demirevskakepova, 1992; Rogers *et al.*, 1996). Even at these lower abundances, Rubisco still accounts for a significant fraction of cellular nitrogen (N) in leaf tissue, particularly in C₃ plants. This has implications for N-limiting conditions and is important for determining the N requirement of plants.

The assumption that Rubisco abundances in phytoplankton should mirror those found in plants is not justified by the values reported in the literature. Further, high concentrations of carboxylating enzyme may not be necessary in organisms that possess a carbon concentrating mechanism (CCM). Raven (1991) has compiled the most comprehensive dataset for Rubisco concentration (defined here as the fraction of Rubisco to total protein by mass) in phytoplankton, showing values from 2% up to 23%, with most of the measurements falling between 4% and 10%. However, these percentages were approximated from a variety of indirect methods so that there is a need for direct quantification. It is evident that reliable measurements of Rubisco in marine phytoplankton are needed to better understand the nutrient and energy requirements of phytoplankton. This is especially important for N-limiting conditions because some studies have suggested that Rubisco acts as an N reservoir in these environments (Falkowski *et al.*, 1989). Rubisco abundance and regulation may also play a role in the

cellular allocation of energy and other nutrients, such as phosphate. Several studies have explored the effect of CO₂ on Rubisco concentrations in phytoplankton with mixed results (Levitan *et al.*, 2010; Losh *et al.*, 2012; McCarthy *et al.*, 2012).

The commercial availability of a global Rubisco antibody (Agrisera, Sweden), which binds to a region of the large subunit of Rubisco that is conserved amongst plants, diatoms, cyanobacteria and dinoflagellates, now allows accurate measurements. Here we present measurements of Rubisco concentrations in several diatoms and other microalgae including haptophyta and a green alga. We tested four experimental conditions: batch cultures of eight microalgae, CO₂-controlled batch cultures of the diatom *Thalassiosira weissflogii*, P-limited and N-limited continuous cultures of the coccolithophore *Emiliana huxleyi* at varying CO₂, and field experiments with natural populations of organisms off the coast of California exploring N limitation and varying CO₂.

Materials and Methods

Batch cultures

Five species of marine diatoms were obtained from either the Provasoli-Guillard National Center for Culture of Marine Phytoplankton (CCMP) or the Culture Collection of Algae at the University of Texas at Austin (UTEX): *Thalassiosira weissflogii* (*Tw*, CCMP 1336), *Thalassiosira oceanica* (*To*, CCMP 1005), *Chaetoceros mulleri* (*Cm*, CCMP 1316), *Skeletonema cf. costatum* (*Sc*, CCMP 1332) and *Phaeodactylum tricorutum* (*Pt*, UTEX 642). Two haptophytes were obtained from the Plymouth Culture Collection of Marine Algae in the UK: *Emiliana huxleyi* (*Eh*, PLY B92/11) and *Isochrysis galbana* (*Ig*, PLY 565). The green alga *Chlamydomonas reinhardtii* was obtained from the Chlamydomonas Resource Centre at the Department of Plant Biology at the University of Minnesota, USA (*Cr*, CC-124). Each of these phytoplankton was grown in triplicate batch cultures in 250-ml sterilized polycarbonate bottles using coastal seawater (background nutrients *c.* 1 μM PO₄³⁻, 20 μM NO₃⁻, 25 μM SiO₄⁴⁻) obtained from the Institute of Marine and Coastal Science (IMCS) at Rutgers University (USA) that had been filter- and microwave-sterilized (seawater did not boil). For diatoms and haptophyta, the coastal seawater was amended to final concentrations of 120 μM NO₃⁻, 10 μM PO₄³⁻ and 50 μM SiO₄⁴⁻, with vitamins and trace metals according to the Aquil recipe (Sunda *et al.*, 2005). *Chlamydomonas reinhardtii* was grown on freshwater minimal medium with 75 μM NH₄⁺, 9 μM PO₄³⁻ and trace metals (Kropat *et al.*, 2011). Cultures were maintained at 20°C under continuous light (*c.* 150 μmol quanta m⁻² s⁻¹) and harvested for protein in exponential and stationary growth phases. 10–200 ml culture was filtered under gentle vacuum onto a 0.2- or 0.8-μm polycarbonate filter, extracted with 500 μl extraction buffer (50 mM Tris at pH 6.8, 2% sodium dodecyl sulfate, 10% glycerol), and frozen at –20°C. Previous work identified that stationary phase was initiated by N limitation.

CO₂-controlled batch cultures of *T. weissflogii* *Thalassiosira weissflogii* was maintained in unialgal batch cultures under

conditions as outlined above and bubbled at various CO₂ concentrations (182 ppm/pH 8.2, 380 ppm/pH 8.0 and 750 ppm/pH 7.8). Cells were pre-acclimated in these conditions for eight generations before being transferred to fresh medium at a concentration of 50 cells ml⁻¹. Cells were grown in this medium and harvested in exponential phase (*c.* 10 000 cells ml⁻¹) before the pH had shifted > 0.05 pH units.

***E. huxleyi* continuous cultures varying CO₂** *Emiliana huxleyi* was maintained in unialgal continuous cultures under either P-limiting (reservoir medium 50 μM NO₃⁻, 100 nM PO₄³⁻) or N-limiting (reservoir medium 20 μM NO₃⁻, 2 μM PO₄³⁻) conditions at various CO₂ concentrations. Growth medium was prepared using 0.2-μm filtered and microwave-sterilized Gulf Stream seawater with added vitamins and trace metals (Sunda *et al.*, 2005), with the exception of 20 nM Zn, 100 nM Fe and 59 nM thiamine for the P-limited cultures. Growth rates of 0.3 d⁻¹ (P limitation) and 0.6 d⁻¹ (N limitation) were set by continuous peristaltic pumping into acid-washed 500-ml glass bottles. For the P-limited cultures, CO₂ was controlled by bubbling pre-mixed CO₂ air (Airgas). For the N-limited cultures, the pH of the growth media was adjusted with either 10% HCl or 1 M sodium hydroxide (NaOH) to achieve steady state pH values in the desired range (7.7–8.4); CO₂ concentrations were calculated using pH, dissolved inorganic carbon (DIC), salinity and temperature using equilibrium constants from Dickson & Goyet (1994), with updates to the carbonic acid equilibria, on the pH_T scale from Lueker *et al.* (2000). DIC samples were submitted to the UC Davis Stable Isotope Facility for analysis (Brandes, 2009). Steady state growth was achieved when cell counts and pH were constant for at least 1 wk. Alkaline phosphate activity was measured to confirm P limitation (Xu *et al.*, 2006). N limitation was confirmed via analysis at the Woods Hole Nutrient Analytical Facility for analysis according to Parsons *et al.* (1984) and showed no detectable nitrate or ammonia (< 0.05 μM) in the culture.

Field experiments

The experimental design is described in detail in Losh *et al.* (2012). Briefly, five experiments were conducted using surface seawater off southern California over a 2-wk period (15–27 October 2008) varying NO₃⁻ and CO₂. In all experiments, 1.0 μM PO₄³⁻, 14 μM SiO₄⁴⁻ and 2 nM iron were added. In the N-limited treatments, no NO₃⁻ was added, while the N-replete treatments received 10 or 20 μM NO₃⁻. Both nutrient treatments had triplicate bottles at three different CO₂ concentrations (Supporting Information Table S1). CO₂ was adjusted using acid/base additions as in Losh *et al.* (2012). After CO₂ and nutrient manipulations, bottles were placed in on-deck incubators for 3–4 d with flow-through seawater (*c.* 16°C) at 20% surface irradiance controlled by neutral density screening. At *t* = 0 and the final timepoint for each bottle, *c.* 2 l water was filtered through a 0.22-μm Sterivex filter cartridge, flash-frozen in liquid N₂, and stored at –80°C until processing.

Protein quantification

Total protein concentration was determined using the BCA protein assay (Pierce, Thermo Scientific, Waltham, MA, USA). Quantitative western blots were performed for RbcL – the large subunit of Rubisco – in all samples and of PsbA – a core reaction center protein in photosystem II – in some samples, as described in Losh *et al.* (2012). Experimental samples (*c.* 1–2 μg total protein) were run alongside a series of three of four RbcL or PsbA standards (Agrisera) on SDS-PAGE gels (250 V, 40 min) and then transferred to a polyvinylidene fluoride (PVDF) membrane (200 mA, 2 h). Membranes were blocked for 1 h with TBST-milk buffer (5% milk powder in Tris-buffered saline containing 0.25% *v/v* Tween-20, pH 7.5) and probed with polyclonal, global anti-RbcL or anti-PsbA (1 : 10 000, 1 h; Agrisera) and a secondary antibody (Pierce, alkaline phosphatase-coupled goat anti-rabbit IgG antibody, 1 : 5000, 1 h) in TBST-milk buffer. Antibody incubations were followed by five washes in TBST buffer. The primary antibodies are designed against peptide tags that are conserved across all oxygenic photosynthesizers so that no bias in affinity is expected even if phytoplankton communities differ (Campbell *et al.*, 2003). For the batch and continuous culture samples, protein bands were visualized by colorimetric analysis with nitro-blue tetrazolium chloride/5-bromo-4-chloro-3-indolylphosphate *p*-toluidine (NBT/BCIP). For the field samples, Immobilon western chemiluminescent AP substrate (Millipore) was used for more sensitive detection. Picomoles of RbcL or PsbA were determined by directly comparing the intensities of the bands using ImageJ (Schneider *et al.*, 2012). Results were only used when samples fell within the linear range of loaded standards.

All tested microalgae contain Form 1 Rubisco with equimolar subunits, 8 RbcL and 8 RbcS subunits per molecule. Picomoles of RbcL obtained from the western blot were converted to μg of RbcL using a molecular weight of 55 kDa, so that μg of RbcS could be calculated using equimolar pmoles and a molecular weight of 15 kDa (Baker *et al.*, 1975). Total μg of Rubisco was then calculated by summing the μg of RbcL and the μg of RbcS. Concentration of Rubisco can then be expressed as a percentage of total μg Rubisco of total protein loaded on the gel. PsbA has only 1 subunit of 39 kDa (Agrisera) and can therefore be calculated directly from pmoles measured on the western blot.

Method validation

The quantitative western blot method was validated against spinach, *Spinacea oleracea* (*Sp*), and against PsbA protein. Three-week-old baby leaf spinach (*Sp*; Blue Moon Acres Farm, Pennington, NJ, USA) was grown in fertilized soil at a temperature of 20°C and 16 h : 8 h, light : dark ambient daylight. Total protein was extracted from 1 cm^2 leaf area using a glass homogenizer and extraction buffer. We obtained values similar to published results, $10.4 \pm 3.2\%$ of total protein (from eight replicates; Goldthwaithe & Bogorad, 1971; Seemann & Berry, 1982; Makino *et al.*, 1992). Although these values are towards the lower end for Rubisco in *Sp*, this could be due to other

quantification methods, specifically Coomassie blue staining which is not specific for Rubisco and may give an overestimation.

PsbA was measured in the diatom batch cultures and was found to match previous measurements of PsbA in the diatom *Thalassiosira pseudonana* (Wu *et al.*, 2011). PsbA ranged from 0.6% to 1.6% of total protein in exponential phase and decreased in stationary phase to 0.5% or less (Fig. S1). We note that Rubisco : PsbA ratios have been used as a proxy for light acclimation and that the calculated Rubisco : PsbA ratios for our batch cultures (data not shown) are within range of previous measurements (Brown *et al.*, 2008).

Statistics

Differences between CO₂ treatments were assessed using a two-sample unequal variance t-test with two tails. To test for significance in the field experiments, data within each experiment were normalized to the mean of a reference CO₂ treatment, for example Expt 1 low CO₂ when comparing Expt 1 intermediate and high CO₂. Normalized values were then pooled from all five experiments and compared as described above (Losh *et al.*, 2012).

Results

Batch cultures

Eight species of microalgae were grown in batch culture and harvested for protein in exponential and stationary growth phases. In all species, Rubisco concentrations were higher in the exponential phase compared to stationary phase. In exponential growth phase when the cells were at their maximum growth rate under nutrient-replete conditions, diatom Rubisco concentrations ranged from $1.4 \pm 0.2\%$ in *Skeletonema cf. costatum* to $3.7 \pm 0.7\%$ in *Chaetoceros mulleri* (Fig. 1, Table 1). Haptophytes displayed concentrations slightly higher than diatoms, ranging from 4.0% to 6.1%, but the green alga, *Chlamydomonas reinhardtii*, had only $2.0 \pm 0.1\%$. In stationary growth phase when the cells had become limited by N, Rubisco concentration decreased but the trend between species remained, with all diatom species displaying Rubisco concentrations of 2% or less, haptophytes with 2.8–3%, and *C. reinhardtii* with $0.80 \pm 0.3\%$.

CO₂-controlled batch cultures of *T. weissflogii* The diatom *Thalassiosira weissflogii* was grown under varying CO₂ in batch cultures. Rubisco concentrations ranged from $4.4 \pm 1.1\%$ at low CO₂ (182 ppm) to $2.9 \pm 0.1\%$ at high CO₂ (750 ppm; Table 2). There is a systematic but not statistically significant trend of decreasing Rubisco concentration with increasing CO₂.

***E. huxleyi* continuous cultures varying CO₂** The coccolithophore *Emiliana huxleyi* was grown in both P- and N-limited continuous cultures varying growth rate and CO₂. Compared to the batch cultures of this organism, Rubisco concentrations were lower, ranging from 0.8% to 1.9% and 2.1% to 2.2% for P limitation and N limitation, respectively (Table 3). Under P limitation, *E. huxleyi* had a lower Rubisco concentration at high CO₂

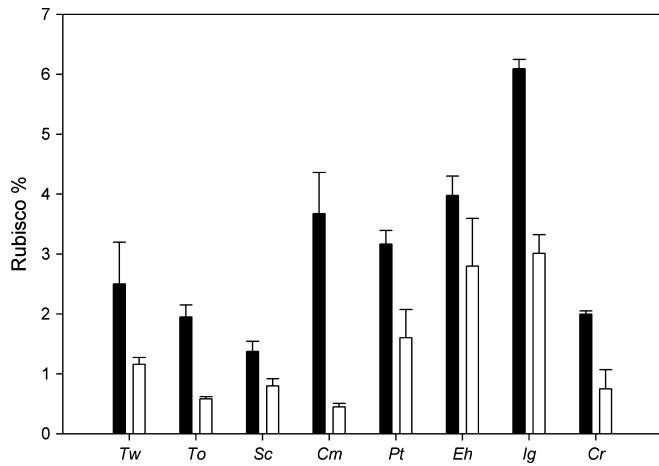


Fig. 1 Rubisco as a percentage of total protein in batch cultures of microalgae. Cultures tested are: *Thalassiosira weissflogii* (Tw), *Thalassiosira oceanica* (To), *Skeletonema costatum* (Sc), *Chaetoceros muelleri* (Cm), *Phaeodactylum tricornutum* (Pt), *Emiliana huxleyi* (Eh), *Isochrysis galbana* (Ig) and *Chlamydomonas reinhardtii* (Cr) at exponential (closed bars) and stationary (open bars) phases of growth. Values are averages of triplicate bottles with error bars representing the standard deviation between replicates. See Table 1 for exact values.

Table 1 Rubisco as a percentage of total protein in batch cultures of eight microalgae (as depicted in Fig. 1)

Species	μ (d^{-1})	Exponential	Stationary
Tw	1.2 ± 0.04	2.5 ± 0.7	1.2 ± 0.1
To	1.4 ± 0.03	2.0 ± 0.2	0.6 ± 0.04
Sc	1.5 ± 0.5	1.4 ± 0.2	0.8 ± 0.1
Cm	1.3 ± 0.1	3.7 ± 0.7	0.5 ± 0.1
Pt	1.5 ± 0.09	3.2 ± 0.2	1.6 ± 0.5
Eh	1.1 ± 0.06	4.0 ± 0.3	2.8 ± 0.8
Ig	0.94 ± 0.01	6.1 ± 0.2	3.0 ± 0.3
Cr	1.1 ± 0.01	2.0 ± 0.1	0.8 ± 0.3

Growth rate (μ) shown is from exponential growth. Data are mean ± SD ($n = 3$).

Table 2 Rubisco as a percentage of total protein in batch cultures of *Thalassiosira weissflogii*

CO ₂ (ppm)	Rubisco%
182	4.4 ± 1.1
396	3.3 ± 0.6
750	2.9 ± 0.1

Data are mean ± SD ($n = 3$).

compared to both low and intermediate CO₂ (P -value < 0.05), although this was not seen under N limitation.

Field experiments

Rubisco was measured in natural populations of organisms off the coast of California in bottle incubations comparing N-limited with N-replete treatments at three different CO₂

Table 3 Rubisco as a percentage of total protein in phosphorus (P)- and nitrogen (N)-limited continuous cultures of *Emiliana huxleyi*

Type of limitation	μ d^{-1}	CO ₂	Rubisco%
P-limited	0.3	100 ppm	1.9 ± 0.4
P-limited	0.3	380 ppm	1.0 ± 0.4*
P-limited	0.3	750 ppm	0.8 ± 0.3*
N-limited	0.6	170 μ atm	2.1 ± 0.7
N-limited	0.6	420 μ atm	2.2 ± 0.4
N-limited	0.6	780 μ atm	2.1 ± 0.5

P-limited data are mean ± SD ($n = 3$) for biological replicates. N-limited data are mean ± SD ($n = 3$) from a single experiment and technical replicates.

*Results significantly different from low CO₂, $P < 0.05$.

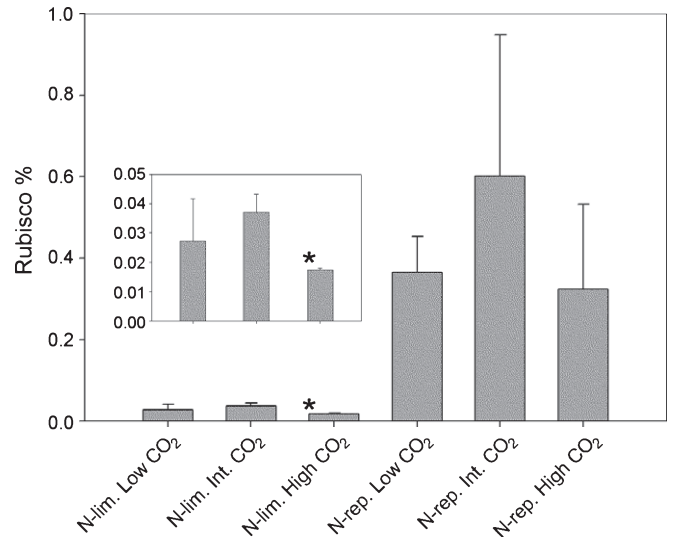


Fig. 2 Rubisco as a percentage of total protein in an N-limited (N-lim) and N-replete (N-rep) field experiment at different CO₂ concentrations. Data shown are from Expt 3, which is representative of the five experiments shown in Table 4. Values are averages of triplicate bottles with error bars representing the standard deviation between replicates. The inset in the panel replots N-limited data to show the effect of CO₂. Differences between CO₂ treatments were assessed using t -tests; P -values < 0.05 are indicated with an asterisk (*). See Fig. S2 for data from Expts 1, 2, 4, and 5.

concentrations (Table S1). In all N-replete treatments, Rubisco concentrations exceeded those of the N-limited treatments by at least a factor of 3 (Figs 2, S2, Table 4), ranging from 0.3% to 2.5%. There was no systematic trend in Rubisco concentration under N-replete conditions. But under N-limited conditions, Rubisco concentrations generally decreased with increasing CO₂. This change is only significant in two individual experiments, 1 and 3 (P -value < 0.05), but becomes significant for the ensemble when the data are normalized and pooled together (see the Materials and Methods section; P -value < 0.05). We note that the Rubisco concentrations measured in the field samples are markedly lower (Fig. 2, Table 4), presumably reflecting the fact that a significant fraction of the biomass was not actively photosynthesizing organisms.

Table 4 Rubisco as a percentage of total protein in all field experiments

	Low CO ₂	Intermediate CO ₂	High CO ₂
N-limited			
1	0.2 ± 0.01	0.2 ± 0.05	0.1 ± 0.02*
2	0.1 ± 0.01	0.06 ± 0.02	0.05 ± 0.02
3	0.03 ± 0.02	0.04 ± 0.01	0.02 ± 0.001*
4	0.3 ± 0.05	0.3 ± 0.08	0.3 ± 0.09
5	0.1 ± 0.01	0.1 ± 0.05	0.08 ± 0.04
N-replete			
1	0.3 ± 0.1	0.5 ± 0.3	0.9 ± 0.3
2	0.4 ± 0.2	0.3 ± 0.03	0.7 ± 0.1
3	0.4 ± 0.09	0.6 ± 0.3	0.3 ± 0.2
4	0.9 ± 0.3	1.4 ± 0.03	1.2 ± 0.3
5	2.5 ± 0.4	2.3 ± 0.5	1.5 ± 0.2

Data are mean ± SD ($n = 3$). Exact CO₂ concentrations, growth data, elemental stoichiometry, photosynthetic pigments, and total and absolute protein concentrations for Rubisco and PsbA are available for these five experiments in Losh *et al.* (2012).

*Results significantly different from intermediate CO₂, $P < 0.05$.

Discussion

The fraction of Rubisco to total protein in all experiments was markedly lower compared to the available data for plants and to the assumed fraction in phytoplankton (Dorner *et al.*, 1957; Pickersgill, 1986). While these concentrations are lower than is commonly claimed in the literature, the results for the batch cultures in exponential growth phase do align well with the majority of the values presented in Raven (1991), except for a few high

Table 5 Theoretical calculation of minimum Rubisco necessary for growth

μ = specific growth rate (s^{-1} or d^{-1})
 X = cell number per l ($cells\ l^{-1}$)
 M = cell biomass ($g\ cell^{-1}$)
 Rub = mol Rubisco per cell ($mol\ Rubisco\ cell^{-1}$)
 $R = K_{cat}^c$ of a Rubisco active site (turnover rate, carbons fixed active site⁻¹ s^{-1})
 C_{min} = concentration of Rubisco as a fraction of total protein
 This can be rewritten as $(Rub) = 6.06 \times 10^{-7}(C_{min} M)$

Assumptions:

All eight Rubisco active sites are active at once
 The molecular weight of Form 1 Rubisco is 550 kDa
 1/3 of carbon fixed by Rubisco is lost as respiration (24 h light, J. Goldman, pers. comm.)
 1/2 of biomass (M) is carbon (Falkowski & Raven, 2007)
 1/3 of biomass (M) is protein (Falkowski & Raven, 2007)

Calculation:

Total carbon fixed per cell in 1 s ($g\ cell^{-1}\ s^{-1}$) = $(Rub) \times 8 \times R \times 12$
 Net carbon fixed (taking into account respiration) = $(Rub) \times 8 \times R \times 12 \times (2/3)$
 Biomass fixed (M) ($g\ cell^{-1}\ s^{-1}$) = $(Rub) \times 8 \times R \times 12 \times (2/3) \times 2$
 Biomass fixed total (MX) ($g\ l^{-1}\ s^{-1}$) = $(Rub) \times 8 \times R \times 12 \times (2/3) \times 2 \times X$
 $C_{min} = 5.5 \times 10^5 (Rub) / (M/3)$
 If we substitute $(Rub) = 6.06 \times 10^{-7} (C_{min} M)$
 Biomass fixed total (MX) ($g\ l^{-1}\ s^{-1}$) = $7.75 \times 10^{-5} C_{min} RMX$

Divide by MX to get μ :

$\mu\ (s^{-1}) = 7.75 \times 10^{-5} C_{min} R$
 $\mu\ (d^{-1}) = 7.75 \times 10^{-5} \times C_{min} R \times 86\ 400$
 $C_{min} = 0.15 \times (\mu/R)$

If we assume a $\mu\ (d^{-1})$ of 1.2 and $R = 3$, $C_{min} = 0.06$ or 6% of total protein

If we assume a $\mu\ (d^{-1})$ of 0.3 and $R = 3$, $C_{min} = 0.015$ or 1.5% of total protein

*We note that this result is sensitive to changes in the proportion of carbon and protein to total biomass.

numbers (Yokota & Calvin, 1985; Newman & Cattolico, 1987; Falkowski *et al.*, 1989). A minimum Rubisco concentration (C_{min}) can be calculated in phytoplankton as a function of the turnover rate of the enzyme (R) and the growth rate (μ) of the organism (see Table 5):

$$C_{min} = 0.15(\mu/R) \quad \text{Eqn 1}$$

(C_{min} , fraction of Rubisco to total protein by mass; μ , day^{-1} ; R , Rubisco K_{cat}^c (expressed as carbons fixed s^{-1} active site⁻¹)). Given an often quoted value of R of $3\ s^{-1}$ and a growth rate of $1.25\ d^{-1}$ (the average of values in Table 1), the calculated C_{min} is 6.3%. This value is on the high end of the concentrations found in our batch cultures (Fig. 1, Table 1). This result could indicate that the turnover rate of Rubisco in phytoplankton may generally be higher than the often quoted $3\ s^{-1}$ (Tcherkez *et al.*, 2006 show a range of 1.2 – $13.4\ s^{-1}$). But in any case, our data suggest that Rubisco concentrations in eukaryotic phytoplankton are near the minimum required to maintain observed growth rates.

Our result also implies that all Rubisco in phytoplankton is active. Analysis of the genomes of two diatoms reveals no homolog for Rubisco Activase (Kroth *et al.*, 2008), suggesting a different regulatory mechanism for Rubisco in diatoms than in plants. This has been demonstrated in experimental studies whereby regulation of Rubisco to changing environmental conditions in diatoms appears to be driven largely by protein content (e.g. in response to light (MacIntyre & Geider, 1996) and CO₂ concentrations (McCarthy *et al.*, 2012)).

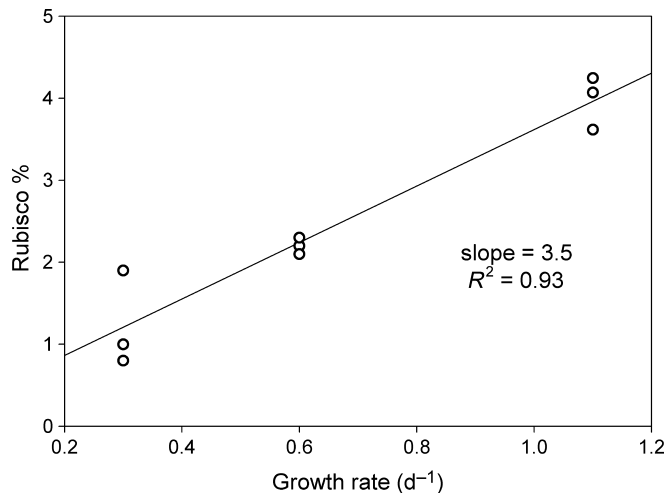


Fig. 3 Rubisco as a percentage of total protein vs growth rate in *Emiliana huxleyi*. Data shown are from batch and continuous cultures (Tables 1, 3). Data for growth rate = 0.6 d⁻¹ are displaced slightly so that all three points are visible. Line shown is a linear regression of the nine data points plotted.

Our experiments also provide insight into several parameters that affect the cellular concentrations of Rubisco in phytoplankton, specifically growth rate and CO₂ concentration. In all species tested, Rubisco concentrations (and PsbA; see Fig. S1) decreased from exponential to stationary growth phase (Fig. 1, Table 1). In the field data, Rubisco concentrations were also much higher in faster growing N-replete cultures than in slower growing N-limited cultures (Figs 2, S2, Table 4). The most direct evidence for an effect of growth rate comes from our *E. huxleyi* data obtained in both batch and continuous cultures, in which lower Rubisco concentrations correspond to slower growth rates, as is expected from Eqn 1 (Fig. 3). Furthermore, the slope of a linear regression fit to our *E. huxleyi* data, 3.5, would correspond to the theoretical value in Eqn 1 for a Rubisco turnover rate of $R = 4 \text{ s}^{-1}$.

Previous studies have measured Rubisco at varying CO₂ under nutrient-replete conditions and have obtained mixed results in the field (Tortell *et al.*, 2000; Israel & Hophy, 2002; Losh *et al.*, 2012). In the laboratory, McCarthy *et al.* (2012) observed an increase in Rubisco concentration with higher CO₂, while Levitan *et al.* (2010) saw no change. In our experiments, Rubisco concentrations decreased at high CO₂ in CO₂-controlled batch cultures of *T. weissflogii* (Table 2), in P-limited continuous cultures of *E. huxleyi* (Table 3), and in most field N-limited experiments (Fig. 2, Table 4), although the trends were not always statistically significant. In all of these experiments, the total protein concentration did not vary with CO₂ (data not shown) so that it is indeed the amount of Rubisco that was changing. This differs from plants where studies have shown no change in Rubisco protein content in response to CO₂ (Campbell *et al.*, 1988) and the response to light is predominantly due to changing the activation state of the enzyme (Perchorowicz *et al.*, 1981).

If confirmed, this decrease in Rubisco concentration with increasing CO₂ in phytoplankton would be an interesting result. It implies that the down-regulation of the CCM is not the only acclimation mechanism to a change in external CO₂

concentration (Rost *et al.*, 2003; Tortell *et al.*, 2008; Wu *et al.*, 2010; Hopkinson *et al.*, 2011). Because the cellular Rubisco concentration is near the minimum necessary, this result seemingly also implies that the regulation of the CCM does not maintain a saturating CO₂ concentration at the site of fixation by Rubisco. For example, an increase in the rate of carbon fixation per enzyme as external CO₂ increases would make it possible to reduce the Rubisco concentration, while maintaining constant rates of carbon fixation and growth.

Contrary to some previous claims, our data indicate that Rubisco is < 6% of total protein in microalgae. Given the low turnover rate of Rubisco, this result implies that phytoplankton have close to the minimum amount of Rubisco necessary to support their growth. Therefore, unlike in some plants, Rubisco is not a dominant sink of cellular N. This has implications for our fundamental understanding of the role of Rubisco in N-limiting environments, and for phytoplankton's response to increasing CO₂.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Fig. S1 PsbA as a percentage of total protein in diatom batch cultures.

Fig. S2 Rubisco as a percentage of total protein in N-limited and N-replete field experiments 1, 2, 4 and 5 at varying CO₂.

Table S1 Final CO₂ concentrations (µatm) in N-limited and N-replete field experiments

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