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Slow carboxylation of Rubisco constrains the rate of carbon fixation during Antarctic phytoplankton blooms

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Summary

- High-latitude oceans are areas of high primary production despite temperatures that are often well below the thermal optima of enzymes, including the key Calvin Cycle enzyme, Ribulose 1,5 bisphosphate carboxylase oxygenase (Rubisco).
- We measured carbon fixation rates, protein content and Rubisco abundance and catalytic rates during an intense diatom bloom in the Western Antarctic Peninsula (WAP) and in laboratory cultures of a psychrophilic diatom (*Fragilariopsis cylindrus*).
- At −1°C, the Rubisco turnover rate, k_{cat}^c, was 0.4 C s^{-1} per site and the half saturation constant for CO_2 was 15 μM (vs *c.* 3 C s^{-1} per site and 50 μM at 20°C). To achieve high carboxylation rates, psychrophilic diatoms increased Rubisco abundance to *c.* 8% of biomass (vs *c.* 0.6% at 20°C), along with their total protein content, resulting in a low carbon : nitrogen ratio of *c.* 5.
- In psychrophilic diatoms, Rubisco must be almost fully active and near CO_2 saturation to achieve carbon fixation rates observed in the WAP. Correspondingly, total protein concentrations were close to the highest ever measured in phytoplankton and likely near the maximum possible. We hypothesize that this high protein concentration, like that of Rubisco, is necessitated by slow enzyme rates, and that carbon fixation rates in the WAP are near a theoretical maximum.

Introduction

High-latitude oceans are major contributors to global primary productivity, with the Southern Ocean alone accounting for *c.* 2 Pg C yr^{-1} of global phytoplankton production (Arrigo *et al.*, 2008). Large, seasonal blooms of phytoplankton, in particular diatoms, provide the basis for short but substantial food webs (Ducklow *et al.*, 2007). To date, research on high-latitude primary productivity has focused on the timing and magnitude of phytoplankton blooms (Arrigo & van Dijken, 2003; Montes-Hugo *et al.*, 2009) but there is little understanding of the biochemical adaptations that constrain the rates of carbon (C) fixation and growth during the bloom.

Spring/summer blooms of high-latitude phytoplankton occur while water temperatures remain close to freezing. Due to the thermal sensitivity of enzymes, biological catalytic rates could be severely limited at these cold temperatures. Most enzyme-catalyzed reactions display a temperature coefficient (Q_{10}) between 2 and 3, that is, a two to threefold reduction in rate with a 10°C temperature decrease. As a consequence, enzymatic rates at −1°C during a spring phytoplankton bloom in the West Antarctic Peninsula (WAP) could be 6–20 times slower compared with rates at temperatures of 25°C found in low-latitude oceans. Yet phytoplankton growth rates and primary productivity in polar waters are not an order of magnitude lower than in temperate systems (Prézelin *et al.*, 2000; Smith *et al.*, 2000), demonstrating that phytoplankton have adapted to cold temperatures. Many cold water phytoplankton are psychrophilic; that is, they display an optimal growth temperature of < 15°C (Eddy, 1960).

To overcome slow enzymatic rates at low temperatures, psychrophilic phytoplankton utilize a combination of two strategies (Morgan-Kiss *et al.*, 2006). The first is to evolve cold-adapted enzymes that display thermal optima much lower than those of their mesophilic counterparts (for a review, see Feller & Gerday, 2003). The second approach, for enzymes that cannot be cold adapted, is to maintain high cellular enzymatic rates by increasing abundance of enzyme protein.

It is presently unclear to what extent phytoplankton use one or both of these strategies to optimize C fixation in cold waters. The rate-limiting step of C fixation is often the first enzyme of the Calvin Cycle, Ribulose 1,5 bisphosphate carboxylase oxygenase (Rubisco), which catalyzes the fixation of CO_2 onto ribulose 1,5 bisphosphate (RuBP), producing two molecules of 3-phosphoglycerate. Even at a temperature of 25°C, this enzyme is extraordinarily slow, with a turnover rate (k_{cat}^c) of *c.* 3.4 C s^{-1} per site (Badger *et al.*, 1998). This kinetic rate falls to even lower values when the temperature is decreased. The temperature sensitivity of Rubisco, with a Q_{10} ranging between 2.2 and 2.9 (Sage,

2002), is similar among psychrophilic and mesophilic plants (Sage, 2002), green algae (Devos *et al.*, 1998) and diatoms (Descolas-Gros & de Billy, 1987), suggesting that cold- adaption in Rubisco is minimal. The slow carboxylation rate of Rubisco could thus be an important rate-limiting step for primary production in the high-latitude oceans.

To date, there has been little focus on how the slow carboxylation rates of Rubisco may constrain C fixation in ecologically important, polar phytoplankton. Generally, attention has concentrated on the effect of light limitation and the short growing season on the magnitude of annually integrated primary production. Those factors may not be limiting the rate of C fixation during the austral spring/summer in the WAP as light intensities are often saturating and nutrients near the coast are usually abundant (Smith & Dierssen, 1996).

Elucidating the parameters that constrain growth rates during Antarctic phytoplankton blooms is important for our general understanding of polar ecosystem function, and should be helpful in developing biogeochemical models that assess the consequences of anthropogenic change at high latitudes. We thus measured C fixation rates, C : N ratios and Rubisco content and catalytic rates during a large bloom of diatoms in the WAP during the austral spring of 2012. With supporting measurements in laboratory cultures comparing the psychrophilic (*Fragilariopsis cylindrus*) and mesophilic (*Thalassiosira weissflogii*) diatoms, we quantified the role of Rubisco carboxylation in constraining C fixation rates during a phytoplankton bloom in the WAP.

Materials and Methods

WAP sample collection

Phytoplankton productivity was measured in the WAP at the Palmer Long-Term Ecological Research (LTER) Station. Samples were collected at station B (64.7795 S, 64.0725 W), twice weekly during the austral spring and summer between late October 2012 and late March 2013. Water was pumped gently from a depth of 10 m into 20-l carboys using a monsoon pump (WSP-SS-80-NC; Waterra Pumps Ltd, Ontario, Canada). Twenty-litre carboys were kept in low light and returned to the station laboratories within *c.* 1 h of collection for processing. This water was subsequently sampled for particulate C and nitrogen (N), gross primary production, Chlorophyll chlorophyll *a* (Chl*a*) and total protein.

Particulate organic carbon (POC) and nitrogen (PON) Particulate organic carbon (POC) and nitrogen (PON) samples were collected at each time-point by filtering up to 1 l of 10-m-depth seawater onto precombusted (450°C, 4 h) 25 mm glass fibre filter (GF/F) which were acidified by fumigation with 6 N HCl overnight and dried at 60°C for 2 d. The whole filter was measured for POC and PON using an Elemental Combustion Analyzer (Costech, Valencia, CA, USA) (e.g. Losh *et al.*, 2012) and calibrated against the standard reference material, peach leaf (NIST 2157) (Becker, 1990), which was run in triplicate after every eight samples. C : N ratios were calculated on a mole–mole basis from POC and PON on the filter.

Gross primary productivity Gross primary productivity (GPP) was measured by the production of $^{18}O^{16}O$ uptake in bottle incubations as described in by Goldman *et al.* (2014). Briefly, duplicate gas-tight Pyrex bottles were filled with collected seawater, spiked with $H_2{}^{18}O$ (97.6%; Medical Isotopes Inc., Pelham, NH, USA) for a final enrichment of 412.4‰ and incubated for 5–8 h in a flowing seawater tank maintained near sea surface temperatures, under two layers of neutral density screening (to achieve *c.* 50% of surface photosynthetically active radiation (PAR) intensities). Bottle contents (including an initial time-point with no $H_2{}^{18}O$) were transferred to pre-evacuated (*c.* 1.5 mTorr = *c.* 0.2 Pa) and pre-poisoned (100 µl of $HgCl_2$) 500-ml Leuwers Hapert (LH) flasks. For analysis, the gas in each sample was transferred under vacuum from frozen LH flasks to a stainless steel tube kept in a helium tank. Each tube is was allowed to warm up for 1 h before analysis in a Delta Plus XP mass spectrometer (Thermofisher Scientific, Waltham, MA, USA) (see Emerson *et al.*, 1995). GPP, defined as the amount of oxygen produced from the splitting of water during photosynthesis, was measured as the increased concentration of $^{18}O^{16}O$. GPP was converted to C fixed by using a Photosynthetic Quotient (PQ) based on measured POC : PON ratios and derived from electrons balance:

$$PQ = \frac{\text{mol } O_2}{\text{mol C}} = \frac{\frac{1}{4}\text{mol } e^-}{\text{mol C}} = 1 + 2 * \frac{PON}{POC}.$$

Chlorophyll *a* Chlorophyll *a* was measured in duplicate. One hundred millilitres to one litre of seawater was filtered onto a GF/F and was extracted overnight in 90% acetone at −20°C. The Chl*a* concentration ($\mu g\, l^{-1}$) was determined with a 10-AU fluorometer (Turner Designs, Sunnyvale, CA, USA), measuring the un-acidified and acidified sample to correct for phaeopigments (Welschmeyer, 1994).

Protein quantification One- to four-litre sub-samples from the field, or 50-ml samples from exponentially growing laboratory cultures were filtered onto a 0.2-µm polycarbonate filter, stored in cryovials and snap-frozen in liquid nitrogen. Quantification of Rubisco and PsbA (the core reaction center protein in photosystem II) protein was performed as in Losh *et al.* (2013). Briefly, protein was extracted from filtered biomass using SDS buffer (50 mM Tris-HCl, 2% SDS, 10% glycerol, and 12.5 mM EDTA) and with the addition of 1% β-mercaptoethanol. Total protein in these crude extracts was quantified using the BCA assay according to the manufacturer's instructions (Pierce, Thermofisher Scientific, Waltham, MA, USA).

Quantitative western blots were performed for the large subunit of Rubisco (RbcL) and PsbA using global antibodies and standards (Agrisera, Vännäs, Sweden) which are designed against peptide tags conserved across all oxygenic photosynthesizers so

that no bias in affinity is expected even if phytoplankton communities differ (Campbell *et al.*, 2003). Then, 0.5–1 μg (for RbcL) or 2 μg (for PsbA) of total protein was loaded onto a 12% SDS-PAGE gel (250 V, 40 min) along with a molecular size ladder (Precision Plus, Dual-colour; Bio-Rad, Hercules, CA, USA) and three different concentrations of RbcL or PsbA standards (Agrisera; to generate standard curve). Proteins were transferred onto a polyvinylide fluoride (PVDF) membrane (200 mA, 2 h). The membrane was blocked with TBST-milk buffer (5% milk powder in Tris-buffered saline containing 0.25% v/v Tween-20, pH 7.5) for 1 h and then probed for 1 h each with polyclonal, global anti-RbcL or anti-PsbA (1 : 10 000; Agrisera) followed by a secondary antibody (Pierce; alkaline phosphate-coupled goat anti-rabbit IgG antibody, 1 : 5000, 1 h) in TBST-milk buffer with TBST washing steps in between. Colorimetric determination of bound proteins were performed with nitro-blue tetrazolium chloride/5-bromo-4-chloro-3-indolyphosphate p-toluidine (NBT/BCIP). 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 (Supporting Information Fig. S1). The Rubisco concentration was calculated from the equimolar stoichiometry of the large and small (RbcS) subunits with molecular weights of 55 and 15 kDa, respectively (Baker *et al.*, 1975). The PsbA concentration was calculated from the single subunit with a molecular weight of 39 kDa (Agrisera).

Culture experiments and estimates of *in vivo* Rubisco k_{cat}^c

Thalassiosira weissflogii (CCMP 1336) and *Fragilariopsis cylindrus* (CCMP 1102) were grown in batch culture using 0.2-μm filtered coastal seawater supplemented with Aquil medium (Sunda *et al.*, 2005) under continuous light (*c.* 150 μmol photons m^{-2} s^{-1}) at 20 and 3°C, respectively. Cells were counted with a Coulter Counter Z2 (Beckman Coulter Inc., Fullerton, CA, USA) and harvested during exponential growth by filtering onto a polycarbonate filter and snap-frozen in liquid nitrogen.

Whole-cell, gross C fixation rates were measured in triplicate for *F. cylindrus* from using the short-term (1 h) ^{14}C uptake rates during early exponential growth under the above temperature (3°C) and light (150 μmol photons m^{-2} s^{-1}) conditions. 2 μCi $NaH^{14}CO_3$ (0.38 nM) was added to 100 ml of culture as a tracer for the naturally occurring dissolved inorganic C in the medium, which was measured in a Prisma QMS-200 quadrapole Membrane Inlet Mass Spectrometer (Pfeiffer, Asslar, Germany) at 2031 ± 37 μM. After 1 h of incubation with ^{14}C, cells were filtered onto GF/F filters and inorganic C was degased by acidification with 6 N HCl overnight. Fixed organic C was measured using a scintillation counter (Beckman Instruments Inc.). Carbon fixation rates were normalized to biomass using cell numbers and a measured dry weight per cell. *In vivo* Rubisco k_{cat}^c was calculated by dividing whole-cell ^{14}C fixation rates by the number of Rubisco active sites. Rubisco active sites were quantified by measuring total Rubisco protein and assuming all Rubisco was active *in vivo*, with eight active sites and a molecular weight of 550 kDa.

In vitro Rubisco carboxylation rates and Michaelis–Menten half saturation constants (K_c)

Carboxylation rates of Rubisco at various temperatures (0, 5, 10, 20 and 30°C) were determined in duplicate using crude Rubisco extracts from *T. weissflogii* and *F. cylindrus* (grown at 20 and 3°C, respectively). Crude extracts of Rubisco were obtained by filtering 1 l of exponentially growing culture onto 3 μM polycarbonate filters and snap-frozen in liquid nitrogen. Cells were washed from the filter with 1 ml of ice-cold homogenization buffer (50 mM bicine, 20 mM $MgCl_2$, 1 mM EDTA, 5 mM dithiothreitol, 0.2% Triton X and 10 mg ml^{-1} PVPP, pH 8.0) into a dounce homogenizer and ground to lyse cells. Extract was centrifuged at 13 000 ***g*** at 4°C for 5 min to remove cell debris and a subsample was taken for protein quantification. Rubisco was not activated before the assay as this did not appear to have any influence on activation state (our unpublished results and MacIntyre *et al.*, 1997). In 2-ml, N_2 sparged gas-tight vials, *c.* 10 μg of Rubisco (in 20 μl of crude extract) was added to 510 μl of assay buffer (50 mM bicine, 20 mM, $MgCl_2$, 1 mM EDTA, 5 mM dithiothreitol, 0.1 mg ml^{-1} bovine carbonic anhydrase, and 0.4 mM Ribulose 1,5 bisphosphate (RuBP), pH 8.0, bubbled with N_2 to remove all CO_2 and O_2). The reaction was started by the addition of 9.26 mM $NaH^{14}CO_3$, for 10 min at 0°C, 5 min at 10°C or 2 min at 20 and 30°C and stopped by the addition of 0.5 ml of 6 N HCl. Vials were left overnight to degas all inorganic ^{14}C as CO_2. Organic ^{14}C was counted with a liquid scintillation counter (Beckman Instruments Inc., Fullerton, CA, USA). To confirm there was no non-specific activity, we tested ^{14}C assays with crude extract without RuBP. Increasing incubation times with decreasing temperature was necessary to achieve fixation that was sufficiently above background and appeared to have no effect on rates measured (Fig. S2).

To confirm that the available CO_2 in the assay buffer was saturating Rubisco, we calculated the CO_2 concentration for each temperature and pH (see Table S1). In addition, we measured the Michaelis–Menten half saturation constant for CO_2 (K_c) of Rubisco from *F. cylindrus* and *T. weissflogii* at both 0 and 20°C. This was done using a similar assay to the above method to measure Rubisco carboxylation but with a series of vials containing increasing concentrations of $NaH^{14}CO_3$ from 0.5 to 9.26 mM. From our plotted results we calculated the K_c and maximum reaction rate (V_{max}) from a least squares fit of the Michaelis–Menten curve. Between the temperatures of 0 and 20°C we found CO_2 to be saturating Rubisco. At 30°C we did not measure the K_c; however, according to Badger & Collatz (1977), it is possible that the K_c might be double that of 20°C (*c.* 100 μM). Thus, Rubisco may be undersaturated for CO_2 at 30°C and therefore the Q_{10} was calculated only using the rates between 0 and 20°C.

Results

The bloom

During the austral spring and summer of 2012/2013 in the WAP, we observed an intense phytoplankton bloom early in the season (Fig. 1a), which was dominated by diatoms (Fig. S3 or

Goldman *et al.*, 2014). The high abundance of particulate organic carbon (POC) during the bloom was almost entirely autotrophic based on gross primary production (GPP) measurements (Fig. 1a) and high Chl*a* : C mass ratios (1 : 50; Fig. S4). This phytoplankton bloom was one of the largest recorded in the 20-yr Long-Term Ecological Research (LTER), with Chl*a* concentration exceeding 40 $\mu g\, l^{-1}$ (Goldman *et al.*, 2014), and occurred while water temperatures remained low (*c.* −1°C; Fig. 1b).

Increase in protein content

There are two main physiological mechanisms that enable phytoplankton to maintain high productivity despite slow enzymatic rates at low temperatures. The first is to increase protein abundance; the second is to possess cold-adapted enzymes. While it is likely that WAP diatoms utilize a combination of these strategies, their remarkably low C : N ratio (mean of 4.9 with a standard deviation of 0.7; Fig. 1c) instead of the Redfield ratio of 6.6 suggests an increase in protein content and does not appear to be influenced by the large seasonal drawdown of NO_3^- and CO_2 during the bloom (Fig. S5).

Protein accounts for the bulk (60–80%) of the nitrogen (N) pool in diatoms (Dortch *et al.*, 1984), with the remainder split between DNA, RNA, free amino acids and intracellular NO_3^-. Accurate measurements of protein content in our field samples not being available, we analyzed laboratory cultures of the psychrophilic diatom, *F. cylindrus*. This cold-adapted species, *F. cylindrus*, had a protein content of 58 ± 4% of dry biomass (mg mg^{-1}) (Fig. 2a, Table 1), more than double the 25% dry biomass observed in mesophilic diatoms (Brown, 1991); Fig. 2a). The values we measured in *F. cylindrus* are similar to values of 50% reported by Sackett *et al.* (2013). Taking a conservative value of protein being 50% of dry biomass, we calculate a C : N ratio of *F. cylindrus* within the range of 4.7–5.1 (see Box 1 for details of the calculation). This calculated value matches well with our field observations, and suggests that the low C : N ratios in the field samples result from high protein content.

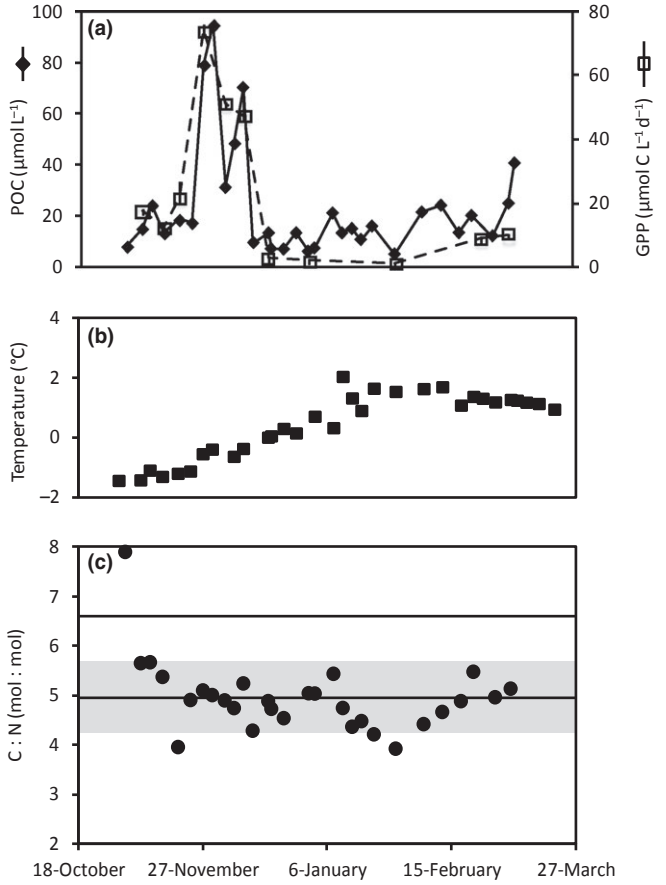

Fig. 1 (a) High primary production during an intense phytoplankton bloom in the Western Antarctic Peninsula during spring/summer of 2012/2013 as indicated by a sharp increase in gross primary production (GPP; open squares) and particulate organic carbon (POC) (closed diamonds). (b) Temperature at 10 m depth over the course of the season, indicating that the bloom occurred while water temperatures remained *c.* −1°C. (c) Carbon : nitrogen (C : N) (mol : mol) ratios of particulate organic matter (closed circles) with a mean of 4.9 (black line) with and a standard deviation of 0.7 (shaded grey area). The Redfield ratio of 6.6 is shown by the thick grey line).

Box 1 Calculation of carbon : nitrogen (C : N) ratio from protein content of diatom biomass at 20 and 0°C

At 20°C, 100 g of biomass in a mesophilic diatom:

is 25–35% protein **(25–35 g of protein)** of which 17% is N[a] **(4.25–5.95 g of N in protein).**
60% of the total N pool is protein **(7.08–9.92 g of total N)**
(Which means 2.83–3.97 g of N is NOT in protein)
50% of biomass is carbon **(50 g of total carbon)**
C : N ratio (mol/mol) ranges from **6.3 to 8.8**

At 0°C, 100 g of biomass of *Fragilariopsis cylindrus*:

is 50% protein **(50 g of protein)** of which 17% is N[a] **(8.5 g of N in protein).**
Assume no other N pools increase i.e. 2.83–3.97 g of N is NOT in protein **(11.33–12.47 g of total N)**
50% of biomass is carbon **(50 g of total carbon)**
C : N ratio (mol/mol) ranges from **4.7 to 5.1**

[a]Sterner & Elser (2002).

Increase of Rubisco

To explore how particular proteins respond to low temperature, we focused on two proteins essential for photosynthesis, Rubisco and PsbA. Rubisco is the carboxylating enzyme responsible for the first step in the Calvin cycle, fixing CO_2 onto 3-phosphoglycerate. PsbA is the core reaction center protein in photosystem II. We found extremely high levels of Rubisco protein throughout the season (Fig. 3a and insert). Rubisco abundance increased during the bloom, from *c.* 13% to a peak of 23% of total protein at the peak of the bloom, with an average concentration during the bloom of 15 ± 3%. We also found similar concentrations of Rubisco (17 ± 3% of total protein) in exponentially growing cultures of *F. cylindrus* at 3°C (Table 1). After the bloom, Rubisco

Table 1 *In- vivo* estimate of the Rubisco turnover rate (k_{cat}^c) in *Fragilariopsis cylindrus* grown at 3°C

Replicates	pmoles of C fixed cell^{-1} s^{-1} (10^{-6})	pmoles of C fixed μg biomass^{-1} s^{-1a}	Rubisco % of total protein (w/w)	pmoles Rubisco sites per μg biomass^{-1b}	Estimated k_{cat}^c (C s^{-1} per site)^c
1	4.4	0.44	18.2	1.32	0.42
2	3.6	0.36	20.4	1.49	0.30
3	3.4	0.34	14.7	1.07	0.40
4	3.5	0.35	13.0	0.95	0.46
Average	**3.73**	**0.37**	**16.59**	**1.21**	**0.39**
SD	**0.46**	**0.05**	**3.34**	**0.24**	**0.07**

Bold values represent the average and standard deviation (SD) for the four replicates.
[a]10 pg dry biomass per cell.
[b]Calculated assuming eight active sites per molecule, a molecular weight of 550 kDa and using the percentage of biomass weight that is Rubisco (from Fig. 2).
[c]Estimated k_{cat}^c calculated by dividing the measured, whole cell carbon fixation rates (third column, pmoles C fixed per μg biomass per second) by the number of active sites of Rubisco (fifth column, pmoles of Rubisco sites per μg of biomass), taking into account that only 80% of the active sites are saturated by CO_2.

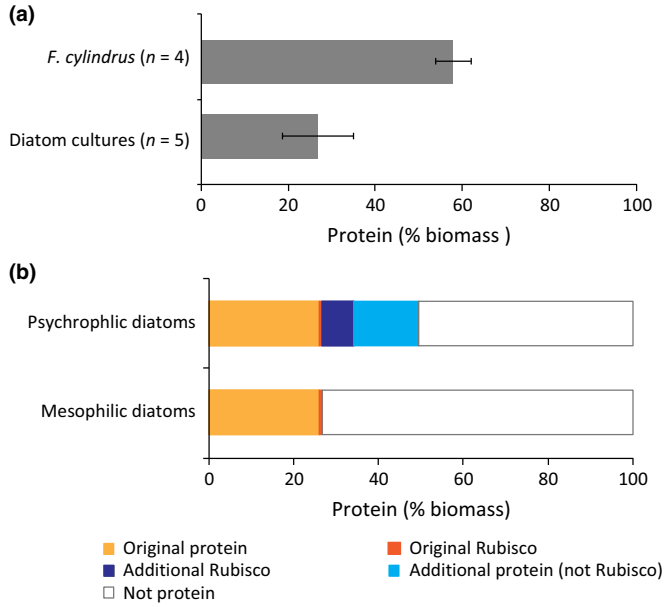

Fig. 2 (a) Measured protein concentrations in biomass of mesophilic (Brown, 1991) and psychrophilic (this study; *Fragilariopsis cylindrus*, four biological replicates) diatoms. Error bars, ± SD. (b) Diagrammatic representation of the protein and Rubisco concentrations in psychrophilic and mesophilic diatoms. The orange bar and red bar together represent total protein found in the mesophilic diatoms, with the red bar denoting Rubisco protein. The dark blue bar and light blue bar together represent the additional protein found in psychrophilic diatoms, with the dark blue bar representing additional Rubisco.

concentrations decreased and remained between 5% and 10% of total protein throughout the rest of the season. This decrease in Rubisco abundance also coincided with a shift from a community that was almost entirely diatoms to a mixed population of cryptophytes, *Phaeocystis* sp. and diatoms (Fig. S2 or Goldman *et al.*, 2014) and presumably attributable to an increase in non-autotrophic particulate matter.

The concentrations of Rubisco found in WAP phytoplankton are significantly higher than those observed in field samples from warmer climates (*c.* 1% of total protein; Losh *et al.*, 2012) and the 2–3% Rubisco found in exponentially growing cultured diatoms at 20°C (Losh *et al.*, 2013). Taking into account the increase of total protein under low temperature (Fig. 2a), we calculated that Rubisco in psychrophilic diatoms accounts for *c.* 8% of dry biomass (based on a protein content of *c.* 50% biomass and a Rubisco content of *c.* 16% protein) compared with only 0.62% in mesophilic diatoms (Fig. 3b, based on a protein content of *c.* 25% biomass (Brown, 1991) and a Rubisco content of *c.* 2.5% protein (Losh *et al.*, 2013)). Rubisco thus accounts for *c.* 36% of the additional protein content in psychrophilic compared with mesophilic diatoms (Fig. 2b). By contrast, PsbA showed concentrations similar to those found in other field studies, < 0.5% total protein, and there was no trend with biomass or temperature (Fig. 3a).

Our findings agree with studies in the plant, *Arabidopsis thaliana*, where cold acclimation from 23 to 5°C almost triples protein content and dramatically increases the abundance Calvin Cycle enzymes, enabling the plant to recover the photosynthetic capacity to that found at warmer temperatures (Strand *et al.*, 1999). Likewise, measurements of *in vitro* Rubisco carboxylase activity at 25°C were found to be seven times higher per cell in cultures of the mesophilic diatom, *Skeletonema costatum*, grown at 3°C compared with 18°C, presumably indicative of increased Rubisco protein (Mortain-Bertrand *et al.*, 1988). Psychrophilic green algae have double the Rubisco concentration found in their mesophilic counterparts, presumably to compensate for the slow catalytic rate (Devos *et al.*, 1998). In comparison, the light light-harvesting pathway, which includes PsbA, is not influenced by temperature (Ensminger *et al.*, 2006).

Temperature sensitivities of *in vitro* Rubisco kinetics

To determine whether the measured increase in protein is sufficient to fully compensate for the slow catalytic activity of Rubisco, we measured the *in vitro* carboxylation rates of crude Rubisco extracts from both *T. weissflogii* and *F. cylindrus* at 0, 10, 20 and 30°C (Fig. 4). Our measured carboxylation rates, when normalized to 30°C, agree extraordinarily well with similar measurements from crude Rubisco extracts from different psychrophilic and mesophilic diatom species performed by Descolas-Gros & de Billy (1987), suggesting that our results truly represent differences between mesophilic and psychrophilic

diatoms and are not attributable to species-specific differences. Both mesophilic and psychrophilic diatoms have similar carboxylation rates at temperatures of 15°C. However, there appears to be a break point in the temperature relationship (common in many enzymes; Feller & Gerday, 2003). The carboxylation rate of the mesophilic *T. weissflogii* decreased 23-fold between 20 and 0°C compared to with only a 10-fold reduction in *F. cylindrus*. This suggests that there is a degree of cold adaptation in psychrophilic Rubisco.

Rubisco from *F. cylindrus* has had a Q_{10} of *c.* 2.6, fitting within the Q_{10} values found in plants (2.2–2.9; Sage, 2002). Turnover rates (k_{cat}^c) were not calculated from the crude extracts since as, even under optimal conditions, *in vitro* carboxylase rates of diatom Rubisco are always significantly lower than *in vivo* rates (MacIntyre *et al.*, 1997). However, if we take the measured k_{cat}^c from a diatom, *Phaeodactylum tricornutum*, (Whitney *et al.*, 2001), of 3.4 C s^{-1} per site at 25°C and apply our calculated Q_{10} of 2.6 for *F. cylindrus* Rubisco, we estimate a k_{cat}^c of 0.31 C s^{-1} per site at 0°C.

Along with reducing carboxylation rates, decreasing temperatures have also been shown to decrease the Michaelis–Menten half saturation constant for CO_2 (K_c) in higher plants (Badger & Collatz, 1977). However, no information is available on K_c of diatom Rubisco at 0°C. We thus measured the K_c of Rubisco in crude extracts of *T. weissflogii* and *F. cylindrus* (grown at 20 and 3°C, respectively). At 20°C, Rubisco from both the two species showed identical K_c values near 50 ± 7 μM (Fig. 5). At 0°C, these values decreased markedly to *c.* 13 ± 3 and *c.* 17 ± 1 μM, respectively (Fig. 5).

To verify whether our calculated *in vitro* k_{cat}^c is applicable to observed *in vivo* carboxylation rates in psychrophilic diatoms, we estimated *in vivo* k_{cat}^c by dividing measured, cellular C fixation rates by the number of active sites of Rubisco. The number of Rubisco active sites were calculated from the amount of Rubisco protein, a molecular weight of 550 kDa and assuming all eight sites per molecule are active and nearly saturated with substrate, at 80% CO_2 saturation (see Kranz *et al.* (2014), and full RuBP saturation, (Losh *et al.*, 2013).

Gross C fixation rates of exponentially growing *F. cylindrus* cultures were determined in 1-h ^{14}C uptake experiments (Table 1). Rubisco content was determined as a percentage of

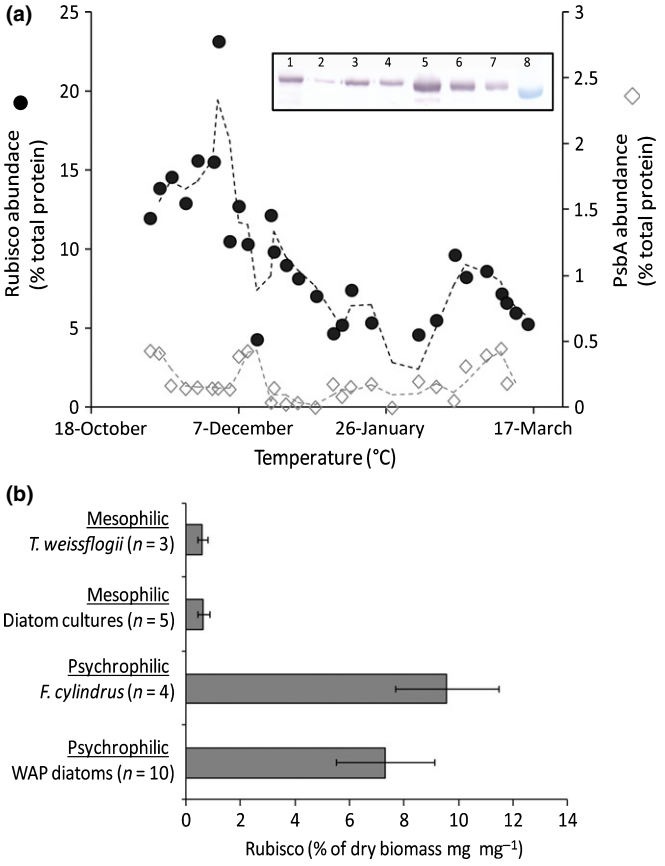

Fig. 3 (a) The abundance of Rubisco (closed circles) and PsbA (open diamonds) as measured as percent total protein weight in particulate organic matter, with dashed lines denoting a three-point moving average. (Inset) Western blot for the large subunit of Rubisco (RbcL). Lanes are as follows: 1–4, 1 μg of total protein from samples 18 February, 28 January, 14 January and 11 January 2013, respectively; 5–7, global standard for RbcL (spinach) at 3, 1.5 and 0.75 pmoles, respectively, to generate the standard curve; 8, molecular weight protein ladder. (b) Rubisco concentrations as a percentage of dry biomass based on total protein content and Rubisco content as a percent total protein. Values for mesophilic diatoms were taken from Losh *et al.* (2013). The number of samples (*n*) represents biological replicates and the errors bars, ± SD. *T. weissflogii*, *Thalassiosira weissflogii*; *F. cylindrus*, *Fragilariopsis cylindrus*; WAP, Western Antarctic Peninsula.

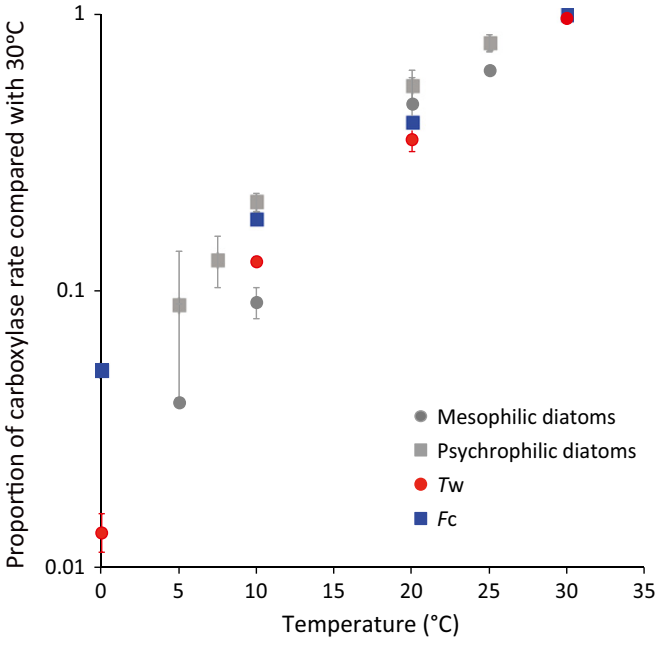

Fig. 4 *In vitro* carboxylation rates of crude Rubisco extracts of the mesophilic (*Thalassiosira weissflogii,* (Tw); red circles) and psychrophilic (*Fragilariopsis cylindrus,* (Fc); blue squares) diatoms. Data are from two biological replicates at 0, 10, 20 and 30°C. Error bars, ± SD. These results were compared to with those of Descolas-Gros & de Billy (1987) for the psychrophilic diatoms *Nitzschia turgidouloides* and *F. kerguelensi* (grey gray squares) and mesophilic diatoms *Skeletonema costatum* and *Phaeodactylum tricornutum* (grey gray circles). Rates were normalized for each species to the rate observed at 30°C.

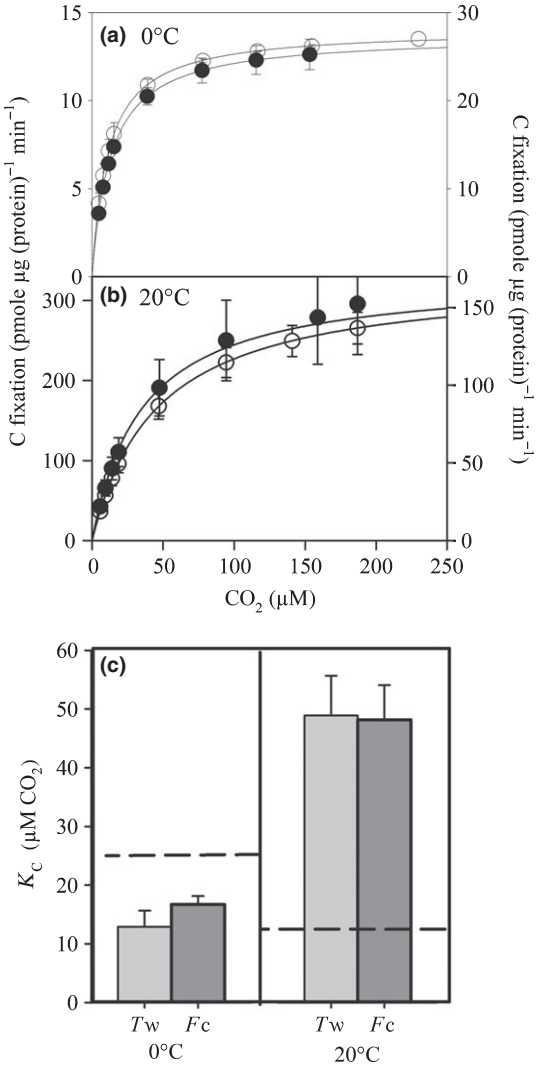

Fig. 5 Michaelis–Menten curves showing the increase of carbon fixation rates with increasing CO_2 concentrations in crude Rubisco extracts from *Thalassiosira weissflogii* (*Tw*, open circles, left *y*-axis) and *Fragilariopsis cylindrus* (*Fc*, closed circles, right *y*-axis) at (a) 0°C and (b) 20°C. Michaelis–Menten half saturation constant for CO_2 (K_c) values of Rubisco calculated from (a) and (b) for *Tw* and *Fc* are shown in (c) along with the concentration of dissolved CO_2 in air-equilibrated seawater (dashed line). Measurements were done performed in duplicate; error bars, +1 SD.

total protein and converted to percentage biomass taking the conservative value of 50% protein content per cell (Table 1). From this, we calculated 1.2 ± 0.2 pmoles of Rubisco active sites per µg biomass and a k_{cat}^c of 0.39 ± 0.07 C s^{-1} per site (Table 1). This value is in very good agreement with our estimated *in vitro* calculation. Several lines of evidence thus suggest that *F. cylindrus*, growing at low temperatures has a Rubisco k_{cat}^c of *c.* 0.3–0.4 C s^{-1} per site.

In the WAP diatoms during the bloom, gross C fixation rates were measured directly in the field using ^{18}O incubations (see the Materials and Methods section or Goldman *et al.*, 2014) and normalized to Chl*a* (Table 2). Rates were converted per unit biomass by using a measured C to Chl*a* ratio (Fig. S5) and an assumption that 50% of the biomass is C (Sterner & Elser, 2002). The resulting gross C fixation rates, as well as the measured Rubisco abundance, were close to those found in *F. cylindrus*. Assuming that 50% of the biomass is protein, we calculated 1.15 ± 0.2 pmoles Rubisco active sites per µg biomass and a k_{cat}^c, of 0.40 ± 0.07 C s^{-1} per site (Table 2). This may be a slight overestimation of k_{cat}^c as a result of a possible diel cycle of Rubisco protein (Granum *et al.*, 2009) and difference in sampling times for Rubisco protein (late morning) and gross C fixation rates (midday).

Our estimated *in vivo* k_{cat}^c values from psychrophilic diatoms in the laboratory and in the field agree remarkably well with the values extrapolated from *in vitro* rates at 25°C using a Q_{10} of 2.6 (Fig. 6). The k_{cat}^c from psychrophilic diatoms seems to align closely with those of C_4 plants, and C_3 plants from cold climates, but is significantly higher than that observed in C_3 plants from warm climates (Fig. 6). This is not surprising, as diatoms have effective C-concentrating mechanisms to saturate Rubisco with CO_2, even at cold temperatures (Tortell *et al.*, 2010; Kranz *et al.*, 2014), and their Rubisco may have been optimized for rapid turnover rather than substrate specificity (Christin *et al.*, 2008).

Discussion

Diatoms in the WAP often produce intense blooms enabled by their high C fixation and growth rates despite temperatures well below the thermal optimum of most enzymes. In particular, the enzyme responsible for C fixation, Rubisco, has a turnover rate (k_{cat}^c) that decreases dramatically with temperature. To compensate for these slow rates, diatoms up-regulate Rubisco abundance. Rubisco is not the only protein up-regulated, and the increase of Rubisco and other proteins doubles cellular protein content in psychrophilic compared with mesophilic diatoms, which results in a low C : N of *c.* 5 in cold waters.

The *in vivo* Rubisco turnover rate, derived from cellular C fixation rates and Rubisco abundance, closely matches the calculated *in vitro* rates. This indicates that, in psychrophilic diatoms, all Rubisco must be active and that the active sites are nearly saturated with substrate (as assumed in the calculations). Saturation by CO_2 agrees well with the findings of Kranz *et al.* (2014) for psychrophilic diatoms in the field but there is little known about RuBP saturation in phytoplankton. RuBP regeneration is often the rate-limiting step of C fixation in plants at low temperatures (Bernacchi *et al.*, 2003). Our results imply that the large Rubisco concentration measured in the natural WAP population is about the minimum necessary to achieve the observed C fixation rate.

Our results provide a mechanistic explanation for the high productivity observed in the WAP (Fig. 1a; Goldman *et al.*, 2014). However, the maintenance of these high rates requires a large proportion of cellular content to be devoted to protein, up to 50% of biomass. This is more than double the protein content of mesophilic diatoms and is near the highest ever reported in phytoplankton (71% in *Spirulina* sp., 63% in *Synechococcus* sp., 61% in *Euglena* sp., 58% in *Chlorella* sp., and 56% in *Anabaena* sp.; Becker, 1994). Psychrophilic diatoms, with their high protein

Table 2 *In- vivo* estimate of the Rubisco turnover rate (k_{cat}^c) in Western Antarctic Peninsula (WAP) phytoplankton

Date	GPP (μmol CO_2 μg chl^{-1} d^{-1})[a]	GPP converted (pmol CO_2 μg $biomass^{-1}$ s^{-1})[b]	Rubisco % of total protein (w/w)	pmoles Rubisco sites μg $biomass^{-1}$[,c]	Estimated k_{cat}^c (C s^{-1} per site)[d]
7- November	3.11	0.33	12	0.87	0.47
14- November	2.55	0.27	15	1.09	0.31
19- November	2.56	0.27	13	0.95	0.36
27- November	3.16	0.33	16	1.16	0.36
4- December	2.90	0.31	11	0.80	0.48
10- December	2.45	0.26	10	0.73	0.44
Average	2.79	**0.29**	**12.83**	**0.93**	**0.40**
SD	0.31	**0.03**	**2.32**	**0.17**	**0.07**

Bold values represent the average and standard deviation (SD) for the four replicates.
[a]Gross primary production (GPP) in WAP phytoplankton during the bloom as measured by ^{18}O incubations and converted to C fixation rates.
[b]GPP rates normalized to unit biomass using a 50 : 1 C : Chl*a* ratio (Supporting Information Fig. S5).
[c]Assuming biomass is 50% protein based on *Fragilariopsis cylindrus* cultures.
[d]Estimated k_{cat}^c calculated by dividing measured, whole cell carbon fixation rates by the number of active sites of Rubisco, taking into account only 80% of the active sites are saturated by CO_2.

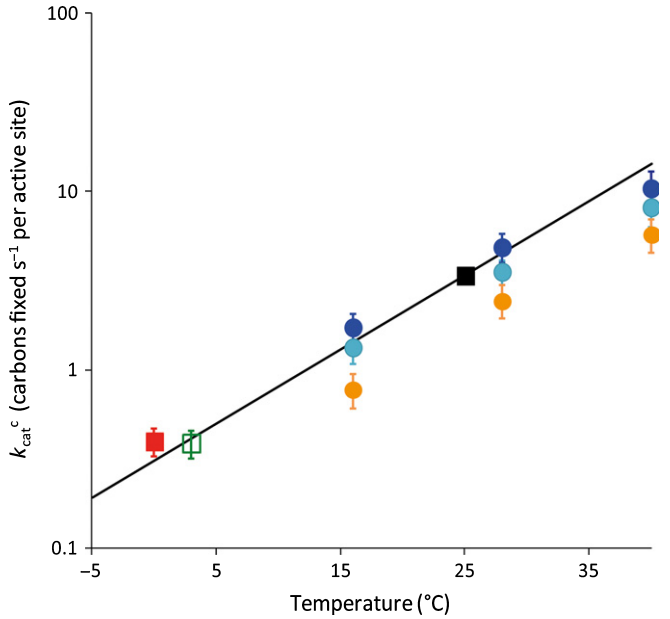

Fig. 6 Turnover rates (k_{cat}^c; C s^{-1} per site) of Rubisco at varying temperature. Circles are measured k_{cat}^c rates taken from Sage (2002) of C_3 plants from cool climates (light blue), C_3 plants from warm climates (orange) and C_4 plants (dark blue). The k_{cat}^c measured from the diatom, *Phaeodactylum tricornutum* at 25°C (Whitney *et al.*, 2001) was used in conjunction with a temperature coefficient (Q_{10}) value of 2.6 (from Fig. 4) to create a temperature response curve for diatom Rubisco (black line). Estimated *in vivo* k_{cat}^c values from for *Fragilariopsis cylindrus* at 3°C and Western Antarctic Peninsula (WAP) diatoms at 0°C are shown indicated by the open green and closed red squares, respectively.

content, have a significantly larger requirement for N compared to with their mesophilic counterparts. This is generally not a problem in polar Southern Ocean waters where nitrate is often present at high concentrations. Even during the large bloom observed, nitrate was not completely utilized (Fig. S4). The large protein content also raises an interesting question regarding N partitioning in the cell, as many photosynthetic proteins show a diel pattern and are degraded at night (including Rubisco in diatoms; Granum *et al.*, 2009). The fate of cellular N during these diurnal cycles is poorly understood. Furthermore, high protein content and low C : N imply a proportionally smaller polysaccharide plus lipid reserve, which could have implications on for the over-wintering strategies of psychrophilic diatoms.

It is unlikely that psychrophilic phytoplankton could elevate protein levels contents much higher than observed. Cellular integrity and the necessity to maintain cell metabolism requires a minimum concentration of lipids and carbohydrates, and the low C : N values in the WAP are among the lowest ever observed in marine phytoplankton (Sterner & Elser, 2002). If this high protein content is the result of increasing concentrations of enzymes, whose turnover rates are decreased at low temperatures, as is the case for Rubisco (which accounts for *c.* 36% of the increase in total protein), then diatoms of the WAP may be fixing C near their theoretical maximum rate. This upper constraint for C fixation could have important implications for ecosystem productivity and global climate models.

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Supporting Information

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

Fig. S1 Original Western western blots for RbcL and the corresponding standard curve.

Fig. S2 The effect of incubation times on the *in vitro* carboxylation activity of crude Rubiso extracts at different temperatures.

Fig. S3 Community composition based on HPLC pigment data.

Fig. S4 Carbon to Chl*a* ratios in particulate matter during the bloom in the WAP.

Fig. S5 Inorganic CO_2, dissolved inorganic nitrogen (DIN) and dissolved inorganic phosphate during the 2012/2013 season in the WAP.

Table S1 Concentrations of CO_2 and the K_c of Rubisco at different temperatures

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