LETTER

Divergent physiological and molecular responses of light- and iron-limited Southern Ocean phytoplankton

Sarah M. Andrew,1* Robert F. Strzepek,2 Spencer M. Whitney,3 Wah Soon Chow,3 Michael J. Ellwood1

1Research School of Earth Sciences, Australian National University, Canberra, Australian Capital Territory, Australia; 2Australian Antarctic Program Partnership (AAPP), Institute for Marine and Antarctic Studies, University of Tasmania, Hobart, Tasmania, Australia; 3Research School of Biology, Australian National University, Canberra, Australian Capital Territory, Australia

Scientific Significance Statement
Phytoplankton growth and distribution in the Southern Ocean are often limited by low iron concentrations or insufficient light supply to drive photosynthesis. Iron is a key component in many of the proteins involved in energy generation and metabolism, but we still lack a comprehensive understanding of the molecular strategies underlying the well-studied physiological responses to changing iron and light. Our results show that an ecologically relevant Southern Ocean diatom invests a large proportion of its protein resources into Rubisco, much more than other diatoms with the same growth rate. We suggest that uncoupled protein abundances involved in energy capture and energy synthesis may permit this species to dedicate important resources to diffusive carbon uptake rather than relying solely on active carbon uptake.

Abstract
It has recently been shown that Southern Ocean phytoplankton species have evolved to optimize their light-harvesting potential without increasing the high iron-requiring proteins used for photosynthesis. We measured molecular and physiological responses of phytoplankton cultures under a combination of iron and light conditions. While iron-replete cultures mostly increased biovolume, photochemical efficiency (Fv/Fm) and the relative abundance of photosystem II (PSII) and Cytochrome b6f protein compared to iron-limited cultures, light also regulated cellular chlorophyll a content and played a role in controlling PSII protein abundance. Investment of protein resources into the carbon fixing enzyme Ribulose 1,5-bisphosphate carboxylase oxygenase (Rubisco) was species-specific, but increased growth rates correlated with increased investment into Rubisco for all species. Our results suggest that Proboscia inermis uses a divergent molecular strategy to compete for nutrients, light, and CO2 in the Southern Ocean.

*Correspondence: sarah.andrew@unc.edu

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The Southern Ocean is a region of high nutrients yet low chlorophyll concentrations because of growth-limiting irradiance and low iron (Fe) concentration (Boyd et al. 2010). Fe plays an essential role in photosynthesis, as a cofactor of many photosynthetic protein complexes like photosystem II (PSII), Cytochrome b_{6f}, and photosystem I (PSI). Southern Ocean phytoplankton have been the focus of many studies to understand how they are able to photosynthesise optimally under low light and low Fe conditions (Strzepek et al. 2012). This relatively well-studied combination has revealed systematic differences between “model” phytoplankton species (typically coastal, temperate isolates) and Southern Ocean phytoplankton (Moreno et al. 2018; Strzepek et al. 2019).

It has been shown that temperate coastal and oceanic diatoms increase intracellular Fe concentrations and Fe : C ratios with decreasing light, but this does not happen in Southern Ocean diatoms or the haptophyte *Phaeocystis antarctica* (Strzepek et al. 2012). These polar species acclimate to low irradiances by increasing the size of their photosynthetic units under lower light conditions (Strzepek et al. 2019). In contrast, under low light conditions, many coastal phytoplankton species increase their photosynthetic unit number, increasing the cellular concentrations of Fe-rich protein complexes (Strzepek et al. 2019). In addition, when irradiance exceeds growth requirements, marine phytoplankton employ a range of photoacclimation strategies to reduce energy intake—such as reducing light-harvesting potential and inducing mechanisms that dissipate or divert excess energy—so that it balances the energy required for carbon fixation and metabolism (Arrigo et al. 2010). To maintain this balance between energy capture and cellular production would suggest that organisms need to maintain constant photosynthetic protein stoichiometries, even under Fe stress (Macey et al. 2014). Nevertheless, there is evidence that remodeling photosynthetic protein stoichiometry can occur when cells are Fe limited (Strzepek and Harrison 2004; Strzepek et al. 2019).

Recent studies have sought to understand how biochemical pathways are regulated under Fe limitation (Nunn et al. 2013; Boyd et al. 2016). Polar extremes have influenced unique adaptations in diatoms to reduce the high Fe requirements generally associated with photosynthesis (Moreno et al. 2018). For example, diatoms can substitute complexes with high Fe requirements for other proteins that do not contain Fe, such as flavodoxin for ferredoxin (La Roche et al. 1996) and copper-containing plastocyanin for Cytochrome c_{6} (Peers and Price 2006). By determining the differential protein regulation under controlled conditions, key adaptations can be identified and used to highlight the ability of phytoplankton to grow under certain environmentally relevant conditions. We present the molecular responses (the relative abundance of PSII, Cytochrome b_{6f} complex, adenosine triphosphate [ATP] synthase, and Rubisco proteins) of Southern Ocean phytoplankton and the temperate diatom *Phaeodactylum tricornutum* grown under a combination of light and Fe conditions.

**Methods**

Cultures of the Southern Ocean haptophyte *P. antarctica* (clone SX9), and the diatoms *Chaetoceros flexuosus* and *Probosia inermis* (Strzepek et al. 2012; Andrew et al. 2019), were selected due to their importance in the Southern Ocean. The temperate, model diatom *P. tricornutum* (CS-29, Australian National Algae Supply Service) was also cultured to compare responses of specialized vs. cosmopolitan diatoms to low and Fe supply. Physiological measurements of growth, pigment and elemental composition, and photo-physiology are described in the Supporting Information.

**Culturing conditions and experimental design**

Southern Ocean isolates were maintained at 3°C under constant high (200 μmol photons m^{-2} s^{-1}) and low (25 μmol photons m^{-2} s^{-1}) irradiance, and maintained as semi-continuous batch cultures by dilution. The temperate model diatom *P. tricornutum* has greater light requirements than Southern Ocean phytoplankton (Geider et al. 1985), thus was grown under constant irradiance at high (250 μmol photons m^{-2} s^{-1}) and low (30 μmol photons m^{-2} s^{-1}) light at 20°C (Supporting Table S1). All cultures were cultured in microwave-sterilized artificial seawater medium Aquil (Price et al. 1989), and grown in triplicate 1-liter polycarbonate bottles (Nalgene).

Southern Ocean cultures were grown in Fe replete medium prepared with a filter-sterilized Fe : ethylenediaminetetraacetic acid (EDTA) complex (1 : 1), which was added to Aquil containing 10 μM EDTA to obtain a total Fe concentration of 58 nM. High Fe (Fe+) and low Fe (Fe−) media for *P. tricornutum* were prepared following the same method to achieve final concentrations of 200 nM and 2 nM total Fe (Greene et al. 1991). Iron-limitation in the Southern Ocean phytoplankton was induced by varying the Fe concentrations, and the siderophore desferrioxamine B mesylate (DFB) was added to reduce growth by ~50% for each species. Low Fe media for the Southern Ocean cultures was prepared using a premixed solution containing 3.5 nM FeCl_{3} complexed with 40 or 400 nM of DFB and added to Aquil medium containing 10 μM of EDTA to buffer the other trace metals as described in Strzepek et al. (2011).

**Determination of PSII, Cytochrome b_{6f}, ATP synthase, and Rubisco concentrations**

Total soluble protein (TSP) was determined by colorimetric detection (Bradford Assay) against bovine serum albumin standards (Sigma-Aldrich; see Supporting Information). Total protein from an equivalent number of cells per treatment were separated on 4–12% NuPage Bis-Tris Gels (Invitrogen), then blotted onto polyvinylidene difluoride membrane for 1 h at 150 mA (16 V). After blocking (5% w/v skim milk powder in tris-buffered saline [TBS]), membranes were incubated with the primary antibody (PsbA AS05 084, D1 subunit of PSII; PetC AS08 330, Rieske Fe-S protein of Cytochrome b_{6f}; and AtpB AS05 085, ATP synthase; Agrisera) at a dilution of 1:500 and incubated at room temperature for 1 h. Membranes were then washed 3× in TBS-Tween (TBS + 0.05% Tween 20) and incubated with horseradish peroxidase-labeled secondary antibodies (Anti-Rabbit or Anti-Mouse IgG, 1:3000) for 30 min at room temperature. Membranes were washed 3× in TBS-Tween and developed with enhanced chemiluminescence substrate (GE Healthcare) at room temperature for 15 min. Chemiluminescent signals were captured on an amershamfluorography film (GE Healthcare) and the relative band intensity quantified using ImageJ.
1:5000 for 1 h with agitation and then stored at 3°C overnight. Membranes were rinsed again with TBS, then incubated with a secondary antibody (goat anti-rabbit IgG horse radish peroxidase conjugated, Bio-Rad) diluted to 1:10,000, for 3 h at room temperature with gentle agitation. Membranes were washed again with TBS, and blots were visualized with Clarity Western ECL Substrate (Bio-Rad) and imaged using the ChemiDoc MP imaging system (Bio-Rad) and Image Lab software (Bio-Rad). Unprocessed blots are provided as Supplemental Figs. S1–S6.

Cellular Rubisco content was quantified by chromatographic separation of Rubisco bound $^{14}$C carboxyarabinitol bisphosphate (CABP) from unbound $^{14}$C-CABP (Whitney et al. 1999). Radioactivity in each sample was measured using a Tri-Carb 2800TR Liquid Scintillation Analyzer (PerkinElmer). Rubisco concentration was expressed as a % (w/w) of the TSP.

**Statistical analysis**

Interactive effects of Fe and light were statistically analyzed by two-way ANOVA, followed by the Tukey post hoc test using the functions aov and TukeyHSD in R 3.5.1 (R Core Development Team 2016). The relationship between physiological and protein responses was assessed using a principal component analysis (PCA) on a scaled correlation matrix and plotted using the prcomp and autoplot functions in the ggfortify package. All testing was done at the 95% confidence

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**Fig. 1.** Experimental setup and physiological relationships between (A) specific growth rate, (B) Chl $\alpha$ normalized to carbon, (C) cellular carbon per liter cell volume, (D) cellular nitrogen per liter cell volume, (E) cell volume ($L = 10^{-15} L = \mu m^3$), and (F) surface area to volume ratio. Values represent the mean and standard deviation of three biological replicate samples.
Results

Physiological responses to iron and light

Like other Fe and light limitation studies, the growth rates of all species were significantly reduced \((p < 0.05)\) by Fe limitation under all light irradiances (Fig. 1A; Supporting Table S2; Greene et al. 1991; Strzepek et al. 2012). Carbon and nitrogen quotas per liter cell volume (mol L\(^{-1}\)) were generally lower under low Fe supply, and the effect of light individually and interactively was significant only in cultures of \(P.\ antarctica\) in which both cellular C and N increased approximately twofold under low light and Fe-limited treatments (Fig. 1C,D; \(p < 0.05\)). The chlorophyll (Chl) \(a\) concentration normalized to carbon (Chl : C, mmol mol\(^{-1}\)) for each species also decreased under Fe limitation or high light, thus were highest for all species in the low light, high Fe treatments (Fig. 1B; \(p < 0.05\)).

The cell volumes of all species in this study spanned three orders of magnitude and responded differently to changes in Fe and light (Fig. 1E,F; Supporting Table S2). \(P.\ inermis\) significantly increased its biovolume under Fe-limiting conditions \((p < 0.05)\) and was observed to undergo sexual reproduction under Fe limitation. Diatoms are expected to reduce their biovolume as a response to Fe limitation, while also increasing SA : V (Strzepek et al. 2011). This reduction in nutrient and energy quotas per cell, relative to nutrient uptake rates, is hypothesized to be an advantageous strategy to alleviate Fe limitation (Marchetti and Cassar 2009). Cultures of \(P.\ antarctica\) and \(C.\ flexuosus\) were observed to significantly reduce biovolume under Fe limitation \((p < 0.05)\), like previous

![Figure 2](image1.png)

**Fig. 2.** The inverse correlation observed between the photochemical efficiency of PSII \((F_v/F_m)\) and the functional absorption cross-section of PSII \((\sigma_{PSII})\) is more significant at (A) low light than (B) high light. Shapes represent different species; open and closed symbols are for low- and high-iron cultures, respectively.

![Figure 3](image2.png)

**Fig. 3.** PCA of the species responses to different Fe and light treatments. Biological triplicates for each treatment are denoted as shapes, while species are shown by color. (A) Physiological responses including growth rate, cellular chlorophyll, C, N, \(F_v/F_m\), and sigma. PC1 represents 36.33% of the variation for placement in the data set and PC2 28.84% of the variation. (B) Protein responses including PSII, Cytochrome \(b_{6}f\), ATP synthase, and Rubisco. PC1 represents 41.54% of the variation for placement in the data set and PC2 31.25% of the variation.
observations (van Oijen et al. 2004; Luxem et al. 2017), but SA : V did not increase \((p < 0.05)\). The opposite response was observed for \(P. inermis\), where biovolume increased and SA : V decreased under Fe limitation. This relationship has been reported previously for some Southern Ocean diatoms, including \(P. inermis\) (Strzepek et al. 2011) and is suggested to be an Fe stress response on division (Meyerink et al. 2017). Surprisingly, Fe limitation had no significant effect on biovolume in cultures of \(P. tricornutum\), despite similar light, temperature, and/or Fe conditions used in other studies (Kudo et al. 2000; Allen et al. 2008).

Iron limitation caused an increase in the functional absorption cross-section of PSII \((\sigma_{PSII})\) and a decrease in PSII photochemical efficiency \((Fv/Fm)\) (Fig. 2; \(p < 0.05\)). Unexpectedly, it was observed that \(\sigma_{PSII}\) increased slightly under low Fe in cultures of \(P. antarctica\) even though \(Fv/Fm\) was reduced under Fe limitation (Fig. 2A; \(p < 0.05\)). As previously observed, we found an inverse relationship between \(\sigma_{PSII}\) and photochemical efficiency of PSII \((Fv/Fm)\) at subsaturating irradiances (Fig. 2A). However, this relationship is not expected under growth-saturating irradiances because other photoprotective mechanisms, such as nonphotochemical quenching, reduce the cells’ light-harvesting potential (Alderkamp et al. 2013; Fig. 2B).

We performed a PCA to examine the differences in physiological and protein variables across the different species and treatments (Fig. 3). Physiologically, species clustered into a cold-water group vs. the temperate diatom \(P. tricornutum\) along the second axis. This is driven by higher growth rates, \(Fv/Fm\), and SA : V of \(P. tricornutum\) (Fig. 3A). Despite similar trends in physiology, \(P. inermis\) showed clear differences in its underlying protein responses compared to other Southern Ocean phytoplankton and \(P. tricornutum\) (Fig. 3B). While \(P. tricornutum\) clustered with \(C. flexuosus\) and \(P. antarctica\), allocation to Rubisco and PSII differed in \(P. inermis\) compared to the other species, suggesting unique molecular differences in the way \(P. inermis\) responds to changes in light and Fe.

**Intracellular variations in protein regulation**

As expected, Fe-limited cells acclimated to low light conditions had lower Chl : C and PSII relative abundance (PsbA, D1 subunit of PSII) compared to Fe replete cells, suggesting that Fe limitation impedes Chl \(a\) synthesis or results in the downregulation of pigment and protein synthesis (Figs. 1, 4; \(p < 0.05\)). The effect of high light had a significant effect on PSII in all species except for \(P. inermis\) (\(p < 0.05\)). It is expected that pigments and PSII are downregulated under high light to protect against damage from excess light energy, as observed in \(C. flexuosus\) and \(P. tricornutum\); but this is not observed in \(P. antarctica\) (Fig. 4). Trimborn et al. (2014) observed that in contrast to Southern Ocean diatoms, \(P. antarctica\) does not possess energetically well-connected PSII reaction centers. The significance of this result is unclear, but the increased PSII abundance and the high \(\sigma_{PSII}\) observed in the HL Fe+ cultures...
of *P. antarctica* may suggest different photoprotection strategies that enable them to photosynthesize optimally under high light (Figs. 2, 4).

Under Fe limitation, the content of the Cytochrome b$_{6}$f complex was significantly lower compared to the high Fe treatments (Fig. 4; $p < 0.05$). The downregulation of Cytochrome b$_{6}$f observed under Fe limitation in our study is comparable to the previously reported 80–94% reduction in the Cytochrome b$_{6}$f complex in *P. tricornutum* (Glover 1977; Greene et al. 1991). Interestingly, Cytochrome b$_{6}$f was not detected in the high light and Fe replete treatment for *P. antarctica*. A large reduction in Cytochrome b$_{6}$f expression would severely reduce electron transport between PSII and PSI, restricting NADPH synthesis (Anderson et al. 1997). Under such conditions, in which the plastoquinone (PQ) pool would be highly reduced, electrons could be transferred from PQH$_{2}$ to oxygen via plastoquinol terminal oxidase (PTOX; Peltier et al. 2010; Stepien and Johnson 2018), or possibly the primary quinone acceptor Q$_{A}$ in PSII (Cleland and Grace 1999).

Rubisco content as a percentage of TSP ($w$ $w^{-1}$) ranged from $\sim$ 1–3% for cultures of *P. antarctica* and *C. flexuosus*, $\sim$ 5–9% in cultures of *P. tricornutum*, to $\sim$ 7–10% for cultures of *P. inermis* (Fig. 4). Species protein investment into Rubisco responded differently to light or Fe: *P. inermis* and *P. tricornutum* decrease Rubisco under Fe limitation, *C. flexuosus* decreases Rubisco under low light conditions regardless of the abundance of Fe, but Fe and light had no effect on Rubisco abundance in cultures of *P. antarctica* ($p > 0.05$). Previous studies have shown that Rubisco concentration is highly variable in marine phytoplankton, and can reflect growth phase as well as nutrient availability (Losh et al. 2013).

**Discussion**

Apart from *P. inermis*, increases in PSII corresponded with increases in ATP synthase (Fig. 5A; $R^{2} = 0.57$) supporting the hypothesis that the capacity for ATP production correlates with the production of photosynthetic energy (Macey et al. 2014). The inverse trend between PSII and ATP synthase observed in *P. inermis* (Fig. 5B; $R^{2} = 0.62$) is underpinned by little variation in ATP synthase content in response to treatment while PSII levels responded strongly to Fe and light ($p > 0.05$). The lack of variation in ATP synthase content in *P. inermis* may alternatively stem from normalizing to cell...
number. When PSII and ATP synthase are normalized to TSP, not cell number, the ATP synthase content increases with PSII for *P. inermis* ($R^2 = 0.52$; Supporting Fig. S8). Due to the large variation in the cellular soluble protein contents among the *P. inermis* replicate cultures, it is not clear if this result is meaningful. Quantitative measures of PSII and ATP synthase in additional *P. inermis* culture treatments will help to understand the strategies employed by this species for energy generation and metabolism.

Under Fe replete conditions, the Cytochrome *b*$_f$ content increased with ATP synthase content ($R^2 = 0.31$; Supporting Fig. S7). Thus, electron transport through PSII, the Cytochrome *b*$_f$ complex, and PSI was coupled with ATP synthesis. By contrast, under Fe limitation, the Cytochrome *b*$_f$ was very low or not detectable. Even when not detectable by our method, however, Cytochrome *b*$_f$ was likely to have been present to some extent to enable growth under Fe limitation (Fig. 1A). Similarly, although not measured, PSI is expected to be present to some extent to allow the formation of NADPH that, together with ATP, drives carbon assimilation. Indeed, in their review, McDonald et al. (2011) envisaged some NADPH production under Fe limitation, despite upregulation of PTOX-dependent reduction of oxygen that diverts excess electrons from the PQ pool. Given that PTOX is located on the thylakoid stroma interface, away from the PSI water oxidation site in the lumen (Lennon et al. 2003), electron transfer from PQH$_2$ to oxygen will not create a proton gradient to support ATP synthesis. However, if PTOX was coupled with a proton pump (such as the Fe-free light-driven proton pump proteorhodopsin, which has been reported to be upregulated in Fe-limited diatoms), it may contribute to the pH gradient under low Fe conditions (Marchetti et al. 2015; Strzepek et al. 2019). Regardless, the protons from the oxidation of water associated with PSI will support ATP synthesis and, in turn, fuel carbon assimilation.

*P. inermis* invests a large proportion of its soluble protein into Rubisco at 3°C (~10% w w$^{-1}$; Fig. 4), which is greater than any of the other Southern Ocean species in this study. Rubisco is notorious for its slow catalytic rate and low affinity for CO$_2$ (Andrews and Lorimer 1987). Marine phytoplankton have therefore developed carbon concentrating mechanisms (CCMs) to increase the concentration of CO$_2$ at the site of carboxylation (Badger et al. 1998; Reinfelder 2011). CCMs are energetically costly to operate and maintain (Raven et al. 2008), but are beneficial in a marine environment where CO$_2$ availability to Rubisco would otherwise be low and its alternative reactivity with O$_2$ poses an energetic and carbon cost through photorespiration (Raven et al. 2014). Without explicit kinetic measurement of *P. inermis* Rubisco, it could be assumed the increased resource allocation to Rubisco production in *P. inermis* may compensate for slow catalytic rates at low temperatures (Young et al. 2015). As the catalytic properties of diatom Rubisco are substantially more variable than previously considered (Young et al. 2016), a kinetic survey of Southern Ocean species may help interpret the differences in their metabolic adaptation.

In combination with several previously measured morphological and physiological characteristics, we observed that *P. inermis* may have unique nutrient uptake mechanisms that differ from other Southern Ocean phytoplankton (Kemp et al. 2000; Annett et al. 2010; Strzepek et al. 2011). Growth rates and Rubisco concentration are positively correlated for all species; however, this relationship is more significant when *P. inermis* is excluded (Fig. 5C; $R^2 = 0.71$). Maximum growth rates of *P. antarctica* are supported with greater than fivefold less Rubisco (0.43 d$^{-1}$ at 1.6% Rubisco) than *P. inermis* (0.39 d$^{-1}$ at 10.0% Rubisco), suggesting important differences in their energy and carbon acquisition strategies. Yet, this does not seem to increase cellular C or N quotas in *P. inermis*. Indeed, it has been observed that *Proboscia* species produce uniquely negative δ$^{13}$C particulate organic carbon (Henley et al. 2012). Whether this stems from a passive, rather than an active, carbon uptake mechanism remains to be experimentally tested.

Our data show that while some photosynthetic protein ratios may be maintained under Fe or light limitation, this does not seem to be true for all species, or all proteins (Figs. 4, 5). We found that Cytochrome *b*$_f$ and ATP synthase were only correlated under high Fe conditions, while PSII and ATP synthase were correlated under all conditions, except for *P. inermis*. This suggests that photosynthetic protein ratios probably vary between species in response to different environmental conditions and differences in their cellular metabolism, providing diversity among microalgae to adjust to the temporal nutrient fluctuations in the Southern Ocean (Boyd et al. 2010). Considering this information, it would be useful to measure CCM activity on a greater variety of Southern Ocean marine phytoplankton to fully understand the natural diversity in metabolic adaptation and improve our appreciation of carbon cycling in the Southern Ocean.

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