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Excessive irradiance and antioxidant responses of an Antarctic marine diatom exposed to iron limitation and to dynamic irradiance

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ABSTRACT

The synergistic effects of iron limitation and irradiance dynamics on growth, photosynthesis, antioxidant activity and excessive PAR (400–700 nm) and UV (280–400 nm) sensitivity were investigated for the Antarctic marine diatom *Chaetoceros brevis*. Iron-limited and iron-replete cultures were exposed to identical daily irradiance levels, supplied as dynamic (20–1350 μmol m^{-2} s^{-1}) and constant (260 μmol m^{-2} s^{-1}) irradiance. After acclimation, growth, maximal quantum yield of PSII (F_v/F_m), pigment composition, and the activities of the antioxidant enzymes superoxide dismutase (SOD), ascorbate peroxidase (APX) and glutathione reductase (GR) were determined. Then, excessive irradiance sensitivity was assessed by monitoring pigment composition, F_v/F_m and viability loss during a single excessive PAR and UV treatment.

Iron limitation reduced growth rates, F_v/F_m dynamics, and cellular pigments pools. Cellular pigment concentrations were higher under dynamic irradiance than under constant irradiance but this difference was less pronounced under iron limitation compared to iron-replete conditions. SOD and APX activities increased during dynamic irradiance under iron limitation, suggesting increased radical formation around PSII. Despite these physiological differences, no effects on growth were observed between constant and dynamic irradiance cultivation in iron-limited and iron-replete cells. The applied culturing conditions did not affect glutathione reductase activity in *C. brevis*. F_v/F_m and xanthophyll de-epoxidation dynamics during excessive irradiance were not different for iron-limited and replete cells and viability loss was not found during excessive irradiance. This study revealed photoacclimation differences between iron-limited and iron-replete *C. brevis* cultures that did not affect growth rates and excessive irradiance sensitivity after acclimation to constant and dynamic irradiance.

1. Introduction

The combination of iron limitation and deep wind driven vertical mixing can suppress primary productivity in vast parts of the Southern Ocean, leaving much of the dissolved nitrate, phosphate, and silicate unused for algal growth [1,2]. These frequently co-occurring conditions both affect the photoacclimation state of algae. Vertical mixing in the water column can mediate strong irradiance fluctuations, from excessive irradiance near the water surface to complete darkness below the euphotic zone. These dynamics require contrasting acclimation pathways such as increased light harvesting under low irradiance as well as increased photo protection when irradiance exceeds the photosynthetic requirements [3,4]. The effects of iron limitation on photoacclimation are well documented. When iron becomes limiting, algae respond by reducing the abundance of iron rich cellular components such as cytochrome b_6–f and PSI complexes [5,6]. This impairs the electron transport capacity during photosynthesis and leads to a strong reduction in cellular light harvesting pigments and decreased growth rates [7,8]. How iron limitation affects photosynthesis and growth under dynamic irradiance remains unknown. Cytochrome b_6–f complexes are crucial in the buildup of a proton gradient across the thylakoid membrane, which activates protective heat dissipation via the xanthophyll cycle during excessive irradiance. Therefore, it was suggested that iron-limited algae were less able to cope with rapid irradiance fluctuations than those under iron-replete conditions [6]. Furthermore, van Leeuwe and Stefels [9] observed reduced xanthophyll de-epoxidation under high irradiance in iron-limited *Phaeocystis antarctica*. Therefore, a reduction in cytochrome b_6–f complexes may affect the efficiency of the xanthophyll cycle, and thus the protective down regulation of photosynthesis during excessive irradiance. Consequently, iron-limited algae could be more vulnerable to excessive irradiance due to increased formation of reactive oxygen species (ROS). ROS are typically formed as byproducts of electron transport and cellular metabolism. Environmental stress such as excessive PAR and UV exposure near the water surface can significantly enhance ROS

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formation, inhibiting photosynthesis and threatening viability [10]. Cellular ROS concentrations are controlled by an elaborate antioxidant network. The superoxide radical is converted enzymatically to H_2O_2 by superoxide dismutase (SOD), which is subsequently neutralized to H_2O by ascorbate peroxidase (APX). Glutathione is used to regenerate ascorbate, which is the substrate of APX in the latter reaction. The redox status of glutathione is regulated by glutathione reductase (GR). Iron limitation was found to increase ROS production and superoxide dismutase (SOD) activity in marine diatoms [11]. Nevertheless, a preliminary study indicated that iron-limited algae were less sensitive to excessive PAR and UV radiation exposure than iron-replete algae [10]. Furthermore, numerous studies demonstrated that iron-limited algae accumulated more protective xanthophyll cycle pigments relative to their light harvesting pigments compared to iron-replete cells [7]. This emphasizes that the effect of iron limitation on the regulation of photosynthesis is not completely understood, let alone under dynamic irradiance as experienced in the field. Therefore, the responses of pigments, chlorophyll fluorescence and crucial components of the antioxidant network were studied for the Antarctic diatom *Chaetoceros brevis* under simulated dynamic and constant irradiance for iron-limited and iron-replete conditions, followed by excessive (UV) irradiance treatments, during which F_v/F_m, xanthophyll cycling and viability were monitored.

2. Materials and methods

2.1. Cultivation and experimental design

Iron-replete cultures of *C. brevis* (CCMP 163) were grown as batch cultures in autoclaved sea water of 35 PSU enriched with *f*-2 nutrients [12]. Iron-limited cultures were grown in sea water of 35 PSU, collected from the Southern Ocean, enriched with *f*-2 nutrients (without iron), and then run over a Chelex-100 column (Chelex, Rochester, NY, USA) to remove iron (except the silicate stock). The polycarbonate cultivation vessels of iron-limited cells were washed with 1 N HCl and handled in a clean room to prevent iron contamination, whereas 10 μM EDTA (final concentration) was added to bind remaining iron. Iron-limited and replete cultures were grown at 4.5 °C in a cooled culture cabinet under 75 μmol m^{-2} s^{-1} photons PAR and a 16–8 h L–D cycle for several months during which the medium was regularly replaced. Acclimation to dynamic and constant irradiance was achieved by inoculating duplicate transparent polycarbonate erlenmeyers (2 L) with 30 mL culture, giving a total of eight culture vessels. The dynamic irradiance set-up was as described in van de Poll et al. [4]. Dynamic irradiance simulated vertical mixing over 4 h cycles, resulting in irradiance oscillations between 20 and 1350 μmol photons m^{-2} s^{-1} (Fig. 1), whereas constant irradiance was kept at 260 μmol photons m^{-2} s^{-1} during the 16 h light period. The daily irradiance dose was similar for constant and dynamic irradiance. All cultures were maintained at 4.5 °C by cryostat controlled water baths. After inoculation cell numbers were followed for 35 and 15 days, for iron-limited and replete cultures, respectively. Iron-replete cultures were refreshed with new medium once. On the last day, samples for F_v/F_m, pigments, antioxidant activities and excessive irradiance sensitivity were obtained. After the experiments, the remaining iron-limited cultures were supplied with iron to test if this enhanced pigmentation, fluorescence and growth.

2.2. Growth

Two milliliter subsamples were obtained from the cultures for cell counts and immediately processed. Cell concentrations were determined on a Coulter MXL flow cytometer as in van de Poll et al. [13]. The mean growth rate of each replicate was calculated from linear regression of 6–9 natural log transformed cell concentrations plotted against time.

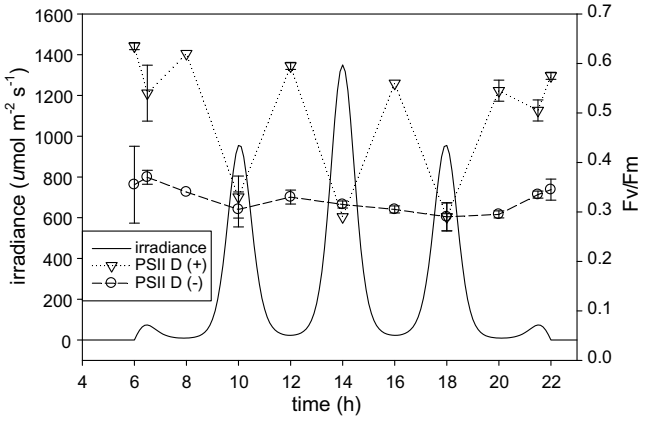

Fig. 1. Irradiance and maximal quantum yield of PSII (F_v/F_m) of iron-limited (−) and replete (+) *Chaetoceros brevis* during the course of the dynamic (*D*) irradiance treatment. Mean and standard deviations are shown for two replicates.

2.3. Maximal quantum yield of PSII: F_v/F_m

To expand measuring capacity, two fluorometers were used to determine F_o and F_m after 5 min dark adaptation and the F_v/F_m was calculated as $(F_m–F_o)/F_m$. On the last day of the experiment, a dual modulated fluorometer with integrating sphere (Photosystem Instruments, Chech Republic, red excitation light; >670 nm) was used to measure F_v/F_m of iron-replete and iron-limited cultures under dynamic irradiance. A pulse amplitude modulation fluorometer (water PAM, Walz, Germany, blue excitation light; peak around 450 nm) was applied to measure F_v/F_m during excessive irradiance (simulated surface irradiance, SSI) in 5 mL subsamples, see below.

2.4. Pigment composition

Samples (75 mL) for pigment composition (one from each bottle, two replicates in total) were filtered on 25 mm GF/F (Whatman) by vacuum, frozen in liquid nitrogen and stored at −80 °C. Filters were freeze-dried (48 h) followed by pigment extraction in 3 mL 90% cold acetone (v/v, 48 h, 4 °C) after van Leeuwe et al. [14]. Pigments were resolved by HPLC (Waters 2690 separation module, 996 photodiode array detector) with a C_{18} 5 μm DeltaPak reversed-phase column (Waters) and identified by retention time and diode array spectroscopy. For quantification, standards of chlorophyll *a*, fucoxanthin, diadinoxanthin, and diatoxanthin were used (DHI, Denmark). Cellular pigment concentrations were calculated from cell counts (flow cytometer), sample volume and extraction volume. Chlorophyll *c*1, 2 and β carotene were detected but not quantified in the present study.

2.5. Antioxidant activity: SOD activity

One hundred to two hundred milliliters of culture was filtered on polycarbonate filters (47 mm, 2 μm pore size) by vacuum and stored at −80 °C for SOD activity analysis. The Riboflavin/NitroBlue Tetrazolium (RF/NBT) method [15], modified by Janknegt et al. [16], was used to measure SOD activity. Filters were suspended in 0.75 mL buffer (50 mM KH_2PO_4, pH 7.8, 0.1 mM EDTA, 0.1% Triton X-100, 2% PVP, and Complete protease inhibitor cocktail). Cells were disrupted by sonication (2* 30 s pulses, 4 °C), and extracts

were centrifuged (20.000*g*, 4 °C, 25 min), and transferred to a clean vial. SOD activity was defined as the amount of sample required for 50% inhibition of NBT reduction (V_{50}). NBT reduction was measured in reaction mixtures (1.5 mL) with 0, 12.5, 25, and 50 μL extract, SOD buffer (50 mM KH_2PO_4, 0.1 mM EDTA, and 0.25% Triton X-100), 2 mM riboflavin (Sigma), and 57 μM NBT (Sigma). After 30 min dark incubation (4 °C) the reaction mixtures were illuminated from above for 15 min (Philips TLD/18W, 199 μmol photons m^{-2} s^{-1}). Afterwards, absorbance was measured at 560 nm on a Cary 3E UV/Vis double beam spectrophotometer (Varian, Middelburg, The Netherlands). The V_{50} was calculated by regression using the linear part of a natural semi-log curve. The specific activity of SOD (U/mg protein) was calculated according to $1/(V^*_{50}[\text{protein}])$. Protein concentrations in the extracts were determined in duplicate according to Bradford [17].

2.6. Antioxidant activity: APX and GR activity

Sampling, filtration and extract preparation for ascorbate peroxidase (APX) and glutathione reductase (GR) activities was as for the SOD activity (see above). APX activity was determined spectrophotometrically by the consumption of ascorbic acid, for 3 min in quartz cuvette containing 2.4 mL buffer (50 mM KH_2PO_4, pH 7; 0.1 mM EDTA), 150 μL 10 mM ascorbic acid (Merck, Darmstadt Germany), and 400 μL cell extract. Reaction mixtures were incubated (5 min) at 4 °C before 20 mM H_2O_2 was added. The rate of ascorbate consumption was measured at 290 nm on a Cary 3E UV/Vis double beam spectrophotometer at 4 °C. Specific activity (U) of APX was calculated according to: $(-\text{slope} * \text{volume}_{\text{tot.}}/\varepsilon * \text{volume}_{\text{extr.}})/[\text{protein}]$ of which $\varepsilon = 2.8\ \text{mM}^{-1}\ \text{cm}^{-1}$.

GR activity was determined by its ability to convert oxidized glutathione (GSSG) into its reduced form (GSH) using NADPH as a reducing agent. NADPH consumption was measured spectrophotometrically at 340 nm for 3 min in a 3 mL quartz cuvette with 1 mL buffer, 150 μL, 10 mM GSSG (Merck, Darmstadt Germany), 300 μL cell extract and 50 μL, 2.5 mM NADPH (Merck, Darmstadt Germany). Before NADPH addition, the reaction mixture was incubated for 5 min at 4.5 °C. Specific activity of GR (in U/mg prot) was calculated according to: $(-\text{slope} * \text{volume}_{\text{tot.}}/\varepsilon * \text{volume}_{\text{extr.}})/[\text{protein}]$ of which $\varepsilon = 6.22\ \text{mM}^{-1}\ \text{cm}^{-1}$.

2.7. Excessive irradiance sensitivity

Three hundred fifty milliliter portions of the cultures were transferred to 1 N HCl washed quartz vessels and exposed to simulated surface irradiance (SSI: 448, 50, and 0.6 W m^{-2} PAR, UVA, and UVB, respectively) as in van de Poll et al. [10]. Excessive irradiance effects on pigment composition and F_v/F_m were examined by taking samples before, directly after 20 min of SSI, and after 20 min of SSI followed by 60 min in low (10 μmol photons m^{-2} s^{-1}) irradiance. In addition, viability loss during prolonged SSI exposure (4 h) was investigated in a separate experiment using 25 mL of culture for each replicate. Subsamples for the viability assay were obtained every hour during 4 h SSI exposure (see below). During SSI exposure temperature was maintained at 4.5 °C.

2.8. Viability loss during excessive irradiance

One milliliter subsamples were incubated for 30 min in darkness with 10 μL SYTOX (molecular probes) solution that was 100 times diluted in MilliQ. SYTOX can enter and stain the DNA of cells with compromised membranes, a non-reversible condition that characterizes non-viable cells. SYTOX emits green fluorescence after excitation at 488 nm. The presence of cellular green fluorescence of SYTOX was assessed flow cytometrically. For each data point at least 30×10^3 individual cells were analyzed.

2.9. Statistics

Differences between groups of replicates were tested for significance with a two factor (iron-replete, iron-limited, and constant, dynamic irradiance) ANOVA and a LSD post hoc test.

3. Results

3.1. Growth

Mean growth rates of iron-replete cultures (followed for 15 days) were significantly higher (~60%) than those of iron-limited cultures (followed for 35 days) ($p = 0.0001$, Fig. 2A). On the last day of the experiment all cultures were still in exponential growth. There was no significant difference in growth between static and dynamic irradiance under iron-limited and iron-replete conditions.

3.2. F_v/F_m during dynamic irradiance

On the last day of the experiment, F_v/F_m was significantly higher for iron-replete than for iron-limited cells. Moreover, iron-replete cells showed a stronger response in F_v/F_m under dynamic irradiance than iron-limited cells (Fig. 1). For iron-limited cells, changes in F_v/F_m were minimal during the irradiance dynamics.

3.3. Pigment composition

Significant differences in pigment composition were found between iron-limited and iron-replete, and between dynamic and constant irradiance conditions. Total pigments (pg per cell) were 3-fold higher under iron-replete than iron-limited conditions for dynamic irradiance, whereas there was a 2-fold difference between

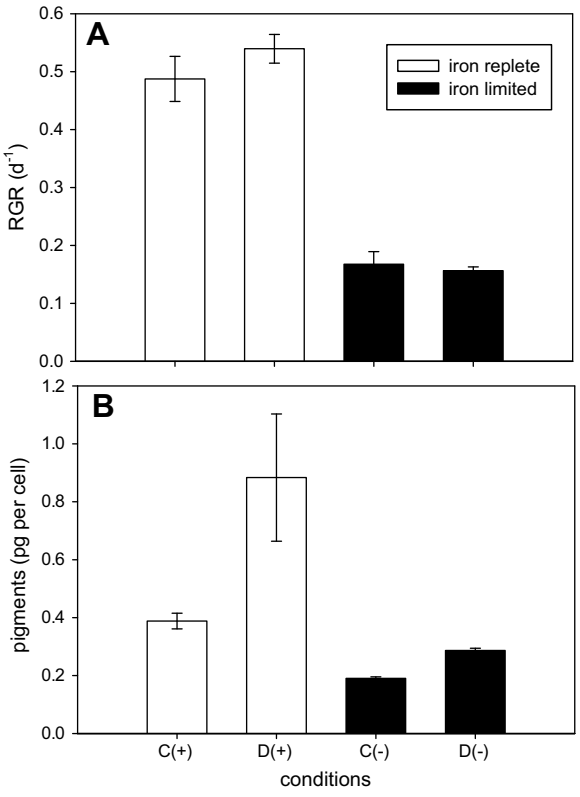

Fig. 2. Relative growth rates (A) and cellular pigment concentrations (B) of iron-limited (−) and replete (+) *Chaetoceros brevis* grown under dynamic (*D*) and constant irradiance (*C*). Mean and standard deviations are shown for two replicates.

iron-replete and iron-limited conditions under constant irradiance (Fig. 2B). Cellular light harvesting (fucoxanthin and chlorophyll *a*) pigment contents were significantly higher for dynamic irradiance than for constant irradiance ($p = 0.01$, Table 1). Cellular concentrations of both pigments were significantly lower during iron limitation ($p = 0.005$). There was also a significant interaction between irradiance and iron availability for fucoxanthin ($p = 0.036$) and chlorophyll *a* ($p = 0.037$), showing different responses of the main light harvesting pigments under dynamic and constant irradiance during iron-replete and limited conditions. The cellular pool of protective pigments (diadinoxanthin and diatoxanthin) was significantly different for irradiance and iron conditions. Iron-replete cells contained more protective pigments than iron-limited cells, whereas cells grown under dynamic irradiance possessed a higher pool than those grown under constant irradiance. For the protective pigments there was no interaction between irradiance and iron conditions. The ratio of protective relative to light harvesting pigments was significantly higher for constant irradiance grown cells than for dynamic irradiance grown cells ($p = 0.0001$), and was significantly higher for iron-limited cells compared to iron-replete cells. There was no interaction between irradiance and iron conditions for this ratio.

3.4. Antioxidant capacity

Significant differences in SOD and APX activities were not found between dynamic and constant irradiance grown cells under iron-replete conditions (Fig. 3). During iron limitation SOD and APX activity was increased 3-fold for cultures exposed to dynamic irradiance, but lower for those exposed to constant irradiance, compared to iron-replete activity levels. APX activity followed a similar pattern as SOD activity, and the activity of both enzymes was significantly correlated (R^2: 0.88, $n = 8$, results not shown). Under iron limitation, APX activity was 2-fold enhanced under dynamic irradiance, but 10-fold reduced under constant irradiance. Significant differences in glutathione reductase were not found between any of the tested conditions (results not shown). The mean specific activity of glutathione reductase was 2.4 (±0.4) U per mg protein for eight replicates.

3.5. Excessive irradiance sensitivity: F_v/F_m

Absolute F_v/F_m values were lower for the water PAM compared to those obtained with the PSI fluorometer, but the relative differences between the conditions were similar. As expected, there was a strong effect of iron on F_v/F_m: the highest F_v/F_m was found for iron-replete cells grown under dynamic irradiance. F_v/F_m was lower for iron-limited cells, whereas significant differences between constant and dynamic irradiance cultivation were not observed (Fig. 4). After 20 min SSI treatment F_v/F_m was significantly reduced to 23 and 58% of the pre-exposure values for iron-limited and replete conditions, respectively. Nearly complete recovery was found after 60 min in low irradiance for all cultivation conditions.

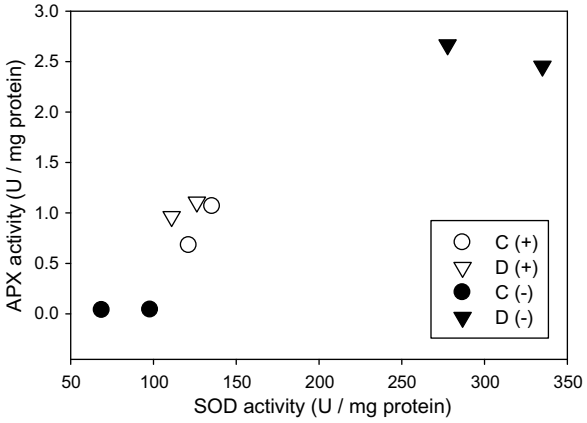

Fig. 3. Activities of the antioxidant enzymes ascorbate peroxidase (APX) and superoxide dismutase (SOD) for *Chaetoceros brevis* grown under dynamic (*D*) and constant irradiance (*C*) under iron-limited (−) and replete (+) conditions. The activities were determined for two replicates.

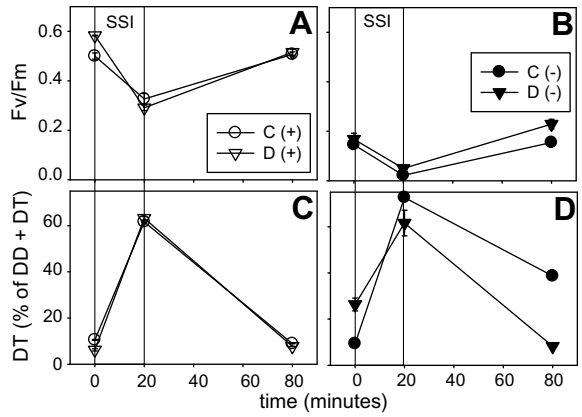

Fig. 4. Dynamics of F_v/F_m (A, B) and the concentration of diatoxanthin relative to the diadino + diatoxanthin pool (C, D) during 20 min excessive irradiance exposure (SSI) and 60 min recovery in low irradiance for iron-replete (A, C) and limited (B, D) *Chaetoceros brevis* grown under dynamic (*D*) and constant irradiance (*C*). Mean and standard deviations are shown for two replicates.

3.6. Excessive irradiance sensitivity: pigment composition

Apart from the de-epoxidation state of diadinoxanthin there were no significant changes in pigment composition after 20 min SSI and 60 min recovery in low irradiance (not shown). Before the SSI treatment 8–24% of the protective pigments was in the form of diatoxanthin (Fig. 4). Directly after SSI this was 64–74%, whereas this was reduced to 8% after 60 min recovery in low irradiance, except for iron-limited cells that were grown in constant irradiance (39%).

Table 1
Cellular concentrations of chlorophyll *a*, fucoxanthin, and the pool of diadinoxanthin and diatoxanthin of iron-limited (−) and replete (+) *Chaetoceros brevis* grown under dynamic (*D*) and constant irradiance (*C*)

	C (+)	*D* (+)	*C* (−)	*D* (−)
Chlorophyll *a*	0.130 (0.007)	0.353 (0.068)	0.048 (0.002)	0.083 (0.003)
Fucoxanthin	0.070 (0.003)	0.198 (0.037)	0.027 (0.001)	0.053 (0.004)
Diadino + diatoxanthin	0.093 (0.015)	0.155 (0.038)	0.058 (0.008)	0.076 (0.010)
Ratio (P/LH)	0.470 (0.079)	0.285 (0.045)	0.783 (0.139)	0.565 (0.089)

The ratio between protective (diadinoxanthin, diatoxanthin) and light harvesting (chlorophyll *a*, fucoxanthin) is also shown (P/LH). Mean and standard deviations are shown for two replicates.

3.7. Excessive irradiance sensitivity: viability loss

The number of non-viable cells was low in iron-replete and iron-limited cultures before SSI exposure, with no difference between dynamic and static irradiance cultivation (mean 2.5 ± 0.5% of cells non-viable, results not shown). The proportion of non-viable cells did not change significantly during 4 h SSI exposure (4 h SSI: mean 2.5 ± 0.9% of cells non-viable).

4. Discussion

Chaetoceros brevis is a common open ocean species from the Southern Ocean with a low iron requirement due to its small size (5 μm) [18]. Cultivation of this species under iron free conditions revealed some well described effects of iron limitation: a reduction in cellular pigments, a reduced ability to profit from irradiance, and highly reduced growth rates. During our experiment, we simulated vertical mixing by fluctuations between high (1350 μmol photons m^{-2} s^{-1}) and low irradiance (20 μmol photons m^{-2} s^{-1}) and compared growth and physiology of *C. brevis* with that under saturating, non fluctuating irradiance (260 μmol photons m^{-2} s^{-1}). Obviously, irradiance patterns that algae experience in the water column are more complex than those during our treatments. Despite the large range in irradiance, no differences in growth rates were observed between the constant and dynamic treatments under iron-limited and replete conditions. Nevertheless, significant physiological differences were found in pigment composition, F_v/F_m, and antioxidant activity. This showed that the algae had photoacclimated efficiently to both conditions, thereby mitigating potential effects of irradiance limitation and photo inhibition. Furthermore, this agrees with recent findings for iron-limited diatom assemblages from the North Pacific that displayed clear physiological responses after iron addition, whereas changes in productivity were not observed [19].

The temporal irradiance fluctuations experienced during the dynamic irradiance treatment increased cellular pigment content more than 3-fold compared to constant irradiance, under iron-replete conditions. This is consistent with previous results for *Thalassiosira weissflogii* and *Emiliania huxleyi* grown under the same conditions [4], showing that dynamic irradiance acclimated algae displayed more low irradiance acclimation characteristics than those acclimated to constant irradiance of equal daily dose. Furthermore, these results imply that vertical mixing increases cellular iron demand. Moreover it suggests that algae have a higher iron requirement under natural irradiance conditions (dynamics caused by diurnal cycle, position in the water column and weather) than under the constant irradiance conditions normally used in the lab. As previously demonstrated, reduced light availability increased the cellular demand of iron rich components [5,20,21].

Differences in cellular pigment content and F_v/F_m between dynamic and constant irradiance were significantly smaller under iron limitation, suggesting a reduced acclimation potential to prevailing irradiance conditions. However, the absence of effects on growth of iron-limited cells during dynamic and constant irradiance indicated that other phenomena compensated for this reduction in light harvesting. For example, the disproportionate down-scaling of light harvesting capacity during dynamic irradiance and iron limitation could be compensated by reduced self-shading of pigments, thereby increasing the light harvesting efficiency of the remaining pigments. Therefore, the interaction between iron and irradiance treatments could have originated from reduced self shading as was also suggested by Geider et al. [7]. Furthermore, relatively high, saturating irradiance was applied in both treatments, whereas vertical mixing can reduce the irradiance that algae experience, and impose light limitation when algae are mixed below the euphotic zone. Possibly, differences between dynamic and constant irradiance would be more pronounced during iron limitation and light limitation.

Previously, it was suggested that iron limitation enhanced excessive irradiance effects such as photo inhibition [7,6,22]. However, our research suggests that iron-limited *C. brevis* was not more vulnerable to excessive irradiance exposure than under iron-replete conditions. Nevertheless, the activities of the antioxidant enzymes SOD and APX were markedly enhanced for iron-limited cells grown under dynamic irradiance. The increased activities of both enzymes are indicative of enhanced superoxide formation during dynamic irradiance. We propose that increased cellular antioxidant activity is triggered by the periodic over-reduction of the photosynthetic electron transport chain during fluctuations in PSII excitation. Evidence for elevated ROS during iron limitation was reported previously for diatoms [11]. Elevated ROS may be due to impairment of the PSI and cytochrome b_6–f complexes, which are particularly iron rich and their relative abundance can be reduced in response to iron limitation [6]. An imbalance between PSII and cytochrome b_6–f complexes could reduce the efficiency of the PSII electron cycle and therefore mediate increased over-reduction of PSII [23]. This scenario leads to enhanced superoxide radical formation, which requires increased scavenging by SOD and APX. In contrast to dynamic irradiance, a significant decrease in APX and SOD activities was found during iron limitation and constant irradiance. We hypothesize that the absence of irradiance fluctuations reduced PSII excitation pressure and therefore did not trigger elevated antioxidant activity. Because no growth reduction was found between dynamic and constant irradiance during iron limitation, it appeared that elevated antioxidant activity compensated the increased superoxide formation during the former condition. Elevated antioxidant activity can increase cellular iron demand because peroxidases like APX and superoxide dismutase (SOD) can contain iron cofactors. However, the iron cofactor in the latter enzyme can be substituted by manganese (Mn) under iron limitation in *T. weissflogii* [11]. In our research SOD and APX activities were normalized to the protein content from the extracts. Normalization to chlorophyll *a* has been reported by Sunda et al. [24], which would accentuate SOD and APX activity differences due to the large iron limitation mediated reduction in cellular chlorophyll *a*.

Iron-limited cells have a highly reduced light harvesting capacity, but this is accompanied by a much smaller reduction in the protective xanthophyll pigments pool. As a result, the ratio of protective relative to light harvesting pigments was higher during iron limitation of dynamic and constant irradiance grown cells. Previous experiments with low irradiance cultivated *C. brevis* showed pronounced viability loss during excessive irradiance. However, these cells had a 6-fold lower ratio of protective relative to light harvesting pigments than the cultures with the lowest ratio from the current experiment (iron-replete cells grown in dynamic irradiance) and a 16-fold difference with the iron-limited cells that were grown under constant irradiance. Consequently, *C. brevis* was relatively insensitive to the harmful effects of excessive irradiance under all of the current cultivation conditions, as shown by fast F_v/F_m recovery and the lack of viability loss after excessive irradiance.

Although the dynamic irradiance treatment covered almost the entire irradiance range that algae experience in the water column, F_v/F_m of iron-limited *C. brevis* showed surprisingly little response during the irradiance fluctuations. In contrast, F_v/F_m was strongly regulated under iron-replete conditions during the dynamic irradiance regime, which coincided with xanthophyll (de-) epoxidation cycles (results not shown). The low F_v/F_m under iron limitation at the start of the excessive irradiance exposure experiments coincided with low xanthophyll de-epoxidation. Therefore, low F_v/F_m

values during iron limitation did not appear to be the result of xanthophyll cycle activity. The excessive irradiance exposure data showed no clear differences in xanthophyll cycle dynamics for iron-limited and replete cells after and during recovery from excessive irradiance. Furthermore, F_v/F_m recovery after 20 min simulated surface irradiance was not clearly different for iron-replete and limited cells. Previous research on *P. antarctica* reported reduced xanthophyll cycle activity under iron limitation during a dynamic irradiance treatment [9]. Presumably, the proton gradient over the thylakoid membrane develops slower in iron-limited cells, thereby suppressing xanthophyll de-epoxidation. However, during our experiments we found no evidence for this and this issue should be verified in future research. The similar PSII response of iron-limited and replete cells and the lack of viability loss during excessive irradiance indicated that xanthophyll de-epoxidation could play a role in photo protection, regardless of iron availability.

In summary, PSII dynamics were reduced and SOD and APX activities were enhanced when iron-limited cells were grown under dynamic irradiance, in comparison to iron-replete cells. However, growth rates of iron-limited and replete *C. brevis* were not different under dynamic irradiance compared to saturating, non-fluctuating irradiance. This indicates that the increased antioxidant activity compensated for decreased non photochemical quenching efficiency around PSII. Furthermore, xanthophyll cycle activity was still induced in iron-limited cells under excessive irradiance. Thus, the observed physiological changes secured maintenance of growth during dynamic irradiance and photo protection against excessive irradiance.

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