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# Effects of iron limitation on photosynthesis and carbohydrate metabolism in the Antarctic diatom *Chaetoceros brevis* (Bacillariophyceae)

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Iron, one of the structural elements of organic components that play an essential role in photosynthesis and nitrogen assimilation of plants, is available at extremely low concentrations in large parts of the Southern Ocean's surface waters. We tested the hypothesis that photosynthesis is the primary target of iron stress in phytoplankton living in this specific environment, resulting in a reduced carbohydrate production. Cultures of a small Antarctic diatom, *Chaetoceros brevis*, were exposed to two different photon irradiances under iron-rich and iron-poor conditions. Under both light regimes growth rate was reduced only slightly by iron starvation, as expected because the iron requirement of a small-celled species such as *C. brevis* is low. Even so, iron-starved cells differed markedly from iron-replete cells: for low and high irradiance, respectively, they had a 20 and 27% lower content of light-harvesting pigments (chlorophyll *a* and $c_2$ and fucoxanthin), a 8 and 15% decrease in light absorption and a 15 and 17% decrease in quantum yield of photosystem II. The diurnal production of water-extractable carbohydrates was reduced by 28 and 31%, which resulted in a low supply of energy and carbon skeletons from these storage products. This may well have influenced protein synthesis. The nocturnal consumption of carbohydrates was also reduced, which, together with the almost proportional decrease in cellular C and N content, suggests that the C and N metabolism were tightly tuned in iron-stressed cultures. The decrease in C and N content correlated with a decrease in cell volume. Our results indicate that iron limitation is likely to affect the ability of phytoplankton to maintain high rates of protein synthesis within the deep wind-mixed layer of the Southern Ocean. In addition, growth at the surface could be inhibited by too much light: iron-poor cultures of *C. brevis* grown at low irradiance showed enhanced sensitivity to photoinhibition.

**Key words**: carbohydrates, diatoms, iron, photosynthesis, phytoplankton, pigments, Southern Ocean

## Introduction

Recent *in situ* iron fertilization experiments (Boyd *et al*., 2000; Dalton, 2002; Gervais *et al*., 2002) have confirmed the evidence obtained earlier that phytoplankton growth in the Southern Ocean is limited by iron (De Baar & Boyd, 2000). Iron plays a crucial role in processes in plants that require electron transfer reactions, including photosynthesis and nitrogen assimilation. One of the most noticeable effects of iron deficiency on photosynthesis is a decrease in cellular content of chlorophyll (chl) *a* and other pigments involved in light harvesting (Glover, 1977; Doucette & Harrison, 1990; Greene *et al*., 1991; Van Leeuwe & Stefels, 1998). Iron limitation also reduces the synthesis of proteins of the photosynthetic apparatus, like the

D1-protein in photosystem II (PSII) (Greene *et al*., 1992; Vassiliev *et al*., 1995). In addition, the functioning of the Photosynthetic Electron Transport (PET) chain is affected by a decrease in iron-sulphur complexes, e.g. ferredoxin (Erdner *et al*., 1999; McKay *et al*., 1999). As a consequence, photosynthetic efficiency is reduced in iron-limited cells (Geider *et al*., 1993; Davey & Geider, 2001).

Iron is also a structural component of the metallo-enzymes that are involved in nitrate uptake, nitrite and nitrate reductase (Gao *et al*., 1993). The activity of nitrate reductase is low in iron-deficient phytoplankton (Timmermans *et al*., 1994, 1998). If this impairs the ability to metabolize nitrate, it could lead to high cellular carbohydrate : protein and carbon : nitrogen (C : N) ratios due to a decrease in cellular nitrogen content and the accumulation of glucan. These are well-known features of nitrogen-limited phytoplankton cells

(Myklestad, 1988). So far, however, no increase in C : N ratios in iron-deplete phytoplankton has been observed (Greene *et al.*, 1991; Maldonado & Price, 1996; Davey & Geider, 2001). In fact, Rueter and Ades (1987) had already suggested that nitrogen assimilation of iron-deplete cells of the freshwater alga *Scenedesmus quadricauda* (Chlorophyceae) was not as strongly affected as photosynthesis, because the cells incorporated a large fraction of total carbon into proteins, as measured by ^{14}C fractionation.

The effect of iron limitation on C metabolism or N metabolism could be light dependent. In light-limited cells, more iron is needed to enlarge the photosynthetic apparatus in order to increase light absorption (Raven, 1990; Strzepek & Price, 2000). Stefels and Van Leeuwe (1998) also found indications based on dimethylsulphoniopropionate (DMSP) measurements that cells of *Phaeocystis* sp. were energy-limited under low light, low iron conditions, whereas cells were bordering on nitrogen deficiency induced by iron limitation under high light, low iron conditions.

Here we elaborate further on the 'photosynthesis = primary target' – hypothesis by studying carbohydrate metabolism. Carbohydrates are the first products of the Calvin-Benson cycle, the activity of which is dependent on ATP and NADPH generated by photosynthesis. Under saturating light conditions, a significant part of the carbon fixed by phytoplankton is stored as polysaccharides (Vårum *et al.*, 1986). For diatoms the main storage carbohydrate is β-1,3-glucan, which is easily extracted in water (Myklestad, 1988). This glucan can facilitate continued protein synthesis in darkness by the provision of energy and carbon skeletons (Cuhel *et al.*, 1984; Granum & Myklestad, 2001). We investigated the hypothesis that photosynthesis is the primary target of iron limitation, which would lead to a reduction in the diurnal water-extractable carbohydrate production. We studied this at two different irradiances in laboratory experiments with the Antarctic diatom *Chaetoceros brevis*.

## Materials and methods

### Cell culture

The single celled Antarctic diatom *Chaetoceros brevis* (no. CCMP 163, diameter approx. 4 $\mu$m) was cultured in a 8 h : 16 h light : dark regime in filtered (0.2 $\mu$m) natural seawater medium. The iron concentration was 15 nM (measured by organic ligand solvent extraction followed by atomic absorption spectrophotometry: Nolting and De Jong, 1994); 140 $\mu$M NaNO$_3$, 7.5 $\mu$M K$_2$HPO$_4$ and 60 $\mu$M Na$_2$SiO$_3$ were added. The medium was supplemented with vitamins and trace metals (excluding iron) as described by Admiraal and Werner (1983). Except for

silicate, all major nutrients were cleaned from iron contamination with a chelex column (Aquil). Low iron availability was further achieved by adding 1 mM Na$_2$-EDTA.

### Experimental set-up

Three cultures were maintained separately for several months at 4°C, with regular transfer of the cells into fresh medium. Several weeks before the start of the experiment, subsets of the cultures were placed at two different photon irradiances (light source TL-Biolux, Philips), 25 and 100 $\mu$mol m$^{-2}$s$^{-1}$. Each of these six cultures was used to inoculate two 3-l polycarbonate Fernbach bottles with 1.5 l medium, which were placed at the pre-adapted irradiance. The cultures were covered with parafilm and were gently shaken several times a day to facilitate gas exchange between the medium and the air. After 1 week, 1 $\mu$M iron (FeCl$_3$) was added to one of these bottles (picked at random). At the moment of iron addition (t = 0), cell density in the cultures was 6 – 7 × 10$^7$ l$^{-1}$.

At $t$ = 0, 3 and 5 days, samples were taken for cell volume, cell number and polysaccharide concentration, at the beginning and at the end of each light period. In the morning, *in vivo* chl *a* fluorescence parameters (see below) were measured. At the end of the light period, samples were taken for pigment analysis and POC/PON analysis (also described below). At the end of the experiment, samples were taken for *in vivo* light absorption measurements.

### Cell counts

Cell numbers and cell volume were determined with an automated particle counter (Coulter, model ZM). The count tube (diameter 30 $\mu$m) was calibrated with polystyrene particles (Coulter no. 9966067, diameter 8.7 $\mu$m). Samples were diluted with artificial seawater to a cell concentration of 20 – 60 cells per $\mu$l; 200 $\mu$l was counted per sample. Growth rates were calculated by fitting the model for exponential growth:

$$N_t = N_0 e^{\mu t}$$

where N is cell number at different times, t is time in days and $\mu$ is growth rate (in d$^{-1}$). The regression coefficient (r$^2$) of the fitted curves varied between 0.9910 and 0.9999 (n = 9).

### Pigment analysis

Samples of 20 – 100 ml were filtered through 25-mm GF/ F Whatman filters under gentle vacuum pressure ( < 200 mm Hg). The filters were frozen in liquid nitrogen immediately after filtration and stored at − 80°C. Later, filters were freeze-dried and extracted in 90% acetone for 48 h. Pigments were separated and quantified by HPLC according to Kraay *et al.* (1992). The HPLC system consisted of a Waters 2690 Separations Module and a Waters 996 Photodiode Array Detector (Milford, MA, USA). A Waters DeltaPak (Milford, MA, USA) reversed-phase column (C$_{18}$, 5 $\mu$m, 100 Å, 150 × 3.9 mm, fully end capped) was used. Pigment standards were obtained from DHI Water &

Environment (Hørholm, Denmark). For $\beta$-carotene no standard was available; the conversion factor applied was taken from zeaxanthin because both pigments have similar absorption characteristics (Jeffrey *et al.*, 1997).

## In vivo *absorption spectra measurements*

The *in vivo* spectral absorption cross section ($a*_\lambda$, in $m^2$ $mg^{-1}$ chl *a* or in $m^2$ $cell^{-1}$, $\lambda = 350 - 700$ nm) was determined on cell suspensions in a Varian-Cary 3E double beam spectrophotometer equipped with an integrating sphere. Spectra were corrected for background noise. The spectrally averaged absorption coefficient for photosynthetically available radiation (PAR) normalized to chl *a*, $a*_{PAR}$ (in $m^2$ $mg^{-1}$ chl *a*), was calculated from the convolution of $a*_\lambda$ ($400 - 700$ nm) with the emission spectrum for the lamps used in our experiments (Dubinsky *et al.*, 1986).

## Chlorophyll a *fluorescence measurements*

*In vivo* chl *a* fluorescence was measured with a pulse-amplitude modulated fluorometer (PAM 2000, Walz, Effeltrich, Germany). Between 10 and 100 ml of culture was filtered through a 10-mm GF/F filter at a vacuum pressure less than 300 mm Hg. The filtration was stopped before the filter dried up to prevent cells from being damaged. The filter was quickly transferred to a custom-made cuvette filled with seawater (cooled to 4°C) and the fibre optic probe was inserted into the cuvette. After application of a 5-s far-red pulse, the algae were darkened for 5 min, before a second far-red pulse was applied. Then the minimal fluorescence ($F_0$) and the maximal fluorescence ($F_m$) were measured to determine the maximum quantum yield of photosystem II (PSII) (Schreiber *et al.*, 1994): $F_v:F_m = (F_m - F_0)/F_m$, where $F_v$ is the variable fluorescence.

## Photosynthesis-irradiance response curves

After another far-red pulse, the algae on the filter were exposed to 10 irradiances up to 370 $\mu mol$ $m^{-2}$ $s^{-1}$. The actinic light was provided by the red LED (650 nm) of the fluorometer. The effective quantum yield of PSII ($\Phi PSII$) was determined each time after 30 s light exposure. The relative PSII electron transport rate (rETR) at each irradiance was calculated as: $rETR = \Phi PSII \cdot irradiance$ (Genty *et al.*, 1989).

The photosynthesis-irradiance response curve (as rETR vs irradiance) was fitted following recommendations of Frenette *et al.* (1993). If no inhibition was observed, the model of Webb *et al.* (1974) was used:

$$rETR = rETR_{max}\left(1 - e^{-\alpha\frac{E}{rETR_{max}}}\right)$$

where $rETR_{max}$ = maximum relative electron transport rate under optimal light, $\alpha$ = initial slope, $\beta$ = photoinhibition constant, E = irradiance. If a curve showed inhibition, the exponential function of Platt *et al.* (1980) was applied:

$$rETR = rETR_s = \left(1 - e^{-\alpha\frac{E}{rETR_s}}\right)\left(e^{-\beta\frac{E}{rETR_s}}\right)$$

where $rETR_s$ = maximum, potential relative electron transport rate in the absence of photoinhibition under optimal light. In this case, $rETR_{max}$ was calculated as follows:

$$rETR_{max} = rETR_s\left(\frac{\alpha}{\alpha + \beta}\right)\left(\frac{\beta}{\alpha + \beta}\right)^{\frac{\beta}{\alpha}}$$

The light saturation parameter $E_k$ was calculated as $rETR_{max}/\alpha$.

## POC and PON analysis

Samples of $20 - 100$ ml were filtered through pre-combusted (350°C) 10-mm GF/F Whatman filters under gentle vacuum pressure ($< 200$ mm Hg). The filters were stored at $-80°C$ until analysis. The filters were analysed for particulate carbon (POC) and nitrogen (PON) on a CHNS-analyser type EA 1110 (Interscience, Breda, The Netherlands).

## Carbohydrate analysis

Samples of $20 - 100$ ml were filtered through pre-combusted (350°C) 25-mm GF/F Whatman filters under gentle vacuum pressure ($< 200$ mm Hg). The filters were stored at $-20°C$ until analysis. They were extracted in water (Milli-Q) in sealed glass test tubes placed in a water bath for 1 h at 80°C. Afterwards, the tubes were cooled and centrifuged for 5 min at 715 *g*. The polysaccharide concentration was determined with the colorimetric TPTZ-method described by Myklestad *et al.* (1997), with minor modifications. D( + )-Glucose was used as the standard.

## Statistics

Differences between treatments were tested with multi-factor analyses of variance (ANOVA), following the procedure described by Underwood (1997). Factors included were iron, light and, where applicable, the number of days after iron addition and the time of day (beginning/end light period). To examine the strength of a relationship between two variables, Pearson correlation coefficients were calculated.

## Results

### Growth rate and cell volume

Iron and light availability both had a significant effect on growth rate (Table 1). Maximum growth rates were measured for cells cultured at high irradiance and high iron conditions. The combination of low iron and low irradiance resulted in the lowest growth rates. Growth rates of iron-enriched cultures were approximately 6% higher than those of iron-limited cultures, both at low and high irradiance. At the end of the experiment, iron-limited cells were $12 - 14\%$ smaller than iron-replete cells (Table 1).

### Pigment content

As expected for a diatom, cells contained chl *a* and *c₂*, fucoxanthin, diadinoxanthin, diatoxanthin and *β*-carotene. Diatoxanthin was detected only in *C. brevis* cells exposed to high irradiance. Cells exposed to a low irradiance had higher total pigment content than those exposed to a high irradiance, under both iron-rich and iron-poor conditions (Table 2). Highest diadinoxanthin contents were recorded for iron-replete cells exposed to a high irradiance (0.08 pg cell$^{-1}$). A reduction in cellular content of all pigments was observed as a result of iron limitation. There was a significant interaction between the effect of iron and light for the cellular content of all pigments except *β*-carotene (Table 2). At the end of the experiment, the chl *a* content of the iron-stressed cells was reduced by approximately 16% at high irradiance and 27% at low irradiance. There was a significant interaction between the factors iron and light for the ratios of chl $a : C$ and chl $a : N$; these ratios were reduced only by iron limitation at low irradiance (Table 1). There was a small but significant effect of

**Table 1.** Physiological characteristics of the Antarctic diatom *Chaetoceros brevis* grown in batch cultures under high and low light with and without iron addition

|  | Fe+ LL | Fe- LL | Fe+ HL | Fe- HL | ANOVA Iron $p$ | Light $p$ | Iron/light $p$ |
|---|---|---|---|---|---|---|---|
| $\mu$ (d$^{-1}$) | 0.34 | 0.32 | 0.41 | 0.39 | 0.005 | < 0.001 | 0.679 |
| Cell volume ($\mu m^3$) | 51.2 | 44.0 | 48.7 | 43.0 | < 0.001 | 0.089 | 0.441 |
| a*$_{PAR}$ (m$^2$ mg chl $a^{-1}$) | 0.023 | 0.028 | 0.034 | 0.036 | 0.002 | < 0.001 | 0.050 |
| $F_v : F_m$ | 0.67 | 0.57 | 0.54 | 0.45 | 0.001 | < 0.001 | 0.882 |
| $C : N$ (g g$^{-1}$) | 6.05 | 6.28 | 6.55 | 6.82 | 0.005 | < 0.001 | 0.762 |
| Chl $a : C$ (g g$^{-1}$) | 0.040 | 0.034 | 0.020 | 0.020 | 0.018 | < 0.001 | 0.027 |
| Chl $a : N$ (g g$^{-1}$) | 0.21 | 0.18 | 0.11 | 0.12 | 0.088 | < 0.001 | 0.027 |
| CHO (pg C cell$^{-1}$ h$^{-1}$) | 0.18 | 0.13 | 0.26 | 0.18 | 0.007 | 0.010 | 0.449 |

Fe + : iron-replete; Fe-: iron-deplete; LL: 25 $\mu$mol photons m$^{-2}$ s$^{-1}$; HL: 100 $\mu$mol photons m$^{-2}$ s$^{-1}$. CHO: net diurnal water-extractable polysaccharide production rate. All other abbreviations are explained in the text. Except for growth rate, measurements are made at the end of the experiment (t = 5). Values represent the mean of triplicate cultures. The significance of the factors iron and light, and their interaction were tested with 2-way ANOVAs (see Methods).

**Table 2.** The (relative) pigment content of *Chaetoceros brevis* cells grown for 5 days in batch cultures under high and low light with and without iron

|  | Fe+ LL | Fe- LL | Fe+ HL | Fe- HL | ANOVA Iron $p$ | Light $p$ | Iron/light $p$ |
|---|---|---|---|---|---|---|---|
| **Pigment content (pg cell$^{-1}$)** | | | | | | | |
| Chlorophyll *a* | 0.50 | 0.36 | 0.27 | 0.23 | < 0.001 | < 0.001 | < 0.001 |
| Chlorophyll *c₂* | 0.10 | 0.08 | 0.06 | 0.04 | < 0.001 | < 0.001 | 0.014 |
| Fucoxanthin | 0.29 | 0.22 | 0.17 | 0.13 | < 0.001 | < 0.001 | 0.006 |
| Diadinoxanthin | 0.06 | 0.04 | 0.08 | 0.06 | < 0.001 | < 0.001 | 0.020 |
| Diatoxanthin | 0* | 0* | 0.0021 | 0.0013 | — | — | — |
| *β*-carotene | 0.0108 | 0.0058 | 0.0087 | 0.0044 | < 0.001 | 0.002 | 0.329 |
| **Pigment ratios (chlorophyll a$^{-1}$)** | | | | | | | |
| Chlorophyll *c₂* | 0.20 | 0.21 | 0.21 | 0.19 | < 0.001 | < 0.001 | < 0.001 |
| Fucoxanthin | 0.59 | 0.61 | 0.63 | 0.58 | 0.010 | 0.077 | < 0.001 |
| Diadinoxanthin | 0.12 | 0.12 | 0.31 | 0.26 | 0.001 | < 0.001 | 0.001 |
| Diatoxanthin | 0 | 0 | 0.0077 | 0.0056 | — | — | — |
| *β*-carotene | 0.022 | 0.016 | 0.032 | 0.019 | < 0.001 | < 0.001 | 0.007 |

For explanation of abbreviations see Table 1. Values represent the mean of triplicate cultures. The significance of the factors iron and light and their interaction were tested with 2-way ANOVAs (see Methods). *Below detection limit.

iron and light on the chl $c_2$ to chl $a$ ratio and the fucoxanthin to chl $a$ ratio (Table 2). The diadinoxanthin to chl $a$ ratio and the $\beta$-carotene to chl $a$ ratio were both higher at high than at low irradiance. For both ratios, there was a significant interaction between iron and light: they were reduced more by iron limitation at high irradiance. The ratio of diatoxanthin to diadinoxanthin was higher at high (0.023) than at low irradiance (0) but was not influenced by iron deficiency.

### In vivo *absorption spectra*

At the end of the experiment, cell-specific absorption was highest for cells exposed to low irradiances (Fig. 1A). Cellular absorption capacity was higher for iron-replete than iron-deficient cells, at both low and high irradiance (8 and 15%, respectively). The difference in absorption was most pronounced at 430 and 675 nm. The light absorption per unit chl $a$ was highest for cells cultured at high irradiance (Fig. 1B). The absorption coefficient ($a*_{PAR}$) was significantly affected by iron and light limitation and varied between 0.02 and 0.04 $m^2$ mg chl $a^{-1}$ (Table 1). There was a significant interaction between iron and light: $a*_{PAR}$ increased more in response to iron limitation at low irradiance than at high irradiance.

### *Chlorophyll* a *fluorescence*

There was a significant effect of iron concentration and irradiance on the maximal quantum yield of photosystem II ($F_v : F_m$) (Table 1). At the end of the experiment, the ratio $F_v : F_m$ was higher for cells exposed to a low irradiance than for those receiving a high irradiance. $F_v : F_m$ was lower for iron-deficient cultures than for iron-replete cultures, at both low and high irradiance (15 and 17%, respectively).

### *Photosynthesis-irradiance response curves*

PE-curves measured at t = 0, 3 and 5 days after iron addition are presented in Fig. 2. At t = 0, the maximum relative rate of photosynthesis ($rETR_{max}$) was highest for cells pre-cultured at high irradiance. Differences between the PE-curves of the iron-replete and iron-deplete treatments gradually increased over the course of the experiment (Fig. 2A – C). At t = 5, $rETR_{max}$ and the light saturation parameter $E_k$ ($rETR_{max}/\alpha$) were significantly affected by iron limitation (Table 3). There was no significant effect of iron availability on $\alpha$, but there was a significant interaction between iron and light for this parameter. The algae growing at low irradiance and low iron showed inhibition if exposed to high irradiance. There also was a significant interaction between iron and light for the photoinhibition parameter ($\beta$): an increase in $\beta$ due to iron limitation was observed only at low irradiance.

### *POC and PON*

There was a significant effect of iron concentration and irradiance on the cellular carbon content (ANOVA, iron: $p = 0.002$, light: $p < 0.001$, iron $\times$ light: $p = 0.54$). Carbon content was higher at high irradiance than at low irradiance (Fig. 3A). During the course of the experiment the carbon content in iron-deficient cells decreased relative to that of iron-replete cells at both irradiances. The cellular nitrogen content was also significantly affected by iron and light limitation (ANOVA, iron: $p < 0.001$, light: $p < 0.001$, iron $\times$ light: $p = 0.32$), in a qualitatively similar manner (Fig. 3B). At the end of the experiment, the C : N ratio was highest in cultures grown at high irradiance (Table 1) and was slightly (4%) higher in iron-deplete cultures than in iron-replete cultures.
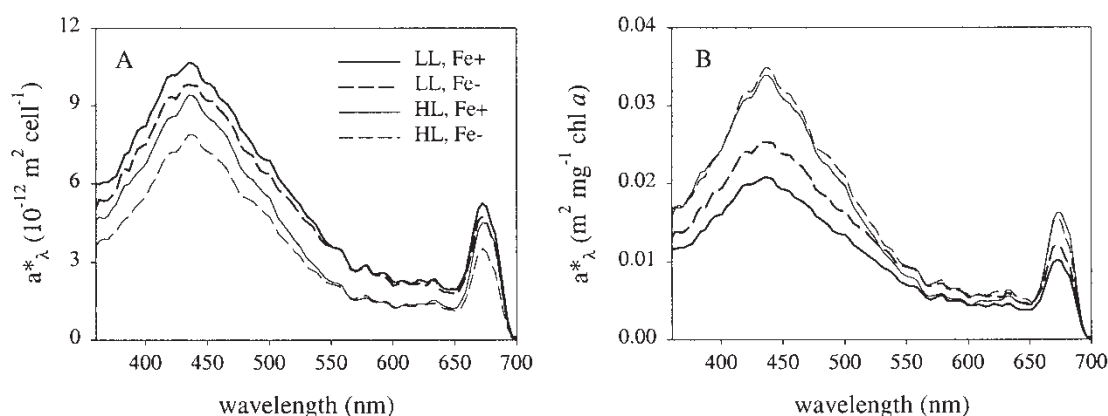


**Fig. 1.** *In vivo* absorption spectra of *Chaetoceros brevis* at the end of batch growth experiments under various conditions. (A) Absorption normalized to cell number; (B) Absorption normalized to chlorophyll $a$ concentration. For abbreviations in key see Table 1.
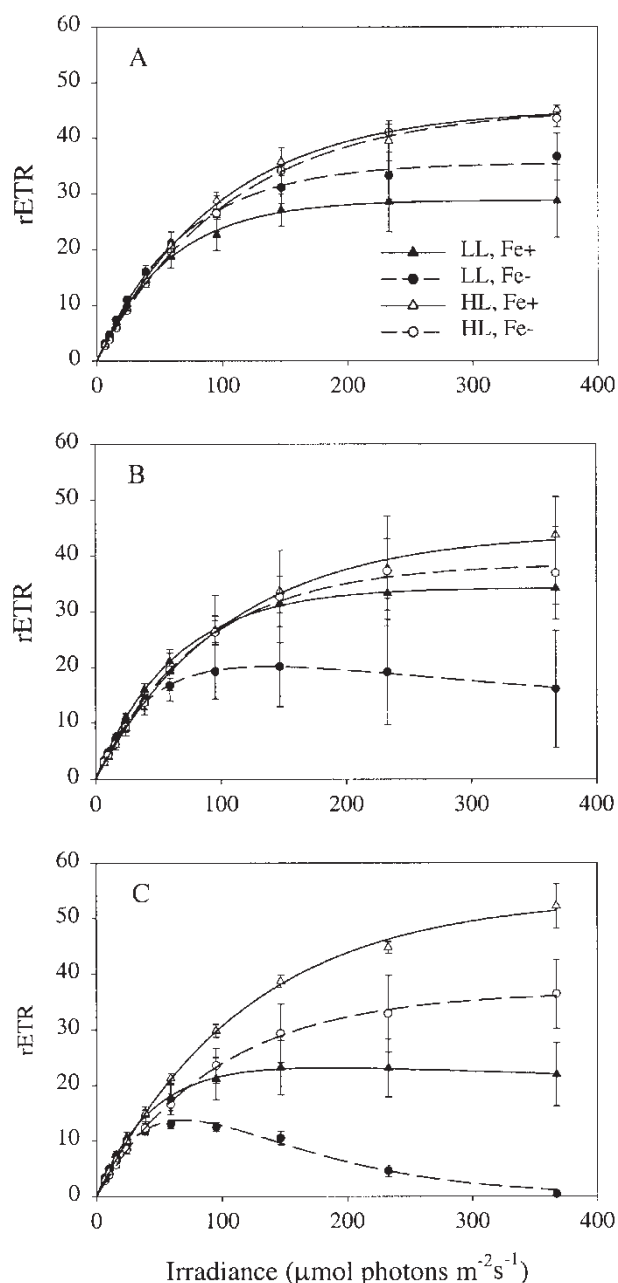
**Fig. 2.** Photosynthesis-irradiance response curves of *Chaetoceros brevis*, measured by PAM fluorometry 0 (A) 3 (B) and 5 (C) days after the start of the experiment. For abbreviations in key see Table 1.

## Polysaccharides

The net diurnal cellular production of polysaccharides was highest at high irradiance (Fig. 4). Over the course of the experiment, production was lower in iron-deplete cultures at both irradiances, but remained unchanged in iron-replete cells. After 5 days, the production was higher in iron-enriched cultures for both irradiances (Table 1). The percentage increase in polysaccharide quota during the light period was highest in iron-replete cultures: 370% at low irradiance and 330% at high irradiance. In iron-deplete cultures the increase was 220 and 290%, respectively. There was a significant effect of iron and light on the net nocturnal cellular polysaccharide consumption (ANOVA, iron: $p = 0.012$, light: $p = 0.003$, iron $\times$ light: $p = 0.13$). The decrease in the total polysaccharide concentration in the cultures during the dark period was around 70% for iron replete cells, at both irradiances. In iron-deplete cultures the decrease was around 50% (low irradiance) and 60% (high irradiance). About 20% of total carbon was polysaccharide (assuming the polysaccharides mainly consist of glucose, which contains 40% C by weight). The cellular carbon quota at the end of the day correlated with the cellular polysaccharide C quota, irrespective of the treatments (Fig. 5, linear correlation if all data are pooled: $r = 0.71$, $n = 34$). The rate of diurnal polysaccharide production correlated positively with the PE-curve parameters $rETR_{max}$ ($r = 0.75$, $n = 34$) and the rETR at the cultured irradiance ($r = 0.83$, $n = 34$).

## Discussion

### Effects of iron limitation on photosynthesis and carbohydrate metabolism

Low iron concentrations led to a decrease in the amount of light harvesting complexes in cells of *Chaetoceros brevis*, accompanied by a reduced
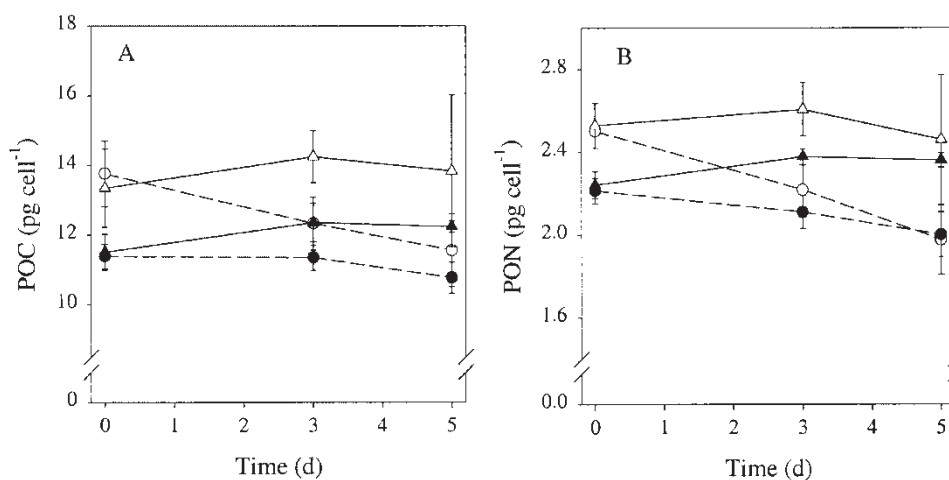
**Table 3.** Photosynthesis-irradiance curve parameters for *Chaetoceros brevis* cells grown for 5 days in batch cultures under high and low light with and without iron

|  | Fe+ LL | Fe- LL | Fe+ HL | Fe- HL | Iron $p$ | Light $p$ | Iron/light $p$ |
|---|---|---|---|---|---|---|---|
|  |  |  |  |  | ANOVA | | |
| $rETR_{max}$ | 23.5 | 13.7 | 54.3 | 36.4 | 0.002 | < 0.001 | 0.250 |
| $\alpha$ | 0.54 | 0.56 | 0.45 | 0.38 | 0.310 | < 0.001 | 0.029 |
| $\beta$ | 0.02 | 1.09 | 0.01 | 0.02 | 0.002 | 0.002 | 0.002 |
| $E_k$ ($\mu$mol photons m$^{-2}$s$^{-1}$) | 41.9 | 25.2 | 121 | 95.6 | 0.027 | < 0.001 | 0.586 |

Values represent the mean of triplicate cultures. Significance of the factors iron and light and their interaction were tested with 2-way ANOVAs (see Methods).

**Fig. 3.** Time course of the cellular carbon (A) and nitrogen content (B) of *Chaetoceros brevis* grown under various conditions. Symbols and lines as in Fig. 2.
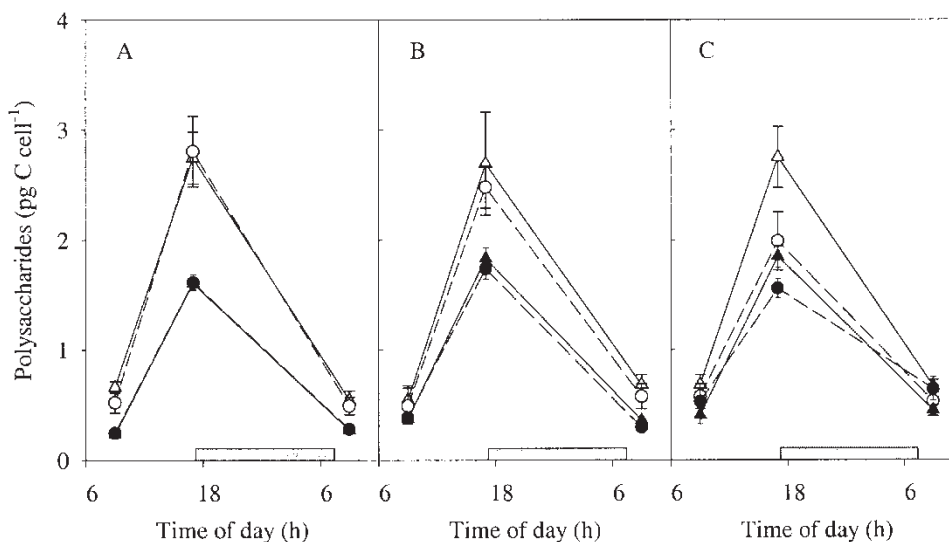


**Fig. 4.** Diel variations in water-extractable polysaccharide content of *Chaetoceros brevis* cells under various conditions after 0 (A), 3 (B) and 5 (C) days from the start of the experiment. Symbols and lines as in Fig. 2.

capacity to absorb light and a lower efficiency of the PET chain. These changes in photochemical characteristics are evidence that photosynthesis was the prime target of iron starvation, but the decline in the diurnal production of water-extractable carbohydrates is even stronger evidence for this notion. Indeed, iron-limited phytoplankton cells have physiological characteristics of cells that are energy limited, with a reduced metabolism. In contrast, cells that are nitrogen-limited tend to accumulate storage polysaccharides (Sakshaug & Holm-Hansen, 1977; Geider & La Roche, 1994). In fact, the diel change in the polysaccharide content and not the overall polysaccharide content was found to be an indicator of iron stress.

Cellular carbon and nitrogen quota both decreased in response to iron limitation. A decrease in C and N has also been observed in iron-limited

*Phaeodactylum tricornutum* (Greene *et al.*, 1991), *Thallasiosira weissflogii* (Milligan and Harrison, 2000) and *Actinocyclus* sp. (Muggli *et al.*, 1996). The decrease in carbon content can be ascribed to a reduction in the rate of carbon assimilation due to the affected photosynthesis. The reduced build-up of water-extractable polysaccharides in the iron-stressed cells at t = 5 supports this notion. Cellular nitrogen quota might be affected directly by iron limitation through changes in the activity of enzymes involved in nitrogen assimilation, or more indirectly by changes in the supply of photosynthetically derived reductant to these enzymes or by the supply of carbon skeletons. Milligan and Harrison (2000) found a reduction in the activity of nitrate and nitrite reductase in iron-stressed cells of the marine diatom *Thalassiosira weissflogii* that, however, did not limit N assimilation: the decrease in
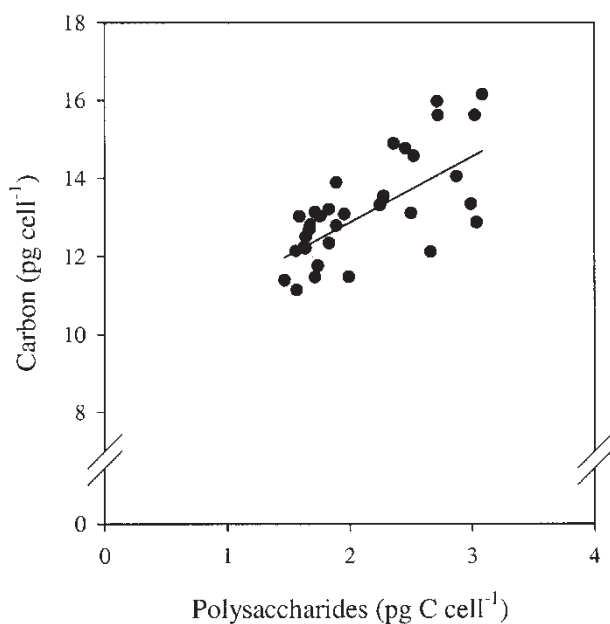
**Fig. 5.** Relationship between cellular water-extractable polysaccharide C content and total carbon content. Data for all treatments were pooled. The solid line is the principal axis (y = 1.7x + 9.2). Note break in y-axis.

cellular N quota was allegedly caused by a diminished supply of NADPH for nitrite reduction, causing excretion of nitrite. Interestingly, no significant change in the total carbohydrate pool in response to iron limitation was observed by Milligan and Harrison (2000). A difference between our study and that of Milligan and Harrison (2000) is that we focussed on the water-extractable fraction, not the total carbohydrate content. A bulk measurement includes both storage glucan and structural carbohydrates of the cell membrane. The latter are of course not available for dark protein synthesis and hardly ever show diel variability. Our data indicate that limitation of nitrogen assimilation by restricted carbohydrate supply cannot be ruled out because the diurnal production of water-extractable polysaccharides was low in iron-limited cells. The ratio of C : N increased only slightly, which, combined with the observed decrease in carbohydrate consumption during the dark period in iron-stressed cells, suggests that C and N-metabolism were tightly coupled. This may have been the result of a diminished supply of NADPH for nitrite reduction, as suggested by Milligan and Harrison (2000). Further investigations should answer this question. The reductions in cellular pigment, C and N content in iron-stressed cells are probably related to the decrease in cell size. A decrease in cell size is commonly observed in iron-stressed phytoplankton cells and is suggested to be an adaptation to low iron availability (Geider & La Roche, 1994). Interestingly, cellular pigment content decreased

more than cell volume and C and N content, especially at low irradiance, leading to a decrease in chl *a* : C and chl *a* : N. This has been observed previously by Greene *et al.* (1991).

A decrease in carbohydrate synthesis due to iron limitation has also been observed in higher plants. In citronella (*Cymbopogon winterianus*), the amount of $^{14}C$ incorporated into an operationally defined sugar fraction was found to be reduced (Srivastava *et al.*, 1998), and in sugar beet (*Beta vulgaris*) net diurnal starch accumulation rate per unit of leaf area was severely reduced upon iron stress (Arulanantham *et al.*, 1990). The starch accumulation rate correlated well with the rate of photosynthesis. All this corroborates our finding of a high correlation between the carbohydrate accumulation rate and the electron transport rate.

Growth rate was strongly reduced by light limitation but only little by iron limitation. A possible explanation for the small impact of iron limitation on growth rate is that *C. brevis* has lower iron requirements for growth than larger diatoms such as *C. dichaeta* (Timmermans *et al.*, 2001). Hence, iron availability was probably not limiting at the start of the experiment. However, iron-deplete cultures presumably ran into iron limitation by their own growth and consumption of the available iron, as indicated by an increased impairment of photosynthesis with time. Given the observed impact of iron limitation on PE-characteristics and carbohydrate production, more pronounced effects on growth can be anticipated under prolonged iron stress.

### Combined effects of light and iron

An expected enhancement of the effects of iron limitation on growth rate due to a decrease in iron-use efficiency at low light was not observed even though the cellular chl *a* content was more strongly affected by iron limitation at low irradiance than at high irradiance. This discrepancy may be explained by the stronger increase in the absorption coefficient a*$_{PAR}$ in response to iron deficiency at low irradiance, which would counteract the effects of the decrease in pigment content at this irradiance. An increase in a*$_{PAR}$ due to iron limitation has also been observed by Greene *et al.* (1991) in the diatom *Phaeodactylum tricornutum* and by Van Leeuwe and De Baar (2000) in the prasinophyte *Pyramimonas* sp.. It has been attributed to a decrease in pigment content, which reduces the 'package-effect', i.e. the shading of thylakoid membranes (Berner *et al.*, 1989).

The ratio of the accessory light harvesting pigments (chl $c_2$ and fucoxanthin) to chl *a* varied little among the different experimental treatments. The ratios of diatoxanthin + diadinoxanthin to chl

*a* and of diatoxanthin to diadinoxanthin were highest at high irradiance. Synthesis and de-epoxidation of diadinoxanthin are processes that play a photoprotective role by increasing dissipation of excitation energy in photosystem II (Olaizola *et al.*, 1994). An increase in the ratio of diatoxanthin to diadinoxanthin in response to iron limitation has been observed in a few studies, where it was presented as an adaptive response to enhanced radical formation caused by iron limitation (Geider *et al.*, 1993; Van Leeuwe & Stefels, 1998). In *C. brevis*, however, radical formation did not seem to be a major problem at the selected irradiances, as indicated by the very low cellular content of diatoxanthin. Apparently, iron-deplete cells preferentially invested in light harvesting capacity, not in photoprotection, as indicated by the decrease in the ratio of diatoxanthin + diadinoxanthin to chl *a*. Notably, *β*-carotene, another pigment that can function as a photoprotector (Middleton & Teramura, 1993), also decreased relative to chl *a* in response to iron limitation.

Iron limitation affected the maximum quantum yield of photosystem II ($F_v : F_m$) and the maximum relative electron transport rate ($rETR_{max}$) to a similar extent at both irradiances. The light utilization efficiency ($\alpha$) was not affected by iron limitation, in agreement with the findings of Greene *et al.* (1991). Van Leeuwe and De Baar (2000) and Davey and Geider (2001) found a decrease in $\alpha$ (based on $^{14}C$ measurements). Apparently, in these studies the decrease in the package effect in iron-limited phytoplankton cells did not fully compensate for the decrease in the quantum yield of photosynthesis (see Greene *et al.*, 1991). In our study, there was a significant interaction between iron and light for $\alpha$, which was probably related to the stronger decrease in the package effect at low irradiance.

In agreement with the observed reduction in photoprotective pigments, our PE curves showed a strong inhibition at high irradiance for iron-limited cells, especially if cultured at low irradiance. This suggests that these cells are sensitive to photo-inhibition if exposed to high irradiance. Inhibition has not been observed in PE-curves based on $^{14}C$ incorporation measurements in earlier studies using similar irradiances (Greene *et al.*, 1991; McKay *et al.*, 1997), but longer incubation periods were used (usually 30 min to 1 h). Moreover, PE curves measured by PAM cannot be compared to $^{14}C$ incorporation because different processes are measured by these methods (electron transport and carbon assimilation, respectively).

Some caution is needed in the quantitative comparison of the impact of iron between the two irradiances. Analysis of the interaction between iron and light availability is complicated by the possible increase in photo-redox cycling of Fe-EDTA chelates at high irradiance which may increase the availability of iron to phytoplankton (Hudson & Morel, 1990). Furthermore, since growth rate differed between the two irradiances and iron availability was probably related to cell number (see above), the changes in the availability of iron through the course of time may have differed between the light treatments.

## Conclusion and ecological considerations

Iron stress appears primarily to affect photosynthesis in *C. brevis*, causing a reduction in the diurnal production of water-extractable carbohydrates. This may have implications for phytoplankton of the Southern Ocean, where cells experience frequent changes in irradiance due to deep mixing caused by high surface wind stress (Mitchell *et al.*, 1991). Under such conditions, the carbohydrate pool can serve as an energy buffer or reserve, allowing cells to store radiation energy during the short periods spent near the surface where light is available at photosynthetically saturating levels; in this way an energy and carbon source, and thus time to adapt, is provided when irradiance decreases (Post *et al.*, 1985). Our results suggest that this process is affected in iron-limited diatom cells. Near the surface, cell growth may also be limited but in a quite different way, namely by too much light: iron-stressed cultures of *C. brevis* which had been exposed to low irradiance showed enhanced sensitivity to photoinhibition.

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