Effect of iron limitation on cells of the diatom *Cyclotella meneghiniana* Kützing*

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Abstract

The response of the Baltic diatom *Cyclotella meneghiniana* to iron deficiency was examined. The following growth parameters were measured: cell number, chlorophyll *a* and protein content. The results demonstrate the ability of this diatom to grow well with minimal iron availability; however, the rate of growth fell markedly at the lowest iron(III) concentration. The results of spectrophotometric chlorophyll *a* measurements and protein assays using the Lowry and Bradford methods indicated a significant decrease in their quantities. Iron may therefore be an important regulatory factor controlling the growth of diatom *C. meneghiniana* in an aquatic ecosystem.

1. Introduction

Iron is undoubtedly the most important transition metal in seawater, partly because of the demand for iron in the metabolic cycles of phytoplank-

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ton cells, and also because of the relatively low abundance of iron in the surface water. The biological uptake of iron depends strongly on its chemical speciation. Iron occurs at extremely low concentrations in oxygenated seawater at pH∼8 primarily as thermodynamically stable iron(III), which is highly reactive with respect to hydrolysis, adsorption and complex formation. Inorganic iron(III) exists in solution as mononuclear iron hydrolysis species that are sparingly soluble and precipitate as ferric hydroxide aggregates (Sunda & Huntsman 1995, Sunda 2001). As a result, iron in the form of oxides and hydroxides is biologically unavailable for algal uptake.

Recent studies have shown that about 99.97% of the dissolved iron in surface water is organically bound and that the concentrations of organic iron chelators far exceed the concentration of dissolved iron. Nevertheless, it is available only to microorganisms that are equipped with a specific high-affinity iron-acquisition system (Neilands 1974, Gledhill & van den Berg 1995, Rue & Bruland 1995).

The concentrations of dissolved Fe in oceanic waters range from approximately 1 nM down to values as low as 20 pM in surface waters of remote high-nitrate, low-chlorophyll (HNLC) regimes (Gordon et al. 1982, Landing & Bruland 1987, Martin et al. 1989, 1991, Bruland et al. 1994). These low concentrations of bioavailable iron are thought to be the factor limiting phytoplankton growth in sea water (Millero et al. 1995, Sunda & Huntsman 1995). Recently, the hypothesis has been put forward that iron limits nitrogen fixation in the pelagic North Atlantic Ocean (Michaels et al. 1996). The results of Stal et al. (1999) appear to confirm this idea. In this particular case iron limited nitrogen fixation and the growth of two Baltic diazotrophic cyanobacteria – *Aphanizomenon* spp. and *Nodularia* spp. Similarly, the non-availability of iron prevented the picoplanktonic species *Synechococcus* spp. from utilising nitrate. Iron is an important component of ferredoxin, an electron donor for nitrate reduction. Timmermans et al. (1994) have shown that iron limitation severely retards this process.

According to Pempkowiak et al. (2000) the concentration of dissolved iron in Baltic Proper surface water reached a level of 1 μg l^{-1} (18 nM) and was higher than in oceanic waters. This is understandable, since the Baltic Sea belongs to a semi-enclosed shelf water area influenced to a large degree by land and rivers. Despite the higher total iron concentrations in nearshore waters, the phytoplankton there is potentially iron-limited (Wells et al. 1995) because some of these species require more iron than oceanic forms (Brand et al. 1983, Murphy et al. 1984). According to recent work, many species of phytoplankton that thrive in coastal environments

grow best only in much higher concentrations of iron in comparison with phytoplankton adapted to pelagic conditions (Sunda & Huntsman 1995). Therefore, even at comparatively high concentrations, iron seems to exert an important influence on the physiology and biochemical composition of marine organisms.

Laboratory and field experiments have provided considerable evidence that the availability of iron controls the productivity, species composition, and the trophic structure of planktonic communities in much of the ocean. The bulk of the evidence supporting the iron limitation hypothesis is derived not only from numerous studies of individual marine algae species (e.g. Harrison & Morel 1986, Greene et al. 1991, Trick & Wilhelm 1995, Doucette et al. 1996, McKay et al. 1997, van Leeuwe & Stefels 1998, Kosakowska 1999, Boye & van den Berg 2000, Milligan & Harrison 2000, Soria-Dengg et al. 2001, Timmermans et al. 2001, Paczuska & Kosakowska 2003, Kosakowska et al. 2004), but also from mesoscale experiments in the ocean, particularly in the HNLC areas (e.g. Martin & Fitzwater 1988, Boyd et al. 1996, Coale et al. 1996, Timmermans et al. 1998, Behrenfeld & Kolber 1999, Boyd & Law 2001, Öztürk et al. 2002, Tsuda et al. 2003).

In addition, many of these studies indicate that iron as a component of some essential metabolic enzymes becomes extremely important in limiting phytoplankton biomass. Because of the high alkalinity and salinity of the marine environment the bioavailability of iron to phytoplankton communities may be severely restricted and thereby exert a crucial influence on their biochemical and physiological functions, such as respiratory and photosynthetic electron transport, chlorophyll synthesis, nitrate and nitrite reduction, nitrogen fixation, sulphate reduction, and detoxification of reactive oxygen species (Raven 1988, Milligan & Harrison 2000, Sunda 2001).

Some marine microalgae replace the iron-sulphur redox protein ferredoxin with the non-iron functional equivalent containing flavodoxin in order to overcome iron deficiency (La Roche et al. 1995, 1996, Doucette et al. 1996, Erdner & Anderson 1999, Erdner et al. 1999). Furthermore, many marine microorganisms secrete specific strong-iron ligands (siderophores) to take up iron under iron-limited conditions (Trick et al. 1983, Reid et al. 1993, Wilhelm & Trick 1994, Kosakowska 1999, Kosakowska et al. 1999, Barbeau et al. 2001, Soria-Dengg et al. 2001).

To sum up, previous findings have demonstrated that iron limitation is responsible for a low growth rate in many marine phytoplankton species and for the release of specific iron-complexing ligands, and more so for significant biochemical changes in pigment composition.

In the present work, we focused on the brackish phytoplankton species *Cyclotella meneghiniana*, one of the many representatives of planktonic centric diatoms in the Baltic. We chose this particular micro-organism in order to investigate whether iron deficiency affects population growth, and/or modifies chlorophyll *a* (chl *a*) production and protein content.

2. Materials and methods

Abbreviations:

TCA – trichloroacetic acid, FW 163.4,

DOC – deoxycholic acid (3α,12α-dihydroxy-5β-cholan-24-oic acid) sodium salt, FW 414.6,

BSA – protein standard (from bovine serum albumin), mol. wt. approx. 66 kDa,

EDTA – ethylenediaminetetra-acetic acid, FW 292.24.

Phytoplankton culture and maintenance

The eukaryotic brackish phytoplankton species examined during this study was the axenic diatom *C. meneghiniana* Kütz. SAG 1020-1a obtained from the culture collection at the University of Göttingen (Schlösser 1994). *C. meneghiniana*, whose cells reach a size in the 10–30 μm range, is a cosmopolitan species in the euphotic surface waters of the whole Baltic Sea. It exists seasonally in the Southern Baltic in algal communities in autumn and winter, and is a quantitatively important component of the blooming species (Pankow et al. 1990).

The cultures were maintained in a sterile modified f/2 medium (Guillard 1975) based on artificial seawater (Lyman & Fleming 1940) diluted four times to salinity 8.75 PSU.

Diatom cells were grown at a constant temperature of 22 ± 1°C and under continuous illumination with 100 μmol m^{-2} s^{-1} irradiance (LiCor equipped with two sensors SPQA2005 and Q21859) supplied by cool-white fluorescent lamps. These conditions of lighting, temperature and salinity were maintained during all the experiments.

Experimental procedures

Sterile techniques were used to minimise bacterial contamination: culture vessels were sterilised by heating at 160°C, whereas the applied media were autoclaved at 121°C for 30 min. In addition, all laboratory glassware was washed in 3M HCl to remove surface iron contamination.

Inoculum preparation

The diatom cultures utilised during this study were of 2 types: iron-replete and iron-starved. The iron-replete cells used as the control were grown in f/2(+Fe) medium rich in iron(III) complexed by EDTA ($c_{Fe} = 1.2 \times 10^{-5}$ mol dm^{-3}). Simultaneously, the iron-starved cultures were grown in f/2(–Fe) medium with iron concentration $< 10^{-8}$ mol dm^{-3}. Such an iron-poor medium was obtained by passing it through an activated Chelex-100 Na^+ ion exchange resin (BioRad) to reduce trace metal background and contamination (Davey et al. 1970).

To remove iron adsorbed extracellularly on cell surfaces, the iron-starved cultures were transferred to 'fresh' f/2(–Fe) medium nine to ten times during consecutive 10 days. Similarly, the iron-replete diatom cells were washed with 'fresh' f/2(+Fe) medium, i.e. 9 or 10 times at daily intervals. Before being put to use in the experiments, both diatom cultures were examined for bacterial purity.

Experiment

Preconditioned sterile iron-starved cultures were used as the initial inoculum in three consecutive experiments to which different concentrations of iron(III) ion were supplied: $< 10^{-8}$, 10^{-8}, 10^{-7}, 10^{-6}, 10^{-5} mol dm^{-3} and iron(III) at a concentration of 1.2×10^{-5} mol dm^{-3} complexed by EDTA. Simultaneously, the iron-replete control culture was incubated in f/2(+Fe) medium with iron(III) complexed by EDTA ($c_{Fe} = 1.2 \times 10^{-5}$ mol dm^{-3}). All variants of the iron concentrations were conducted in 5 replicates.

A stock solution of iron(III) chloride (1000 mg Fe in 15% HCl, Titrisol® Merck) was used to prepare the serial experimental dilutions. The starting cell number in the investigated cultures was calculated at 8×10^4 cells cm^{-3}.

During the 10-day experiments the growth of the diatom population was monitored by microscope cell counts every other day. Cell densities were determined by 5 replicates of samples preserved in Lugol for each iron concentration variant in a Bürker haematocytometer. Cell numbers are expressed in the text as the mean ± 6–27% SD.

At the end of each experiment the cultures were sampled for chlorophyll *a* and protein content determinations. Cells were collected by gentle filtration onto Whatman GF/C glass-fibre filters and stored deep frozen at –80°C until extracted. The filters used for protein determination were precombusted (450°C, 4 h) prior to filtration.

Chl *a* was determined on 5 replicates of each iron concentration variant. Cells collected on the filters were extracted in 90% acetone according to the procedure of Strickland & Parsons (1968). Chl *a* concentrations in

the extracts were measured spectrophotometrically and calculated with the equations of Jeffrey & Humphrey (1975). The chl *a* contents are expressed in the text as the mean ± 12–31% SD.

Samples for protein determination were prepared by a modified TCA precipitation technique (Peterson 1977, 1983, Clayton et al. 1988). Initially, cells collected on the filters were ground in 5 cm^3 1M NaOH. After extraction (12 h, 4°C) the samples were centrifuged to remove residual cell and filter debris. 5 cm^3 TCA (22% w/v H_2O) and 1.2 cm^3 DOC (0.15% w/v H_2O) were used to precipitate the soluble proteins in the supernatant. Proteins in the pellet fraction were assayed with the modified procedure of Lowry et al. (1951) and Bradford (1976). The pellets were resuspended in 0.1 M NaOH and subsamples taken to determine protein. The absorbances at $\lambda = 650$ nm (Lowry method) and at $\lambda = 595$ nm (Bradford method) were measured in a Beckman DU 68 spectrophotometer. The estimated protein content was determined by comparison with a standard protein BSA curve. It is important to note that these protein data represent only the soluble protein fraction.

The data on precipitated protein content obtained by the Lowry and Bradford assays are given in the text by the respective means of ± 8–25% SD and ± 3–11%.

All results were evaluated statistically using an analysis-of-variance program.

3. Results

Diatom cell growth at different iron concentrations

The effect of iron limitation can be clearly seen by monitoring the cell number of the experimental diatom population (see Table 1). Growth curves of the *C. meneghiniana* cultures with different iron concentrations are illustrated in Fig. 1. For the iron-starved cultures, data from the control culture are included for comparison. Not only the iron-replete control culture but also the iron-starved diatom cells incubated at lower iron concentrations demonstrated reproducible growth. Nevertheless, it was evident that growth of the iron-starved diatom cells cultured at non-complexing iron concentrations was significantly reduced in comparison with the control culture. After 10 days of incubation the control culture exhibited maximum growth (cell number approx. 8.1×10^5 cells cm^{-3}). Compared with the control, the iron-starved culture diminished the cell number to 4.1×10^5 cells cm^{-3} at the lowest iron concentrations ($\leq 10^{-8}$ mol dm^{-3}). Consequently, the cell number declined with decreasing iron concentration in the medium and was 50% lower in the iron-starved cultures than in the control.

Table 1. Cell number in cultures of *Cyclotella meneghiniana* during incubation and chlorophyll *a* content on the last day of experiments for different iron concentrations. (The data are the means of 15 replicates ± SD)

Iron(III) concentration [mol dm^{-3}]	Kind of cells	Cell number × 10^5 cm^{-3}					Chlorophyll *a* [μg cm^{-3}]
		Incubation time					
		2 days	4 days	6 days	8 days	10 days	10 days
1.2×10^{-5} (Fe+EDTA)	replete cells (control)	3.03 ± 0.62	5.28 ± 0.67	5.84 ± 0.37	6.54 ± 0.45	8.06 ± 0.82	1.53 ± 0.20
1.2×10^{-5} (Fe+EDTA)	iron-starved cells	3.07 ± 0.84	5.80 ± 0.89	6.64 ± 0.47	7.08 ± 0.74	8.58 ± 1.51	1.67 ± 0.41
10^{-5}		2.31 ± 0.48	4.11 ± 0.65	4.43 ± 0.66	4.29 ± 0.35	5.01 ± 0.50	0.84 ± 0.10
10^{-6}		2.20 ± 0.80	4.04 ± 0.71	4.06 ± 0.38	3.92 ± 0.45	4.30 ± 0.59	0.73 ± 0.11
10^{-7}		2.19 ± 0.59	4.04 ± 0.66	4.38 ± 0.66	4.22 ± 0.89	4.28 ± 0.74	0.19 ± 0.07
10^{-8}		1.93 ± 0.49	2.64 ± 0.62	2.89 ± 0.48	3.36 ± 0.62	4.03 ± 0.86	0.13 ± 0.04
$< 10^{-8}$		1.72 ± 0.42	2.15 ± 0.38	2.80 ± 0.52	3.26 ± 0.44	4.10 ± 0.87	0.12 ± 0.03

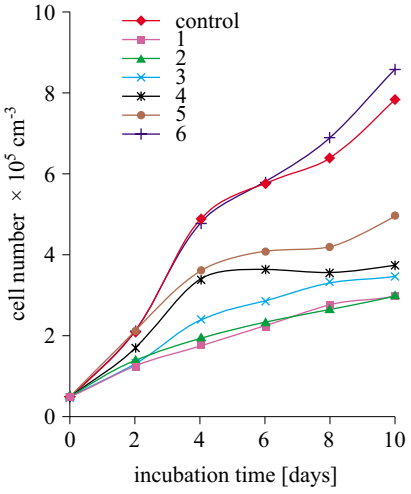

Fig. 1. Growth curves of Fe-starved cells of *Cyclotella meneghiniana* in relation to different iron concentrations: 1–$< 10^{-8}$; 2–10^{-8}; 3–10^{-7}; 4–10^{-6}; 5–10^{-5} Fe mol dm^{-3}; 6–1.2×10^{-5} mol dm^{-3} Fe+EDTA. Control: Fe-replete cells at 1.2×10^{-5} mol dm^{-3} Fe+EDTA

In addition, in the course of the experiments the growth curves of *C. meneghiniana* at other iron(III) ion concentrations – 10^{-7}, 10^{-6} and 10^{-5} mol dm^{-3} – did not differ significantly from each other and were also at a lower level in relation to the control. The iron-starved diatom cultures yielded a traditional growth curve with initial exponential growth for the first 6 days, with subsequent cessation of growth in a stationary phase. Only in one case was this tendency not so clear-cut, i.e. when the iron-starved cultures of *C. meneghiniana* were grown in the medium with iron complexed by EDTA; in this case their cell number exceeded the control level (Fig. 1).

Effect of iron on the chlorophyll level

Measurements of chlorophyll *a* at the end of the serial experiments (after 10 days) showed an apparent response of the diatom cells demonstrating that they were dependent on the availability of iron in the medium. The chl *a* results in Table 1 indicate that the lower the non-complexing iron concentration in the medium, the more the chl *a* concentration in the iron-starved diatom population is reduced. The diatom cells grown at the lowest $c_{Fe} \leq 10^{-8}$ mol dm^{-3} reduced the chl *a* content to 0.12 μg cm^{-3} as compared to the control, which achieved 1.53 μg cm^{-3}. *Ipso facto*, the iron-limited diatom cells showed a nearly 13-fold decrease in chlorophyll *a* concentration in relation to the control cultures, for which the availability of iron complexed by EDTA was optimal. Moreover, the reduction in chl *a* content in the iron-starved population was very conspicuous, except when iron complexed by EDTA was available to the iron-starved cells.

Iron limitation was confirmed by a drop in cellular chlorophyll *a* content (Fig. 2). The cells grown at iron concentrations between $\leq 10^{-8}$ and 10^{-7} mol dm^{-3} reduced the chl *a* content to a range of 0.3–0.5 pg per cell as compared to 1.9 pg per control cell. Furthermore, at the other iron concentrations – 10^{-6}, 10^{-5} mol dm^{-3} – regardless of the form of the available iron, the chl *a* per cell almost reached the control level and was 4–6 times higher than in the iron-starved cells grown at the lowest $c_{Fe} \leq 10^{-8}$ mol dm^{-3}.

The decline was evident in the iron-limited cultures. Chl *a* per cell in the iron-starved cultures fell with decreasing iron concentration and amounted in the medium with $c_{Fe} \leq 10^{-8}$ mol dm^{-3} to between 14% and 20% of the control.

Furthermore, in the course of the experiments the *C. meneghiniana* cells incubated under Fe-limited conditions became discoloured and pale in comparison with the control cells at optimal iron concentration, a symptom of chlorosis, which is also indicative of a change in the chlorophyll content.

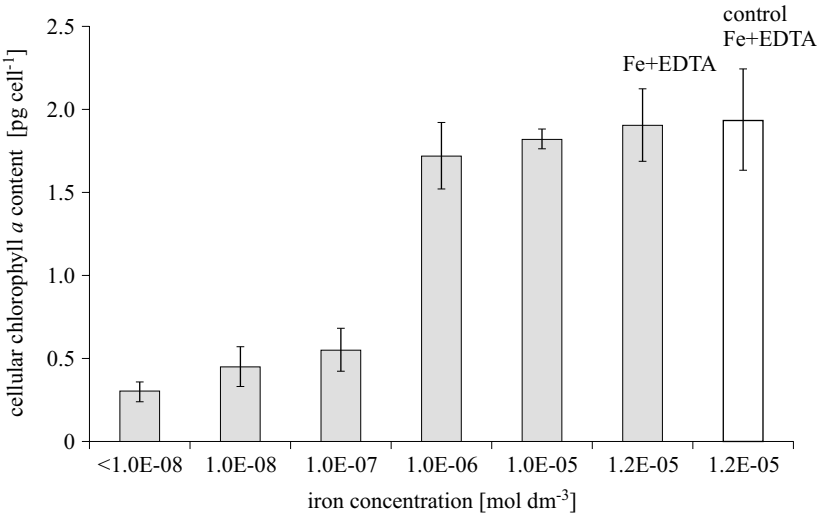

Fig. 2. Cellular chlorophyll *a* content in iron-starved cultures of *Cyclotella meneghiniana* vs iron concentration. The data are the means of 10–15 replicates ± SD. Control: replete cells grown in the presence of EDTA-complexed iron(III)

Effect of iron on protein content

The effect of iron concentration on the relative level of the soluble protein fraction was investigated by measuring the TCA-precipitated protein of diatom cells. The *C. meneghiniana* population generally showed a decrease in the soluble protein fraction in response to the reduced iron concentration in the medium. The quantity of protein determined by Lowry's method dropped from the maximum 18 μg cm^{-3} in the control culture to the minimum 11 μg cm^{-3} in the iron-limited cells at $c_{Fe} \leq 10^{-8}$ mol dm^{-3}.

Bradford's assay also confirmed this tendency; however, the respective results were only 14 μg cm^{-3} and 8 μg cm^{-3}, 20–30% lower than with the Lowry method. The soluble protein fraction in the diatom cells in relation to different iron concentrations for the Lowry and Bradford methods are compared in Fig. 3.

The data set out in Table 2 give the protein content per cell of *C. meneghiniana* according to the different iron concentrations determined by the Lowry and Bradford assays. The quantity of soluble protein in the cells did not noticeably change with decreasing iron concentration in the medium and appeared to be independent of it. Comparison of the soluble protein content in the iron-limited culture and the control yielded respectively 19 and 18 pg per cell (Lowry's method) or 14 and 16 pg per cell (Bradford's method).

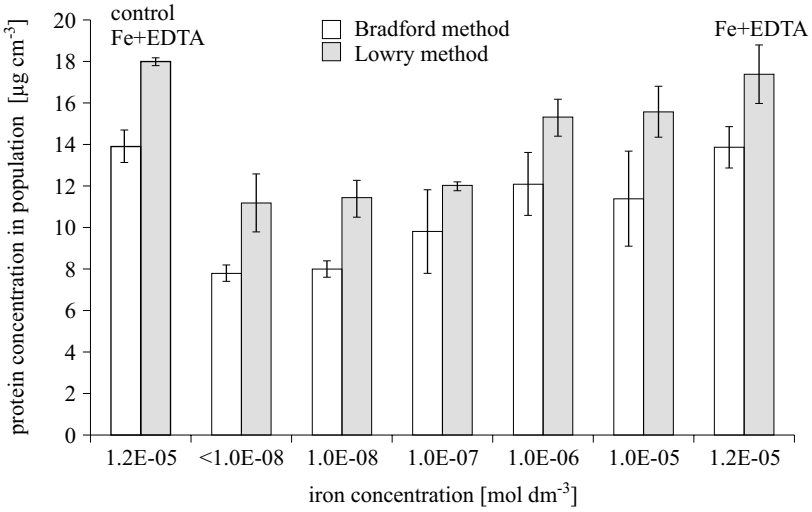

Fig. 3. Protein content in an iron-starved population of *Cyclotella meneghiniana* vs iron concentration (means $\pm$SD, $n = 10$). Control: replete cells grown in the presence of EDTA-complexed iron(III)

Table 2. Soluble fraction of protein per cell of *Cyclotella meneghiniana* in relation to different iron concentrations. The results were determined by 5 replicates of both the Bradford and Lowry assays

Iron(III) concentration [mol dm^{-3}]	Protein content [pq cell^{-1}] methods	
	Lowry	Bradford
1.2×10^{-5} (complexed iron) control	18 ± 2	16 ± 2
1.2×10^{-5} (complexed iron)	18 ± 8	16 ± 2
10^{-5}	22 ± 5	20 ± 4
10^{-6}	24 ± 6	22 ± 5
10^{-7}	23 ± 5	17 ± 7
10^{-8}	21 ± 3	16 ± 3
$< 10^{-8}$	19 ± 5	14 ± 2

4. Discussion

The responses to iron limitation by numerous representatives of the marine phytoplankton include significant physiological and biochemical

changes in their growth characteristics, cell metabolism, pigment and protein composition, as demonstrated by Greene et al. 1991, Sunda & Huntsman 1995, Takeda & Obata 1995, Trick & Wilhelm 1995, van Leeuwe & Stefels 1998, Kosakowska 1999, Kudo et al. 2000, Mann & Chisholm 2000, Davey & Geider 2001, Paczuska & Kosakowska 2003 and Kosakowska et al. 2004.

Our observations of the Baltic diatom *C. meneghiniana* support the hypothesis that iron limitation exerts a significant influence on marine phytoplankton. According to the findings of the present study, the result of depressing the iron concentration was a decrease in chlorophyll *a* and soluble protein content, as well as reduced growth of *C. meneghiniana* in relation to control cultures grown under optimal iron conditions.

The population of this diatom incubated at different iron concentrations demonstrated reproducible growth; however, the cell number declined with decreasing iron concentrations in the medium. We observed that growth of *C. meneghiniana* remained at a low level in the iron-limited cultures, the growth rate of which was half that of the iron-replete control cells under iron-rich conditions. The growth diminution of the iron-starved cells in comparison with the control culture utilising iron complexed by EDTA indicated that iron(III) ion concentrations were insufficient to satisfy the growth requirements of the iron-starved cells. Hence, iron uptake was reduced by the level of uncomplexed iron and was not enough to sustain growth at the same high level as in the case of the iron-replete control cells.

This leads to the assumption that not only the form of iron but also its concentration affect the population of *C. meneghiniana* by retarding its growth. This is consistent with a common trend among other phytoplankton species. For example, Sunda & Huntsman (1995) noted diminishing growth rates with decreasing iron concentrations not only in the large oceanic diatoms *Thalassiosira pseudonana* and *Thalassiosira oceanica* but also in other oceanic and coastal phytoplankton species they examined. A similar effect on the growth of the diatom *Phaeodactylum tricornutum* was recorded by Hayward (1968) and Kudo et al. (2000).

A further effect of iron limitation on *C. meneghiniana* emerged from chlorophyll *a* measurements. The lower iron concentrations caused a marked reduction in the chlorophyll *a* content in its cells. The iron-limited cells displayed a remarkable drop in chl *a* per cell, which fell below 80% in relation to non-iron-limited cells.

This tendency is in good agreement with findings based on data for other diatoms, for example, *P. tricornutum*, in which a decrease in chl *a* content for Fe-stressed cells to 30% of the figure for Fe-replete cells at 20°C was recorded (Kudo et al. 2000). Similar trends in chlorophyll *a* response to

low iron stress occurred among species examined by Sunda & Huntsman (1995). The diatom *Thalassiosira weissflogii* exhibited a 60% decrease in cellular chl *a* : carbon ratios with decreasing iron while the other two diatom species – *T. pseudonana, T. oceanica* – showed 34% and 54% declines, respectively. A decline in chl *a* accumulation by *Chaetoceros muelleri* under iron limitation was also observed by Davey & Geider (2001). The results of Kosakowska (1999) showed that at low iron concentrations of 0.01 –0.5 μM the chlorophyll *a* content in *Chlorella vulgaris* was approximately 30% less and in *Anabaena variabilis*, *Chlorella kessleri* and *Synechocystis aquatilis* 20% less in comparison with the pigment concentration at optimum iron levels – 20–50 μM.

Compared with the cellular chl *a* data of *P. tricornutum* – 272 in Fe-replete cells and 84 fg cell^{-1} in Fe-stressed cells – our data for *C. meneghiniana* yielded 1900 and 300 fg cell^{-1}, respectively (Kudo et al. 2000). The results of our own studies of *P. tricornutum* (SAG1090-1a) also indicated that iron(III) reduced the cellular chlorophyll *a* content from 109.99 at an optimum iron level (10 μM) to 27.14 fg per cell under iron-deficient conditions (0.001 μM) (Kosakowska et al. 2004).

The findings of many authors suggest that iron deficiency is responsible for the breakdown of cellular chlorophyll *a* content in phytoplankton cells. Despite the fact that the pigment itself does not contain iron, there is both a direct and an indirect demand for iron by the enzymes involved in the chl *a* biosynthetic pathway. Iron may be affected directly since it participates as a cofactor of certain enzymes like coproporphyrinogen oxidase, which catalyses the conversion of Mg protoporphyrin to protochlorophylide (Spiller et al. 1982). Furthermore, precursor production such as δ-aminolevulinic acid may be iron-regulated by aconitase and Fe-S protein (Yu & Miller 1982). A reduction in iron availability may simply reduce the cellular abundance and activity of these enzymes and thus reduce the rate of chlorophyll *a* synthesis (Davey & Geider 2001).

On the other hand, iron limitation may indirectly affect the net chl *a* biosynthesis rate because iron is required to regulate the redox state of some of the enzymes responsible for the synthesis of peripheral light-harvesting pigment-protein complexes (Escoubas et al. 1995). Any reduction in the synthesis of these proteins will affect the chl *a* content in cells, because this pigment does not accumulate in their absence (Mortain-Bertrand et al. 1990). In addition, the cellular chl *a* content also depends on the quantity of iron participating in the photosynthetic electron transport chain, particularly at the PSI and PSII reaction centres (12Fe/PSI, 3Fe/PSII).

Limitation of synthesis at these photosynthetic reaction centres by iron availability will also reduce cell pigment accumulation (Raven 1990, Davey & Geider 2001).

As was the case with the results of chl *a* and cell number, the soluble protein fraction measurements indicated that iron limitation tended to cause a reduction in the protein content of *C. meneghiniana* cells as well. The respective quantities of soluble protein in the iron-stressed cells assayed by the Lowry and Bradford methods fell to 60% and 57% of the control. This trend was not confirmed by the quantities of protein per diatom cell. These figures appeared not to depend on iron concentration and did not differ significantly from each other. The cellular protein data attained average values: 21 or 17 pg cell^{-1} determined by the Lowry or Bradford assay, respectively, and were 2 or 3 times higher than the protein results of the diatom *T. pseudonana*, which were similarly determined by the TCA precipitation technique using BSA as standard. According to Berges et al. (1993), the protein data of *T. pseudonana* yielded 8 or 5 pg cell^{-1}, respectively, using the same two assays.

Furthermore, our data on the soluble protein content of *C. meneghiniana* cells assayed by Lowry's method were 20–30% higher than with Bradford's assay. This difference in measurements between the two methods is consistent with the suggestions of other authors. They confirm that the Bradford assay is less sensitive than the Lowry to the class of small peptides (Chiapelli et al. 1979, Mayer et al. 1986, Berges et al. 1993). Extending this argument, if TCA precipitates the class of small peptides to which the Lowry but not the Bradford assay is sensitive, this could explain the differences in estimated protein in the diatom cells reported in this study.

All in all, it is clear from the measurements of cell number, chlorophyll *a* and the soluble protein fraction in the diatom *C. meneghiniana* that iron limitation inhibited its growth and modified its biochemical composition. Our findings therefore appear to support the hypothesis that iron may be an important nutrient limiting phytoplankton growth in sea water. The low availability of iron for phytoplankton communities has important ecological consequences, including the limitation of primary productivity (Kolber et al. 1994, Wells et al. 1994, Cullen 1995, de Baar et al. 1995), nitrate reduction (Timmermans et al. 1994) and nitrogen fixation in the marine environment (Michaels et al. 1996, Falkowski 1997, Paerl 1997). According to Stal et al. (1999), iron limitation appears to be the key to the nitrogen cycle in the Baltic Sea: on the one hand, it may give rise to more intensive denitrification by removing bound nitrogen, on the other it inhibits nitrogen fixation by diazotrophic cyanobacteria. The investigation of iron limitation in other species of Baltic phytoplankton allows a better understanding of

the physiological limitation of essential metabolic processes in their cells, and also the elucidation of an important feedback mechanism between the chemistry of iron in the environment and its biological utilization.

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