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Qualitative and quantitative composition of pigments in *Phaeodactylum tricornutum* (Bacillariophyceae) stressed by iron

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Abstract

The effect of Fe(III) deficiency on qualitative and quantitative changes in pigment composition in *Phaeodactylum tricornutum* Bohlin was demonstrated by HPLC and AAS. Maximum content of pigments showed the diatom cells incubated at the optimum iron concentration, i.e., 10 μM. The contents of chlorophyll *a*, chlorophyll $c_1 + c_2$, fucoxanthin, diadinoxanthin and β,β-carotene were 109.99, 20.16, 40.39, 1.29 and 1.48 fg per cell, respectively. The results obtained showed that Fe(III) affected qualitative and quantitative pigment composition in *P. tricornutum*. The content of individual pigments, proportions between accompanying pigments and their ratios to chlorophyll *a* were important indicators of phytoplankton response to iron stress. The strong reduction in β,β-carotene content, several times (2–5) increase in diadinoxanthin level as compared to β,β-carotene, and high amount of diadinoxanthin in relation to chlorophyll *a* were observed in algae growing at very low Fe(III) concentrations, 0.001 and 0.01 μM. The data suggested that phytoplankton pigments could be a potential physiological marker.

Introduction

Low iron concentration or its limited bioavailability in algal and cyanobacteria environment cause, among others, growth reduction and a decrease in chlorophyll *a* content and CO_2 assimilation rate. In consequence of iron deficiency a severe inhibition of enzymatic processes is observed.

Laboratory experiments point out at an essential role of iron in regulation of biochemical processes in phytoplankton organisms. This element participates in biosynthesis of a protoporphyrine precursor, δ-aminolevuline acid, therefore it takes part in chlorophyll formation. It is a component of cytochromes b and c, electron-transport molecules in respiratory chain and photosynthesis. It is also a constituent of the other photosynthetic electron transporter – ferredoxine. As an element of nitrogenase it takes part in N_2 assimilation and nitrate reduction. Moreover, it is an activator of peroxidase and catalase, enzymes protecting cells against harmful influence of peroxides (Glover 1977; Anderson & Morel 1982; Guikema & Sherman 1983; Sandmann 1985; Rueter & Ades 1987; Raven 1988; Doucette & Harrison 1991; Greene *et al*. 1991, 1992; Geider *et al*. 1993; Geider & La Roche 1994; Surosz & Kosakowska 1996; Kudo & Harrison 1997; Kosakowska 1999; Kudo *et al*. 2000; Davey & Geider 2001).

Environmental investigations also show a significant role of iron in phytoplankton growth in oceanic water, especially in Equatorial Pacific Ocean (Martin *et al*. 1994; Coale *et al*. 1996), Southern Ocean (Martin *et al*. 1990; de Baar *et al*. 1999) and Polar Southern Ocean (Boyd *et al*. 2000; Boyd & Law 2001). These regions are characterized by high nutrient concentration (nitrates, phosphates and silicates) and low chlorophyll (HNLC) content. It is presumed that low chlorophyll level together with high nitrate concentration could be, among others, a consequence of water 'contamination' by zooplankton. Strong turbulences inducing mixing of phytoplankton layers up to the critical depth could also be a reason of limiting

light influence on phytoplankton growth. It is assumed that apart from the above mentioned factors phytoplankton growth could depend on microelement level as well.

'Iron hypothesis' proposed by Martin (Martin 1990) has been verified in the above environment investigations. Introduction of 0.4 μM Fe(II) into oceanic waters resulted in an increase in phytoplankton photosynthetic activity. Maximum primary production, ca. 10^{-5} μg C dm^{-3} d^{-1}, showed 3- or 4-fold increase as compared to the production before iron addition and 3-fold increment in chlorophyll content was also noted (Martin *et al*. 1994). In May 1995 similar experiments were carried out in Pacific region. Fe(II) concentration introduced to the water was 0.2 μM. The results were in accordance with those of the first experiment (Coale *et al*. 1996). In 1999 the next experiment, termed SOIREE-SOUTHERN OCEAN IRON RELEASE EXPERIMENT, was performed (Boyd *et al*. 2000; Boyd & Law 2001). Mass and wide-spread phytoplankton development as a result of Fe(II) introduction was observed. The findings suggest that iron could limit phytoplankton productivity in oceanic waters.

The first reports on the specific changes in the content and composition of pigments in cyanobacteria cells incubated under iron deficiency were those of Öquist (1971, 1974). The later data showed that an increase in Fe(III) concentration, in the range of 0.005 to 42 μM, resulted in an increment in chlorophyll *a* content in *Oscillatoria tenuis*. The highest level of chlorophyll *a* was noted at 4.7 μM. Higher concentrations caused a slight decrease in the pigment content. In concentration ranges of 0.0051 to 0.031 and 0.41 to 4.7 μM a distinct, 2- or 3-fold reduction in chlorophyll *a* level as compared to its maximum content was observed (Trick *et al*. 1995).

Similar effects were found in the case of iron influence (0.41–42 μM) on phycocyanin content in *O. tenuis* population. Experiments *in vivo* revealed that phycocyanin, analogously as chlorophyll *a*, reached its maximum level, at 4.7 μM. On the other hand, at very low iron concentrations (0.031 μM and lower) phycocyanin content in cyanobacteria cells was constant whereas the chlorophyll *a* level per one cell showed a downward tendency (Trick *et al*. 1995). In experiments *in vivo* it was also found that in *Anacystis nidulans* R2, *O. tenuis* and *Synechocystis* sp. PCC 7002 populations the phycocyanin:chlorophyll *a* ratio (PC/Chl$_a$, ABS$_{625}$/ABS$_{678}$) increases under iron deficiency (Guikema & Sherman 1984; Trick *et al*. 1995; Wilhelm & Trick 1995).

The results of the own studies showed that Fe(III) in the range of 0.01 to 50 μM stimulated chlorophyll *a* production in *Chlorella vulgaris*, *C. kessleri*, *Anabaena variabilis* and *Synechocystis aquatilis* populations. Maximum chlorophyll *a* content was noted at 20–50 μM. Above this concentration range a gradual decrease in chlorophyll *a* level was observed in green algae as well as cyanobacteria cultures, and at ca. 500 μM the growth was completely stopped. At low iron concentrations, 0.01–0.5 μM, chlorophyll *a* content in *C. vulgaris* population was ~30% and in *A. variabilis*, *C. kessleri* and *S. aquatilis* cultures less than 20% in comparison with the pigment concentration at optimum iron level, 20–50 μM. Total growth inhibition was noted at 500 μM (*C. kessleri* and *S. aquatilis*) and 5,000 μM (*C. vulgaris* and *A. variabilis*) of iron(III) (Surosz & Kosakowska 1996; Kosakowska 1999).

The data on qualitative and quantitative composition of photosynthetic pigments in phytoplankton organisms are scarce and conflicting. Wilhelm and coworkers (1996) pointed out at a significant decrease in chlorophyll a, β-carotene and zeaxanthin content with a decrease in Fe(III) from 4.7 to 0.031 μM in *Synechococcus sp*. PCC 7002 population. Further reduction in iron concentration, to 0.0051 μM, resulted in an increase in chlorophyll *a* content to the level corresponding to ca. 50% of its maximum amount observed at 42 μM. This dependence was not noted in β-carotene and zeaxanthin. Kudo *et al*. (2000) showed that in *P. tricornutum* NPCC no 738 incubated in Fe(III)-stressed conditions (0.002 μM) chlorophyll *a* concentration was reduced by 50% as compared to the cells growing in Fe-replete medium (2 μM).

Understanding of the mechanisms regulating phytoplankton growth in iron(III) deficient conditions is of great importance in determining marine environment productivity and photosynthetic activity of phytoplankton. Adaptation of phytoplankton organisms to grow in iron-stressed habitat leads to the changes at cellular level. The studies of algal response to Fe(III) shortage will help clarify the course of many biochemical processes by which adaptation comes into being.

Determination of biochemical composition of an organism at different culture conditions is a crucial point in investigations of adaptative mechanisms. The aim of the present work was to estimate the influence of iron(III) on qualitative and quantitative pigment

composition in the common representative of marine planktonic diatom, *P. tricornutum* SAG 1090-1a.

Material and methods

Organism

Axenic culture of planktonic diatom, *P. tricornutum* 1090-1a, was used in the experiments. The strain was obtained from the collection of algal cultures at the University of Göttingen.

The works connected with setting up the experiments were done in axenic conditions, in a chamber with laminar air flow. Sterility was controlled by inoculation on the organic medium solidified with agar. Then the samples were incubated for 7 days at 37 °C. To reduce iron contamination, glassware and polyethylene equipment were pre-treated with 6N HCl for several days and finally rinsed with deionized water.

Incubation medium

The diatom was incubated on modified mineral f/2 medium (Schlösser 1994). The weighed portions of individual salts were dissolved in redistilled water. Iron was excluded from the original medium. To purify macroelement solutions and sea water of bi- and trivalent metal ions, they were passed through a column filled with Chelex-100 Na^+ resin (Bio-Rad) (Davey *et al*. 1970). Then microelement solution and vitamins were added. pH of the medium was adjusted to 8.0 with 1 M KOH. The medium was sterilized at 121 °C for 30 min. Iron concentration in the medium was ca. 0.001 μM.

Inoculum preparation

The modified f/2 medium was inoculated with *P. tricornutum* culture growing on complete original medium. The cell suspension was preincubated for 10 days at 22 $\pm$ 0.1 °C under continuous illumination (Philips 20W fluorescent lamps) at an irradiance of 85 μmol m^{-2} s^{-1} (LiCor equipped with a spherical sensor SPQA 2005 and unidirectional sensor Q21859) (Geider *et al*. 1985; Mc Kay *et al*. 1997). To remove iron ions from the cell surface, the culture was washed with fresh mineral medium, i.e., 8 or 9 passages at daily intervals. After 10 days the preculture was used as inoculum.

Preparation of ferric(III) chloride solutions

The stock solution of ferric(III) chloride (Titrisol, Merck, 1 g Fe^{3+} in 100 ml) was diluted with deionized water. This solution was also used as a standard in absorption atomic spectroscopy (AAS).

Algae were incubated at Fe-deficient and Fe-optimum concentrations, less than 1 μM and 1–10 μM, respectively. The final iron concentrations in modified f/2 medium were in the range of 0.001–10 μM. The inoculum was 2×10^5 cells per 1 ml. The cultures were carried out for 10 days at 20 $\pm$ 0.1 °C and under continuous illumination at an intensity of 85 μmol m^{-2} s^{-1}. Fe concentration in the control cultures was the same as in the original f/2 medium, i.e., 10 μM. All experimental variants were run in triplicate. Iron concentrations in cultivation medium were determined in absorption-atomic spectroscope (Video 11E, Thermo Jarell Ash) in Laboratory of Marine Biogeochemistry, Institute of Oceanology, Polish Academy of Sciences (Pempkowiak *et al*. 2000).

Chemical and biological measurements

Qualitative and quantitative pigment composition in *P. tricornutum* was determined by HPLC. From each experimental variant three samples of 10 ml were taken and filtered through Whatman GF/C glass filters. The filters with algal cells were freezed and stored at -25 °C prior to analysis. The population density was measured by the cell number determined in a Bürker chamber after 10 days of cultivation.

Extraction and chromatographic analysis

The frozen cells on the filters were placed in 3 ml of 90% acetone and homogenized by grinding and sonication for 2 min at 20 kHz using an ice-cooled ultrasonic homogenizer (Cole Palmer) and extracted in darkness at 4 °C for 2 h. The extract was then centrifuged for 20 min at 5 °C and 3700 *g* (Beckman GS-6R) to remove cellular particle debris. The supernatant was subjected to chromatographic analysis.

The pigments were separated using the RP-HPLC technique. The system was equipped with an Hewlett Packard HP 1050 pump, an HP 1046 fluorescent detector, and an HP 1050 diode array detector, connected via a precolumn with a LichroCART^{TM} Hypersil ODS analytical column (dimensions: 250 $\times$ 4 mm, particle size: 5 μm, Merck).

0.5 ml of clarified extract was mixed with 0.5 ml of ion-pairing reagent (1M ammonium acetate) prior

to injection. After 5 min mixing and equilibration, 0.1 ml of the solution was injected into the chromatographic column (Mantoura & Llewellyn 1983; Wright *et al.* 1991). The pigments were separated in a gradient mixture of methanol, 1M ammonium acetate and acetone. The composition of solvents varied from 100% A (80:20 methanol : 1 M ammonium acetate, v/v) to 100% B (60:40 methanol : acetone, v/v) along a 10 min linear gradient followed by a 15 min 100% B isocratic hold with a 0.8 flow rate. After 25 min of analysis the solvent composition was returned to the initial conditions over 10 min, which allowed the system equilibrium to be restored before the next sample injection. This gradient allowed for an adequate resolution of all dominant pigments (Mantoura & Llewellyn 1983; Barlow *et al.* 1993; Stoń & Kosakowska 2002). The eluted pigments were detected using and absorbance detector set at λ = 440 nm (in the absorbance spectrum of each pigment, chlorophylls and carotenoids reveal some absorbance amount in this wavelength) and a fluorescence detector set at $\lambda_{ex} = 431$ nm and $\lambda_{em} = 660$ nm to confirm the presence of chloropigments.

Qualification and quantification

The qualitative and quantitative analysis of the pigment content in the samples was performed using commercially available pigment standards. High purity reference pigments isolated from reference monocultures were obtained from Water Quality Institute, Denmark. The pigment standards (chlorophyll *a*, chlorophyll *c1*, chlorophyll *c2*, fucoxanthin, diadinoxanthin and β-carotene) were subjected to chromatographic analysis in order to obtain calibration curves, detection limits and absorption spectra. Qualitative analysis was based on a comparison of the retention times and the absorbance spectra of eluted peaks with those obtained for the standards (Wright & Shearer 1984). Identification was confirmed by co-injection and on-line diode array spectra. The cellular pigment contents were expressed in fg cell^{-1}. The results were evaluated statistically using analysis of variance program.

Results and discussion

The influence of iron(III) on qualitative and quantitative pigment composition in *P. tricornutum* was presented on Figure 1 and in Table 1. HPLC analysis showed that the acetone extracts from the diatom

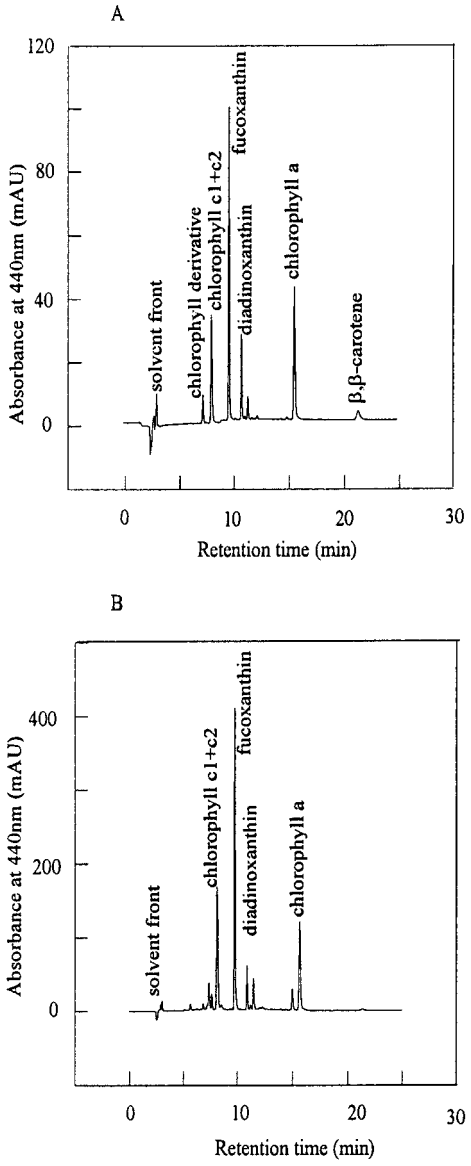

Figure 1. Representative, reverse-phase HPLC chromatogram of pigments extracted from *P. tricornutum* cells after incubation in the medium with 0.5 μM (A) and 0.001 μM (B) of iron (III).

cells contained the following pigments: chlorophyll *a*, chlorophyll *a* derivative, chlorophyll $c_1 + c_2$, fucoxanthin, diadinoxanthin and β,β-carotene. The results obtained indicated that iron(III) affected qualitative pigment composition. In the extracts of *P. tricornutum* cells incubated in Fe-deficient conditions a considerable decrease in peak height (retention time: 22 min) corresponding to β,β-carotene or even its absence was noted (Figure 1).

Table 1. Pigment content in *P. tricornutum* cells incubated at different iron(III) concentrations. Data are means of 9 replicates ± SD.

Concentration of iron(III)	Pigment concentration (fg cell^{-1})				
(μM)	Chlorophyll *a*	Chlorophyll $c_1 + c_2$	Fucoxanthin	Diadinoxanthin	β,β-carotene
0.001	27.14 ± 1.96	6.29 ± 1.34	14.52 ± 1.17	1.20 ± 0.03	0.22 ± 0.02
0.01	49.65 ± 1.33	11.25 ± 1.08	25.50 ± 1.36	1.19 ± 0.09	0.52 ± 0.03
0.1	39.41 ± 0.43	9.06 ± 0.04	20.67 ± 1.52	0.81 ± 0.01	0.64 ± 0.03
0.5	50.94 ± 1.20	13.10 ± 2.44	24.04 ± 1.56	0.91 ± 0.01	0.71 ± 0.04
0.1	60.87 ± 1.74	12.29 ± 1.21	26.49 ± 1.89	1.02 ± 0.03	0.82 ± 0.06
10	109.99 ± 1.23	20.17 ± 0.98	40.39 ± 2.11	1.79 ± 0.08	1.49 ± 0.07

It was also found that iron(III), at the concentration range of 0.001–10 μM, influenced the pigment contents per single cell of *P. tricornutum* (Table 1). The maximum pigment concentrations showed the cells incubated at optimum iron level. The amounts of chlorophyll *a*, chlorophyll $c_1 + c_2$, fucoxanthin, diadinoxanthin and β,β-carotene were 109.99, 20.17, 40.39, 1.79 and 1.49 fg cell^{-1}, respectively. On the other hand, the cells exposed to iron-deficient conditions (0.001 μM) were characterized by the lowest pigment contents: chlorophyll *a* − 27.14, chlorophyll $c_1 + c_2$ − 6.29, fucoxanthin −14.52, diadinoxanthin −1.2 and β,β-carotene −0.22 fg cell^{-1}.

A decrease in iron concentration below 10 μM was accompanied by a reduction in chlorophyll *a* and accessory pigment contents: chlorophyll $c_1 + c_2$ as well as photosynthetic (fucoxanthin) and photoprotective (diadinoxanthin and β,β-carotene) carotenoids as compared to the control sample. The results of quantitative determinations of pigments in the cells exposed to 0.01 μM (Fe-deficiency) were worthy of notice. At this iron concentration chlorophylls *a*, $c_1 + c_2$ and fucoxanthin showed ca. 25% increase in relation to their levels in the cells treated with tenfold higher iron concentration. In the same samples the difference in diadinoxanthin content was even more pronounced (ca. 45%).

The results indicated that a decrease in iron(III) concentration in the incubation medium resulted in a gradual reduction in β,β-carotene content in *P. tricornutum* cells. Its lowest level, 0.22 fg cell^{-1}, was noted at 0.001 μM (Figure 2). The content of the other photoprotective carotenoid, diadinoxanthin, was decreased in diatom cells incubated at 1 and 0.1 μM of iron(III) as compared to the control culture. However, at very low Fe concentrations, 0.01 and 0.001 μM, the diadinoxanthin level was again increased and was about 1.2 fg cell^{-1} (ca. 70% of the control sample). It was found that irrespective of iron concentration the amount of diadinoxanthin in *P. tricornutum* cells was always higher than that of β,β-carotene (Figure 2). The differences in the photoprotective pigment contents were the greatest at 0.001 and 0.01 μM of iron.

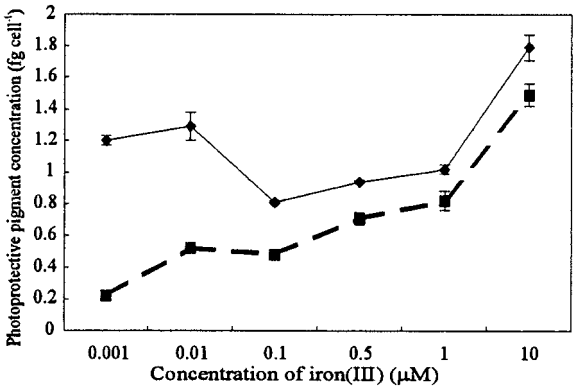

Figure 2. The contents of photoprotective pigments, β,β-carotene and diadinoxanthin, in *P. tricornutum* cells exposed to different iron(III) concentrations. Data are means of 9 replicates ± SD.

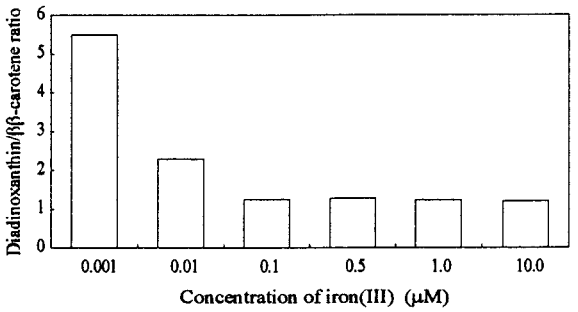

Figure 3. Diadinoxanthin/β,β-carotene ratios in *P. tricornutum* cells incubated at different iron(III) concentrations.

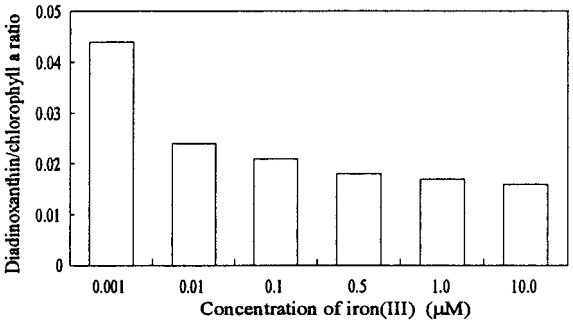

Figure 4. Diadinoxanthin/chlorophyll *a* ratios in *P. tricornutum* cells incubated at different iron(III) concentrations.

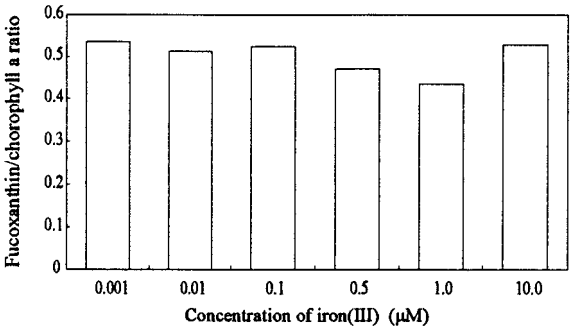

Figure 5. Fucoxanthin/chlorophyll *a* ratios in *P. tricornutum* cells incubated at different iron(III) concentrations.

In cells incubated at Fe concentration range of 0.1–10 μM the diadinoxanthin : β,β-carotene ratios were constant (ca. 1.25). On the other hand, at very low iron level, 0.001 and 0.01 μM, they were much higher, 2.3 and 5.5, respectively (Figure 3). Similarly, the diadinoxanthin:chlorophyll *a* ratios (Figure 4) reached the maximum value at the lowest Fe concentration and showed 3-fold increase in relation to the control culture.

It could be presumed that an increase in diadinoxanthin concentration in *P. tricornutum* cells at the lowest iron level (0.001 μM) made up for a drastic decrease in β,β-carotene content (Figure 2). Therefore, diadinoxanthin constituted the main carotenoid responsible for chlorophyll *a* photoprotection.

Irrespective of iron(III) concentration in the incubation medium fucoxanthin : chlorophyll *a* ratio was between 0.43 and 0.55 (Figure 5). This value was typical of diatoms and is applied as potential environmental chemotaxonomic marker (Jeffrey & Vesk 1997).

The chlorophyll $c_1 + c_2$: chlorophyll *a* ratios in *P. tricornutum* cells growing both at deficient and optimum iron concentrations were constant, 0.19–0.23 (Figure 6).

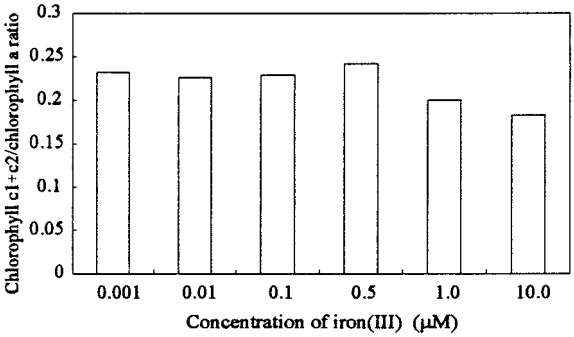

Figure 6. Chlorophyll $c_1 + c_2$/chlorophyll *a* ratios in *P. tricornutum* cells incubated at different iron(III) concentrations.

Wilhelm *et al.* (1996) estimated the contents of some pigments in *Synechococcus sp.* PCC 7002 population by HPLC method. They observed a significant reduction in chlorophyll *a*, β-carotene and zeaxanthin levels with a decrease in Fe(III) concentration from 4.7 to 0.031 μM. However, the lower iron concentration, 0.0051 μM, resulted in an increase in chlorophyll *a* content corresponding to ca. 50% of its maximum value (at 42 μM). Such situation was not noted in β-carotene and zeaxanthin. Kudo *et al.* (2000) found a 50% reduction in chlorophyll *a* level in *P. tricornutum* NPCC738 cells incubated in Fe-stressed conditions (0.002 μM) as compared to the population growing in Fe-replete environment (2 μM).

Laboratory and field investigations showed that the contents of individual pigments and/or accesory pigments to chlorophyll *a* ratios could undergo changes according to the taxonomic composition of phytoplakton organisms and their physiological status determined by nutrient concentration, temperature, intensity and spectral composition of light and photoperiod (Roy 1988; Heath *et al.* 1990; Geider *et al.* 1993; Reigman & Rowe 1994).

Literature data indicated that the amounts of various pigments in 16 strains of *Emiliania huxleyi* (*Prymnesiophyceae*) were genetically dependent. Significant differences were noted in the contents of typical prymnesiophyte pigments, fucoxanthin and 19′-haxanoylofucoxanthin (Stolte *et al.* 2000). The authors also studied the effect of nitrogen, phosphorus and light intensity deficiency on qualitative and quantitative pigment composition in *E. huxleyi* strain L. They found that nitrogen deficiency caused a considerable decrease in total pigment content and inhibition of photosynthetic activity of the alga. On the contrary, in

phosphorus deficient conditions a high pigment content, 2–3 times higher than in the algae incubated in nitrogen deficient medium, was noted. It was accompanied by the changes in the ratios of light harvesting (fucoxanthin) to photoprotective (diadinoxanthin and diatoxanthin) carotenoids. The changes observed were probably related to the fact, that a part of diadinoxanthin pool was a precursor of fucoxanthin synthesis (Stolte *et al*. 2000).

According to Jefferey & Vesk (1997), accessory pigments would be applied as main diagnostic markers, typical of particular taxonomic groups. Since the pigment contents and/or their ratios could depend on the environmental factors, it was suggested that they may be also used as physiological markers of phytoplankton organisms (Stolte *et al*. 2000).

The results obtained showed that iron(III) could modify qualitative and quantitative composition of *P. tricornutum* pigments. It was also demonstrated that the contents of individual pigments as well as their ratios were important indicators of phytoplankton response to iron stress. A drastic drop in β,β-carotene concentration, 2–5 times higher diadinoxanthin:β,β-carotene ratio and high diadinoxanthin content in relation to chlorophyll *a* noted at very low iron(III) level (0.001 μM). It could be suggested that *P. tricornutum* cells incubated at Fe-deficient conditions increased diadinoxanthin synthesis in recompense for a decrease in β,β-carotene content. Therefore it may be assumed that diadinoxanthin constitutes the main photoprotective carotenoid under iron stress. The present investigations supported the hypothesis on the application of phytoplankton pigments as potential physiological markers.

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