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COPPER COMPLEXES WITH THE PORPHYRIN RING
OF CHLOROPHYLL IN PHYTOPLANKTON CELLS

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Natural plankton samples were collected within June – November 1988 in the Southern Baltic. Species composition was determined and chlorophyll extracts from the samples were analyzed for chlorophyll and copper content. The Cu content in extracts from those samples dominated by phytoplankton was considerable, especially during bloom (Cu/Mg ratio of up to 0.1), in contrast to samples with zooplankton predominance (zero Cu/Mg ratio). The data confirm results of previous studies on the blue-green alga *Anabaena variabilis*, proving the formation of a copper-chlorophyll complex in algal cells cultured in a high Cu concentration medium. Effects of such factors as light, pH, Cu and Fe concentrations in the medium on Cu incorporation were studied in batch cultures of the blue-green algae *A. variabilis* and *Anacystis nidulans*. The experiments showed replacement of Mg by Cu to occur in live algal cells, the process requiring light to occur. Moreover, certain Cu concentrations stimulate an increase in chlorophyll content of the blue-green algal cultures. Fixation of some molecules of chlorophyll by copper such that the cell has to produce new chlorophyll molecules in order to function normally cannot be excluded.

INTRODUCTION

Copper is an essential element for the normal growth and physiological activity of unicellular algae, but at higher concentrations it alters the basic cell functions (Kowalewska 1988a). The main physiological effects caused by high copper concentrations are: 1 – effects on growth rate (Morel and Morel-Laurens 1983), and 2 – effects on photosynthesis (Steemann Nielsen and Wium-Andersen 1971). That is why copper chemistry in the environment is connected with algal blooms, a phenomenon still intriguing to scientists.

The method of cyanobacterial bloom inhibition by adding copper to lake water has been known since the beginning of this century. Concentrations used ranged most often within 20–1000 $\mu\text{g}/\text{dm}^3$ (Gachter et al. 1973).

All the toxic effects of copper on unicellular algae depend to a considerable extent on chemical speciation of the element. It is generally accepted that only ionic copper is toxic for algae. It is hence difficult to determine a concentration level above which copper affects normal functions of algal cells, as it forms various associations with different inorganic and organic ligands occurring in the environment (Kowalewska 1988b).

Those effects depend also on an organism exposed, because various species differ considerably in their resistance to toxic effects of copper on growth and photosynthesis. Predominantly, green algae are less sensitive, their growth being even stimulated at low Cu concentrations, while blue-greens are the most sensitive algae (Sueur et al. 1982). Sensitivity of diatoms and dinoflagellates varies greatly from species to species. Some authors contend that variation in resistance to copper induces the so-called "succession of species" (Granéli et al. 1986; Morel and Morel-Laurens 1983).

Earlier work carried out on the blue-green alga *Anabaena variabilis* (Kowalewska et al. 1987; Kowalewska and Hoffmann 1989) has indicated that copper replaces magnesium in the chlorophyll porphyrin ring at high Cu concentrations. This hypothesis was put forward in the literature many years ago, but was not supported by any experimental evidence (Azeez and Banerjee 1987; Gross et al. 1970; Wu and Lorentzen 1984).

In the present work, marine plankton was studied with respect to a possibility of copper-chlorophyll complex formation in cells. Besides, effects of such factors as light, pH, Cu and Fe concentrations in the medium on copper incorporation were studied on laboratory-grown cultures of blue-green algae.

MATERIALS AND METHODS

Location of sampling stations

Location of sampling stations is presented in Fig. 1. Plankton samples were collected within June – November 1988 in the Southern Baltic. An aliquot of each sample was formalin-preserved for species identification, the remainder being frozen until further analysis in the laboratory.

Laboratory cultures of algae

The studies concerned two blue-green species: *Anabaena variabilis* and *Anacystis nidulans*, both originating from the Institute of Oceanology collection. The algae were cultured in 50 cm^3 of Bold's basal medium, BBM (Bischoff and Bold 1963) in 100 cm^3

Sampling stations

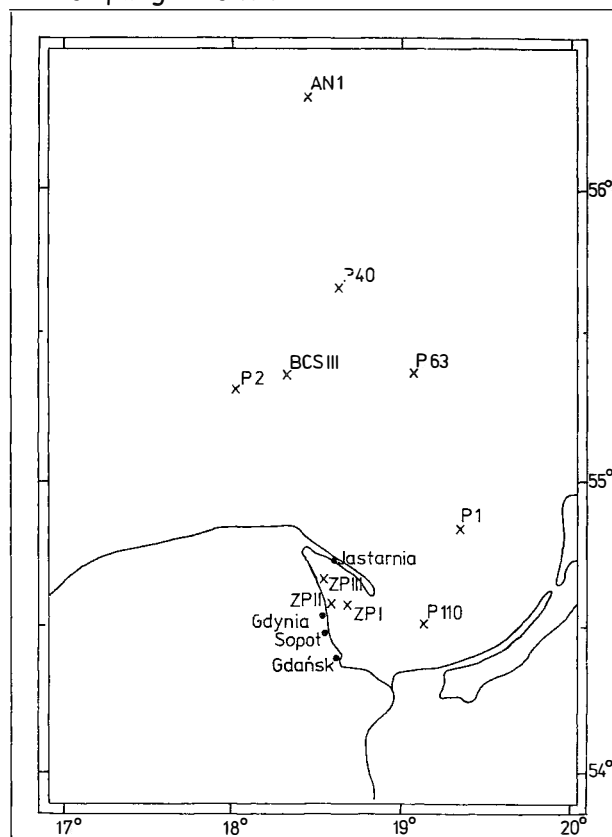


Fig. 1. Location of sampling stations in the Baltic Sea in 1988

total volume conical flasks at constant illumination ($5 \mu\text{E}/\text{m}^2 \text{ s}$) at 25°C for one week. Subsequently, in the case of *Anabaena variabilis* whose cells settled on the bottom of the flask, the medium was decanted and 100 cm^3 of fresh BBM (prepared with or without iron) were added to the culture. In the case of *Anacystis nidulans*, a 5 cm^3 aliquot was transferred to a conical flask containing 10 cm^3 of fresh BBM prepared with or without iron. Then, in both cases, i.e. *Anabaena variabilis* and *Anacystis nidulans* cultures, 5 cm^3 aliquots were collected for an initial chlorophyll content assay. The remainder of each sample was divided into two portions and the stock solution of CuSO_4 was added to the two samples; pH of the sterilized BBM with CuSO_4 added at a concentration of the order of $1 \text{ mg}/\text{dm}^3$ was 5; pH 8 was obtained by adding a KOH solution. Such a double set of cultures was prepared twice, i.e. each analysis at the same experimental conditions was run four times.

Determination of copper and chlorophyll content

An algal sample was filtered through a glass-fibre Whatman GF/C filter. The filter was washed with redistilled water and extracted with 10 cm³ acetone, following which the sample was centrifuged and the supernatant decanted. The filter was washed with acetone (2 cm³) and centrifuged again. The combined acetone fractions were transferred to a separation funnel with hexane (2 cm³) and redistilled water (2 cm³) was added. After mixing, chlorophyll and its derivatives were in the hexane layer.

Chlorophyll was determined by magnesium assays, as each molecule of the compound contains a single Mg ion; 2 cm³ of the hexane layer were heated in a quartz beaker and, after evaporation of the solvent, the sample was mineralized with 3 cm³ HNO₃ (3.5 N). The residue was dissolved in 3 cm³ of redistilled water. Magnesium and copper were determined in flame atomic absorption spectroscope (Instrumental Laboratory, Type 151). Sensitivity of the method was 0.05 µg Cu/cm³ and 0.001 µg Mg/cm³, i.e. about 0.0003 µMole chlorophyll.

RESULTS AND DISCUSSION

Tables 1 and 2 summarize the results of copper and magnesium assays in the plankton samples collected from the Baltic Sea. Table 3 presents the species composition. In the samples containing mainly phytoplankton, the copper content is noticeable,

Table 1

Copper and magnesium contents in the plankton samples dominated by phytoplankton,
collected in the Baltic Sea in 1988

| No. | Station | Month of collection | Cu (µg) | Mg (µg) | Cu/Mg µMole/µMole |
|-----|---------|---------------------|---------|---------|----------------------|
| 1 | BCS3X | June | 0.204 | 1.234 | 0.062 |
| 2 | P 110 | June | 0.068 | 5.949 | 0.004 |
| 3 | ZP I | July | 0.090 | 0.710 | 0.047 |
| | | | 0.085 | 0.916 | 0.034 |
| | | | 0.090 | 0.548 | 0.061 |
| | | | | | } 0.05 ±0.013 |
| 4 | ZP I | September | 0.201 | 3.808 | 0.020 |
| 5 | ZP II | September | 0.105 | 2.389 | 0.016 |
| 6 | ZP II | October | 0.651 | 4.196 | 0.058 |
| 7 | ZP I | November | 0.237 | 0.999 | 0.089 |
| | | | 0.335 | 1.248 | 0.101 |
| | | | | | } 0.10 ±0.010 |

Table 2

Copper and magnesium contents in the plankton samples dominated by zooplankton, collected in the Baltic Sea in 1988

| No | Station | Month of collection | Cu (μg) | Mg (μg) | Cu/Mg $\mu\text{Mole}/\mu\text{Mole}$ |
|----|---------|---------------------|----------------------|----------------------|--|
| 1 | AN 1 | June | 0 | 0.367 | 0 |
| 2 | P 40 | June | 0 | 0 | — |
| 3 | P 1 | June | 0 | 0.301 | 0 |
| 4 | P 63 | June | 0 | 0.030 | 0 |
| 5 | BCS3X | June | 0 | 0.320 | 0 |
| 6 | P 2 | June | 0 | 0.130 | 0 |

Table 3

Samples of the plankton dominated by phytoplankton, collected in 1988:
species composition

| No | Station | Month of collection | Main classes | % | Dominant species |
|----|---------|---------------------|---|--------------|---|
| 1 | BCS3X | June | <i>Cyanophyta</i> | 99.8 | <i>Aphanizomenon flos-aquae</i> |
| 2 | P 110 | June | <i>Cyanophyta</i> | 99.9 | <i>Aphanizomenon flos-aquae</i> |
| 3 | ZP I | July | <i>Cyanophyta</i> | 90.0 | <i>Aphanizomenon flos-aquae</i> <i>Nodularia spumigena</i> |
| 4 | ZP I | September | <i>Chlorophyta</i> <i>Bacillario-phyceae</i> | 47.8 37.2 | <i>Cladophora</i> sp. <i>Coscinodiscus grani</i> |
| 5 | ZP II | September | <i>Chlorophyta</i> <i>Bacillario-phyceae</i> | 83.4 | <i>Cladophora</i> sp. |
| 6 | ZP II | October | <i>Bacillario-phyceae</i> | 90.6 | <i>Coscinodiscus grani</i> |
| 7 | ZP I | November | <i>Bacillario-phyceae</i> | 99.4 | <i>Coscinodiscus grani</i> |

especially in those samples collected during a bloom (e.g. ZP 1 November). In such cases, the Cu/Mg ratio reached even 0.1. In the light of the previous results (Kowalewska et al. 1987; Kowalewska and Hoffmann 1989) we may suppose that the cause lies

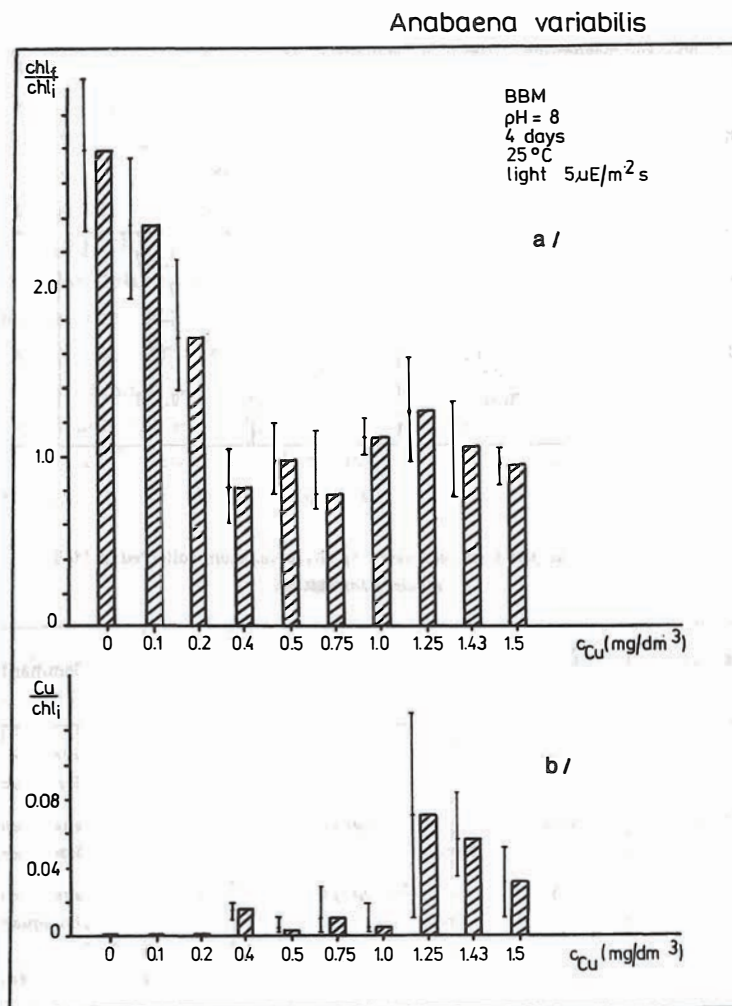


Fig. 2. *Anabaena variabilis* batch cultures in BBM, at pH = 8, 25°C, exposed to light of $5 \mu\text{E}/\text{m}^2 \text{ s}$: a) chlorophyll to initial chlorophyll ratio ($\text{chl}_f/\text{chl}_i$) vs copper concentration in medium (C_{Cu}); b) copper to initial chlorophyll ratio (Cu/chl_i) vs copper concentration in medium (C_{Cu})

in the copper-chlorophyll complex formation. The samples containing mainly zooplankton showed no copper or the contents were negligible, which confirms the above suggestion. The observed Cu/Mg ratios equal to, for instance, 0.1 means that in one out of 10 chlorophyll molecules the Mg ion was replaced by Cu. The above results are the first data concerning the natural plankton whereby the presence of copper in the chlorophyll extract was found. The subsequent part of the work deals with the question whether copper was incorporated in live algal cells and, if so, under what conditions the substitution could occur.

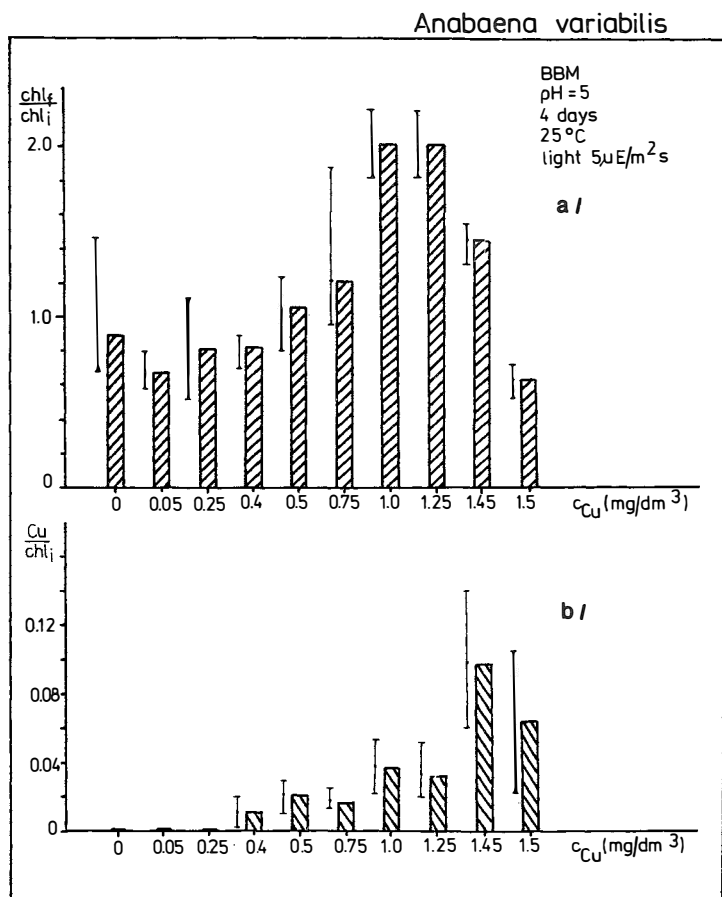


Fig. 3. *Anabaena variabilis* batch cultures in BBM, at pH = 5, 25 °C, exposed to light of 5 $\mu\text{E}/\text{m}^2\text{s}$: a) chlorophyll to initial chlorophyll ratio ($\text{chl}_f/\text{chl}_i$) vs copper concentration in medium (C_{Cu}); b) copper to initial chlorophyll ratio (Cu/chl_i) vs copper concentration in medium (C_{Cu})

Figs 2–7 present results of the studies on effects of pH, light, Cu and Fe contents in the medium on the copper content in the blue-green algae chlorophyll extract. Figs 2–5 show results obtained for *Anabaena variabilis*, while Figs 6 and 7 illustrate the data for *Anacystis nidulans*.

Based on the above results, one observes firstly that both in the case of *Anabaena variabilis* and *Anacystis nidulans*, no copper was found in the chlorophyll extracts of cultures kept in darkness (Figs. 5b and 7b). The observation confirms the literature data pointing to the fact that toxic effects of copper occur only in illumination (Azeez et al. 1987; Steemann Nielsen and Wium-Andersen 1971).

For both *Anabaena variabilis* and *Anacystis nidulans*, all the light-exposed cultures showed a decrease, and then again an increase to the maximum, in the chloro-

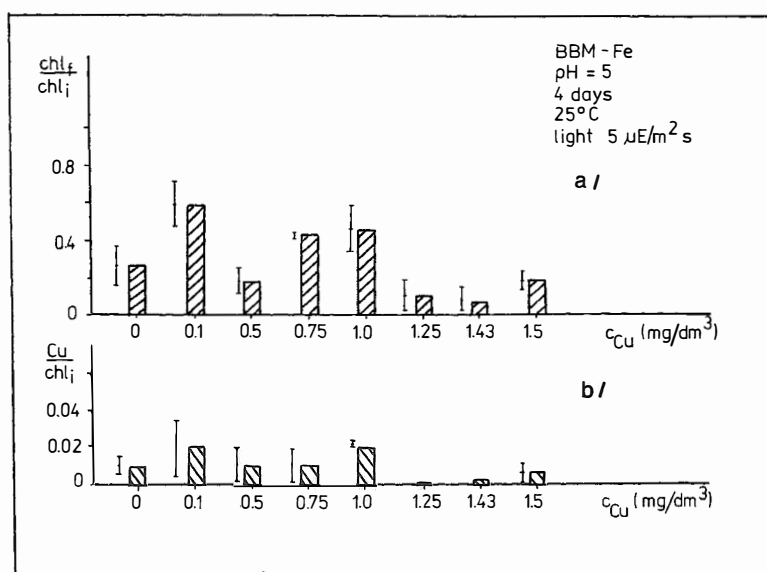
Anabaena variabilis

Fig. 4. *Anabaena variabilis* batch cultures in BBM - Fe, at pH = 5, 25°C, exposed to light of 5 $\mu E/m^2 s$: a) chlorophyll to initial chlorophyll ratio ($\frac{chl_f}{chl_i}$) vs copper concentration in medium; b) copper to initial chlorophyll ratio ($\frac{Cu}{chl_i}$) vs copper concentration in medium (C_{Cu})

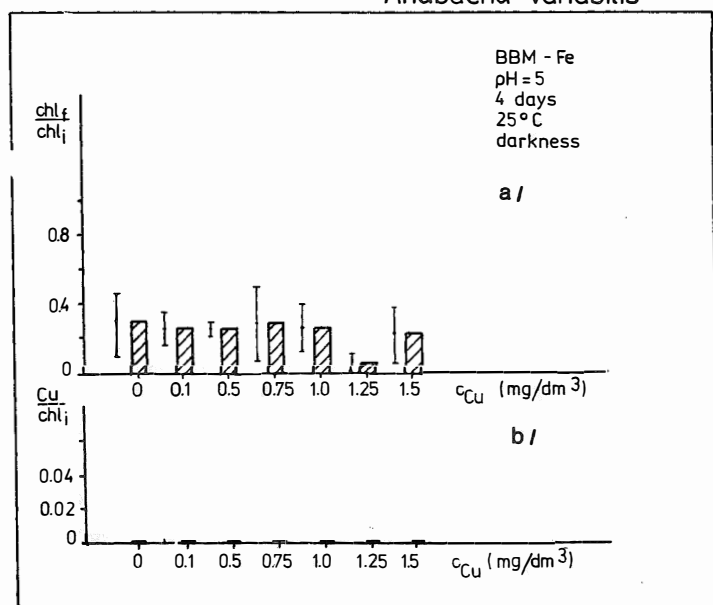
Anabaena variabilis

Fig. 5. *Anabaena variabilis* batch cultures in BBM - Fe, at pH = 5, 25°C, darkness: a) chlorophyll to initial chlorophyll ratio ($\frac{chl_f}{chl_i}$) vs copper concentration in medium (C_{Cu}); b) copper to initial chlorophyll ratio ($\frac{Cu}{chl_i}$) vs copper concentration in medium (C_{Cu})

Anacystis nidulans

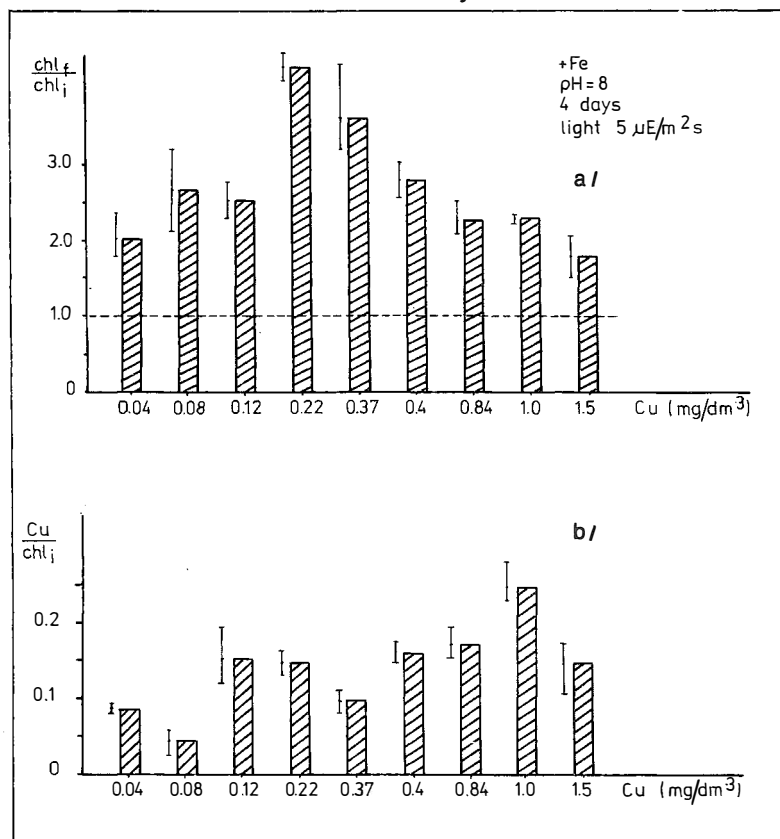


Fig. 6. *Anacystis nidulans* batch cultures in BBM, at pH=8, 25°C, exposed to light of $5 \mu E/m^2 s$: a) chlorophyll to initial chlorophyll ratio (chl_f/chl_i) vs copper concentration in medium (C_{Cu}); b) copper to initial chlorophyll ratio (Cu/chl_i) vs copper concentration in medium (C_{Cu})

phyll content to the initial chlorophyll content ratio (chl_f/chl_i) in relation to copper concentrations in the medium (C_{Cu}) (Figs 2a, 3a, 4a, 6a), but the function shifted along the abscissa depending on the presence in the medium of components affecting copper speciation. pH and Fe concentration are the major parameters which can change the equilibrium.

Two pH values were tested: pH 5 at which copper should occur in solution mainly in the form of Cu^{2+} when considering the inorganic species only; and pH 8 typical of the surface seawater, when the equilibrium is shifted towards the inorganic complexes $Cu(OH)_2$ and $CuCO_3$, that is when the amount of free Cu^{2+} ions is proportionally lower. Comparison of the results for *Anabaena variabilis* at pH 5 and 8 (Figs 2a and 3a) shows that the copper concentrations at which the chl_f/chl_i vs. C_{Cu} function

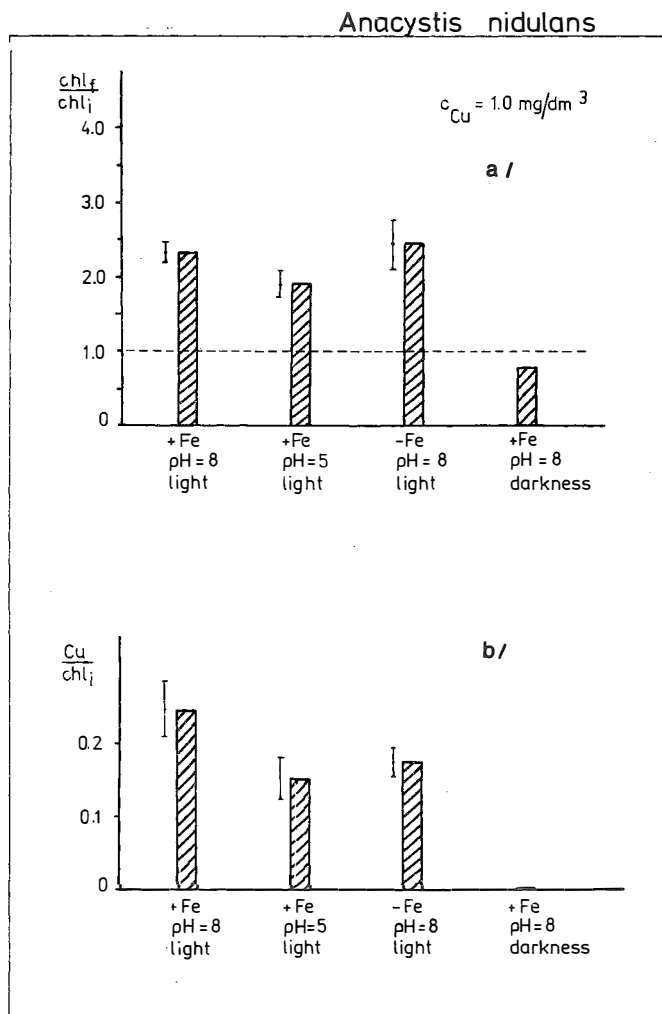


Fig. 7. *Anacystis nidulans* batch cultures in BBM with or without iron, copper concentrations in medium $C_{Cu} = 1.0 \text{ mg/dm}^3$, exposed to light of $5 \mu\text{E/m}^2\text{s}$ or kept in darkness: a) chlorophyll to initial chlorophyll ratio ($\frac{chl_f}{chl_i}$), b) copper to initial chlorophyll ratio ($\frac{Cu}{chl_i}$)

attains its maxima are $1.0\text{--}1.25 \text{ g/dm}^3$ and 1.25 mg/dm^3 , respectively, which is in agreement with the opinion mentioned at the beginning of ionic copper being more toxic to algae. The results obtained for *Anabaena variabilis* cultures kept in the medium with (Fig. 3a) and without (Fig. 4a) iron as well as those of *Anacystis nidulans* (Figs 6a and 7a) led to a similar conclusion, i.e. that copper is less toxic in the presence of iron, as the cultures were able to withstand much higher copper concentrations.

Based on the results presented, we may judge *Anabaena variabilis* to be more resistant to copper than *Anacystis nidulans* because the $\text{chl}_f/\text{chl}_i$ vs. C_{Cu} function reaches its maximum in the latter case at much lower Cu concentrations (cf Figs 2a and 6a), i.e. 1.25 mg/dm^3 and 0.22 mg/dm^3 , respectively.

Along with the maximum of the $\text{chl}_f/\text{chl}_i$ vs. C_{Cu} function, an increase in the copper content in the chlorophyll extract can be observed (Figs 2b, 3b, 4b, 6b). With reference to the previous studies on *Anabaena variabilis* (Kowalewska and Hoffmann 1989) which demonstrated the formation of the copper-chlorophyll complex in a high Cu concentration medium we may suppose that the copper found in the chlorophyll extract is fixed in such a complex. This conclusion, when applied to the present results, implies that within a certain copper concentration range, some chlorophyll molecules are complexed with copper and new molecules of chlorophyll have to be produced within the cells of the plant organisms studied to enable them to develop normally. Moreover, the results presented suggest that copper is incorporated in the chlorophyll porphyrin ring in live cells.

CONCLUSIONS

– The copper content in chlorophyll extracts from plankton samples dominated by phytoplankton, collected in the Baltic Sea, was substantial (Cu/Mg ratios of up to 0.1) in contrast to samples dominated by zooplankton in which the ratio was null; it means that copper forms a complex with chlorophyll in phytoplankton cells.

– The experiments on laboratory cultures of two blue-green algal species, *Anabaena variabilis* and *Anacystis nidulans*, showed that the replacement of magnesium by copper occurs in live cells and that the process requires light to occur.

– Within a certain concentration range, copper stimulates the increase in chlorophyll content in blue-green algal cultures, which is possibly caused by copper fixing some chlorophyll molecules so that the cell has to produce new molecules of chlorophyll to function normally.

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