Copper toxicity on the marine microalga *Phaeodactylum tricornutum*: effects on photosynthesis and related parameters

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Abstract

Essential heavy metals, as copper, can be toxic for microalgae at high concentrations. Copper affected growth and other parameters closely related to photosynthesis of the marine diatom *Phaeodactylum tricornutum*. A copper concentration of 0.10 mg l^{-1} provoked about 50% growth reduction and 1 mg l^{-1}inhibited the growth. Copper also interfered with photosynthesis and ATP production. A copper concentration of 0.5 mg l^{-1} reduced in a 50% the photosynthetic rate. Therefore, growth is more affected by copper than photosynthesis. Results of chlorophyll *a* fluorescence obtained by flow cytometry showed that copper's inhibitory effect on PS II activity is located on its oxidizing side. The lower copper concentration assayed provoked a significant decrease in the cellular pool of ATP. Pigment analysis by HPLC showed that copper affected the pigment pattern of *P. tricornutum*. Important changes were observed for chlorophyll *a* and its allomer: chlorophyll *a* proportion decreased while its allomer increased with the copper concentration, being maximum at 1 mg Cu l^{-1}. The study of the intracellular pH by flow cytometry revealed that *P. tricornutum*cells exposed to 0.5 and 1 mg Cu l^{-1} showed an intracellular pH higher than control cultures cells, explaining the high proportion of the chlorophyll *a* allomer in these cells.

Keywords

Copper toxicity; Phaeodactylum tricornutum; Photosynthesis; Flow cytometry

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1. Introduction

Heavy metals are among the most frequently identified pollutants in aquatic environments. From a biological point of view, heavy metals can be divided in two categories: essential and non-essential. However, essential heavy metals have been also reported to be toxic at high concentrations. Many of these metals have a direct influence on various physiological and biochemical processes. Heavy metals are known to interfere with a variety of photosynthetic functions (Clijsters and Van Assche, 1985; Mohanty and Mohanty, 1988; Murthy et aI., 1989).

Copper is an essential micronutrient for growth, metabolism and enzyme activities of various algae, cyanobacteria and other organisms; however, it is also a proven inhibitor of algal growth at high concentrations (Erickson, 1972).

Different aspects of copper toxicity on micro algae were evaluated in the marine diatom *Phaeodactylum tricornutum*. Parameters closely associated with photosynthesis, such as radioactive carbon assimilation, ATP concentration and photosynthetic pigment pattern, were evaluated, besides growth rate, the classical parameter utilized in toxicity assays.

2. Materials and methods

Algae cultures. *Phaeodactylum tricornutum* Bohlin (Bacillariophyceae) (isolated from Ria de Arousa waters by Dr. J. Fabregas, University of Santiago, Spain) was cultured in batch conditions in natural seawater filtered through a 0.45 µm Millipore filter; after that, this filtered seawater was newly filtered through an active carbon filter to eliminate organic chelating substances, and autoclaved at 120°C for 60 min. The assays were carried out in this raw, unenriched, sea water, with no inorganic nutrients added. Copper concentrations assayed were 0.05, 0.10, 0.50 and 1 mg Cu l^{-1}, added as copper chloride; control cultures without copper were also included. The free copper ion concentration in each culture was quantified (Abalde et aI., 1994), showing that in this range of concentrations, all copper added to the cultures appeared as free copper ion. Salinity of seawater was $35\%_0$ and the initial pH of the cultures was 7.6. Cultures were grown in KIMAX test tubes, containing 40 ml of medium. The tubes were previously rinsed with nitric acid and washed several times with redistilled water. Cultures were maintained at 18 ± 1°C and 140 µmol photon m^{-2} s^{-1}, with a dark: light cycle of 12: 12 h. Initial cell density was 24 x 10^4 cells m l^{-1}. All experiments were carried out in triplicate.

Measurement of growth. Growth of the microalgal cultures was measured by counting culture aliquots in a Neubauer hemocytometer. Microalgal growth is characterized by a sigmoid or logistic function. The form of the logistic growth function used at the present work was (Schanz and Zahler, 1981):

$$y(t) = KI[1 + B \exp(-zt)]$$

where $y(t)$ represents the cell density at time t and K its ultimate limiting value ('carrying capacity'). B is a biologically unimportant constant, and its value was calculated by the following equation: $B = (K\text{-}y_0)/ K\text{-}y_0$. The parameter z is related with growth rate. All the parameters of the equation were fitted by non-linear regression using Marquadt's algorithm.

Measurement of photosynthesis. The effect of copper on photosynthesis was determined by estimation of carbon fixation, recording the uptake of ^{14}C from $NaH^{14}CO_3$ ^{14}C-bicarbonate (Amersham, 674 µCi mg^{-1}) was added to the micro algal suspensions in culture tubes to give an activity of 0.01 µCi mg^{-1}. Incubation periods tested were 0.5, 1,2 and 4 h, and the photosynthetically fixed radioactivity in microalgal cells was counted in a LKB scintillation counter with Readysafe (Beckman) as scintillator.

Determination of ATP. ATP extraction was carried out using the method of Holm-Hansen (1970), as described by Larsson and Olsson (1979), after 8 h of copper exposure. ATP concentration was determined using a commercial bioluminescence assay CATP bioluminescence CLS', Boehringer-Mannheim) based in the luciferin-luciferase reaction. The constant light signal produced in this reaction was measured in a LKB scintillation counter.

Chlorophyll a. The analysis of chlorophyll a was carried out after 24 h of copper exposure. Pigments were extracted in acetone 90% at 4°C, in dark conditions, and concentrated by evaporation of the solvent. Dry extracts of pigments were resuspended in 100 µl of methanol (HPLC grade). The HPLC analysis of photosynthetic pigments was carried out as Wright et al. (1991).

Chlorophyll *a* was also determined spectrophotometrically; the concentration was calculated using the formula of Jeffrey and Humphrey (1975).

Flow cytometry determinations. Chlorophyll *a* fluorescence and intracellular pH were determined after 8 and 24 of copper exposure by flow cytometry (FCM), using a FACScan flow cytometer (Becton Dickinson Instruments), equipped with an argonion excitation laser (488 nm). Propidium iodide (final concentration: 60 µM) was used to counterstain the DNA of dead cells. Forward light scatter (FSC), which can be correlated with the size of the cell, and the fluorescence of the propidium iodide (PI), which can be correlated with the viability of the cells, were used to set gating levels. In the study of chlorophyll *a* fluorescence, aliquots of micro algal cultures were directly analyzed in the flow cytometer. The chlorophyll *a* fluorescent emission was collected in the FL3 (660 nm) channel.

The FCM technique used for the determination of variations in the intracellular pH is based on the pH-dependent emission intensity of a fluorescein derivate, 2'-7'-dichlorofluorescein (DC F) (Vissier et aI., 1979). A suspension of *P. tricornutum* cells (cell density: 24 x 10^4 cells ml^{-1}) were stained with this fluorescent compound and analyzed in the flow cytometer. Final DCF concentration achieved was 20 µM; the green fluorescent emission of this compound was collected in the FLI (530 nm) channel.

Data analysis. Data were statistically analyzed by an one-way analysis of variance (ANOVA) and, when differences observed were significatives, means were compared by the multiple range Duncan test, at a level of significance of 0.05.

For each cytometric parameter investigated, 104 events (cells) were analyzed per condition and fluorescence measurements were in the logarithmic scale. Data collection was performed using the list mode. The mean of fluorescence for any given population was provided by the instrument software (LYSIS II program; Becton Dickinson Instruments).

Data on chlorophyll *a* fluorescence and intracellular pH of P tricornutum cells were expressed as a percentage (%) of the fluorescence of the control cells according to the equation of Reader et al. (1993):

$$\%F = 100 - [100\ (F_c - F_t) / F_c]$$

where $\%F$ is the percentage of fluorescence of the *P tricornutum* cells; F_c the mean fluorescence of control cells; and F_t mean fluorescence of copper-treated cells.

3. Results

3.1. Growth

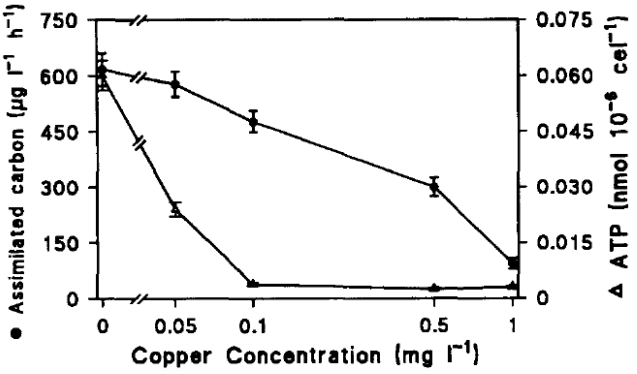
Phaeodactylum tricornutum grew in all copper concentrations assayed except in cultures with 1 mg Cu l^{-1} (Table I). Maximum cell density was reached in control cultures, with 149.25 x 10^4 cells ml^{-1}; maximum cell density achieved in cultures with 0.05 mg Cu l^{-1} (135.00 x 10^4 cells ml^{-1}) was not significantly different than that obtained in control cultures ($P < 0.05$). A copper concentration of 0.10 mg Cu l^{-1} provoked an strong decrease in growth, and maximum cell density obtained in these cultures was about a 50% lower than those reached in control cultures (Table 1).

Microalgal growth is characterized by a sigmoid or logistic function and the parameters of their mathematical functions are also shown in Table 1. The growth of the cultures with I mg Cu l^{-1} did not fit to the logistic function. The carrying capacity calculated with this function correlated well with the maximum cell density observed experimentally (Table 1).

Table 1: Maximum cellular densities (expressed in cells x 10^4 ml^{-1}), logistic growth function parameters and cellular content in chlorophyll *a* (pg $cell^{-1}$) obtained in cultures of *Phaeodactylum tricornutum* exposed to different copper concentrations, expressed as mg l^{-1}

		Logistic function parameters				
Cu	Maximum density	*K*	*B*	*z*	*r*	Chlorophyll *a*
Control	149.25 ± 11.93	157.40	7.67	1.59	0.99	0.06 ± 0.003
0.05	135.00 ± 14.25	147.00	6.85	1.29	0.99	0.08 ± 0.004
0.10	71.62 ± 9.87	76.62	2.41	1.03	0.99	0.11 ± 0.009
0.50	37.50 ± 1.80	36.50	0.53	0.75	0.85	0.08 ± 0.003
1.0	24.00 ± 3.00	–	–	–	–	0.09 ± 0.006

Fig, I. Photosynthetic rate as assimilated carbon (µg l^{-1} $^{h-1}$) and intracellular ATP content (nmol x 10^{-6} $cell^{-1}$) of *Phaeodactylum tricornutum* cells exposed to different copper concentrations (mg l^{-1}),



3.2. Photosynthesis

Photosynthesis, expressed as ^{14}C-bicarbonate assimilated (µg C 1^{-1} h^{-1}), decreased when copper concentration increased (Fig. 1). After 4 h of metal exposure, copper concentrations of 0.05 mg l^{-1} and higher provoked a strong decrease in the photosynthetic rate. In control cultures the photosynthetic rate was 617.81 µg C l^{-1} h^{-1}, whereas in cultures with I mg Cu l^{-1} this rate was 94.28 µg C l^{-1} h^{-1}, significantly lower than that obtained in control cultures.

3.3. ATP

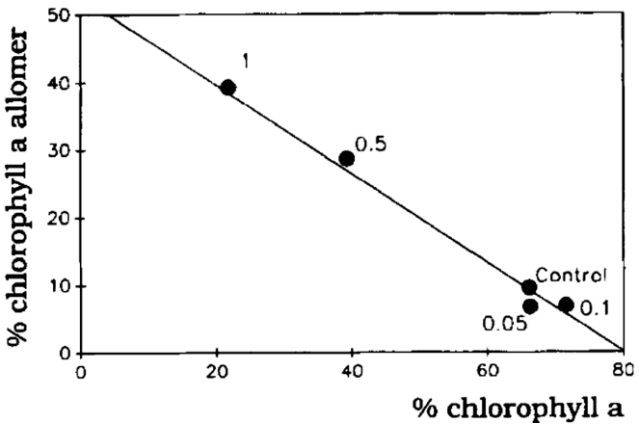
Results obtained showed that the ATP content of *Phaeodactylum tricornutum* cells was drastically affected by copper (Fig. I). Intracellular ATP concentration in control cultures was 0.060 nmol ATP x 10^{-6} cells; this value decreased when copper concentration increased, being 0.004 nmol x 10^{-6} cells in cultures with 0.1 mg Cu l^{-1}. There were no significant differences between these cultures (0.1 mg Cu 1 l^{-1}) and cultures with higher copper concentrations.

3.4. Chlorophylls

Cell contents of chlorophyll *a* of *Phaeodactylum tricornutum*, calculated from spectrophotometric data, are shown in Table 1. Chlorophyll a content increased when copper concentration increased to 0.1 mg Cu l^{-1} (0.11 pg $cell^{-1}$). Copper concentrations higher than 0.1 mg l^{-1}, provoked a decrease in chlorophyll a content (Table 1).

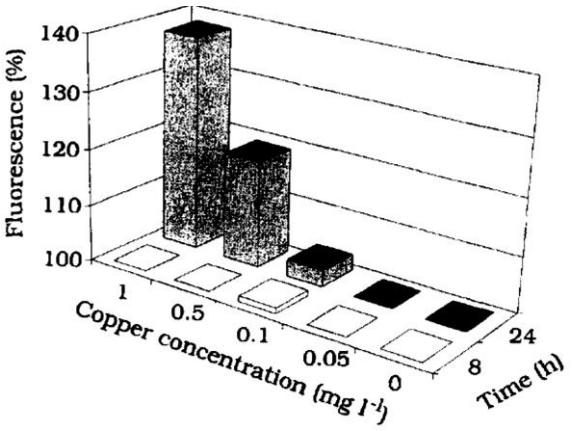
The analysis of pigments by HPLC showed that copper affected the pigment pattern of *P tricornutum* after 24 h of copper exposure. In the figure 2 are represented the chlorophylls expressed as percentage of the total pigments resolved by HPLC (chlorophylls, xanthophylls and carotenoids). Important changes were observed for chlorophyll *a* and its allomer. The proportion of the chlorophyll *a* allorner increased when copper concentration increased in the medium, while the proportion of chlorophyll a decreased (Fig. 2). The maximum proportion of chlorophyll a was observed in cultures with 0.1 mg Cu l l^{-1} (70.54%), whereas the proportion of the chlorophyll *a* allomer in these cultures was 6.97%. Higher concentrations of copper provoked an inversion in the proportions of both pigments: chlorophyll a decreased while its allomer increased, being maximum at 1 mg Cu l^{-1} (39.31 %).

Fig. 2. Relationship between chlorophyll *a* and its allomer (expressed as percentage of total pigments) in *Phaeodactylum tricornutum* cells exposed to different copper concentrations (mg l^{-1}) (r = 0.99).



The proportions of chlorophyll a and its allomer showed a negative correlation (Fig. 2). There were not significant differences in this ratio among control cultures and cultures with 0.05 and 0.10 mg Cu l^{-1}, but higher concentrations of copper provoked an inversion of this ratio.

Fig. 3. Chlorophyll a fluorescence after copper exposure in *Phaeodactylum tricornutum* cells. Data on chlorophyll *a* fluorescence are expressed as the percentage of the fluorescence of control cells, according to the equation of Reader et al. (1993). Results are the means of three replicates.



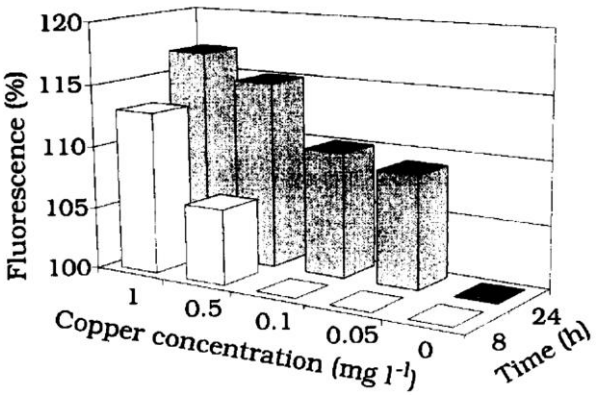


Fig. 4. Intracellular pH after copper exposure in *Phaeodactylum tricornutum* cells. 2'.7'-dichlorofluorescein was used as the fluorescent probe to evaluate intracellular pH. Data on intracellular pH are expressed as the percentage of the fluorescence of control cells, according to the equation of Reader et al. (1993). Results are the means of three replicates.

3.5. Chlorophyll a fluorescence

The study of chlorophyll a fluorescence by flow cytometry showed a significant increase in this fluorescence in *Phaeodactylum tricornutum* cells exposed to 0.5 and I mg Cu l^{-1}, after 24 h of metal exposure (Fig. 3). If the percentage of chlorophyll a fluorescence in control cultures is 100%, the fluorescence increase in affected cells was 19% and 38%, in cultures with 0.5 and 1 mg Cu 1^{-1}. respectively. After 8 h of copper exposure the differences were not significant.

3.6. Intracellular pH

As copper concentration increased in the medium, an increase of the intracellular pH was observed in cells of *Phaeodactylum tricornutum* analyzed for this purpose by flow cytometry (Fig. 4). Cells exposed to 0.5 and I mg Cu l^{-1} were the most affected after 24 h of metal exposure; in these cultures, the fluorescence emitted by the cells, stained with the fluorescein derivate used, accounted for 115 and 117% than the fluorescence observed in the stained control cultures cells (Fig. 4).

4. Discussion

One of the most studied toxic effect of copper on microorganisms is the effect on growth. Copper concentration that affects growth in micro algae is largely variable and depends on the species assayed, cell density, composition of the medium, physical culture conditions, etc. (Whitton, 1968; Stauber and Florence, 1985a). At the present work, a copper concentration of 0.10 mg 1^{-1} provoked about 5O% growth reduction of *Phaeodactylum tricornutum*, and 1 mg l^{-1} inhibited the growth (Table I). At the cell membrane, copper may interfere with cell permeability or the binding of essential metals (Sunda and Huntsman, 1983). Following copper transport into the cytosol, copper may react with -SH enzyme groups and free thiols (e.g. glutathione), disrupting enzyme-active sites and cell division (Stauber and Florence, 1985b; Florence and Stauber, 1986).

Copper may also exert its toxicity in subcellular organelles, interfering with photosynthesis in the chloroplasts (Overnell, 1975) and ATP production (Viarengo et aI., 1981). Photosynthesis inhibition by copper has been observed previously in other diatoms (Erickson, 1972; Overnell, 1975; Stauber and Florence, 1987). As growth, copper concentration that affects photosynthesis depends on the species assayed. Wong and Chang (1991) have observed that 0.1 mg Cu 1^{-1} produced a slight inhibition of photosynthesis in *Chlorella pyrenoidosa*, while 0.25 mg 1^{-1} produced total inhibition.

Results obtained in the present work showed that the photosynthesis rate of *P. tricornutum* cultures decreased when copper concentration increased; 0.5 mg Cu 1^{-1} reduced in a 50% the photosynthetic rate (Fig. 1). Therefore, growth is more affected by copper than photosynthesis. This uncoupling between division rate and photosynthesis can be due to copper inhibits the process of cell division indepedently of any effect on the production of new cell material (Stauber and Florence, 1987).

Chlorophyll *a* fluorescence is particularly sensitive to the functioning of photosystem II (PS II). Measurement of the fluorescence of chlorophyll a in intact algal cells provides information on the absorption, distribution and utilization of energy in photosynthesis (Papageorgiou, 1975; Fork and Mohanty, 1986). Algal fluorescence induction phenomena were used to study phytotoxicity (Moody et aI., 1983; Samson and Popovic, 1988). Chlorophyll a fluorescence monitored by the single-laser-based flow cytometer is the maximum fluorescence when the PS II reaction centers are locked in the Q_A - state (Xu et aI., 1990). It is known that the inhibition of the electron flow in the PS II reaction center at the donor side provokes a decrease in the chlorophyll a fluorescence, while if the inhibition is produced in the acceptor side of the PS II, an increase in the chlorophyll a fluorescence is observed (Butler, 1977; Samson et aI., 1988; Murthy et aI., 1990). In accordance with Samson et al. (1988), chlorophyll *a* fluorescence results obtained by flow cytometry (Fig. 3) show that copper's inhibitory effect on PS II activity is located on its oxidizing side, probably because copper inactivates some PS II reactions centers.

Photosynthesis is driven by two photosystems, I and II, leading to the oxidation of H_20 to molecular O_2, to the reduction of a pyridine nucleotide, and to the production of ATP. So any compound which affects photosynthesis, would also be expected to alter the ATP intracellular content. Results obtained showed that minimum copper concentrations assayed (0.05 mg l^{-1}) provoked a significant decrease in the cellular pool of ATP in P tricornutum cells (Fig. 1). This copper concentration had no effect on photosynthesis; this fact can be explained by a higher consumption of ATP in the cell to avoid the toxic effects of copper, maintaining cellular integrity and activating metabolic processes related with cell growth; results obtained showed that growth was not affected by 0.05 mg Cu 1^{-1}, which reduced the ATP content.

The pigment composition of diatoms is a consequence of environmental factors (Klein, 1988). Klein (1988) has suggested that chlorophyll *a* allomer is related to active growth in no stress conditions; the chlorophyll *a*: chlorophyll *a* allomer ratio is lowest during the period of active

growth and increases when growth declines. However, in *Phaeodactylum tricornutum* cultures with 0.5 and 1 mg Cu 1^{-1} the growth was inhibited and cells presented a higher proportion of chlorophyll *a* allomer than chlorophyll a (Fig. 2). Chlorophyll *a* allorner is produced by the oxidation of chlorophyll *a* at alkaline pH. The study of the intracellular pH by flow cytometry revealed that *P tricornutum* cells exposed to 0.5 and 1 mg Cu l^{-1} showed an intracellular pH higher than control cultures cells (Fig. 4), explaining the high proportion of the chlorophyll a allomer in these cells.

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