

Toxic action of copper on the membrane system of a marine
diatom measured by flow cytometry

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ABSTRACT

Flow cytometric measurements were used to investigate the toxic action of copper on some *Phaeodactylum tricornutum* membrane systems. Throughout the time of metal exposure, the percentage of viable cells decreased as copper concentration increased. The forward scatter signal increased as a result of copper exposure. After 72 h of metal exposure, cultures with 0.5 and 1 mg l⁻¹ of copper showed an important increase in the peroxidase activity in comparison with control cells. Cells cultured with copper presented alterations in the membrane potential, increasing as copper concentration increased, after 96 h of metal exposure. Results obtained in this work showed that copper induced a degenerative process in *P. tricornutum* cells, closely related with alterations or disorders in membrane systems.

Keywords:

Flow cytometry; microalgae; viability; forward scatter; peroxidase activity; membrane potential

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 (5). The increasing occurrence of heavy metals, copper included, has stimulated many studies on the toxicity to aquatic microorganisms, and the need for convenient methods for assayed pollutants toxicity has become evident. The response of microalgae to a toxicant is typically measured using population-based parameters (11, 12), such as specific growth rate, biomass, cell yield, chlorophyll fluorescence, and primary production. The bulk population based endpoints used in algal toxicity tests did not supply information on the distribution of responses among the individual cells within the population. Flow cytometry is an alternative to the standard algal population-based endpoints, since it allows the rapid and quantitative measurement of responses of individual algal cells to a toxic stress.

Microorganisms, and microalgae in particular, are the first organisms affected by heavy metals discharges in aquatic environments (9) because they are directly in contact with the medium, separated only by the cytoplasmic membrane and the cell wall. Cellular membranes are selective, dynamic barriers that play an essential role in

regulating biochemical and physiological events, so any alteration produced in the environment provokes changes in microorganisms membranes.

The present work studies the effect of different copper concentrations on growth and different parameters closely related to cellular membranes, in the marine microalgae *Phaeodactylum tricornerutum*. Flow cytometric measurements were used to investigate the mode of toxic action of copper to some *P. tricornerutum* membrane systems.

MATERIALS AND METHODS

Algal Cultures

Phaeodactylum tricornerutum Bohlin (Bacillariophyceae) (isolated from Ria de Arousa waters by Dr. J. Fábregas, University of Santiago, Spain) was cultured in batch conditions in seawater filtered through a 0.45 μm Millipore filter, and autoclaved at 120°C for 60 min. Microbiological studies on heavy metals are generally performed in synthetic growth media, the constituents and properties of which can greatly influence the free concentrations, and thus toxicity, of metals. Because of this, the assays were carried out in raw, unenriched sea water, with no inorganic nutrients added. *P. tricornerutum* grows normally in raw sea water, as has been previously shown (3). Salinity of seawater was 35‰ and the initial pH of the cultures was 7.6. Cultures were grown in KIMAX test tubes, containing 40 ml of seawater. The tubes were previously rinsed with nitric acid and washed several times with redistilled water. Cultures were maintained at $18 \pm 1^\circ\text{C}$ and $140 \mu\text{mol photon m}^{-2} \text{s}^{-1}$, with a darklight cycle of 12:12 h. Initial cell density was 2.4×10^5 cells ml^{-1} . 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. All experiments were carried out in triplicate.

Measurement of Growth

Growth of the microalgal cultures was measured by counting daily culture aliquots in a Neubauer hemocytometer, during the 96 h of copper exposure. Growth rate, expressed as doublings day^{-1} , was calculated using the following formula:

$$\mu = (\ln N_t - \ln N_0) / \ln 2 (t - t_0)$$

where N_t , is the cell density at time t and N_0 is the cell density at time 0.

The most common parameter used in toxicity assays is the EC_{50} , i.e., the concentration of the tested substance which decreases the growth rate by 50%. The easiest way to obtain an EC_{50} , value is the graphic interpolation; however, in order to obtain a confidence interval for the EC_{50} , value, a statistical method, such as probit analysis, should be used. Probit analysis of growth data was carried out using the SPSS-PC + software.

Flow Cytometry Determinations

Forward scatter and cell viability, peroxidase activity, and membrane potential were determined during copper exposure (96 h) by flow cytometry (FCM), using a FACScan flow cytometer (Becton Dickinson Instruments, San Jose, California), equipped with an argon-ion excitation laser (488 nm). Fluorescence signals were collected at 90° to the light beam, split by a dichroic mirror, and detected by photomultiplier tubes (PMT). Scattered light was removed from fluorescence measurements using a 515 nm laser blocking pair. Autofluorescence from chlorophyll *a* was separated from the green and orange fluorescences of the different fluorochromes, using short pass filters. The interval of fluorescence collected by the different PMT were 530-560 nm for the green fluorescence (FL1 channel), 560-590 nm for the orange fluorescence (FL2), and 660-700 nm for the red fluorescence (FL3). Chlorophyll *a* red fluorescence histograms were used to set gating levels, excluding particles without red fluorescence, which are obviously non-algal particles.

Cell viability. The fluorescence of cells stained with propidium iodide (PI; Sigma Chemical Co.) was measured to study cell viability. PI is a fluorescent dye that intercalates with double-stranded nucleic acids to produce red fluorescence when excited by blue light. It is unable to pass through intact cell membranes; however, when the cell dies the integrity of the cell membrane fails, PI is able to enter and stain the nucleic acids (14). In this way, PI can be used to discriminate between live nonfluorescent cells and non-viable fluorescent cells; the orange fluorescent emission of this compound was collected in the FL2 channel (560-590 nm). aliquots of 2.4×10^5

cells ml^{-1} were stained with PI to a final concentration of $60 \mu\text{M}$, during an incubation period of 20 min.

This staining procedure was also applied in the study of cell volume and of membrane potentials.

Forward scatter. Since this signal (FSC) can be correlated with the size of the cell (19), aliquots of microalgal cultures, stained with PI, were analyzed to study the possible changes in cell volume. Only viable cells were analyzed.

Peroxidase activity. Flow cytometry techniques have important advantages over conventional biochemical assays of enzyme activities, particularly as cells can be assayed under near physiological conditions (19). Dihydroethidium, also called hydroethidine (HE; Molecular Probes, Inc.), is a chemically reduced fluorophore. Cytoplasmic dihydroethidium has blue fluorescence, but when intracellular peroxidases, in combination with reactive oxygen species (peroxide and superoxide), catalyze the oxidative reaction, ethidium, a highly red fluorescent product, is obtained (2, 8). The orange fluorescent emission of this compound was also collected in the FL2 channel. Aliquots of 2.4×10^5 cells ml^{-1} were stained with HE to a final concentration of 10.3 mM . The incubation time was 30 min.

Membrane potential. Flow cytometry was first demonstrated to be applicable to analysis of membrane potential by Shapiro et al. (20), and the techniques used subsequently are fundamentally unaltered. The dyes used for this purpose are lipophilic to permit passage of lipid bilayers, and are positively charged as the interior of the cell and of the mitochondria are negative; once the cells are equilibrated with the probe, depolarization (decrease in potential difference) will cause release of the dye into the medium, and hyperpolarization (increase in potential difference) will cause uptake of the dye (15). The dyes used were 3,3'-dihexyloxacarbocyanineine, abbreviated DiOC₆(3) (Sigma Chemical Co.), and rhodamine 123 (Rh123; Sigma Chemical Co.) (8). Final concentrations used were $0.35 \mu\text{M}$ for DiOC₆(3) and $26 \mu\text{M}$ for Rh123. A centrifugation step is necessary in the staining procedure with Rh123, to eliminate the excess of dye in the medium. The green fluorescent emission of these compounds were collected in the FL1 channel (530-560 nm). When these dyes were used as membrane potential probes, PI was also added at concentrations described above, allowing non-

viable cells to be gated out of analyses on the basis of its orange fluorescence (FL2 channel).

Data Analysis

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

For each cytometric parameter investigated, 10^4 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).

Since results obtained by flow cytometry are qualitative, they are treated in a special way, making possible the comparison of data. Except in the study of the viability, data were expressed as a percentage (%) of the fluorescence (or forward scatter signal) of the control cells according to the equation of Reader et al. (16):

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

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.

RESULTS

Growth

Copper affected the growth of the marine diatom *Phaeodactylum tricornutum* (Fig. 1). There are not significant differences between control cultures, without copper, and cultures with 0.05 mg l^{-1} of copper ($P < 0.05$), with growth rates of 1.16 and 1.10 doublings day^{-1} , respectively. As copper concentration increased in the medium, the growth decreased; a copper concentration of 1 mg l^{-1} did not allow the growth of this diatom, with a growth rate close to 0. (Fig. 1). The EC_{50} , of copper for growth was 0.208 and 0.231 mg l^{-1} , after 48 and 96 h, respectively.

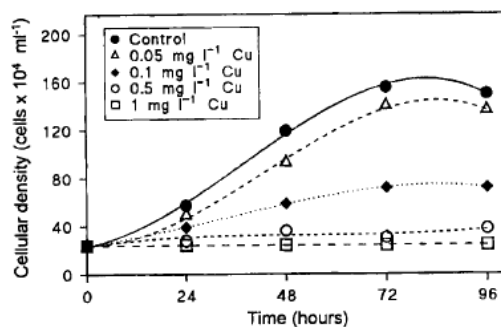


Fig. 1. Growth curves of *P. tricornutum* cultures with different copper concentrations (mg l^{-1}). Results are the means of three replicates.

Cell Viability

The evolution of cell viability did not show important variations in the first 24 h of copper exposure, but the proportion of viable cells decreased after 48 h in cultures with 0.5 and 1 mg l^{-1} (Table 1). After 96 h of copper exposure, the percentage of viable cells decreased to 76, 14 and 8% in cultures with 0.10, 0.50, and 1 mg l^{-1} , respectively.

Forward Scatter

Copper provoked an increase in the FSC signal of *P. tricornutum* cells (Figs. 2 and 3). The highest copper concentration assayed, 1 mg l^{-1} , provoked an important increase in FSC after 24 h of copper exposure, while the effect of the remaining copper concentrations were not visible until 48 or 72 h of metal exposure (Fig. 3). After 96 h of copper exposure, differences in forward scatter between control and all cultures with copper occurred, being maximum for cultures with 1 mg l^{-1} of copper, which provokes an increase of 49% in FSC, calculated as described before. This increase in forward scatter is correlated with an increase in the volume of the cells, observed using an optical microscope (unpublished data).

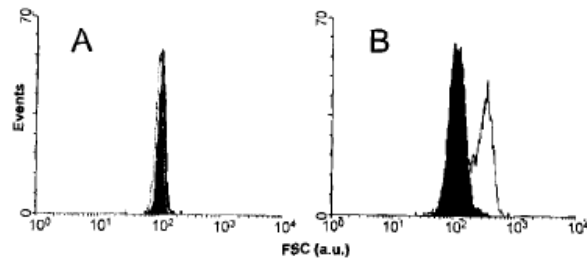


Fig. 2. Typical overlay of FSC signal histograms showing the profiles of a control sample (solid histogram) and a sample with the maximum copper concentration assayed (1 mg l^{-1}), after 8 and 96 h of culture (A and B, respectively).

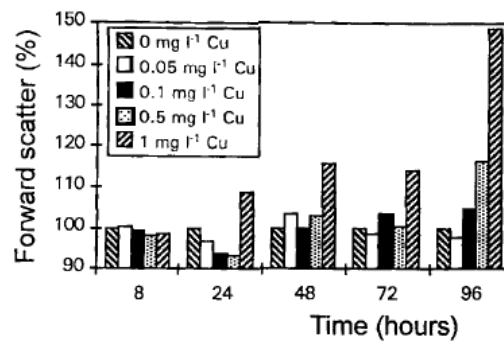


Fig. 3. Forward scatter, after copper exposure, of *P. tricornutum* cells. Data are expressed as the percentage of the FSC signal of control cells, according to the equation cited in the text. Results are the means of three replicates.

Peroxidase Activity

Figure 4 represents data on the changes observed in the peroxidase activity during the 96 h culture, expressed as percentage of the hydroethidine fluorescence of the control cells, according with the equation described by Reader et al. (16). After 48 h of copper exposure, viable cells exposed to copper showed a higher peroxidase activity (Fig. 4). After 72 h of copper exposure, only cultures with 0.5 and 1 mg l^{-1} of copper presented an important increase in the peroxidase activity respect to the control cells, while differences after 96 h were not relevant (Fig. 4).

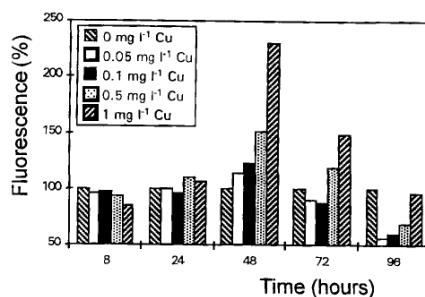


Fig. 4. Variations in the peroxidase activity after copper exposure of *P. tricornutum* cells. Hydroethidine was used as the fluorescent probe to evaluate the peroxidase activity. Data are expressed as the percentage of the fluorescence of control cells, according to the equation cited in the text. Results are the means of three replicates

Membrane Potential

Possible variations in membrane potential were measured after 24 and 96 h of copper exposure, using DiOC₆(3). After 24 h of culturing, differences in membrane potential were not found using this fluorochrome (Figs. 5 and 6); but after 96 h of metal exposure, all cells cultured with copper presented alterations in the membrane potential, which increased as copper concentration increased (Fig. 6).

The lipophilic cationic dye rhodamine 123 has been used for investigations of mitochondrial structure and function; it accumulates in energized mitochondria as a result of their membrane potential (18). Copper has also provoked an increase in the membrane potential studied using Rh123, following the same pattern described for the assays carried out using DiOC₆(3) (Fig. 7): after 96 h of metal exposure, all cultures with copper presented higher membrane potential than control cultures.

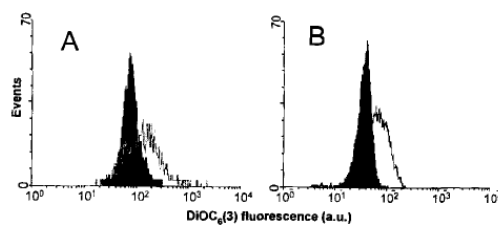


Fig. 5. Typical overlay of 3,3'-diethyloxycarbocyanine fluorescence histograms showing the profiles of a control sample (solid histogram) and a sample with the maximum copper concentration assayed (1 mg l⁻¹), after 24 and 96 h of culture (A and B, respectively).

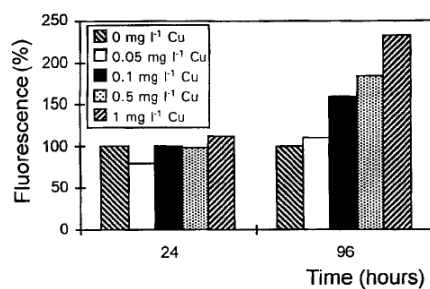


FIG. 6. Variations in the membrane potential after copper exposure of *P. tricornutum* cells, using 3,3'dihexyloxacarbocyanine as the fluorescent probe. Data are expressed as the percentage of the fluorescence of control cells, according to the equation cited in the text. Results are the means of three replicates.

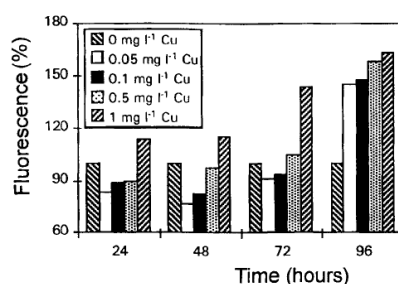


FIG. 7. Variations in the membrane potential after copper exposure of *P. tricornutum* cells, using rhodamine 123 as the fluorescent probe. Data are expressed as the percentage of the fluorescence of control cells, according to the equation cited in the text. Results are the means of three replicates.

DISCUSSION

Some metals play indispensable roles in cell growth and maintenance of metabolic functions, but when their concentrations in the environment increase above a threshold, many cellular changes can be detected as a response to the stress provoked. Results obtained indicated that growth of *Phaeodactylum tricornutum* cultures was affected by copper (Fig. 1).

Copper concentrations assayed provoked an increase in the forward scatter signal of *P. tricornutum* cells detected by flow cytometry, being maximum for cultures with 1 mg l⁻¹ of copper (Figs. 2 and 3). Microscopical analysis of these cells have shown an increase in size, probably due to the incapacity to finish the cell division because of copper exposure. These data can suggest a correlation between the increase in cell size

observed microscopically and the increase in the forward scatter signal. Other authors, using microscopic techniques, have observed an increase in the cellular volume in different microalgal species exposed to high concentrations of different heavy metals (1, 17, 21, 22).

The main characteristic of cell death, whether from senescence, acute stress, or aging, seems to be the loss of the cell's ability to maintain homeostasis (4,6). Cellular membranes are selective, dynamic barriers that play an essential role in regulating biochemical and physiological events. The viability of *P. tricornutum* cells decrease throughout 96 h of copper exposure (Table 1), showing a progressive loss of their membrane integrity, like occurs in the aging or senescence process (23). Whereas senescence represents endogenously controlled degenerative processes leading to death, aging encompasses a wide array of passive or nonregulated, degenerative processes driven primarily by exogenous factors (23).

Membranes could be expected to be highly prone to free radical attack inasmuch as unsaturated fatty acids are major components of most membrane lipid bilayers. The consequences of free radical attack on membranes are numerous and include the induction of lipid peroxidation (10), lysis (7), and fatty acid deesterification (13). Senescence is an active process initiated by some combination of internal and environmental triggers, and membrane deterioration is an early and fundamental feature of this process. Results obtained in this work show important changes in the cytoplasmic membrane. Peroxidase activity increased in cells exposed to the higher copper concentrations assayed (Fig. 4), where cell viability decreased. The peroxidase activity detected seems to be directly correlated to the progressive loss of function and structural integrity of the cell membrane, leading to the cell death or decrease of cell viability.

After 96 h of copper exposure, results obtained by flow cytometry showed that cells cultured with this metal presented an increase in the membrane potentials, increasing as copper concentration increased (Figs. 6 and 7). These changes in the membrane potentials can be associated with alterations provoked by the peroxidation of membrane lipids as consequence of free radical attack (copper in this case), and also with other changes found in these cells like the increase in the intracellular pH (3).

Results obtained in this work showed that copper induced a degenerative process in *Phaeodactylum tricornutum* cells, closely related with alterations or disorders in membrane systems.

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