Response of the marine microalga *Dunaliella tertiolecta* (Chlorophyceae) to copper toxicity in short time experiments

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Heavy metals are introduced into aquatic ecosystems from industrial wastes, agricultural runoff and mining activities. The toxicity of heavy metal ions in solution to microalgae is well known. The essentiality and toxicity of heavy metals has been a subject of extensive research (Stokes 1983). From a biological point of view, heavy metals can be divided into two categories: essential and non-essential. However, essential heavy metals also have been reported to be toxic at high concentrations. Many of these metals have a direct influence on various physiological and biochemical processes including reduction in growth, photosynthesis, and chlorophyll content or inhibition of enzyme activities (Reddy and Prasad 1990).

Microalgae already have been used as biological indicators to assay pollutant toxicity (Hornstrom 1990). Interest in microalgae can be explained because they make up most of the base production of marine ecosystems, a base which would probably cause effects on higher trophic levels if it was disturbed.

To evaluate different aspects of copper toxicity to marine microalgae, growth responses, photosynthetic pigment content, photosynthetic rates, cell viability, and cell volume of the naked marine microalga *Dunaliella tertiolecta* during exposure to selected concentrations of copper were examined.

MATERIALS AND METHODS

Dunaliella tertiolecta (Chlorophyceae) was batch cultured in seawater that had been filtered through a 0.45 11m Millipore filter, autoclaved at 120°C for 60 min and enriched with the growth medium reported by Fabregas et a1. (1986). Salinity of seawater was 35‰ and the initial pH of the cultures was 7.6. Cultures were grown in Erlenmeyer flasks, previously rinsed with nitric acid and washed several times with redistilled water, and containing 50 mL of medium. Cultures were maintained at 18±1 °C and 140 µmol photon m^{-2} S^{-1}, with a dark : light cycle of 12:12 hr. Aliquots of micro algal cultures in logarithmic phases, with a cell density of 2 $x10^6$ cells mL^{-1}, were exposed to different copper concentrations, added as copper chloride. Control cultures without copper also were included. All experiments were carried out in triplicate.

The stock copper solution was prepared by dissolving $CuCl_2$ in redistilled and sterilized water. Various volumes of copper stock solution (1000 mg Cu L^{-l}) were added to the cultures. Because the cupric ion can be precipitated or complexed in the enriched seawater, free copper was determined in each growth medium before inoculation. Seawater enriched with culture medium and different copper concentrations was filtered through 0.45 µm Millipore-MF filters. Filtrates were passed through a column of Ca-Chelex. The column was eluted with HNO_3 and eluates from the column were measured by atomic absorption spectrophotometry (AAS) (Figura and McDuffie 1977). Free copper concentrations obtained in the different cultures were: 8, 12, and 16 mg Cu^{+2} L^{-1}.

Growth of the microalgal cultures was measured by counting culture aliquots in a Coulter Counter model ZM, and the growth rates are expressed in doublings d^{-1}. Pigments were extracted in 90% acetone at 4°C for 24 hr, and the concentrations of chlorophyll *a* and *b* were determined by the formula of Jeffrey and Humphrey (1975), and carotenoids concentration by the formula reported by Strickland and Parsons (1972).

The effect of copper on photosynthesis was determined by estimation of carbon fixation, recording the uptake of HC from NaH_4CO_3. ^{14}C-bicarbonate (Amersham, 674 µCi mg^{-1}) was added to the microalgal suspensions in culture flasks to give an activity of 0.01 µCi mL^{-1}. Incubation periods tested were 0.5, 1, 2, and 4 hr, and the photosynthetically·fixed radioactivity in microalgal cells was counted in a LKB scintillation counter with Readysafe (Beckman) as scintillator.

Data were analyzed statistically by an overall one-way analysis of variance (ANOVA) ($P<0.05$).

Viability and cellular volume were determined using flow cytometric techniques (FCM). Aliquots of micro algal cultures in logarithmic phase were exposed to the different copper concentrations. After 1 hr of exposure, these cultures were analyzed in a FACScan flow cytometer (Becton Dickinson Instruments). Viability was measured by incubation of 500 µL of the culture sample for 5-10 min at room temperature with 10 µL of 3 *mM* propidium iodide (PI) (Sigma) in HBS (REPES buffered saline) (Rothe and Valet 1990); this compound stains non-viable cells and if excited by the blue light of an argon-ion laser (488 nm), give orange fluorescence (530-560 nm) that was measured in the flow cytometer. At least 10^4 cells were measured per sample. Results obtained by flow cytometry were analyzed with LYSIS II program (Becton Dickinson Instruments).

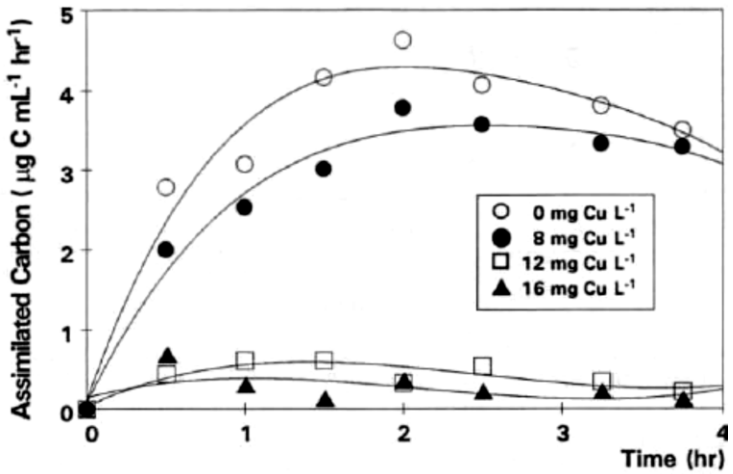

Figure 1. Photosynthetic curves of *Dunaliella tertiolecta* at different copper concentrations.

RESULTS AND DISCUSSION

Copper effects on growth and pigment content of *D. tertiolecta* were determined in 48 hr exposures. Growth in control cultures and in cultures with 8 mg Cu^{+2} L^{-1} was unaffected, with maximum cell densities of 3.17 and 3.28 xl0^6 cells mL^{-1}, respectively, and growth rates of 0.33 and 0.36 doublings d^{-1} (Table 1). These data showed a non-toxic effect of copper on growth at 8 mg Cu^{+2} L^{-1}. Growth was slightly affected in cultures with 12 mg Cu^{+2} L^{-1}cultures, with a cell density of 2. 73 x 10^6 cells mL^{-1}, and growth rate of 0.22 doublings d^{-1} (Table 1). A toxic effect of copper was observed at this concentration and growth was significantly lower than that obtained in the control cultures. Growth was affected strongly in cultures with 16 mg Cu^{+2} L^{-1}, and minimum

cellular densities were obtained (2.02 x10^6 cells mL^{-1}) (Table 1); growth rate was close to 0 (0.01 doublings d^{-1}).

Therefore, the toxic effect of copper on growth of the marine microalga *Dunaliella tertiolecta* was clearly demonstrated in cultures with 12 and 16 mg Cu^{+2} L^{-1}. 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 (Lustigman 1986; Stauber and Florence 1987). The concentrations used here were relatively high in comparison with those reported as toxic for other microalgae (Stauber and Florence 1987). Tolerance to heavy metals is considered highly specific (Visviki and Rachlin 1991) and it has already been observed that the *genus Dunaliella* is able to tolerate substantially higher concentrations of toxic heavy metals than most other marine microalgae (Davies 1976; Gimmler et aI. 1991). Lustigman (1986) used copper concentrations between 5 and 50mg Cu L-l with the same species of microalgae, and Riisgard et al. (1980) studied the effect of 5 and 10 mg Cu L^{-1} on *Dunaliella marina.* In addition to this, copper inhibition of growth is dependent on cell density (Stauber and Florence 1987), leading to decreased heavy metal toxicity when the cell density increases (Rai et al. 1991), and the cell density used in the present work is relatively high.

Table 1. Cellular densities after 48 hr exposure (cells x 10^6 mL^{-1}), growth rates (doublings d^{-1}) and cellular features of *Dunaliella tertiolecta* cells, in cultures exposed to different copper concentrations. *Cellular features values were obtained by flow cytometry and are expressed in percentages. Those cells stained with propidium iodide are named non viables.

Cu (mg L^{-1})	Cellular density (Cell x 10^6 mL^{-1})	Growth rate (doubling d^{-1})	abnormal size* (%)	non viable* (%)
0	3.17±0.10	0.33	1.54	0.78
8	3.28±0.08	0.36	2.32	0.70
12	2.73±0.15	0.22	1.81	1.17
16	2.02±0.01	0.01	21.36	45.36

Other unicellular algae have been reported to respond to toxic levels of metals by depressed cell division rates (Fisher et al. 1981). It has been suggested that copper might prevent the production of methionine which appears necessary for cell division (Davies 1976). It has also been proposed that metals inhibit microalgal cell division by binding reactive thiols on the tubulin molecule, which is important in spindle formation during mitosis (OnreIt 1983), Copper, or other toxic heavy metals, may bind to -SH groups and interfere with a number of associated metabolic pathways, one or more of which are essential for maintaining normal cell division rates (Fisher et aI. 1981).

Real copper concentrations of 8, 12 and 16 mg Cu^{+2} L^{-1} were also used to determine photosynthetic rates; cultures without copper were used as control. Values of

photosynthetic rates (Fig. 1) appeared very similar among cultures without copper (control) and with 8 mg Cu^{+2} L^{-1}, without significant differences between them. However, no ^{14}C-bicarbonate fixation occurred at the remaining copper concentrations assayed. Therefore, the inhibition point for the photosynthesis rate was between 8 and 12 mg Cu^{+2} L^{-1}. Copper inhibition of photosynthesis was observed previously in several species of microalgae. For instance, in the diatom *Nitzschia closterium* photosynthesis was affected at lower copper concentrations, above l00].lg Cu VI, with a cellular concentration of 0.2 $x10^6$ cells mL^{-1} (Stauber and Florence 1987).

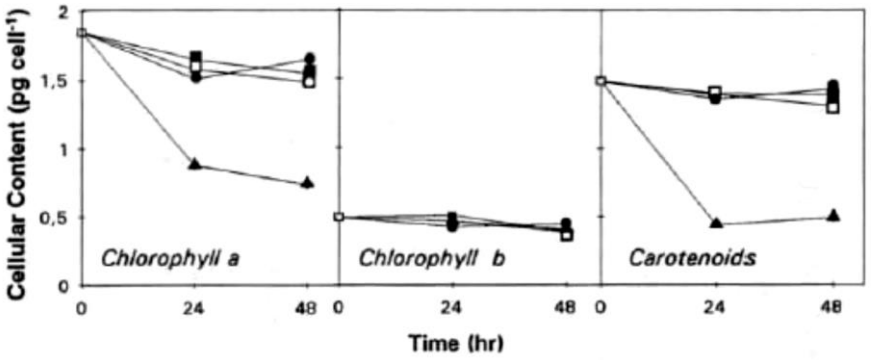

Figure 2. Pigment content (pg $cell^{-1}$) of *Dunaliella tertiolecta* grown at different copper concentrations. (■ 0 mg Cu L^{-1}; □ 8 mg Cu L^{-1}; ● 12 mg Cu L^{-1}; ▲ 16 mg Cu L^{-1})

Cell pigment contents of D. *tertiolecta* cultures during48hr of exposure to different Cu^{+2} concentrations are shown in Fig. 2. Chlorophyll *a* and carotenoid contents were more affected by Cu than chlorophyll *b* content. Chlorophyll *b* content remained constant in the different cultures during the test time with no differences among cultures with the different copper concentrations and control cultures (Fig. 2). Cellular content in chlorophyll *a* and carotenoids also remained constant except in cultures with 16 mg Cu Cu^{+2} L^{-1}; these cultures showed a strong decrease in cell content in chlorophyll *a* and carotenoids after 24 hr of copper exposure. Final pigment content, after 48 hr of copper exposure, was copper concentration dependent, decreasing as copper-concentration increased, with minimum values at 16 mg Cu Cu^{+2} L^{-1}. These values were 3.3 times lower than those obtained in control cultures for chlorophyll *a* and 4.4 times lower for carotenoids, whereas differences accounted for only 1.56 times chlorophyll *b*. Lustigman (1986) observed enhancement of pigment concentrations in *D. tertiolecta* as a result of copper toxicity; however, the duration of the experiments were different (14 days).

Besides these effects, cellular responses of *D. tertiolecta* to copper were also considered after 1 hr exposure. These responses were analyzed by flow cytometry (FCM) using a 1024~channel resolution. Copper exposure induced changes in cellular

volume (Fig. 3). There was a great increase in the cellular volume (FSC in flow cytometric terms) when the copper concentration increased (Table 1), observed by the movement of the population along the *x*-axis in the scattergrams (Fig. 3). The percentage of cells bigger than normal was 21.36 in cultures with 16 mg Cu^{+2} $L^{-1.}$ Changes in cellular volume were quantified defying an area corresponding to normal cellular volume in control cultures, and the percentage of cells into this area was calculated for the different cultures with copper. Normal cells in control cultures (the left-low quadrant in the Fig. 3) accounted to 98.19%; similar percentages occurred at 8 and 12 mg Cu^{+2} L^{-1}, with values of 97.36 and 97.79%, respectively. A strong decrease in the percentage of these normal cells was observed in cultures with 16 mg Cu^{+2} L^{-1}, with only 75.31% of normal cells.

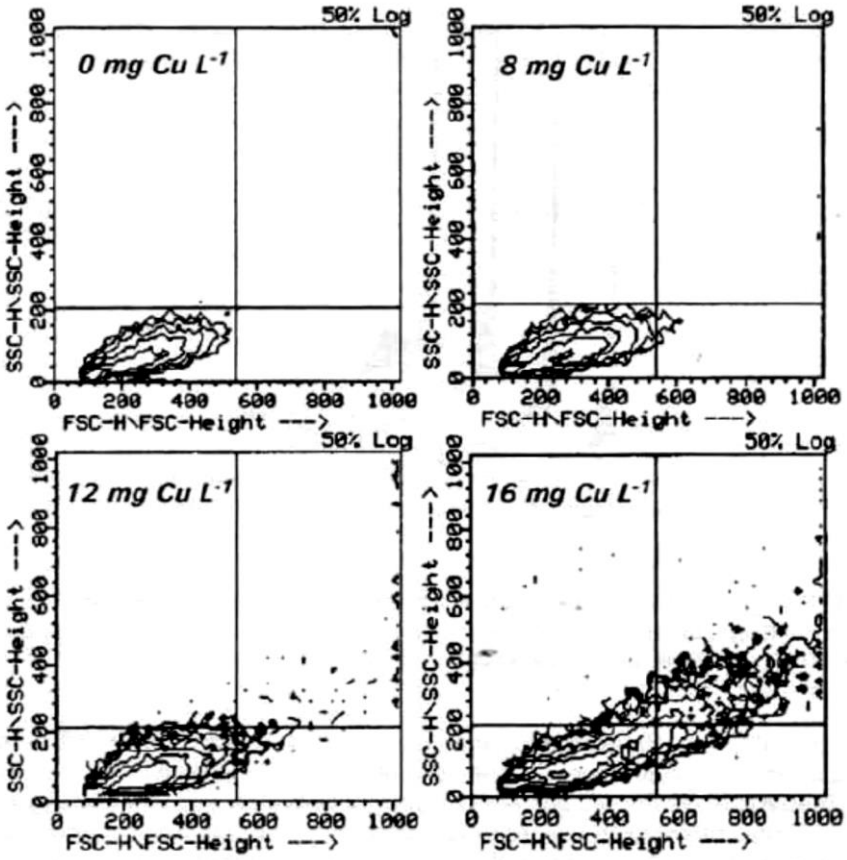

Figure 3. Scattergrams of samples of *Dunaliella tertiolecta* exposed to different copper concentrations. In these figures the intensity of laser light scattered in the forward direction (FSC related to volume) is plotted along the *x*-axis, while the *y*-axis represents the intensity of laser light scattered in the side direction (SSC related with cellular complexity).

Researchers examining the effects of heavy metals on uni-or multicellular algae have reported that, at least for some species, cells respond to toxic levels of metals by increased cell size (Fisher et a1. 1981; Stauber and Florence 1987; Bolanos et al. 1992). Increase in cell volume was noted in cells from several phyla and including

studies with different heavy metals. In the present work an increase in size was observed after only 1 hour; this result is in accordance with those found by Riisgard et a1. (1980) for D. *marina* at a copper concentration of 5 and 10 mg Cu L^{-1}, probably due to the increase of the permeability of cell membrane to Na^{+}. Heavy metals change cell membrane permeability to small cations (OverneII1975).

Cellular viability was determined using the propidium iodide (PI) unability to pass through intact cell membranes. The integrity of the cell membrane fails as cells die, so that PI is able to enter the cell and selectively bind to nucleic acids (Ormerod 1990), In this way, PI fluorescence can be used to discriminate between live non-fluorescent cells and dead or damaged fluorescent cells. As shown in Fig. 4 and Table I, there was an increase in number of non-viable (fluorescent) cells (log PI fluorescence, observed by the movement along the y-axis) with copper concentration. The percentage of non-viable cells at 8 and 12 mg Cu^{+2} L^{-1} and in control cultures were similar, between 0,70 and 1.17 %, whereas 16 mg Cu^{+2} L^{-1} invoked a strong decrease in cellular viability, and the percentage of non-viable cells reached the 45.36 % (Table 1). Most of non-viable cells presented an abnormal size.

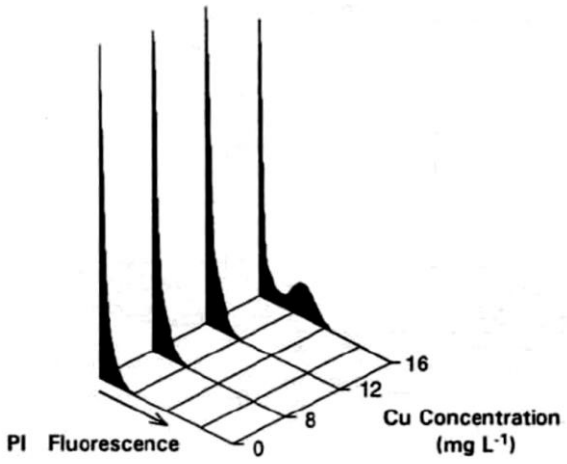

Figure 4. Histograms of samples of *Dunaliella tertiolecta* exposed to different copper concentrations. In these figures the log of PI fluorescent light emission in channels is plotted along de *x*-axis, while the *y*-axis represents the number of cells.

Among the many methods providing an evaluation of toxic effects on cultured cells, viability assays are the oldest and most widespread technique. The major criteria employed in viability assays is the membrane integrity. Cells that have lost the integrity of their plasma membrane, and have become permeable to external compounds, such as dyes and enzymes, are considered to be non-viable and they are not metabolically active (Frankfurt 1990). Measurements of cell viability may be of importance for characterization of cell populations exhibiting spontaneous cell death, such as cells

affected by cytotoxic agents (Frankfurt 1990). Fluorescent exclusion dyes can be used to monitor the viability of cells by FCM. Although viability determinations by FCM correlate well with conventional microscopic counting methods and present some advantages (speed of measurement, improved statistical analysis, and suppression of subjective analysis biases) (Adolphe and Ronot 1986), this technique is not yet used as a routine tool in cellular toxicology. This study provides the efficiency ofthis technique to study cytotoxic effects in marine microalgae.

Results obtained show that high concentrations of copper are toxic to this microalga, leading to depressed photosynthesis rates, cell division rates and pigment concentration. Cellular data also showed that 16 mg Cu^{+2} L^{-1} was toxic to *D. tertiolecta* in short term exposures.

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