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Oxidative stress and antioxidant defenses in two green microalgae exposed to copper

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ABSTRACT

The aim of this work was to assess the effects of 1 week copper exposure (6.2, 108, 210 and 414 μM) on *Scenedesmus vacuolatus* and *Chlorella kessleri*. The strains showed different susceptibility to copper. Copper content was determined in both strains by total X-ray reflection fluorescence analysis (TXRF). In *S. vacuolatus*, the increase of medium copper concentration induced an augmentation of protein and MDA content, and a significant decrease in the chlorophyll *a*/chlorophyll *b* ratio. *S. vacuolatus* showed a significant increase of catalase activity in 210 and 414 μM of copper, and a significant increment of SOD activity and GSH content only in 414 μM of copper. On the contrary, *C. kessleri* did not show significant differences in these parameters between 6.2 and 108 μM of copper. Increased copper in the environment evokes oxidative stress and an increase in the antioxidant defenses of *S. vacuolatus*.

1. Introduction

Contamination of aquatic environments by heavy metals has drawn the attention of a large number of researchers and environmentalists in the last decades. Environmental levels of copper and other metals have been rising over this period as a result of various human activities (Pinto et al., 2003).

Photosynthetic organisms are the main entrance of heavy metals to food chains, accumulating and transferring pollutants to consumers, including humans (Moreno Sánchez and Devars, 1999). Particularly, copper is a trace element essential for the development of photosynthetic species. However, in high concentrations this metal is toxic to most species of microalgae, which can, in turn, present different degrees of susceptibility (Baos et al., 2002; Yan and Pan, 2002). The effects of toxic copper concentrations on microalgae have been extensively reviewed and it is known that the free cation (Cu^{2+}) is the one that performs the toxic effects (Rai et al., 1981; Lobban and Harrison, 1994; Gledhill et al., 1997; Pinto et al., 2003). Some of these effects include the decrease in chlorophyll and accessory pigments content (Rijstenbil et al., 1994), increase in lipid peroxidation (Vavilin et al., 1998) and reduction in growth rate (Laube et al., 1980; Prasad et al., 1998).

It has been reported that the toxicity mechanism of copper involves the generation of reactive oxygen species (ROS) through the intervention of Cu^{+2} ions in Fenton's reaction (Okamoto et al., 2001; Rijstenbil and Gerringa, 2002). The increased levels of ROS produce oxidative damage to macromolecules such as proteins, nucleic acids and lipids, finally leading to the damage of different cellular organelles. Algae chloroplasts are formed by a complex system of membranes rich in polyunsaturated fatty acids, which are potential targets for peroxidation (Halliwell and Gutteridge, 1999).

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Several adaptive responses have developed in algae, which tend to reduce the macromolecular damage evoked by oxidative stress. These responses include metal detoxification and defense mechanisms to remove ROS before they can produce an irreversible damage (Pinto et al., 2003). Detoxification mechanisms involve extracellular or intracellular metal exclusion via complexation with different ligands, and storage into vacuoles (Lobban and Harrison, 1994; Rijstenbil and Gerringa, 2002). Antioxidant defense mechanisms include enzymes that catalyze reactions of ROS scavenging, such as catalase, ascorbate peroxidase, glutathione peroxidase and superoxide dismutase (Pinto et al., 2003; Mallick, 2004), lipophylic compounds and free radical scavengers like carotenes and α-tocopherol, reducers like ascorbate and reduced glutathione (GSH) (Buchanan et al., 2000; Mallick, 2004). The latter compound is the major water-soluble antioxidant in plant and algal cells. It can directly reduce most of the ROS (Noctor et al., 1998) or act as a cofactor or substrate in enzymatic reactions that contribute to control the cell ROS levels (Okamoto et al., 2001; Pinto et al., 2003).

Some algae have the ability to accumulate copper adsorbed in the cell walls and membranes or internalized in the cytoplasm. Internalized copper would be responsible for toxic effects (Stauber and Davies, 2000). Metals accumulated by algal cells are generally analyzed by atomic absorption spectroscopy. An alternative method is total reflection X-ray fluorescence (TXRF) spectrometry after digestion (Barkács et al., 1999). The latter technique allows performing multi-elemental analyses of microsamples (solid and liquid) with detection limits of trace elements in the range from ppb to ppm (Klockenkämper, 1997).

Chlorella spp. and *Scenedesmus* spp. are among the most widely distributed green microalgae, since these genera are found in most aquatic environments in the world. Species of both genera are often used in toxicity tests due to their sensitivity to different contaminants, their relatively short life cycle and the ease to handle them in laboratory cultures (Caux et al., 1996; Muñoz et al., 1996). However, metals (including copper) can produce differential effects on algae species (and even strains within the same species) (Kessler, 1986; Baos et al., 2002; Yan and Pan, 2002). The differential effects may be due to morphological and structural factors such as size, cell volume and presence or absence of a cell wall and mucilaginous sheet and their chemical composition (Schiariti et al., 2004; Levy et al., 2007), and also their physiological mechanisms for metal detoxification. *Scenedesmus vacuolatus* and *Chlorella kessleri* present different sizes and cell wall compositions (Takeda, 1996; Schiariti et al., 2004). In this study, we assess the effect and the response of *S. vacuolatus* strain exposed to concentrations of copper above and below its IC50, compared with the response of a *C. kessleri* strain exposed to the same conditions. Cell density, chlorophyll and protein contents were measured as indexes of metabolic damage. Intracellular and extracellular copper content was analyzed by TXRF as indicative of toxic accumulation, and malondialdehyde (MDA) content was measured as a lipid peroxidation index. Superoxide dismutase (SOD) and catalase (CAT) activities and reduced glutathione (GSH) were measured as intracellular level antioxidant responses.

2. Materials and methods

2.1. Organisms

The BAFC CA10 strain of *C. kessleri* (Trebouxiophyceae, Chlorophyta) was originally isolated from Laguna Verde, Copahue, Neuquén, Argentina (Juárez and Velez, 1993). BAFC CA4 strain of *S. vacuolatus* (Chlorophyceae, Chlorophyta) was originally isolated by Emerson as *C. kessleri*. Both strains are currently kept in the Culture Collection of the Laboratory of Phycology, Departamento de Biodiversidad y Biología Experimental, Facultad de Ciencias Exactas Exactas y Naturales, Universidad de Buenos Aires.

2.2. Chemicals

For toxicity bioassays, Bold's Basal Medium (BBM), pH 5.5 (Bischoff and Bold, 1963), was prepared with different copper concentrations. The control treatment containing 6.2 (already present in the BBM media), 108, 210 and 414 μM of copper, were prepared by adding copper as $CuCl_2 \cdot 2H_2O$ (Schiariti et al., 2004). Copper solutions were prepared by using analytical-grade copper chloride ($CuCl_2 \cdot 2H_2O$). Stock solution of 100 mg L^{-1} was prepared with deionised water. Copper was weighed in a Sartorius 1219 MP analytical balance with 0.0001 g resolution. Solutions of varying concentrations were prepared by diluting the stock solution with BBM medium.

Since abnormal growth of algal strains had been obtained in media lacking chelators (Schiariti et al., 2004), 0.171 mM EDTA was employed in the present bioassays. Due to the presence of EDTA, which reduces the availability of free cation in the culture media, high concentrations of copper were required to display toxic effects on *S. vacuolatus* and *C. kessleri*. The final concentration of free copper cation (Cu^{2+}) in culture solution was estimated in each case using MINEQL$^+$ 4.0 (Table 1). To select the copper concentrations, we performed preliminary assays with *S. vacuolatus* during 7-day exposures. We used three copper concentrations in the final study: one corresponds to the IC50 (210 μM Cu), another was a lower concentration (108 μM Cu) and last was higher (414 μM Cu).

Modeling initial culture media conditions in software MINEQL$^+$ 4.0 indicated that the copper was totally in solution, in all the assayed concentrations.

2.3. Algae cultures

Bioassays were carried out in 250 mL flasks containing 140 mL of medium with initial cell density of 30,000 cells mL^{-1}. An exponential growing phase culture was used as inoculum. Assays were performed by duplicate, and each assay was repeated twice. Suspension cultures were maintained at 23 ± 1 °C, with continuous cool-white fluorescent light illumination (80 μM photons m^{-2} s^{-1}) and agitation in an orbital shaker. After 7 days, cell density (number of cell mL^{-1}) was determined by direct counting, using an Olympus light microscope at 400× with 0.1 mm deep counting hemocytomer (improved Neubauer chamber). Cell suspension was diluted with BBM medium to give an appropriate cell concentration. The counting of at least 25 squares ensured an error less than 10% (Venrick, 1978). Values achieved were expressed as average ± S.D.

The cell volume and surface area of both species were determined by measuring the diameter of at least 30 randomly chosen cells and applying the equations for volume and surface area of a sphere (Hillebrand et al., 1999; Levy et al., 2007).

For measuring different parameters, we used a number of cells that allowed precise and reliable determinations.

2.4. Copper content

Internal and total copper contents were determined in both strains. Cells were harvested by centrifugation at 3000*g* and washed with 0.134 M potassium phosphate buffer. A sample of pelleted cells was used for the determination of total copper. A sub-sample of the same culture was washed with 0.134 M potassium phosphate buffer, and further washed three times with the same buffer containing 5 mM EDTA to remove the copper adsorbed to the cell wall. These cells were then used for the analysis of intracellular copper content. After washing, cells were digested in a microwave oven with nitric acid and hydrogen peroxide (2:1, v/v). A known amount of gallium was added to these dissolutions, as internal standard. Aliquots of 10 μl were placed in a quartz reflector for analysis by total X-ray reflection fluorescence analysis (TXRF) (Prange and Schwenke, 1992). This analysis, was performed with a disperse energy spectrometer equipped with a total reflection module with double reflector and collimator, a Si (Li) detector (CANBERRA model SL30170). An X-ray tube with a molybdenum anode was used as intracellular excitation source. Spectrum evaluation and quantitative analysis were performed using the QXAS software package from IAEA, using least-square regression analysis and calibration curves within the range 1–20 ppm. Results were expressed as micrograms of total Cu and intracellular Cu per 10^6 cells. Total copper = Adsorbed copper+intracellular copper.

Table 1
MINEQL$^+$ estimation of copper concentrations as free divalent cation (Cu^{2+}) and associated with EDTA.

Total added copper (as chloride)	Free copper (Cu^{2+})	Copper associated with EDTA
6.2×10^{-6}	2.12×10^{-12}	6.19×10^{-6}
1.08×10^{-4}	7.71×10^{-10}	1.07×10^{-4}
2.01×10^{-4}	3.42×10^{-5}	1.69×10^{-4}
4.14×10^{-4}	4.67×10^{-5}	1.69×10^{-4}

Concentrations are expressed in moles L^{-1}.