

Research Article

Assessment of catalase activity, lipid peroxidation, chlorophyll-*a*, and growth rate in the freshwater green algae *Pseudokirchneriella subcapitata* exposed to copper and zinc

Paulina Soto¹, Hernán Gaete^{1,3} & María Eliana Hidalgo²

¹Departamento de Biología y Ciencias Ambientales

²Departamento de Química y Bioquímica

³Centro de Investigación y Gestión de Recursos Naturales (CIGREN), Facultad de Ciencias
Universidad de Valparaíso, Valparaíso, Chile

ABSTRACT. In this work, the effect of copper and zinc on green alga *Pseudokirchneriella subcapitata* was evaluated through catalase activity, lipid peroxidation by TBARS essay, growth rate, and the chlorophyll-*a* concentration. Catalase activity increased significantly ($P < 0.05$) in comparison to the control at 0.1 mg L⁻¹ copper and 0.075 mg L⁻¹ zinc, whereas the damage to the cell membrane expressed as nmols/10⁶cell of malondialdehyde increased significantly ($P < 0.05$) at 0.025 mg L⁻¹ copper and 0.1 mg L⁻¹ zinc. On the other hand, a significant ($P < 0.05$) decrease in chlorophyll-*a* concentration was found at 0.075 mg L⁻¹ of both metals. The results showed that catalase activity, lipid peroxidation, and chlorophyll-*a* concentration were more sensitive to metals than the growth rate.

Keywords: toxicity, bioassays, water quality, oxidative damage, *Pseudokirchneriella subcapitata*, Chile.

Evaluación de la actividad de la catalasa, peroxidación lipídica, clorofila-*a* y tasa de crecimiento en la alga verde de agua dulce *Pseudokirchneriella subcapitata* expuesta a cobre y zinc

RESUMEN. En este trabajo, se evaluó el efecto del cobre y zinc en la alga verde *Pseudokirchneriella subcapitata* a través de la actividad catalasa, peroxidación lipídica por el ensayo TBARS, tasa de crecimiento y concentración de clorofila-*a*. La actividad catalasa aumentó significativamente ($P < 0,05$) en comparación al control en 0,1 mg L⁻¹ y 0,075 mg L⁻¹ de cobre y zinc respectivamente, mientras que el daño en la membrana celular expresado en nanomols/10⁶ células de malondialdehído aumentó significativamente en 0,025 mg L⁻¹ y 0,1 mg L⁻¹ de cobre y zinc respectivamente. Por otra parte, hubo una disminución significativa ($P < 0,05$) en la concentración de clorofila-*a* en ambos metales a 0,075 mgL⁻¹. Los resultados mostrados en actividad catalasa, peroxidación lipídica y concentración de clorofila-*a* son parámetros más sensibles que la tasa de crecimiento a los metales.

Palabras clave: toxicidad, bioensayos, calidad de agua, daño oxidativo *Pseudokirchneriella subcapitata*, Chile.

Corresponding author: Hernán Gaete (herman.gaete@uv.cl)

INTRODUCTION

Metals such as copper and zinc are released into the aquatic ecosystems by anthropogenic activities such as agricultural, industrial and mining (Yruea, 2005). In this ecosystems the primary producers play an important role in the structure and functioning of aquatic ecosystems. Any negative effect on them will affect upper trophic levels (Li *et al.*, 2006). Copper is

an essential redox-active transition metal, which acts as a structural element in regulatory proteins, and participates in electron transport in photosynthesis, mitochondrial respiration, oxidative stress responses, cell wall metabolism and hormone signaling (Moreno, 2003; Srivastava *et al.*, 2006). Zinc is essential for the stability of cell membranes in the activation of over 300 enzymes in the metabolism of proteins and nucleic acids (Cakmak & Marschner, 1988). However,

in high concentrations these metals can be toxic to aquatic organisms (Srivastava *et al.*, 2006).

The metals, as other environmental factors (ultraviolet light, extreme temperatures, radiation, environmental pollution, etc.) induce ROS (reactive oxygen species) formation inside the cell (Marshall & Newman, 2002). These ROS are formed under physiological conditions in manageable proportions, while maintaining a balance due to the defensive cellular enzymes (Dröge, 2003). The breakdown of this balance is known as oxidative stress, which results in alterations of the structure-function of organs, systems, or specialized transport (Ames *et al.*, 1993).

The damage caused by oxidative stress in fatty acid rich structures, such as cell membranes, results in lose rigidity, integrity and permeability. This process known as lipidperoxidation, produces carbonylic compounds such as malondialdehyde. Aerobic organisms have developed antioxidant defence mechanisms to prevent cell damage by ROS (Finkel & Holbrook, 2000; Sohal *et al.*, 2002). The enzyme catalase has an active participation in the reactions that reduce the intracellular levels of H₂O₂ formed from the superoxide radical (De Zwart *et al.*, 1999).

Although copper can act at many different levels in the cell, one of the most important mechanisms of copper action in green plants is believed to be the inhibition of electron transfer in the chloroplasts, the destruction of the chloroplast membrane, and inhibition of the photosynthetic pigments formation (Yan & Pan, 2002; Charles *et al.*, 2006).

The microalga of freshwater *Pseudokirchneriella subcapitata* has been used in toxicity bioassays due to their sensitivity to chemicals agents. In these tests, the exposed population rate growth is the response variable determined (NCh2706, 2002). At present, it is known that the molecular alterations are the first detectable, quantifiable responses, allowing the evaluation of exposure to agents at sublethal level. The biomarker concept is based in that the first level of interaction of pollutants with organisms is the molecular-cellular (Huschek & Hansen, 2005).

The studies on oxidative stress caused by metals in microalgae are few, the most are in green plants (Okamoto *et al.*, 1996; Lee & Shin, 2003; Elisabetta & Gioacchino, 2004; Mallick, 2004; Li *et al.*, 2006). The lipidperoxidation's products determination (TBARS), together with the evaluation of the catalase activity are effective methods used to assess oxidative stress (Scandalios, 1997; Nordberg & Arne, 2001). In this research is proposed that the molecular response is more sensitive than the response at the population level in *P. subcapitata* exposed to copper and zinc.

The goal of this study is to evaluate the effect of copper and zinc on *P. subcapitata*, through the activity of catalase, lipidperoxidation by TBARS assay, growth rate and concentration of chlorophyll-*a*.

MATERIALS AND METHODS

The green alga species *Pseudokirchneriella subcapitata*, belonging to the order Chlorococcales, class Chlorophyceae, was acquired in the Laboratory of Phycology of University of Concepción, Chile.

Bioassays

The bioassays were carried out according to the Chilean standard NCh2706 (2002). Prior to the bioassay a nutrient solution consisting of five stock solutions, which contain: micronutrients, macronutrients, Fe-EDTA, trace elements and NaHCO₃ was prepared. The algae was inoculated into 500 mL Erlenmeyer flasks in an equal volume for all samples, from culture stock during the exponential phase of growth. Then, they were enriched with the nutrient concentration to avoid false negatives, and volumes of copper sulphate pentahydrate (CuSO₄.5H₂O) and zinc sulphate heptahydrate (ZnSO₄.7H₂O) were added until a concentration of 0.025, 0.05, 0.075 and 0.1 mg L⁻¹ of Cu and Zn respectively. Finally, the flask was adjusted to 300 mL with distilled water. Additionally, a blank was prepared containing only the inoculum of *P. subcapitata*, nutrients and water. Each treatment and the blank, in triplicate, was exposed to continuous cool white light with a low intensity of 90 ± 10 μmol m⁻² s⁻¹, at 23°C ± 2°C and shaken by hand every day. To have a biomass of microalgae that would make the determinations, the exposure time was 15 days. The cell density (*n*) was determined at the beginning and the end of the bioassay counting directly in a microscope using a Neubauer Bright Line hemacytometer.

The growth rate (*k*) expressed as number of cell duplications was determined from:

$$k = \frac{3,322 \times \log N_n - \log N_0}{tn}$$

Where *tn* is the time elapsed between the start and end of the trial (in days), *N*₀ is the nominal initial cell density and *N*_{*n*} is the density at the end of the test.

Chlorophyll-*a*

To determine the chlorophyll-*a* concentration, 100 mL of the sample was filtered and immersed in acetone (90% V/V) for 20 h in darkness and cold. The absorbance of the solvent was determined at 630, 645

and 665 nm in a spectrophotometer (Cecil CE 2000 model 2041). The chlorophyll-*a* concentration was calculated through the Richards & Thompson (1952):

$$\text{mg chl-}a\text{ m}^3 = \frac{(15,6 E_{665} - 2 E_{645} - 0,8 E_{630}) \times 10}{V}$$

Where E_{665} is the absorbance at 665 nm, E_{645} the absorbance at 645 nm, E_{630} at 630 nm and V the volumen sample leaked.

Oxidative stress parameters

A volumen of 200 mL of each treatment was centrifuged and frozen with liquid nitrogen before grinding it with a mortar. Two mL of buffer 50 mM sodium phosphate (pH 7.0) were added to obtain an approximate volume of 3 mL, 1 mL of which was used to measure thiobarbituric acid reactive species (TBARS) and the remaining 2 mL to determine the enzymatic activity of catalase (CAT).

Determination of thiobarbituric acid reactive species (TBARS)

Lipidperoxidation was determined using the TBARS assay: samples were treated with trichloroacetic acid and then with thiobarbituric acid, the complex formed absorbed at 540 nm, the results are expressed in nanomoles of MDA/ 10^6 cells. MDA content was calculated using the calibration curve of MDA bis dimethylacetal.

Catalase activity (CAT)

The enzymatic activity of catalase was determined, by spectrophotometry following the hydrogen peroxide degradation rates for 1 min every 15 sec, at 240 nm which is accelerated in the presence of enzyme (Ratkevicius *et al.*, 2003; Contreras *et al.*, 2005; Cargnelutti *et al.*, 2006; Hidalgo *et al.*, 2006). The sample (2 mL) was concentrated by centrifugation at 3500 rpm for 15 min. A blank (2.9 mL sodium phosphate buffer pH 7.0 and 100 mL of supernatant) was used. The results are expressed as U/ 10^6 cells (Li *et al.*, 2006). The results were analyzed through the analysis of variance Anova and correlation determined with the program Systat 5.0. For each treatment three replicates were considered.

RESULTS

The growth rate of *P. subcapitata* exposed to copper decreased at 0.075 mg L⁻¹, while in the presence of zinc no statistically significant differences to the control was found in the range of concentration tested (Fig.1), however correlations with metal concen-

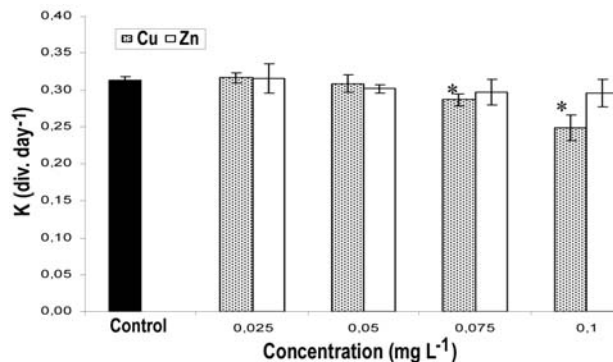


Figure 1. Growth rate of *P. subcapitata* exposed to copper and zinc *: significant difference to the control ($P < 0.05$)(n:4).

Figura 1. Tasa de crecimiento de *P. subcapitata* expuesta a cobre y zinc *: diferencia significativa al control ($P < 0,05$).

trations (Table 1) were observed. The concentration of chlorophyll-*a* decreased significantly at copper concentration 0.05 mg L⁻¹ and zinc concentration 0.075 mg L⁻¹. However an increase of chlorophyll-*a* at 0.025 mg L⁻¹ of copper was observed (Fig. 2). Only the correlation with zinc was significant (Table 1).

The catalase activity increased in comparison to the control in 0.075 mg L⁻¹ and 0.05 mg L⁻¹ of copper and zinc, respectively (Fig. 3). Zinc induced the activation of the antioxidant enzyme catalase at lower concentration than copper. The correlation between metal concentration and catalase activity was significant only for zinc (Table 1). There was significant correlation between lipid damage expressed as malondialdehyde content and catalase activity (Table 1). The lipid damage in the cell membrane was higher with copper than zinc. The damage was observed in exposure to 0.025 mg L⁻¹ copper, while for zinc it was observed at 0.1 mg L⁻¹ (Fig. 4). In this case, the correlation between damage to the cell membrane expressed as nmols/ 10^6 cell of malondialdehyde was significant to copper and zinc (Table 1).

DISCUSSION

Copper was more toxic than zinc in relationship at the rate growth, chlorophyll-*a* content and, lipoperoxidation endpoints, wich is similar to what was reported by Utgikar *et al.* (2004) in *Vibrio fischeri*. However, copper toxicity was lower than what was found by Kaneko *et al.* (2004); these authors reported an IC50 at 0.11 mg L⁻¹ of copper in *Selenastrum capricornutum*, similar values were reported by Bossuyt & Janseen (2004) in the same species, our

Table 1. Correlation between measured parameters.**Tabla 1.** Correlación entre los parámetros medidos.

	Concentration	CatCu	CatZn	ClofCu	ClofZn	MdaCu	MdaZn
CatCu	0.71						
CatZn	0.98*	-					
ClofCu	0.74	-0.53	-				
ClofZn	-0.93*	-	-0.96*				
MdaCu	0.92*	0.93*	-	-0.69			
MdaZn	0.81	-	0.78	-	-0.88*		
μ Cu	-0.88*	-0.92*	-	0.74	-	-0.98*	
μ Zn	-0.90*	-	-0.95*	-	0.90*	-	-0.78

Concentration : metal concentrations; CatCu: catalase activity exposed to copper; CatZn: catalase activity exposed to zinc; ClofCu: Chlorophyll-*a* exposed to copper; ClofZn: chlorophyll-*a* exposure to zinc; MdaCu: MDA production exposed to copper; MdaZn: MDA production exposed to zinc; μ Cu: growth rate exposed to copper; μ Zn: growth rate exposed to zinc. *: indicate significant correlation ($P < 0.05$).

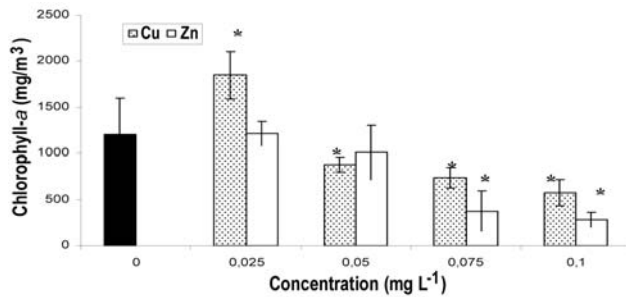
**Figure 2.** Chlorophyll-*a* concentrations in *P. subcapitata* exposed to copper and zinc. *: significant difference to the control ($P < 0.05$).

Figura 2. Concentración de clorofila-*a* en *P. subcapitata* expuesta a cobre y zinc. *: Indica significativamente diferente al control ($P < 0,05$).

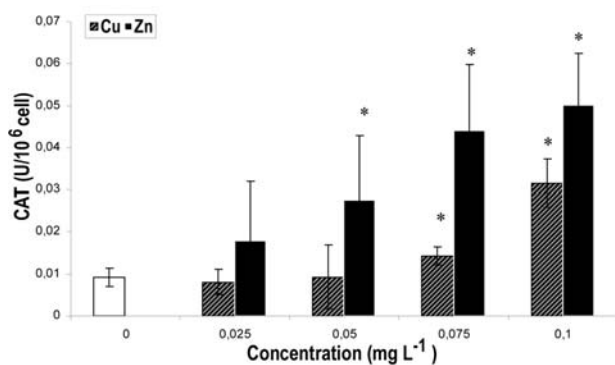
**Figure 3.** Catalase activity in *P. subcapitata* exposed to copper and zinc. *: significant difference to the control ($P < 0.05$).

Figura 3. Actividad catalasa en *P. subcapitata* expuesta a cobre y zinc. *: Indica significativamente diferente al control ($P < 0,05$).

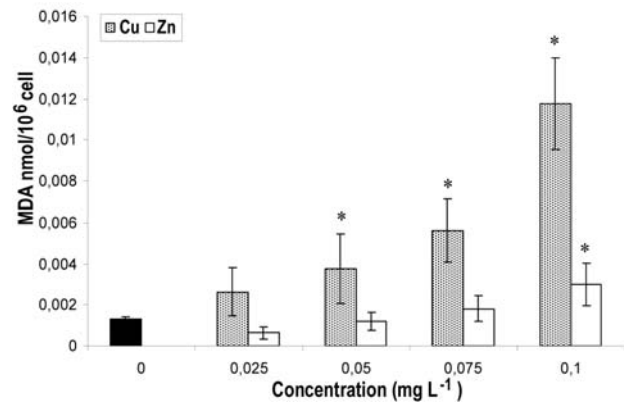
**Figure 4.** Effect of copper and zinc different concentrations on MDA production in *P. subcapitata*. *: Indicate significantly different to the control ($P < 0.05$).

Figura 4. Efectos del cobre y zinc a diferentes concentraciones sobre la producción de MDA en *P. subcapitata*. *: Indica significativamente diferente al control ($P < 0,05$).

study show that at copper concentration similar the inhibition on the rate growth was less to twenty percent. The differences may be due to the chemical characteristics of test water, such as pH, hardness, time exposure, concentration and type of chelating agent in the nutrients used in the these studies. On the other hand, the differences in the effects of the respective metals could be attributed to the redox potential of the metals. Copper has a greater redox potential that zinc ($\text{Cu } E^\circ = 0.16$ and $\text{Zn } E^\circ = -0.76$) which would lead to an increased oxidant effect at lower concentrations.

A manifestation of the hormesis effect was expressed under the Cu exposure. The stimulation of chlorophyll-*a* at the most low concentration of copper was similar to that founded by Knauer *et al.* (1997) and Janssen & Heijrick (2003). Whitton (1970) reported that the algae growth may be stimulated by low metal concentrations and inhibited at high metal concentrations. The parameter chlorophyll-*a* concentration was more sensitive than growth rate. The lowest content of chlorophyll-*a* in the higher concentrations of the metals can be due to the peroxidation of chloroplast membranes. Metals can destabilize the membrane by oxidation due to free radicals formation. Copper alters membrane tilacoides and causes changes in its composition (De Vos *et al.*, 1991), interference with the photosynthetic system and the a decline in the rate of photosynthesis (Cook *et al.*, 1997; Yruela, 2005). Our results differ from those reported by Bossuyt & Janssen (2004), who found a significant increase in chlorophyll-*a* at higher concentrations of metals in *P. subcapitata*. However, these authors used a different medium to that used in the present study and their cultures were acclimated previously to 60 and 100 $\mu\text{g L}^{-1}$, which would explain the differences with our results.

The catalase activity was higher in the highest concentrations tested. Similar results are reported by Li *et al.* (2006) when *Pavlova viridis* was exposed to copper and zinc. Contrarily, Srivastava *et al.* (2006) found that the catalase enzyme is dependant on concentration-time, their results show an increase in catalase activity until the 7 day of exposure with 108% induction. However, catalase activity declines in plants exposed to up 5 and 25 μM copper independently of the exposure duration, indicating a maximum decline of 42% on day 7 at 25 μM , probably due to enzyme degradation at higher free radicals concentration. Tripathi *et al.* (2006) reported less catalase activity at lower concentrations in *Scenedesmus* sp. when exposed to copper and zinc, than the present study. The results in our work suggest that the antioxidant enzyme catalase plays an important role at higher toxicant concentrations in *P. subcapitata*. The antioxidant catalase in *P. subcapitata* was protective at lower concentrations in *P. subcapitata* exposed to zinc compared to copper exposure.

Both metals provoked damage to lipid. MDA content increased significantly from 0.025 mg L^{-1} and 1.0 mg L^{-1} in copper and zinc respectively, indicating rise in lipid peroxidation in *P. subcapitata*. This is similar to that found by Li *et al.* (2006) with the microalga *Pavlova viridis* (Prymnesiophyceae), whose results show that the MDA content is expressed

significantly from 0.5 mg L^{-1} of copper and 3, 25 mg L^{-1} of zinc. The difference with our work can be explained by the different sensibility between species. Similarly, Srivastava *et al.* (2006) reported a gradual increase in the content MDA and significant differences to the control in *Hydrilla verticillata* at the highest concentration of copper tested.

In conclusion, both metals induce the antioxidant activity of catalase. Copper induces lipidperoxidation at lower concentrations than zinc. Copper and zinc cause adverse effects on the physiology of *P. subcapitata* determined through the content of chlorophyll-*a*. The growth rate was less sensitive to the metals that catalase activity, lipidperoxidation and chlorophyll-*a* content. The molecular parameters can be more adequate than growth rate to monitoring the effect in microalgae aquatic ecosystems.

REFERENCES

- Ames, B., M. Shigenaga & T. Hagen. 1993. Oxidants, antioxidants, and the degenerative diseases of aging. *Proc. Nat. Acad. Sci.*, 90: 7915-7922.
- Bossuyt, B. & C. Janseen. 2004. Long-term acclimation of *Pseudokirchneriella subcapitata* (Korshikov) Hidak to different copper concentrations; change in tolerance and physiology. *Aquat. Toxicol.*, 68: 61-74.
- Cakmak, J. & H. Marschner. 1988. Increase in membrane permeability and exudation in roots of zinc deficient plants. *J. Plant. Physiol.*, 132: 356-361.
- Cargnelutti, D., L. Tabaldi, R. Spanevello, G. Jucoski, V. Battisti, M. Redin, C. Blanco, V. Dressler & E. De Moraes. 2006. Mercury toxicity induces oxidative stress in growing cucumber seedlings. *Chemosphere*, 65: 999-1006.
- Charles, A.L., S.J. Markich & P. Ralph. 2006. Toxicity of uranium and copper individually, and in combination, to a tropical freshwater macrophyte (*Lemna aequinoctialis*). *Chemosphere*, 62: 1224-1233.
- Contreras, L., A. Moenne & J. Correa. 2005. Antioxidant responses in *Scytosiphon lomentaria* (Phaeophyceae) inhabiting copper-enriched coastal environments. *J. Phycol.*, 41: 1184-1195.
- Cook, C., A. Kostidou, E. Vardaka & T. Lanaras. 1997. Effects of copper on the growth, photosynthesis and nutrient concentrations of *Phaseolus vulgaris* plants. *Photosynthetica*, 34: 179-193.
- De Vos, C.H. Schat, M. De Waal, R. Vooijs & W. Ernst. 1991. Increased resistance to copper-induced damage of the root cell plasmalemma in copper tolerant *Silene cucubalis*. *Plant Physiol.*, 82: 523-528.

- De Zwart, L., J. Meerman, J. Commandeur & N. Vermeulen. 1999. Biomarkers of free radical damage. Applications in experimental animals and in humans. *Free Radic. Biol. Medic.*, 26: 202-226.
- Dröge, W. 2003. Free radicals in the physiological control of cell function. *Physiol. Rev.*, 82: 47-95.
- Elisabetta, M. & S. Gioacchino. 2004. Copper-induced changes of non-protein tilos and antioxidant enzymes in the marine microalga *Phaeodactylum tricoratum*. *Plant. Sci.*, 167: 289-296.
- Finkel, T. & N.J. Holbrook. 2000. Oxidants, oxidative stress and the biology of aging. *Nature*, 408: 239-247.
- Hidalgo, M., E. Fernández, A. Cabello, C. Rivas, F. Fontecilla & L. Morales. 2006. Evaluation in the antioxidant response in *Chiton granosus* Fremby, 1928 (Mollusca: Polyplacophora) to oxidative pollutants. *J. Mar. Biol. Oceanogr.*, 41: 155-165.
- Huschek, G. & P.D. Hansen. 2005. Ecotoxicological classification of the Berlin river system using bioassays in respect to the european water framework directive. *Environ. Monit. Assess.*, 121: 15-31.
- Janssen, C. & D. Heijrick. 2003. Algal toxicity testing for environmental risk assessment of metals: physiological and ecological considerations. *Environ. Cont. Toxicol.*, 178: 23-52.
- Kaneko, H., A. Shimada & K. Hirayama. 2004. Short-term algal toxicity test based on phosphate uptake. *Water Res.*, 38: 2173-2177.
- Knauer, K., R. Behra & L. Sigg. 1997. Effects of free Cu^{2+} and Zn^{2+} ions on growth and metal accumulation in free-water algae. *Environ. Toxicol. Chem.*, 16: 220- 229.
- Lee, M. & H. Shin. 2003. Cadmium-induced change in antioxidant enzymes from the marine alga *Nanochloropsis oculata*. *J. Appl. Phycol.*, 15: 13-19.
- Li, M., C. Hu, Q. Zhu, L. Chen, A. Kong & Z. Liu. 2006. Copper and zinc induction of lipid peroxidation and effects on antioxidant enzyme activities in the microalga *Pavlova viridis* (Prymnesiophyceae). *Chemosphere*, 62: 565-572.
- Mallick, N. 2004. Copper-induced oxidative stress in the chlorophycean microalga *Chlorella vulgaris*: response of the antioxidant system. *J. Plant. Physiol.*, 161: 591-597.
- Marshall, J.A. & S. Newman. 2002. Differences in photoprotective pigment production between Japanese and Australian strain of *Chattonella marina* (Raphidophyceae). *J. Exp. Mar. Biol. Ecol.*, 272: 13-27.
- Moreno, M.D. 2003. Toxicología ambiental. Evaluación del riesgo para la salud humana. Ed. McGraw-Hill, Madrid. pp. 384.
- NCh2706. 2002. Calidad de agua- Bioassay of growth of algae in freshwater with *Selenastrum capricornutum* (*Raphidocelis subcapitata*). Institute National of Normalization, Chile. 28 pp.
- Nordberg, J. & E. Arner. 2001. Reactive oxygen species, antioxidants, and the mammalian thioredoxin system. *Free Radicals Biol. Medic.*, 31: 1287-1312.
- Okamoto, O., C. Asano, E. Aida & P. Colepicolo. 1996. Effects of cadmium on growth and superoxide dismutase activity of the marine microalga *Tetraselmis gracilis*. *J. Phycol.*, 32: 74-79.
- Ratkevicius, N., J. Correa & A. Moenne. 2003. Copper accumulation, synthesis of ascorbate and activation of ascorbate peroxidase in *Enteromorpha compressa* (L.) Grev. (Chlorophyta) from heavy metal-enriched environments in northern Chile. *Plant. Cell Environ.*, 26: 1599- 1608.
- Richards, F.A. & T.J. Thompson. 1952. The estimation and characterization of plankton populations by pigment analysis. II. A spectrophotometric methods for the estimation of plankton pigment. *J. Mar. Res.*, 156-172.
- Scandalios, J.G. 1997. Oxidative stress and the molecular biology of antioxidant defenses. Cold Spring Harbor Laboratory Press, New York: 890 pp.
- Sohal, R.S., R.J. Mockett & W.C. Orr 2002. Mechanisms of aging: an appraisal of the oxidative stress hypothesis. *Free Radicals Biol. Medic.*, 33: 575-586.
- Srivastava, S.S., R.D. Mishra, S. Tripathi, D. Dwivedi & K. Gupta. 2006. Copper-induced oxidative stress and responses of antioxidants and phytochelatins in *Hydrilla certicillata* (L.F.) Royle. *Aquat. Toxicol.*, 80: 405- 415
- Tripathi, B.N., S.K. Mehta Anshu Amar & J.P. Gaur. 2006. Oxidative stress in *Scenedesmus* sp. during short- and long-term exposure to Cu^{2+} and Zn^{2+} . *Chemosphere*, 62: 538-544.
- Utgikar, V.P., N. Chaudhary, A. Koeniger, H.H. Tabak, J.R. Haines & R. Govind. 2004. Toxicity of metals and metal mixtures: analysis of concentration and time dependence for zinc and copper. *Water Res.*, 38: 3651-3658.
- Whitton, B.A. 1970. Toxicity of heavy metals to freshwater algae: a review. *Phykos*, 9: 116-125.
- Yan, H. & G. Pan. 2002. Toxicity and bioaccumulation of copper in three green microalgal species. *Chemosphere*, 49: 471-476.
- Yruela, I. 2005. Copper in plants. *Braz. J. Plant Physiol.*, 17: 145-156.