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Enhancement of copper toxicity in cultures of *Dictyosphaerium pulchellum* (Chlorophyceae) by mucilage removal

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With 5 figures and 3 tables in the text

Abstract: Extracellular mucilage from cells of *Dictyosphaerium pulchellum* WOOD 1872 was removed by controlled ultrasonication. Batches of exponentially growing cells, with and without mucilaginous capsules, were incubated during 48 hours with 50, 100, 200 and 300 $\mu g . l^{-1}$ of copper (added as copper sulfate). Free divalent cation concentration at the beginning of the experiment was estimated by MINEQL+. Both cellular yield and total chlorophyll content were negatively affected by the increment of copper concentration in the incubation media. Mucilage removal enhanced susceptibility to bleaching, while it did not modify significantly cellular density at the end of the experiment.

Key words copper toxicity, extracellular mucilage, extracellular copper binding, bleaching, *Dictyosphaerium pulchellum*, Chlorophyceae

Introduction

Several toxic effects for copper have been reported in algae, such as reduction of chlorophyll and accessory pigments content, interruption of electron transport in Photosystem II, changes in membrane permeability, reduction of growth rate and enhanced oxidation of proteins, lipids, sterols and free fatty acids (SANDMAN & BÖGER 1980, RAI et al. 1981, STAUBER & FLORENCE 1987, LOBBAN & HARRISON 1994, BARÓN et al. 1995, GLEDHILL et al. 1997, GIRLING et al. 2000, SCHIARITI et al. 2004). Frequently, in algae cations can be immobilized extracellularly through adsorption to (glyco)proteins and acid polysaccharides in mucilage (SUEUR et al. 1982, KAPLAN et al. 1997, GLEDHILL et al. 1999, LOMBARDI & VIEIRA 2000, LOMBARDI et al. 2002). Adsorption of metal ions to the cell surface determines the initial toxicant loading of the cells, by concentrating metals in the proximity of cell

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membrane transporters (FRANKLIN et al. 2002). Differences in cell wall composition may be responsible for differences in susceptibility to copper exposure (PRASAD et al. 1998, SCHIARITI et al. 2004).

Dictyosphaerium pulchellum WOOD 1872 is a cosmopolitan freshwater phytoplankter. Following their liberation from autosporangia, autospores remain attached in bundles by means of the remains of mother cell walls (KOMÁREK & PERMAN 1978) forming up to 64-celled aggregates. Partial hydrolysis of mother cell walls during cell division renders a mucilaginous sheath around the aggregate. In the present work, experimental conditions for mucilage removal by ultrasonication were optimized in order to study the incidence of mucilage on chlorophyll bleaching and cellular yield after 48 hours of exposition to increasing copper concentrations.

Materials and methods

Axenic cultures of *Dictyosphaerium pulchellum* WOOD are maintained in Bold's Basal Medium (BBM) (NICHOLS & BOLD 1965) in the culture collection of the laboratory of Phycology (Departamento de Biodiversidad y Biología Experimental, FCEN, UBA) by successive replication. Suspension cultures were carried out at 23 ± 1 °C, with constant cool-white fluorescent light illumination (60 mmol photons . m^{-2} . s^{-1}) and agitation in shaker.

For toxicity bioassays, BBM was prepared in MilliQ water with the omission of chelator (EDTA) as indicated in EPA (1985) and copper in the micronutrient solutions. The addition of copper (as sulfate) proceeded to make up the following final copper concentrations: 50, 100, 200 and 300 mg . l^{-1} (in BBM50, BBM100, BBM200 and BBM300, respectively) and pH was adjusted to 6.4 at the beginning of the experience. Free divalent copper cation concentration in each case was estimated by MINEQL+ software (SCHECHER & MC AVOY 1994).

All bioassays were carried out with an initial cell density of at least 1 . 10^5 cells per ml using as inoculum a late exponential growing phase culture (duplication time for the exponential phase estimated in 16 hours). Cells were axenically harvested by centrifugation, washed repeatedly and pre-incubated in BBM without copper addition before the onset of the bioassays. The resulting algal suspension (50 ml) was subdivided in two batches (2 x 25 ml), in order to proceed to mucilage removal in one of them under laminar flow hood (see below). Controls (BBM0) (without copper addition) and four replicas were run for each series with (S) and without (NS) mucilage removal.

Controlled mucilage removal was achieved by tip ultrasonication operating at a maximum frequency of 20 KHz with a Vibra Cell Ultrasonicator (Sonics and Materials, USA). Efficiency of mucilage removal was estimated after different time intervals of exposure to ultrasound. Estimation of cell viability after each ultrasonication treatment was done by examination of the capacity for mucilage regeneration after 48 h by microscope observation of at least 100 cells, contrasted with Indian ink.

Histochemical staining was performed according to KRISHNAMURTHY (1999). For digestion with protease, cells were resuspended in 0.2 M phosphate buffer pH 6.8 with the addition of 4 units of Pronase from *Streptomyces griseus* (EC 3.4.24.31, Sigma). Incubation proceeded at 37 °C during 48 h.

Cellular counts were obtained in a Neubauer chamber (standard error under 10 %, VENRICK 1978). Spectrophotometric quantification of chlorophyll content was determined in the cellular pellet of 4 ml of culture suspension. The pellet was resuspended in warm methanol (80 °C) for 30 minutes, keeping it protected from light. The extract was clarified by centrifugation and chlorophyll estimation was done using the trichromatic equations of SCOR-UNESCO (1966).

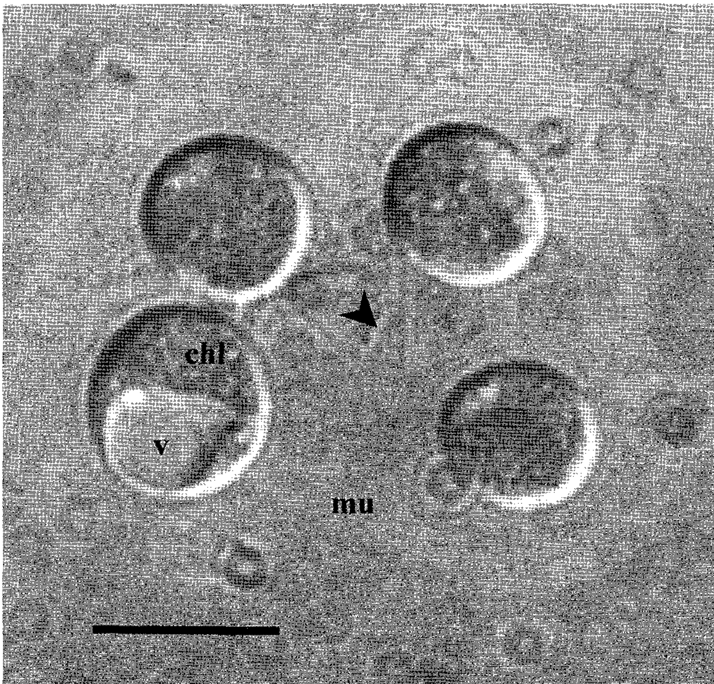


Fig. 1. Nomarski micrograph of tetracellular aggregate of *Dictyosphaerium pulchellum*. **chl** – cupuliform chloroplast; **mu** – mucilage; **v** – vacuole. Arrowhead indicates tracts of mother cell walls. [Scale bar = 10 mm]

The effects of copper concentration on cell density and chlorophyll content were studied by simple linear regression analysis. Differences between slopes for sonicated and non sonicated cells were obtained applying analysis of difference between independent regressions (SOKAL & JAMES 1981).

Results

Algal population at the beginning of the bioassays majorly consisted of tetracellular aggregates of slightly ovoid to spherical cells, joined in bundles by means of the remaining tracts of the mother cell walls. Abundant extracellular mucilage exhibited slightly lobulated borders (Fig. 1). Cells presented a unique cupuliform chloroplast with conspicuous intraplastidial pyrenoid. A prominent vacuole was located in the central invagination of the plast. Purple metachromasia with toluidine blue O and positive staining with ruthenium red suggested acid nature of mucilage components. After digestion with pronase, the percentage of aggregate cells dropped from 50 to 27 % through rupture of cell wall tracts in cellular aggregates, producing the liberation of single cells. No evident changes in mucilaginous capsules could be detected by optical microscopy after enzyme digestion.

Table 1: Percentages of mucilage removal and regeneration after exposure to increasing ultrasonication intervals in *Dictyosphaerium pulchellum.*

Time [sec]	5	10	15	20	25	30	35	40	45	50	55	60
Mucilage removal [%]	10	47	55	70	85	87	96	100	100	100	100	100
Mucilage regeneration after 48 h [%]	100	100	100	100	100	92	87	74	70	61	58	46

Table 2: MINEQL+ estimation of copper concentrations as free divalent cation (Cu^{2+}) and associated in major inorganic salts[a]. Concentrations are expressed in moles . l^{-1}.

	Total added copper (as sulfate)	Cu^{2+}	Total soluble copper	$CuHPO_4$	$Cu(OH)_2$	$CuSO_4$	$Cu_3(PO_4)_2$	CuOH
BBM 0 [b, c]	–	$1.52 . 10^{-19}$	–	$8.13 . 10^{-19}$	–	–	–	–
BBM 50 [b]	$7.90 . 10^{-7}$	$1.20 . 10^{-7}$	$7.90 . 10^{-7}$	$6.42 . 10^{-7}$	–	–	–	–
BBM 100 [b]	$1.59 . 10^{-6}$	$2.42 . 10^{-7}$	$1.59 . 10^{-6}$	$1.29 . 10^{-6}$	–	–	–	–
BBM 200 [b]	$3.17 . 10^{-6}$	$4.83 . 10^{-7}$	$3.17 . 10^{-6}$	$2.58 . 10^{-6}$	$6.36 . 10^{-8}$	$3.54 . 10^{-8}$	–	–
BBM 300 [b]	$4.76 . 10^{-6}$	$7.25 . 10^{-7}$	$4.76 . 10^{-6}$	$3.87 . 10^{-6}$	$9.56 . 10^{-8}$	$5.34 . 10^{-8}$	–	–
BBM 500	$7.90 . 10^{-6}$	$1.08 . 10^{-6}$	$7.07 . 10^{-6}$	$5.74 . 10^{-6}$	$1.42 . 10^{-7}$	$8.09 . 10^{-8}$	$2.77 . 10^{-7}$	–
BBM 750	$1.19 . 10^{-5}$	$1.08 . 10^{-6}$	$7.07 . 10^{-6}$	$5.74 . 10^{-6}$	$1.42 . 10^{-7}$	$8.20 . 10^{-8}$	$1.61 . 10^{-6}$	$2.71 . 10^{-8}$
BBM 1000	$1.59 . 10^{-5}$	$1.08 . 10^{-6}$	$7.07 . 10^{-6}$	$5.74 . 10^{-6}$	$1.42 . 10^{-7}$	$8.29 . 10^{-8}$	$2.94 . 10^{-6}$	$2.71 . 10^{-8}$

[a] The table only shows values above 10^{-8} moles . l^{-1} (except for the control BBM 0).
[b] Culture media selected for experiments
[c] Free divalent copper cation in BBM with EDTA ($1.1 . 10^{-12}$ M) and without EDTA ($9.58 . 10^{-7}$ M) are given as comparison.

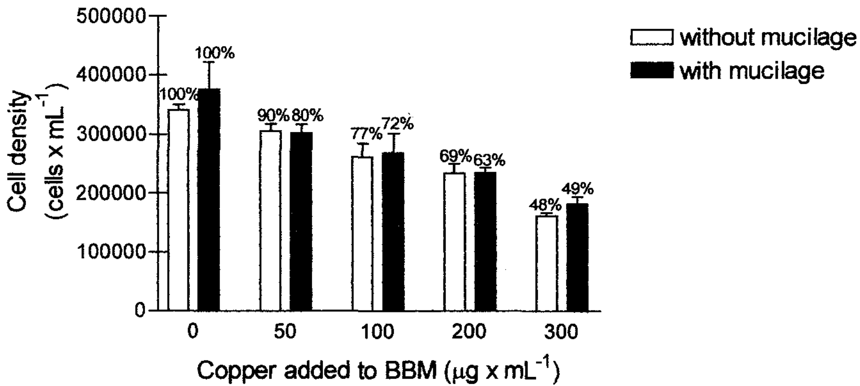
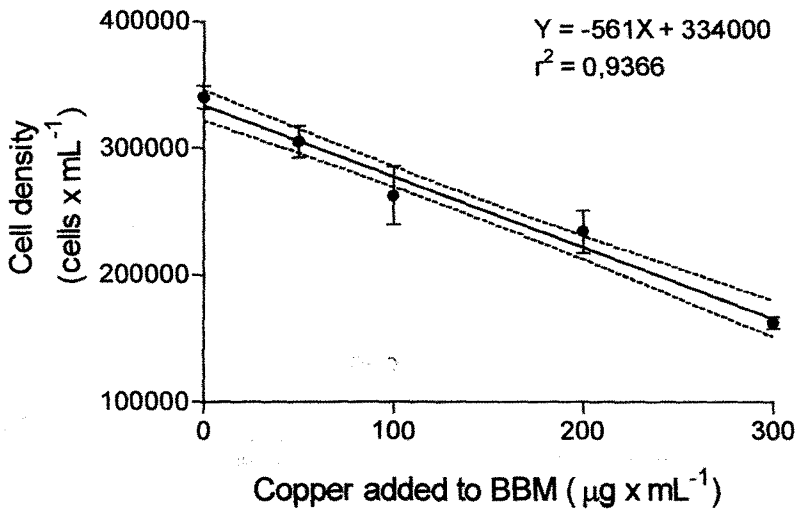


Fig. 2. Final cell density (cells . ml^{-1}) after 48 hours exposure to different copper concentrations in cultures of *Dictyosphaerium pulchellum* with and without preliminary mucilage deprivation. All data correspond to media ± SD. Numbers on top the bars represent values of percentages of the respective controls.

Solubilization of extracellular mucilage increased with ultrasonication time (Table 1). After 40 sec of exposure to ultrasound, all the cells appeared devoid of mucilage, but outlasting sonication treatment over 25 sec resulted in a diminished capacity for mucilage regeneration after 48 hours (Table 1). The capacity for mucilage regeneration was used as an indicator of cellular viability and/or adequate physiological condition to start the bioassays. Thus, even when only 85 % of the cells were deprived of mucilage after 25 sec of ultrasonication, this interval was selected for the preparation of inocula for bioassays, in order to guaranty 100 % cell viability.

Copper concentrations to be assayed were selected in a preliminary experience, employing total added copper concentrations of 50, 100, 200, 300, 500, 750 and 1000 mg . l^{-1}. Copper concentrations above 300 mg . l^{-1} resulted lethal. Estimation of free copper cation by MINEQL+ indicated that in media without EDTA all the added copper sulfate remained soluble up to BBM 300 (Table 2). In absence of EDTA, copper is mainly associated to phosphate ($CuHPO_4$). Note that in BBM with EDTA, free copper divalent cation concentration is less than five orders of magnitude. Free copper cation (which is the toxic species) increased almost linearly in the media used in the present experiments.

After 48 h of exposure to increasing copper concentrations, cellular density was negatively affected (Table 3, Fig. 2). Growth inhibition (expressed as percentage of the control) was always significant ($p<0.05$) in all assays respect the control. For non sonicated cells, significant final cell yield differences ($p<0.05$) were only found among BBM-NS50 and BBM-NS200–300 and between BBM-NS100 and BBM-NS300. For sonicated cells, BBM-S50 differed significantly from the other assays ($p<0.05$) and BBM-S100 and BBM-S200 differed from BBM-S300. Yet, slopes of regression curves obtained with ($b = -561$) and with-


(a)
Y = -561X + 334000
r2 = 0,9366
400000
300000
200000
100000
Cell density
(cells x mL-1)
0
100
200
300
Copper added to BBM (µg x mL-1)

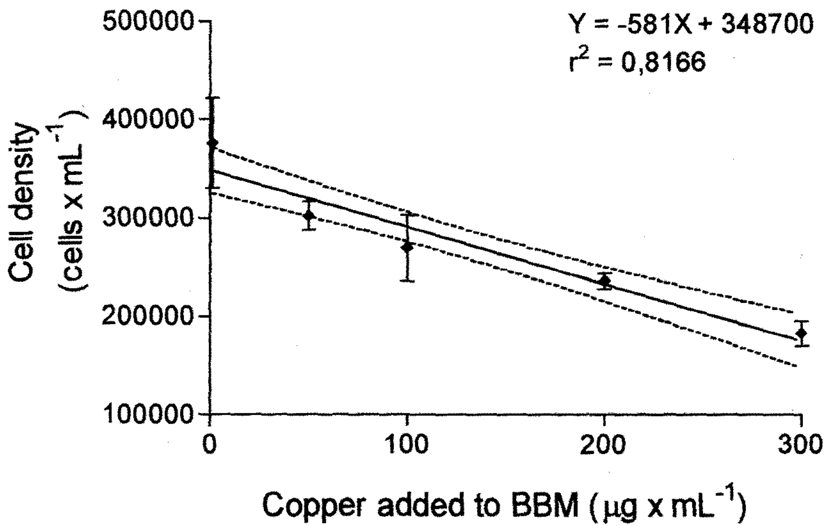

(b)
Y = -581X + 348700
r2 = 0,8166
500000
400000
300000
200000
100000
Cell density
(cells x mL-1)
0
100
200
300
Copper added to BBM (µg x mL-1)

Table 3: Cellular density and total chlorophyll content after 48 hours of exposure to different copper concentrations in cells of *Dictyosphaerium pulchellum* with (S) and without (NS) preliminary mucilage deprivation.

	Cellular density [cells per ml]	Total chlorophyll [pg chl . ml^{-1}]	Total chlorophyll [pg chl . per cell]
BBM-S0	340347 ± 9049	910 ± 48	$2.683 \cdot 10^{-3} \pm 1.875 \cdot 10^{-4}$
BBM-S50	305475 ± 12514	510 ± 22	$1.674 \cdot 10^{-3} \pm 1.411 \cdot 10^{-4}$
BBM-S100	262894 ± 23068	425 ± 44	$1.613 \cdot 10^{-3} \pm 4.524 \cdot 10^{-5}$
BBM-S200	234465 ± 16551	323 ± 38	$1.372 \cdot 10^{-3} \pm 6.070 \cdot 10^{-5}$
BBM-S300	162613 ± 4509	217 ± 26	$1.340 \cdot 10^{-3} \pm 1.318 \cdot 10^{-4}$
BBM-NS0	376325 ± 45744	1135 ± 103	$3.052 \cdot 10^{-3} \pm 4.943 \cdot 10^{-4}$
BBM-NS50	302135 ± 15131	598 ± 92	$1.977 \cdot 10^{-3} \pm 3.124 \cdot 10^{-4}$
BBM-NS100	269097 ± 33309	435 ± 47	$1.628 \cdot 10^{-3} \pm 1.785 \cdot 10^{-4}$
BBM-NS200	135372 ± 7964	315 ± 29	$1.351 \cdot 10^{-3} \pm 1.378 \cdot 10^{-4}$
BBM-NS300	182600 ± 12383	235 ± 35	$1.300 \cdot 10^{-3} \pm 2.118 \cdot 10^{-4}$

out (b = −581) mucilage removal were parallel ($p > 0.05$) (Fig. 3a,b) indicating that preliminary sonication had no incidence whatsoever in enhancing the negative effect of copper on final cell biomass yield. According to the mechanism of cell division in *Dictyosphaerium pulchellum* (KOMÁREK & PERMAN 1978), a reduction in the percentage of aggregates would be expected together with the decrease in final cellular yields. In fact, cellular aggregates diminished from 94 % in BBM0 to 18 % in BBM300. Additionally, cellular morphology was particularly altered at 300 mg . l^{-1} Cu, where bleaching appeared together with a reduction of chloroplast size and altered cupuliform shape. Cells were densely vacuolated and showed different degrees of plasmolyzation.

Total chlorophyll per milliliter diminished with copper concentration (Table 3). Since the decrease in chlorophyll content did not keep a linear relation with the reduction in cell density ($r^2 = 0.4677$), bleaching effect cannot be solely attributed to the reduction in cellular yield. When analyzing variation of chlorophyll content on a per cellular basis (Table 3, Fig. 4), values also decreased with the increment of copper concentration. Contrary to the observation of change in cellular yield, different slopes in regression curves of total chlorophyll per cell with (b = −0.00055) and without (b = −0.00072) mucilage removal ($p < 0.05$) (Fig. 5a, b) indicated that preliminary sonication affected bleaching degree. In fact, for cells deprived of mucilage at the beginning of the experiment, significative differences in chlorophyll content per cell ($p < 0.05$) were detected between BBM-S50 and BBM-S200, while in untreated cells significative differences appear between BBM-NS50 and BBM-NS300.

Fig. 3: Regression curve (confidence belt 95 %) for cell densities as a function of copper concentration in *Dictyosphaerium pulchellum* with (**3a**) and without (**3b**) preliminary mucilage deprivation.

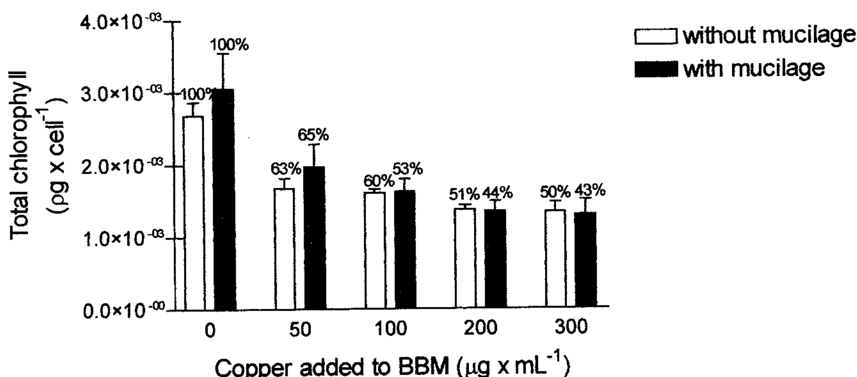


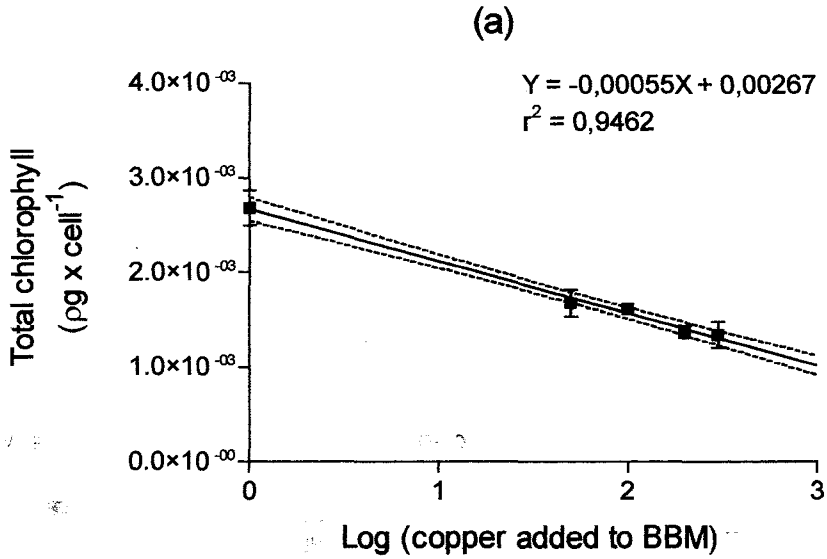
Fig. 4. Final total chlorophyll content per cell [mg per cell] after 48 hours exposure to different copper concentrations in cultures of *Dictyosphaerium pulchellum* with and without preliminary mucilage deprivation. All data correspond to media ± SD. Numbers on top the bars represent values of percentages of the respective controls.

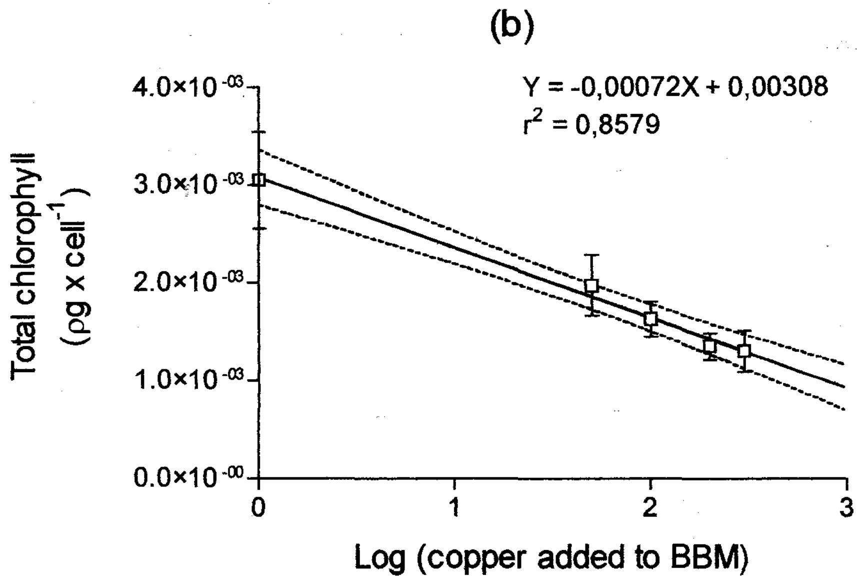
Discussion

Physicochemical form (speciation) of a metal is a critical factor controlling metal bioavailability and toxicity (TWISS 1996). This bioavailability depends upon a series of environmental conditions, such as dissolved oxygen, pH and presence of inorganic and/or organic chelators and ligands (GLEDHILL et al. 1997) which modify the thermodynamic conditions for chemical equilibrium of association-dissociation between free and bound cation. As it has been previously pointed out (STOKES et al. 1973) the omission of chelator (EDTA) in the culture media increased total free divalent cation concentration respect the complete BBM (with EDTA) (Table 2). Thus, toxicity effects are evident at lower added copper concentrations (GEIS et al. 2000). Taking into account that free divalent cation is the toxic species, all the bioassays were carried out in copper concentrations at least 5 orders of magnitude exceeding that of complete BBM. Even so, none of the assayed concentrations resulted lethal in the term of the experiment (48 h).

Omission of EDTA in culture media might hinder the bioavailability of essential nutrients, such as iron (GEIS et al. 2000). Nevertheless, in our 48 h experiments controls showed normal growth. Moreover, incrementing sensibility to copper by the lack of chelator in the media, in our case, had the advantage of stressing the effect of extracellular mucilage as metal ligand and facilitating comparisons between cells with and without mucilage. Copper starving of the cells before the onset of the experiences could have also enhanced metal toxicity by increased uptake of the cation at the beginning of the incubation. Mucilage regen-

Fig. 5. Regression curve (confidence belt 95 %) for total chlorophyll content per cell as a function of copper concentration in *Dictyosphaerium pulchellum* with (**5a**) and without (**5b**) preliminary mucilage deprivation.


(a)
Y = -0,00055X + 0,00267
r² = 0,9462
Total chlorophyll (ρg x cell⁻¹)
4.0×10⁻⁰³
3.0×10⁻⁰³
2.0×10⁻⁰³
1.0×10⁻⁰³
0.0×10⁻⁰⁰
0
1
2
3
Log (copper added to BBM)


(b)
Y = -0,00072X + 0,00308
r² = 0,8579
Total chlorophyll (ρg x cell⁻¹)
4.0×10⁻⁰³
3.0×10⁻⁰³
2.0×10⁻⁰³
1.0×10⁻⁰³
0.0×10⁻⁰⁰
0
1
2
3
Log (copper added to BBM)

eration in mucilage deprived cells cannot be ruled out in the term of the experiment. In fact, mucilage regeneration was used as indicator of cell viability, essential condition to carry out the experiment. But, copper uptake in copper starved cells occurs immediately upon incubation in copper-containing media (HILL et al. 1996). If there is a difference between the initial surfaces exposed to the media (that is to say, cells with or without mucilage), initial conditions for copper uptake and/or adsorption to the surface will differ. Probably, copper membrane transporters are more accesible in pre-sonicated cells and when such cells are incubated in increasing copper concentrations, alterations in metal uptake will appear earlier than in non-sonicated cells (SUNDA & HUNSTMAN 1998).

Algal surfaces (mucilage, cell walls and membranes) can act as ligands for metal ions since they contain several functional groups with different metal affinities. Though chemical characterization was not attempted in the cell wall/mucilage composition of *D. pulchellum*, the presence of negatively charged groups in the cell surfaces can be inferred from the histochemical stains applied. The presence of (glyco)proteins suggested by partial digestion with proteases, indicates the existence of N-ligands for copper. N-ligands have been demonstrated to possess a higher affinity for copper cations than carboxylates (KIEFER et al. 1997) and they would function at lower copper concentrations, while lower affinity carboxylate groups would be saturated at higher metal concentrations. Affinity for different ligands is also implied in copper binding to cell membrane transporters. Recently, FOX & GUERINOT (1998) have characterized a copper transporter that binds the cations to the N-terminal dominium rich in methionine and serine exposed on the external face of the plasmalemma.

Metal toxicity and metal nutrition are often closely interrelated (SUNDA & HUNSTMAN 1998). Coordination sites (in membrane transporters, prosthetic groups of metaloproteins, enzymes or chlorophylls) are seldom specific for only one metal. Metals with similar ionic radii or coordination geometries will compete for the same site. A well known example is the relation between extracellular concentrations of Cu^{2+}, Zn^{2+} and Mn^{2+} (SUNDA & HUNSTMAN 1998). If the concentration of the former two strongly exceed that of the latter, a competition for membrane transporters is established and negative feedback control for copper and zinc uptake will be altered, causing intracellular accumulation of Cu^{2+} and Zn^{2+} on one hand, and Mn^{2+} deficiency, on the other. Excess of intracellular Cu^{2+} can replace Mg^{2+} in chlorophyll molecules, rendering them photosynthetically inactive (KÜPPER et al. 2002). Mn^{2+} deficiency causes impaired water lysis in PSII (BARÓN et al. 1995).

In Chlorophyceae, the major copper proteins are chloroplast plastocyanin and mitochondria cytochrome oxidase, in an approximate 10:1 relation (HILL et al. 1996). Copper active uptake from the medium continues until the saturation of the biosynthetic pathway for plastocyanin is reached. Extrapolating from data for *Chlamydomonas reinhardtii*, in *D. pulchellum* the needs for plastocyanin synthesis operating at a cell density of $3 . 10^5$ cells per ml would occur at approx. 50 nM Cu , which is lower than the concentration employed in our assays.

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The binding capacity of algal surfaces poses an interesting point in the consideration of the initial bioassay rate between cell density and copper concentration. Copper is rapidly adsorbed to algal cells, attaining equilibrium within minutes compared to slower uptake and effects on cell division. Thus, toxicity is proportional to the amount of copper associated with the cells if dissolved copper does not become limiting during the experiment (FRANKLIN et al. 2002). These authors obtain a diminution in copper toxicity for a range of added copper concentrations of 2.5 to 30 mg . l^{-1} with initial cell densities of 10^5 cells per ml. Note that our lower tested copper concentration is 50 mg . l^{-1}, thus no attenuation of copper toxicity would be expected for the cellular densities inoculated.

Copper exposure caused decrease in cellular density. Lower cell division rate is also reflected by the increasing number of single cells in copper stressed cultures. This coincides with the observation of SCHROEVERS & ROEST (1967), who observed a decreasing frequency of aggregates of *Dictyosphaerium pulchellum* under adverse environmental conditions. A tendency from 4-celled to unicellular forms of *Scendesmus* has been also reported with increasing copper concentrations (STOKES et al. 1973).

Increment in copper concentration was accompanied by reduction of chlorophyll content per cell. Bleaching effect due to exposure to high copper concentrations has been repeatedly informed for algae (RAI et al. 1981, STAUBER & FLORENCE 1987, RIJSTENBIL et al. 1994, PRASAD et al. 1998, SCHIARITI et al. 2004). This may be the consequence of the inhibition of chlorophyll biosynthetic pathway (CLIJSTERS & VAN ASSCHE 1985, JONES & JORDAN 1993) or of the synthesis of protein D1, responsible for the assemblage of chlorophyll molecules in the reaction center of the photosystems (PÄTSIKKÄ et al. 1998). Decrease in chlorophyll content kept no lineal relation with decrease in cellular density, differing from the results informed by LAM et al. (1999) for *Chlorella vulgaris*. Mucilage removal at the beginning of the experiment enhanced bleaching effect. As mentioned above, adsorption to extracellular mucilage components delays metal uptake by the cell, since it introduces an additional step of chemical equilibrium metal-ligand within mucilage previous to the corresponding association with membrane transporter. Chlorophyll bleaching implies intracellular uptake of copper, so at a given time interval (48 h) it will result more evident in those cells with less barriers for copper uptake. Copper binding to extracellular ligands in Chlorophyceae has been reported to occur more efficiently during exponential growth (LOMBARDI & VIEIRA 2000). Using different spin labels for electronic paramagnetic resonance, FREIRE-NORDI et al. (1998) calculated their diffusion coefficients through mucilage capsules and cell walls in the desmid *Spondylosium panduriforme* and concluded the capsule plays a role in selectivity as a result of polar interactions with the labels. In short, the more copper is adsorbed to negatively charged polysaccharides in the extracellular mucilage, the less will be accessible to specific membrane transporters for cellular uptake. TWISS et al. (1993) concluded that copper tolerance in a strain of *Scenedesmus acutus* was related with extracellular cell wall binding and exclusion of the metal.

Extracellular mucilage secretion is an active process (note that the longer sonication intervals, which probably affected cell viability, coincide with the incapability of regenerating mucilage after 48 h of incubation). KOMÁREK & PERMAN (1978) mention the presence of pores in the cell wall of *Dictyosphaerium* and relate these to mucilage liberation. Preliminary TEM and fluorescence observations (RODRÍGUEZ et al., unpublished results) indicated an abundant vesicle traffic from Golgi to plasmalemma in actively secreting cells. Nevertheless mucilage extrusion appears more as a process related to cell division rather than as a response to metal stress. When observing the kinetic of regeneration of mucilage in the desmid *Spondylosium panduriforme*, GOUVEA & VIEIRA (1998) concluded that capsule regeneration is genetically determined and independent of environmental conditions.

We conclude that in *Dictyosphaerium pulchellum* controlled mucilage removal by ultrasonication enhanced susceptibility to bleaching, while it did not modify significantly cellular density.

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References

BARÓN, M., ARELLANO, J. B. & LÓPEZ GORGÉ, J. (1995): Copper and photosystem II: A controversial relationship. – Physiol. Plant. **94**: 174–180.

CLIJSTERS, H. & VAN ASSCHE, F. (1985): Inhibition of photosynthesis by heavy metals. – Photosynth. Res. **7**: 31–40.

EPA (1985) (United States Environmental Protection Agency): Short Term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Freshwater Organisms. EPA/600/4-85/014.

FOX, T. C. & GUERINOT, M. L. (1998): Molecular Biology of Cation Transport in Plants. – Annu. Rev. Plant Physiol. Plant Mol. Biol. **49**: 669–696.

FRANKLIN, N. M., STAUBER, J. L., APTE, S. C. & LIM, R. P. (2002): Effect of initial cell density on the bioavailability and toxicity of copper in microalgal bioassays. – Environ. Toxicol. Chem. **21**: 742–751.

FREIRE-NORDI, C. S., VIEIRA, A. A. H. & NASCIMENTO, O. R. (1998): Selective permeability of the extracellular envelope of the microalga *Spondylosium panduriforme* (Chlorophyceae) as revealed by electron paramagnetic resonance. – J. Phycol. **34**: 631–637.

GEIS, S. W., FLEMING, K. L., KORTHALS, E. T., SEARLE, G., REYNOLDS, L. & KARNER, D. A. (2000): Modifications to the algal growth inhibition test for use as a regulatory assay. – Environ. Toxicol. Chem. **19**: 36–41.

GIRLING, A. E., PASCOE, D., JANSSEN, C. R., PEITHER, A., WENZEL, A., SCHÄFER, H., NEUMEIER, B., MITCHELL, G. C., TAYLOR, E. J., MAUND, S. J., LAY, J. P., JÜTTNER, I., CROSSLAND, N. O., STEPHENSON, R. R. & PERSOONE, G. (2000): Delopment of methods for evaluating toxicity to freshwater ecosystems. – Ecotoxicol. Environ. Saf. **45**: 148–176.

Extracellular mucilage secretion is an active process (note that the longer sonication intervals, which probably affected cell viability, coincide with the incapability of regenerating mucilage after 48 h of incubation). KOMÁREK & PERMAN (1978) mention the presence of pores in the cell wall of *Dictyosphaerium* and relate these to mucilage liberation. Preliminary TEM and fluorescence observations (RODRÍGUEZ et al., unpublished results) indicated an abundant vesicle traffic from Golgi to plasmalemma in actively secreting cells. Nevertheless mucilage extrusion appears more as a process related to cell division rather than as a response to metal stress. When observing the kinetic of regeneration of mucilage in the desmid *Spondylosium panduriforme*, GOUVEA & VIEIRA (1998) concluded that capsule regeneration is genetically determined and independent of environmental conditions.

We conclude that in *Dictyosphaerium pulchellum* controlled mucilage removal by ultrasonication enhanced susceptibility to bleaching, while it did not modify significantly cellular density.

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References

BARÓN, M., ARELLANO, J. B. & LÓPEZ GORGÉ, J. (1995): Copper and photosystem II: A controversial relationship. – Physiol. Plant. **94**: 174–180.

CLIJSTERS, H. & VAN ASSCHE, F. (1985): Inhibition of photosynthesis by heavy metals. – Photosynth. Res. **7**: 31–40.

EPA (1985) (United States Environmental Protection Agency): Short Term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Freshwater Organisms. EPA/600/4-85/014.

FOX, T. C. & GUERINOT, M. L. (1998): Molecular Biology of Cation Transport in Plants. – Annu. Rev. Plant Physiol. Plant Mol. Biol. **49**: 669–696.

FRANKLIN, N. M., STAUBER, J. L., APTE, S. C. & LIM, R. P. (2002): Effect of initial cell density on the bioavailability and toxicity of copper in microalgal bioassays. – Environ. Toxicol. Chem. **21**: 742–751.

FREIRE-NORDI, C. S., VIEIRA, A. A. H. & NASCIMENTO, O. R. (1998): Selective permeability of the extracellular envelope of the microalga *Spondylosium panduriforme* (Chlorophyceae) as revealed by electron paramagnetic resonance. – J. Phycol. **34**: 631–637.

GEIS, S. W., FLEMING, K. L., KORTHALS, E. T., SEARLE, G., REYNOLDS, L. & KARNER, D. A. (2000): Modifications to the algal growth inhibition test for use as a regulatory assay. – Environ. Toxicol. Chem. **19**: 36–41.

GIRLING, A. E., PASCOE, D., JANSSEN, C. R., PEITHER, A., WENZEL, A., SCHÄFER, H., NEUMEIER, B., MITCHELL, G. C., TAYLOR, E. J., MAUND, S. J., LAY, J. P., JÜTTNER, I., CROSSLAND, N. O., STEPHENSON, R. R. & PERSOONE, G. (2000): Delopment of methods for evaluating toxicity to freshwater ecosystems. – Ecotoxicol. Environ. Saf. **45**: 148–176.

GLEDHILL, M., NIMMO, M. & HILL, S. T. (1997): The toxicity of copper (II) species to marine algae, with particular reference to macroalgae. – J. Phycol. **33**: 2–11.

– – – (1999): The release of copper – complexing ligands by the brown alga *Fucus vesiculosus* (Phaeophyceae) in response to increasing total copper levels. – J. Phycol. **35**: 501–509.

GOUVÊA, S. P. & VIEIRA, A. A. H. (1998): Estudo sobre as cápsulas mucilaginosas de *Spondylosium panduriforme*. – In: PAIVA AZEVEDO, M. T. (ed.): ANAIS, IV Congresso Latino – Americano. II Reunião Ibero – Americana. VII Reinão Brasileira de Ficologia **1**: 423–437.

HILL, K. L., HASSETT, R., KOSMAN, D. & MERCHANT, S. (1996): Regulated copper uptake in *Chlamydomonas reinhardtii* in response to copper availability. – Plant Physiol. **112**: 697–704.

JONES, R. M. & JORDAN, P. M. (1993): Purification and properties of the uroporphyrinogen decarboxilase from *Rhodobacter sphaeroides*. – Biochem. J. **293**: 703–712.

KAPLAN, D., CHRISTENSEN, D. & ARAD, S. (1987): Chelating properties of extracellular polysaccharides from *Chlorella* sp. – Appl. Environ. Microbiol. **12**: 2953–2956.

KIEFER, E., SIGG, L. & SCHOSSELER, P. (1997): Chemical and spectroscopy characterization of algae surfaces. – Environ. Sci. Technol. **31**: 759–764.

KOMÁREK, J. & PERMAN, J. (1978): Review of the genus *Dictyosphaerium* (Chlorococcales). – Archiv Hydrobiol./Algolog. Studies **20**: 233–297.

KRISHNAMURTHY, K. V. (1999): Methods in Cell Wall Cytochemistry. – 318 pp., CRC Press, Boca Raton, USA.

KÜPPER, H., ŠETLÍK, I., SPILLER, M., KÜPPER, F. C. & PRÁŠIL, O. (2002): Heavy metal-induced inhibition of photosynthesis: targets of in vivo heavy metal chlorophyll formation. – J. Phycol. **38**: 429–441.

LAM, P. K. S., WUT, P. F., CHAN, A. C. W. & WU, R. S. S. (1999): Individual and combinated effects of cadmium and copper on the growth response of *Chlorella vulgaris*. – Environ. Toxicol. **14**: 347–353.

LOBBAN, C. S. & HARRISON, P. J. (1994): Seaweed Ecology and Physiology. – 366 pp., Cambridge University Press. New York.

LOMBARDI, A. T. & VIEIRA, A. A. H. (2000): Copper complexation by Cyanophyceae and Chlorophyceae exudates. -Phycologia **38**: 118–125.

LOMBARDI, A. T., VIEIRA, A. A. H. &. SARTORI, L. A. (2002): Mucilaginous capsule adsorption and intracellular uptake of copper by *Kirchneriella aperta* (Chlorococcales). – J. Phycol. **38**: 332–337.

NICHOLS, H. W. & BOLD, H. C. (1965): *Trichosarcina polymorpha* gen. et sp. nov. – J. Phycol. **1**: 34–38.

PÄTSIKKÄ, E., ARO, E. V., TYSTJÄRVI, E. (1998): Increase in the quantum yield of photoinhibition contributes to copper toxicity *in vivo*. – Plant Physiol. **117**: 619–627.

PRASAD, M. N., DREJ, K., SKAWINSKA, A. & STRATKA, K. (1998): Toxicity of cadmium and copper in *Chlamydomonas reinhardtii* wild-type (WT 2137) and cell wall deficient mutant strain (CW 15). – Bull. Environ. Contam. Toxicol. **60**: 306–311.

RAI, L., GAUR, J. P & KUMAR, H. D. (1981): Phycology and heavy metal pollution. – Biol. Rev. **56**: 99–151.

RIJSTENBIL, J. W., DERKSEN, J. W. M., JERINGA, L. J. A., POORTVLIET, T. C. W., SANDEE, A., VAN DEN BERG, M., VAN DRIE, J. & WINJNHOLDS, J. A. (1994): Oxidative stress induced by copper: defence damage in the marine planktonic diatom *Ditylum brightwelli* grown in continuous cultures with high and low zinc levels. – Mar. Biol. **119**: 583–590.

SANDMAN, G & BÖGER, P. (1980): Copper-mediated lipid peroxidation processes in photosynthetic membranes. – Plant. Physiol. **66**: 797–800.

SCHECHER, W. D. & MC AVOY, D. C. (1994): MINEQL+: A chemical equilibrium program for personal computers (Version 3.01). Software. – 107 pp., Hallowell, Maine Environmental Research.

SCHIARITI, A., JUÁREZ, A. B. & RODRÍGUEZ, M. C. (2004): Effects of sublethal concentrations of copper on three strains of green microalgae under autotrophic and mixotrophic culture conditions. – Arch. Hydrobiol./Algolog. Studies **114:** 143–157.

SCHROEVERS, P. J. & ROEST, A. C. F. (1967): Merkwaardidge verschijnselen bij *Dictyophaerium pulchellum* WOOD. – Gorteria **3**: 224–232.
SCOR-UNESCO (1966): Report Working Group 17. Determination of photosynthetic pigments in seawater. Monographs on Oceanographic Methodology, 1. – UNESCO, Paris.
SOKAL, R. R . & JAMES, R. F. (1981): Biometry: The principles and practice of statistics in biological research. 2nd ed. – 859 pp., Freeman, New York, USA.
STAUBER, J. L. & FLORENCE, T. M. (1987): Mechanism of toxicity of ionic copper and copper complexes to algae. – Mar. Biol. **94**: 511–519.
STOKES, P. M., HUTCHINSON, T. C. & KRAUTER, K. (1973): Heavy metal tolerance in algae isolated from contaminated lakes near Sudbury, Ontario. – Can. J. Bot. **51**: 2155–2168.
SUEUR, S., VAN DEN BERG, C. M. G. & RILEY, J. P. (1982): Measurement of the metal complexing ability of exudates of marine macroalgae. – Limnol. Oceanogr. **27**: 536–543.
SUNDA, W. G & HUNSTMAN, S . A. (1998): Interactions among Cu^{2+}, $Zn^{2+}$and Mn^{2+} in controlling cellular Mn, Zn, and growth rate in coastal alga *Chlamydomonas*. – Limnol. Oceanogr. **43**: 1055–1064.
TWISS, M. R. (1996): The importance of chemical speciation from the bulk solution to the cell surface. – J. Phycol. **32**: 885–886.
TWISS, M. R.; WELBOURN, P. M. & SCHWÄRTZEL, E. (1993): Laboratory selection for copper tolerance in *Scenedesmus acutus* (Chlorophyceae). – Can. J. Bot. **71**: 333–338.
VENRICK, E. L. (1978): How many cells to count? – In: SOURNIA, A. (ed.): Phytoplankton Manual, **1**: 67–180, UNESCO, Paris.

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