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Ultrastructure and X-ray microanalysis of *Euglena gracilis* (Euglenophyta) under chromium stress

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The effect of chromium on the biology of *Euglena gracilis* was studied. The ultrastructural modifications caused by this metal and its location within the cell were analyzed by TEM and EDXA, respectively. The effects of chromium on protein, pigment, and lipid contents were also studied in order to evaluate the metabolic responses to metal exposure. Two strains of *Euglena gracilis,* UTEX 753 (from the Culture Collection of Texas University) and MAT (isolated from the Matanza River), were used in this research. Both were grown in photoauxotrophic and photoheterotrophic conditions and exposed to different metal concentrations. In all treated cells, increases in total protein and lipid contents, changes in chlorophyll amount, and alterations in fine structure were observed, especially with the higher concentration tested. In photosynthetic treated cells, assays showed chloroplast thylakoid disorganization, the presence of cytoplasm lipid globules, and several vacuoles with electron-dense inclusions and remnants of membranes inside. Nuclei presented lobulations, and eventually total fragmentation in some cells treated with the highest chromium concentration was seen, suggesting that chromium cytotoxicity leads to cellular death. The EDXA spectrum showed well-defined Cr and S peaks in the vacuoles containing electron-dense inclusions and remnants of membranes from autotrophic MAT samples. These results indicate that the different defense mechanisms against chromium depend on strain type and culture conditions. The S peak detected in MAT would suggest that sulfur-rich proteins groups play an important role in the detoxification system inducing metal-complex accumulation into vacuoles.

KEY WORDS: Chromium, *Euglena gracilis,* Euglenophyta, Ultrastructure, X-ray microanalysis

INTRODUCTION

In recent years, polluted environments have increased as a consequence of the industrial development associated with human population growth. Waste discharges without pretreatment play an important role in the pollution of aquatic systems. An increase in the concentration of essential and toxic metals may affect phytoplankton composition and richness, altering the steps of the food chain.

It is well known that algal cells exposed to heavy metals may suffer serious morphological and biochemical alterations (Devars *et al.* 1998; Rai & Rai 1998; Okamoto *et al.* 2001). The effects of metals on fine structure have been described for several micro- and macroalgae (Wong *et al.* 1994; Amado Filho *et al.* 1996; Nagel *et al.* 1996; Wong *et al.* 1997; Amado Filho *et al.* 1999; Leonardi & Vásquez 1999). The study of metabolic responses is therefore very important to understanding the cellular defense machinery involved in environmental metal toxicity (Gingrich *et al.* 1984, 1988). In recent years, the process of metal removal from solutions by algal absorption has been improved (Garnham 1997), and macroalgae has been the most common biomass used in this type of bioremediation (Talarico 2002; Andrade *et al.* 2004).

Among the industrial waste discharged into aquatic systems, chromium is one of the environmental contaminants usually released into waters by metallurgical, tanning, and other industries. It has been demonstrated that the cytotoxic effects of this metal on animals and plants can alter aquatic ecosystems (Cervantes *et al.* 2001; Vajpayee *et al.* 2001). Hexavalent chromium is considered the most toxic form of the metal, and it usually associates with oxygen to form chromate (CrO^{2-}_4). This molecule can easily permeate through cell membranes and function as an alternative substrate for the sulfate transport system (Riedel 1985). Hexavalent chromium physicochemical characteristics, pH-dependent equilibrium and redox properties, as well as the thermodynamic and kinetic stability observed in various chromium oxidation states are key elements to understand its behavior in living systems (Cieslak-Golonka 1996; Rocchetta *et al.* 2006b). The reported

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effects of hexavalent chromium on algae cells include pigment content reduction, chloroplast disorganization, mitochondria damage, cytoskeleton alterations, and DNA damage (Wang 1999; Cervantes *et al.* 2001; Rocchetta *et al.* 2003).

Many authors reported the great tolerance to heavy metals showed by algae cells collected from highly contaminated rivers (Devars *et al.* 1998; Rai & Rai 1998). Previous works on the phytoplankton of the Matanza River (Conforti 1991; Conforti *et al.* 1995), one of the most polluted rivers of Buenos Aires, Argentina, showed that euglenoids are among the most important algae groups of this community. For decades, the quality of this river has been deteriorating because of the high discharges of pollutants derived from untreated sewage, solid wastes, and petroleum. The main industrial discharge comes from the tanning industry (Beron 1984). In a previous study, we reported the damage caused by hexavalent chromium on two strains of *Euglena gracilis* Klebs (1883): UTEX 753 (from the Culture Collection of Texas University) and MAT (isolated from the Matanza River). The exposure to different metal concentrations showed a higher resistance for MAT strain (Rocchetta *et al.* 2003). Assays on lipid peroxidation and changes in the fatty acid composition due to chromium exposure revealed differences in the metabolism system of both algal strains (Rocchetta *et al.* 2006a).

Energy dispersive X-ray analysis (EDXA) has been employed systematically in biological sciences to identify chemical elements present in different tissues. This technique is mainly used to localize ions in cellular compartments, to monitor ion distribution changes during physiological processes, to determine the behavior of toxic ions incorporated by cells, or in detoxifying processes (Zierold 1993).

In order to contribute to understanding of the process of metal accumulation by microalgae we determined in two strains of *E. gracilis* the ultrastructural modifications caused by Cr and the localization of this element within the cell by TEM and EDXA, respectively, The effects of chromium on protein, pigment, and lipid contents have also been studied in order to evaluate the metabolic responses to metal exposition.

MATERIAL AND METHODS

Microorganisms, culture conditions, and metal toxicity assays

Bioassays were performed with two photosynthetic strains of *E. gracilis* and their nonphotosynthetic mutants bleached with streptomycin (Ruiz *et al.* 2004). Dr Richard Triemer generously provided one of the strains, UTEX 753, from the Culture Collection of Algae of Texas University, USA. The MAT strain was isolated from the highly polluted Matanza River, Buenos Aires, Argentina. Experimental cultures were grown in organic medium EGM (CCAP 2001), at 24 ± 1°C with cool-white fluorescent continuous light at 150 μE m^{-2} s^{-1} irradiance. Axenicity was monitored plating cultures in a bacterial medium. A new culture was initiated 6 days before each experiment in order to obtain an inoculum in exponential growth.

Experiments were carried out on static cultures containing 150 ml culture medium in 250-ml glass flasks at 24 ± 1°C and under continuous light. Aliquots of stock cultures (wild-type MAT and UTEX and their nonphotosynthetic mutants) containing 10^5 cell ml^{-1} were inoculated in each flask. The $K_2Cr_2O_7$ was added axenically from a 0.1 M stock solution until two dichromate concentrations were obtained: one close to the minimum concentration necessary to obtain 50% growth, IC_{50} (20 μM), and the other, higher (100 μM). Harvesting time for all the cultures was 96 h after metal stress development (U.S. Environmental Protection Agency 1994).

Protein determination

Aliquots of 5-ml culture were harvested by centrifugation at 3700 × *g* for 15 min; washed three times with 0.154 M phosphate buffer, pH 7; and then sonicated. Protein content was evaluated by the Bradford (1976) method using bovine serum albumin as standard.

Chlorophyll quantification

Chlorophyll content was determined following Wellburn (1994) procedure. Cells were harvested filtering 5-ml samples with Whatman GF/C filter papers. Pigments (chlorophylls *a* and *b*) were extracted exposing samples to 80% acetone solution (v/v) for 24 h at 4°C, and optical densities were measured with a UV/VIS JAS-CO 7850 spectrophotometer.

Lipid extraction and quantification

To determine lipid content, culture cells were harvested by centrifugation at 3700 × *g* for 15 min and washed three times with 0.154 M phosphate buffer, pH 7. Total lipids were extracted with chloroform:methanol (2 : 1 v/v) and quantified according to the Bligh and Dyer (1959) method.

Transmission electron microscopy (TEM)

Cells collected by centrifugation at 4500 × *g* for 20 min were fixed in 2.5% glutaraldehyde in 0.1 M Na-cacodylate buffer. Then they were postfixed in 1% osmium tetroxide in 0.1 M Na-cacodylate buffer for 2 h, dehydrated in an acetone series, and embedded in Spurr resin. Ultrathin sections were cut with a diamond knife and stained with uranyl acetate and lead citrate. Later, they were examined using a JEOL 100 CX-II electron microscope at the Centro de Investigaciones Básicas y Aplicadas de Bahía Blanca.

Analytical TEM

For the EDXA, cells were fixed for transmission electron microscopy without osmium tetroxide and were observed in a JEOL 1200 EX microscope equipped with a Noran-Voyager analytical system. The original copper specimen retainer was changed for a graphite one to minimize undesirable X-ray peaks. Typical acquisition data were accelerating voltage, 80 kV; livetime, 300 s; deadtime, 20%; sample tilt angle, 30°; and beam spot size, about 60 nm (diameter). Nonstained ultrathin sections on nylon grids were used for analytical purposes.

Statistical analysis

Mean and standard deviations were obtained from the duplicates of each concentration. Each treatment was carried out in duplicate, and each assay was repeated three times. Data were

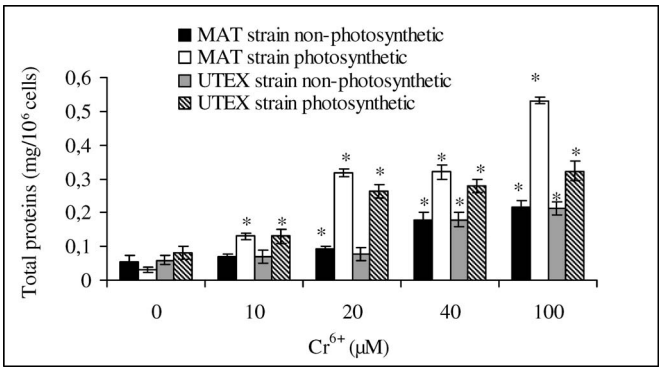


Fig. 1. Total protein content in two strains of *Euglena gracilis* cultured in autotrophic and heterotrophic conditions at different chromium concentrations. * Denotes significant ($P < 0.05$) analysis of variance between control and treated cells.

evaluated by analysis of variance. A value of $P < 0.005$ was considered significant.

RESULTS

Effects of chromium on protein, pigment, and lipid content

Chromium exposure increased the total protein content in all the treated cells in a concentration-dependent manner. The highest values were recorded in the photosynthetic cells ($P < 0.05$) (Fig. 1). It is worth mentioning that the highest chromium concentration assayed (100 μM) led to an 11-fold increase of protein content in MAT green cells with respect to controls, and this was the highest value observed.

Fig. 2 shows the amount of chlorophyll *a* and *b* of both strains cultured in all the conditions. In cells grown in heterotrophic conditions, pigments were detected in both strains since the streptomycin treatment used for bleaching induced the transformation of active chloroplasts into proplastids. However, pigment levels in bleached control cells were lower than in green strains, this difference being significant for chlorophyll *a.* This pigment's content significantly decreased in both photosynthetic strains after the two metal treatments (Fig. 2A), whereas it increased in heterotrophic strains with the highest chromium concentration assayed. Chlorophyll *b* content, on the other hand, significantly increased in all the strains in most of the conditions tested (Fig. 2B).

Total lipid content increased significantly in all strains when they were treated with 40 or 100 μM Cr (Fig. 3). Both wild-type strains showed higher lipid levels in control and treated cells with respect to their respective bleached counterparts. Regarding the strain origin, the UTEX cells showed higher lipid content than MAT cells.

Conventional TEM

No ultrastructural differences between autotrophic UTEX and MAT control cells were detected. Both algal strains presented the typical characteristics of the species *E. gracilis,* among which is the presence of chloroplasts with diplopyrenoids (Figs 4, 5). On the other hand, heterotrophic cells, UTEX and MAT, showed higher paramylon content and a more extensive

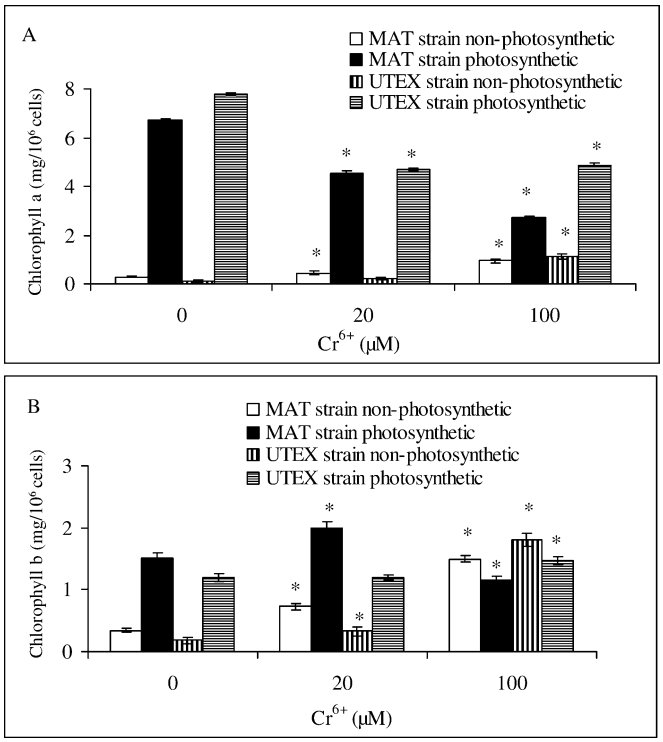


Fig. 2. Effects of chromium on chlorophyll *a* (A) and chlorophyll *b* (B) content in two strains of *Euglena gracilis* cultured in autotrophic and heterotrophic conditions. * Denotes significant ($P < 0.05$) analysis of variance between control and treated cells.

mitochondrial network than green cells (Figs 6, 7). Moreover, proplastids could be observed in the heterotrophic cultures (Fig. 7).

Cells grown in the presence of chromium showed several changes in their fine structure, and these changes were more significant with the highest concentration assayed. In photosynthetic-treated cells, we observed chloroplasts with highly disorganized thylakoids (Figs 8, 9), abundant cytoplasmic lipid globules (Fig. 8), and numerous vacuoles with electron-dense inclusions and remnants of membranes inside (Figs. 9, arrows, 10). Both autotrophic and heterotrophic treated cells showed mitochondrial nets with hypertrophic development (Figs 11, 12). However, only in autotrophic cells mitochondria

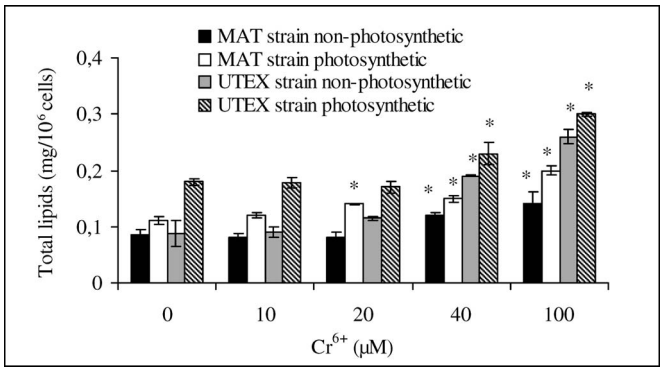
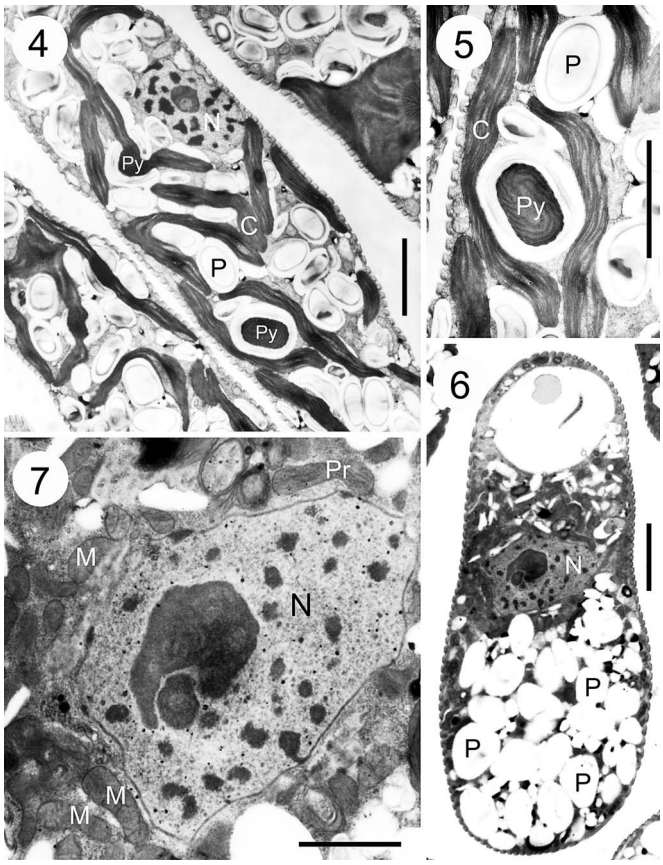


Fig. 3. Total lipid content in two strains of *Euglena gracilis* cultured in autotrophic and heterotrophic conditions at different chromium concentrations. * Denotes significant ($P < 0.05$) analysis of variance between control and treated cells.



Figs 4–7. Transmission electron micrographs of *Euglena gracilis* control cells.

Figs 4, 5. UTEX strain grown in autotrophic conditions.

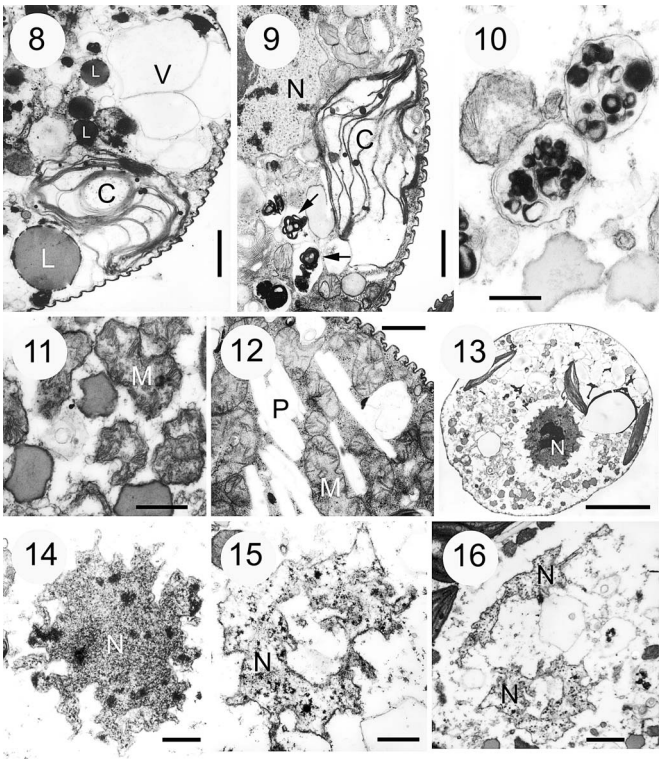
Fig. 4. General view of the cells. Scale bar = 10 μm.

Fig. 5. Detail of a diplopyrenoid. Scale bar = 5 μm.

Figs 6, 7. MAT strain grown in heterotrophic conditions.

Fig. 6. General view of a cell with numerous paramylon grains. Scale bar = 5 μm.

Fig. 7. Detail of a cell portion showing the nucleus, mitochondria, and one proplastid. Scale bar = 10 μm. C, chloroplast; M, mitochondrion; N, nucleus; P, paramylon; Pr, proplastid; Py, pyrenoid.



Figs 8–16. Transmission electron micrographs of *Euglena gracilis* cells treated with 100 μM Cr^{6+}.

Figs 8, 10, 13. MAT strain grown in autotrophic conditions.

Figs 9, 11, 15, 16. UTEX strain grown in autotrophic conditions.

Fig. 12. UTEX strain grown in heterotrophic conditions.

Fig. 14. MAT strain grown in heterotrophic conditions.

Figs 8, 9. Details of a cell portion showing chloroplast disorganization, lipid globules, and numerous vacuoles with electron-dense inclusions and remnants of membranes inside (arrows). Scale bars = 1 μm.

Fig. 10. Detail of vacuoles. Scale bar = 0.5 μm.

Figs 11, 12. Detail of mitochondria. Note in Fig. 11, they present irregular shape and cristae disorganization or loss. Scale bars = 1 μm.

Fig. 13. General view of a cell with a lobulated nucleus. Scale bar = 10 μm.

Fig. 14. Detail of a lobulated nucleus. Scale bar = 0.5 μm.

Figs 15, 16. Details of nucleus fragmentation. Scale bars = 0.5 μm.

presented irregular shape and cristae disorganization (Fig. 11). No changes were observed in the pellicle structure (Figs 9, 12). Nuclei exhibited irregular lobulations (Figs 13, 14) and eventually total fragmentation in some of the cells exposed to the highest chromium concentration (Figs 15, 16).

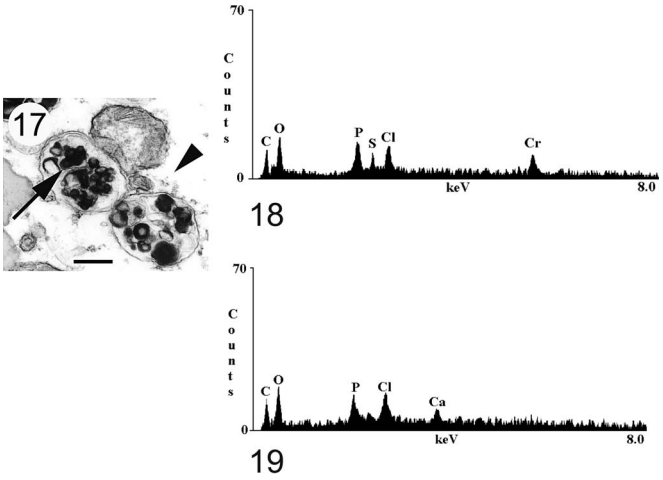
Analytical TEM

Electron probe X-ray microanalysis was performed to evaluate the local distribution of elements within the cell. The only structures that presented characteristic peaks of interest were the cytoplasmatic vacuoles with electron-dense inclusions and remnants of membranes. The EDXA spectrum showed well-defined Cr and S peaks only in the above-mentioned vacuoles of autotrophic MAT samples (Figs 17, arrow, 18). Elements C, O, P, Cl, and Ca were detected not only in these vacuoles but also in other analytical points (Figs 17, arrowhead, 19). X-ray spectra did not show any peak of interest in the chloroplasts of any strain (Fig. 20).

DISCUSSION

The increased production of total lipid and protein contents in chromium treated cells could be directly related to the defense/detoxification system. In a previous report, an increase in the level of saturated fatty acids (SAFAs) has been interpreted as a mechanism to counteract the loss of polyunsaturated fatty acids, especially from chloroplasts and mitochondria, caused by chromium exposure (Rocchetta *et al.* 2006a). In the present work, the augmented lipid content recorded may be correlated with the abundance of lipid globules observed with TEM in chromium-treated cells. Regnault *et al.* (1995) have shown that *E. gracilis* produces storage lipids (SAFAs like C14 and C16) depending on culture conditions and stress situations.

In *E. gracilis,* increased concentrations of cholesterol and phospholipids related to the chloroplast membrane account for



Figs 17–19. *Euglena gracilis* MAT strain grown in autotrophic conditions.

Fig. 17. Vacuoles with electron-dense inclusions and remnants of membranes. The arrow and arrowhead show the area of emission. Scale bar = 0.5 μm.

Fig. 18. X-ray spectrum of vacuole content.

Fig. 19. X-ray spectrum of cytoplasmic region.

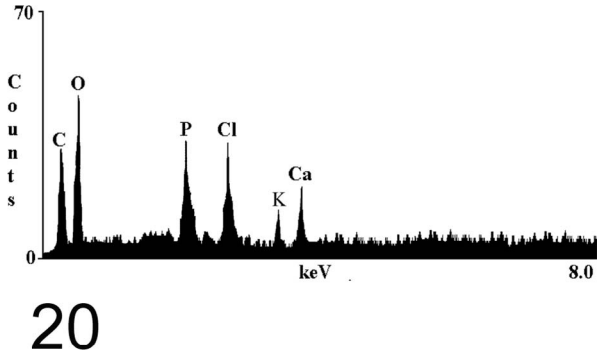


Fig. 20. *Euglena gracilis* MAT strain grown in autotrophic conditions. X-ray spectrum of chloroplasts.

the cadmium-induced increase in total lipid content (Einiker-Lamas *et al.* 1996), indicating that chloroplasts may be the primary target of heavy metal toxicity (Duret *et al.* 1986). The same total lipid increase has been observed in *Euglena* cells exposed to concentrations close to the IC_{50} of copper and zinc (Einicker-Lamas *et al.* 2002).

Several authors have reported cell division damage suffered by algal samples treated with hexavalent chromium (Fasulo *et al.* 1982; Rocchetta *et al.* 2003). This damage involves replication blocking and secondary inhibition of translocation and transcription processes (Wong & Trevors 1988; Cieslak-Golonka 1996; Cervantes *et al.* 2001). The nucleus fragmentation observed in *E. gracilis* treated with the highest chromium concentration shows that the cytotoxicity of this metal leads to cellular death.

Chloroplasts seem to be the organelles most affected by chromium since even the lowest concentration tested produced thylakoid disorganization. Although chlorophyll *a* content decreases in treated green cells, there is an increase of the same pigment in bleached cells, possibly as a response to lowered energy availability. In accordance, the great mitochondria net development detected in the same treated cells seems also to respond to impaired metabolic energy production. A recent molecular biology study, showing overexpression of several chloroplast and mitochondria enzymes (Ferreira *et al.* 2006), gives further support to this idea. Higher ATP production could translate into more energy to protein activation or synthesis, needed for defense/detoxification mechanisms. In the same fashion, the increase in total protein content in all strains with increasing metal concentration possibly reflects defense/detoxification protein synthesis.

The presence of vacuoles with internal membranes indicates drastic changes in cellular ultrastructure due to the autophagic process previous to cellular death. Carbon-starved cells of *E. gracilis* and *E. granulate* revealed lysosome or autophagic vacuole formation, containing mitochondria and chloroplast remnants to provide materials for basic metabolism (Leedale & Buetow 1976).

Studies carried out by Roderer (1979) demonstrated that several heavy metals, like chromium, were able to change cellular permeability by binding membrane protein SH groups. Thus, ionic transport would be altered, affecting cell ion concentrations.

The electron-dense deposits observed in vacuoles from treated cells could be related to a detoxifying system. Moreover, we have detected chromium and sulfur peaks in the EDXA spectrum of MAT green cells restricted to these organelles. The colocalization of chromium and sulfur peaks would suggest that the positively charged metal would bind to negatively charged phosphate or thiol groups from sulfur-rich proteins within these dense inclusions (Hamer 1986).

Previous studies with copper and zinc show that those metals are also stored in vacuoles (Einiker-Lamas *et al.* 2002). Experimental evidences suggest that an unspecific membrane protein could transport cadmium by ionic channels into chloroplasts (Hinkle *et al.* 1987; Fuks & Homble 1995). A study on cadmium toxicity has pointed chloroplasts as the primary structures for the storage of this metal (Mendoza-Cozatl & Moreno Sánchez 2005). In our study, Cr was not detected in intracellular compartments such as chloroplasts (Fig. 20), mitochondria, or starch granules. It was detected only in the dense inclusions of vacuoles from MAT cells grown in autotrophic conditions. These results suggest either that the UTEX strain has a different defense mechanism against chromium or that Cr concentration accumulated within the cells of this strain is beyond the detection limit of the our X-ray system. However, the fact that the MAT strain is much more tolerant to Cr than UTEX (IC_{50} 24 vs 2.4 μM) (Rocchetta et al. 2003) favors the former hypothesis. Our study suggests that the main chromium detoxification mechanism of *E. gracilis,* at least in the MAT strain, is based on chelating proteins and cytoplasmatic sequestering, with metal complex accumulation into vacuoles.

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