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Chromium Toxicity to *Euglena gracilis* Strains Depending on the Physicochemical Properties of the Culture Medium

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Chromium can be found in several oxidation states being the most stable and common forms trivalent Cr(III) and hexavalent Cr(VI), which present different chemical properties (Bagchi *et al.*, 2002). This metal is an essential element for some organisms in relation with some metabolic pathways (Hamilton & Wetterhahn, 1987). Discharges of this metal into surface water increase its concentration in the environment due to the application of this metal in diverse industries, such as the metallurgical and tanning industries.

Hexavalent chromium is an environmental contaminant whose cytotoxic effects in animals and plants are well documented (Cervantes *et al.*, 2001; Vajpayee *et al.*, 2001). It is considered the most toxic form of this metal, usually associates with oxygen to form chromate (CrO_4^{2-}). This molecule can easily go through cell membranes, as an alternative substrate for the sulfate transport system (Riedel, 1985). Cr(VI) 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 the interaction of Cr(VI) in living systems (Cieslak-Golonka, 1996).

Many authors reported the great tolerance to heavy metals showed by algae cells collected from highly contaminated rivers (Rai and Rai, 1998, Devars *et al.*, 1998). Previous works on the phytoplankton of the Matanza River, one of the most polluted rivers of Buenos Aires, Argentina, showed that the euglenoids were one of the most important groups of this community. For decades, the quality of this River has been deteriorating due to the high discharges of different pollutants derived from untreated sewage, solid wastes and petroleum. The most important industrial discharge was produced by tanning (Conforti, 1991, Conforti *et al.* 1995). In a previous paper (Rocchetta *et al.*, 2003), we demonstrated the damage produced by hexavalent chromium on two strains of *Euglena gracilis;* UTEX 753 (from the Culture Collection of the Texas University), and MAT (isolated from the Matanza River). Both were cultured in Buetow mineral medium with a neutral pH (Buetow, 1982). Exposure to different metal concentrations showed that MAT had higher resistance than UTEX.

Based on this information, we decided to compare chromium toxicity on these

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strains using a mineral medium but with a lower pH (Buetow, 1982) to evaluate the possible impact from some acid industrial effluents, related with the leather treatment, discharged into the River.

MATERIALS AND METHODS

All the experiments were carried out on axenic cultures of *Euglena gracilis* strains UTEX 753 (from the Culture Collection of Algae of the Texas University), and MAT (isolated from the Matanza River, Ruiz *et al.*, 2004.). Experimental cultures were grown in a mineral medium with B_{12} vitamin (0.5 μg/L), Cramer and Myers, initial pH 5 (Buetow, 1982), at 24 ± 1 °C, under continuous light. Axenicity was monitored plating the cultures in a bacteria broth medium. A new culture was started 6 days before each experiment in order to obtain an inoculum in exponential growth.

Aliquots of stock cultures from both strains containing 10^5 cells/ml were inoculated in each flask $K_2Cr_2O_7$ MERCK (analytical purity), was added axenically from a 0.1 M stock solution to a total nominal concentration of 20, 100, 200 and 400 μM Cr(VI). Each treatment was done in duplicate and each assay was repeated three times. Assays were performed on static cultures containing 125 ml culture medium in 250 ml glass flasks with a manual agitation twice per day. Controlled conditions were used with a temperature of 24 ± 1°C, with cool-white fluorescent continuous light (150 $\mu E.m^{-2}.s^{-1}$ irradiance) and lasted 96 hours. The experiments were carried out according to EPA protocols (U.S. Environmental Protection Agency, 1985).

Cellular density was evaluated using a Neubauer chamber, and the error was less than 10%, α 0.05. Results were expressed as cells/ml.

Total sugars were measured spectrophotometrically using the Dubois *et al.* (1956) procedure standardized with glucose. Cells were harvested centrifuging 5 ml culture for 20 min at 3,700 x g. Each sample was mixed with 2 ml distilled water in a 15-ml borosilicate glass tube before Dubois reagents were added. The spectrophotometric analysis was carried out at 490 nm with a UV/VIS JAS-CO 7850 spectrophotometer.

Paramylon (β-1,3- glucans, Euglenida storage carbohydrates) was extracted and purified according to Kiss and Triemer (1988). For this determination, 40 ml culture was centrifuged a 3,700 x g and the cells were washed twice with phosphate buffer. Pellet was frozen overnight. A solution of 2% SDS (w/v) and 0.125 M buffer tris was added, and then the suspension was shaken to mix and incubated for 30 min at 37°C. Paramylon granules were recovered by centrifugation for 20 min at 3,700 x g. The treatment was repeated until a translucid solution was obtained. The paramylon granules obtained were washed twice with hot glass distilled water (70° C). After the second wash, granules were put on glass fiber filters (APFC type, Millipore), and dried overnight at 90°C for weight determination.

Chlorophyll content was determined following Devars *et al.* (1992) procedure. Cells were harvested filtering 5 ml sample through Whatman GF/C filter papers. Pigments were extracted with 80% acetone solution (vol/vol) for 24 h at 4°C, and optical densities were measured with a UV/VIS JAS-CO 7850 spectrophotometer.

Aliquots of 5 ml culture were centrifuged, washed twice 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.

For lipid content determination, culture cells were harvested by centrifugation for 15 min at 3,700 x g, and washed three times with 0.154 M phosphate buffer, pH 7. Total lipids were extracted with chloroform:methanol (2:1 v/v), and then quantified according to the Bligh and Dyer method (1959).

Lipid peroxidation was measured in terms of malondialdehyde (MDA) content determined by the thiobarbituric acid reactive substances (TBARS) method (Vavilin *et al.*, 1998), using the equations of Hodges *et al.* (1999). Cells (125 ml culture) were harvested by centrifugation and the pellet was washed three times.

Chromium uptake was determined harvesting 20 ml culture. The pellet was washed three times with distilled water, and then each washed fraction together with the supernatant was digested with concentrated nitric acid. Both the amount of chromium adsorbed and the amount of total chromium uptake were determined. Total chromium concentration was measured using a SHIMADZU 6800 atomic absorption spectrophotometer (Kyoto, Japan) equipped with an autosampler ASC 6100. A Hamamatsu hollow cathose lamp was employed as radiation source at 357.9 nm with a slit width of 0.2 nm and 6 mA lamp current. Working solutions of chromium were prepared by appropriate dilution of a stock standard solution of Cr 1000 mg/L (trace to SRM from NIST) from Merck Chemical. These solutions were used as standards for obtaining the calibration curve. Appropriate blanks controls were conduced during all analytical methodology. The detection limit based of three times the standard deviation of the blank was estimated to be 0.020 mg/L.

Light microscopic examinations were used to observe the motility and the shape on living cells. It was carried out with an OLYMPUS B201 photomicroscope.

Mean and standard deviations were obtained from the duplicates of each concentration. The results were compared applying an analysis of variance (ANOVA) with Tuckey's test and the significant differences were obtained using a $p<0.05$ (Sokal and Rohlf, 1984). This analysis was performed with the STATISTICA program. The minimum concentration that produced 50% growth (IC_{50}) was obtained with the Probit Algae program (Walsh *et al.*, 1987). The Student $t(\alpha, n-1)$-test, α 0.05, was used to compare the two values of IC_{50} (Sokal and Rohlf, 1984).

RESULTS AND DISCUSSION

Hexavalent chromium inhibition on cell growth in a time and dose dependent

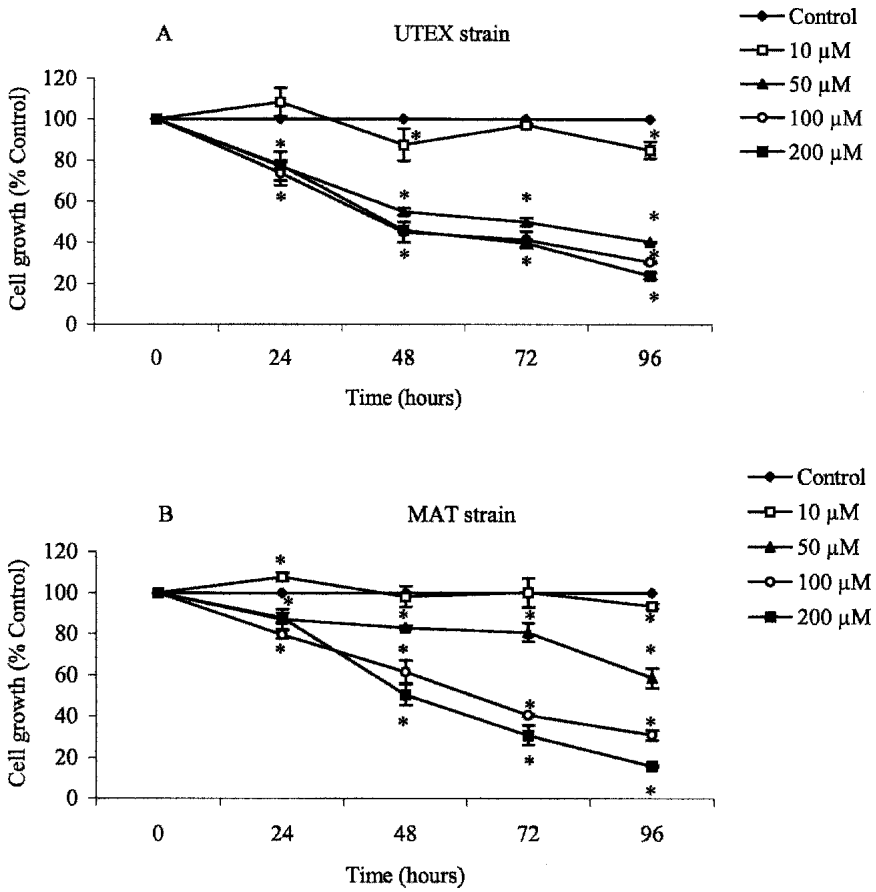

Figure 1. Effect of hexavalent chromium on cell growth in a time and dose dependent manner in two strains of *Euglena gracilis*. Cell proliferation is represented as a percentage with regards to the control value. The graphed concentrations are nominals. (A) MAT strain, isolated from a highly polluted river, (B) UTEX strain, from the Culture Collection of Algae of the Texas University. Initial cell density was the same for both strains (10^5 cells/ml). Data are means of three different experiments with standard deviations. *Denotes significant difference between control and treated cells, ANOVA, Tuckey test, $p<0.05$.

High malondialdehyde (MDA) levels revealed the existence of lipid peroxidation events (Figure 5). MAT control cells showed significant higher basal levels than UTEX cells. This could be explained by the adaptation to an oxidative environment devised to protect the intracellular membrane structure and cellular function. This adaptation would involve the reduction of polyunsaturated fatty acid content in the biomembrane, thus producing higher MDA values (Watanabe

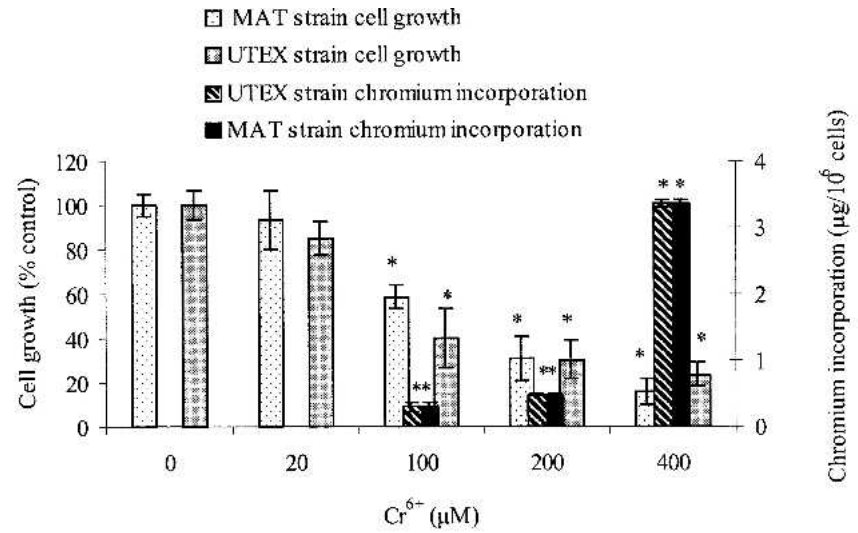

Figure 2. Effect of different chromium concentrations on cell growth, and total chromium incorporation in two strains of *Euglena gracilis*. Cell proliferation is represented as a percentage of the control value. The graphed concentrations are nominals Data are means of three different experiments with standard deviations. *Denotes significant difference between control and treated cells, ANOVA, Tuckey test, $p<0.05$.

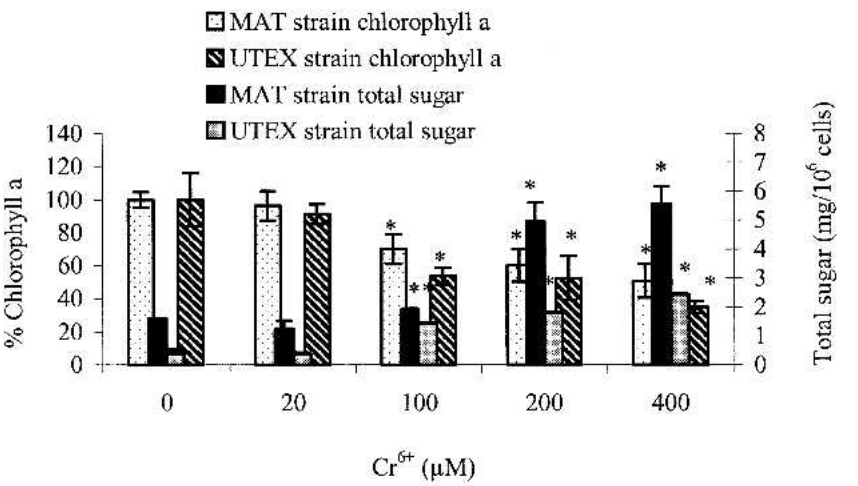

Figure 3. Effect of chromium on chlorophyll a and total sugar content in two strains of *Euglena gracilis*. Chlorophyll content is represented as a percentage of the control value. The graphed concentrations are nominals Data are means of three different experiments with standard deviations. *Denotes significant difference between control and treated cells, ANOVA, Tuckey test, $p<0.05$.

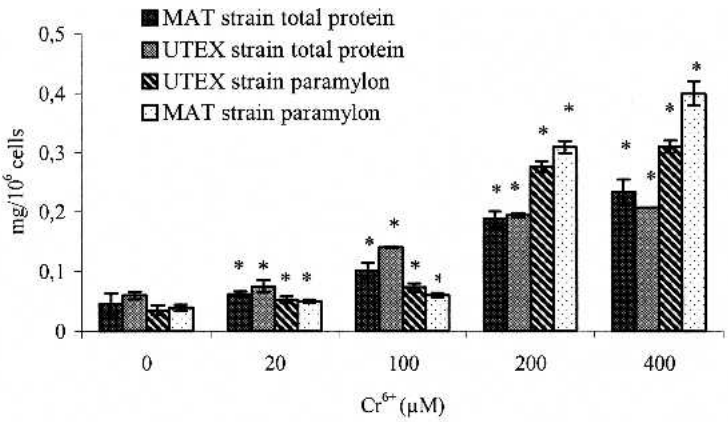

Figure 4. Effect of chromium on paramylon and total protein content in two strains of *Euglena gracilis*. Both parameters are expressed as mg/10^6 cells. The graphed concentrations are nominals Data are means of three different experiments with standard deviations. *Denotes significant difference between control and treated cells, ANOVA, Tuckey test, $p<0.05$.

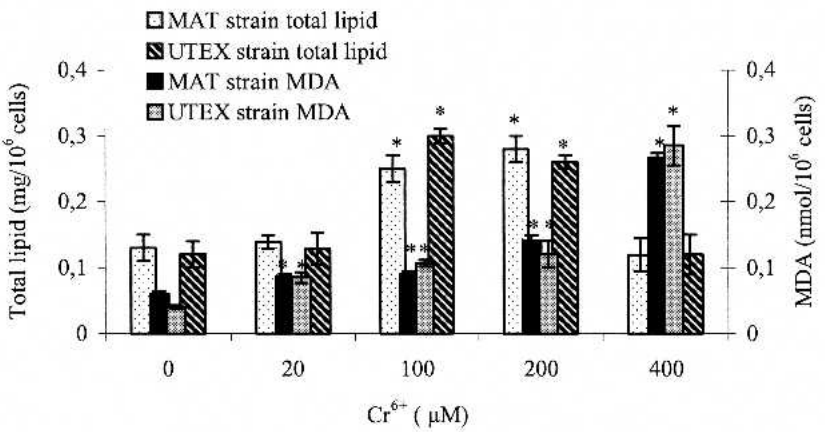

Figure 5. Effect of hexavalent chromium on total lipid content, and malondialdehyde (MDA) level as lipid peroxidation indicator in two strains of *Euglena gracilis*. The graphed concentrations are nominals Data are means of three different experiments with standard deviations. *Denotes significant difference between control and treated cells, ANOVA, Tuckey test, $p<0.05$.

and Suzuki, 2002). In both strains treated cells, a significant increase was observed from 20 μM concentration.

Figure 2 shows that chromium was not incorporated by cells at the lowest metal concentration assayed. In spite of this, both strains showed lipid oxidation (Fig. 5)

and an increase of the protein and paramylon content (Fig. 4). This could be related to damage in the plasmatic membrane produced by the metal adsorbed. The amount of chromium attached to the extracellular membrane was determined in the washed fractions by atomic absorption spectrophotometry. Both 20 μM Cr(VI) treated strains showed the same amount of chromium adsorbed (0.126 mg/10^6 cells). The highest chromium concentration assayed (400 μM) lead to a 7.1-fold increase of MDA content in UTEX, whereas the increase was 4.5-fold in MAT with respect to the controls. These results suggest higher oxidative damage in the commercial strain lipid content.

Alterations in cell motility and shape were observed in the presence of 200 μM Cr(VI) by light microscopy. In a previous work using Buetow medium, great morphological damage had been reported at lower chromium concentrations (26 μM), including loss of flagella and color, and high amounts of paramylon grains in the cells.

It is clear that the significant toxic effect exerted by chromium in cells cultured in Cramer and Myers medium was different from the one observed in the bioassay performed using Buetow medium (Rocchetta *et al.*, 2003). Chromium uptake in cells grown in Cramer and Myers occurred at high metal concentrations, thus leading to greater IC_{50} values for both strains, showing a higher value the strain isolated from a highly polluted river (MAT). Despite of this result, we could observe only few differences between strains. The differences in chromium toxicity observed in both studies are related to the culture media used, which basically have a different pH. In aqueous solutions, Cr(VI) exists as oxoforms in a variety of species depending on pH and Cr(VI) concentration (Cieslak-Golonka, 1996). In the concentrations used in our bioassays, the major ion for a neutral pH (Buetow medium) is chromate (CrO_4^{-2}). This molecule can easily go through cell membranes, being an alternative substrate for the sulphate transport system (Haglund, 1997). In the case of a lower pH (Cramer and Myers medium), the most common ion is the hydrochromate form ($HCrO_4^-$), which is incorporated by the cell with more difficulty (Cieslak-Golonka, 1996). This may account for the different levels of toxicity observed in the same strains.

The present study shows the different levels of hexavalent chromium toxicity depending on the physicochemical properties of the culture medium used. There is a difference between both strains with the highest chromium concentration treatment. MAT strain showed the greatest levels in the carbohydrate amount, while the malondialdehyde content was higher in UTEX cells indicating great oxidative damage in lipid content. MAT cells showed higher MDA basal levels, which could indicate a higher lipid sensibility against oxidative damage. However, MAT MDA increase was lower with the metal treatment, respect to its control, than UTEX, suggesting a better adaptation to an oxidative environment. As both strains presented quite similar variations at the chromium concentrations assayed, it is possible that they have a similar toxicity/detoxification mechanism. Further investigations are necessary to understand the relation between the higher resistance showed by MAT strain and the detoxification or adaptation systems that it may have developed.

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