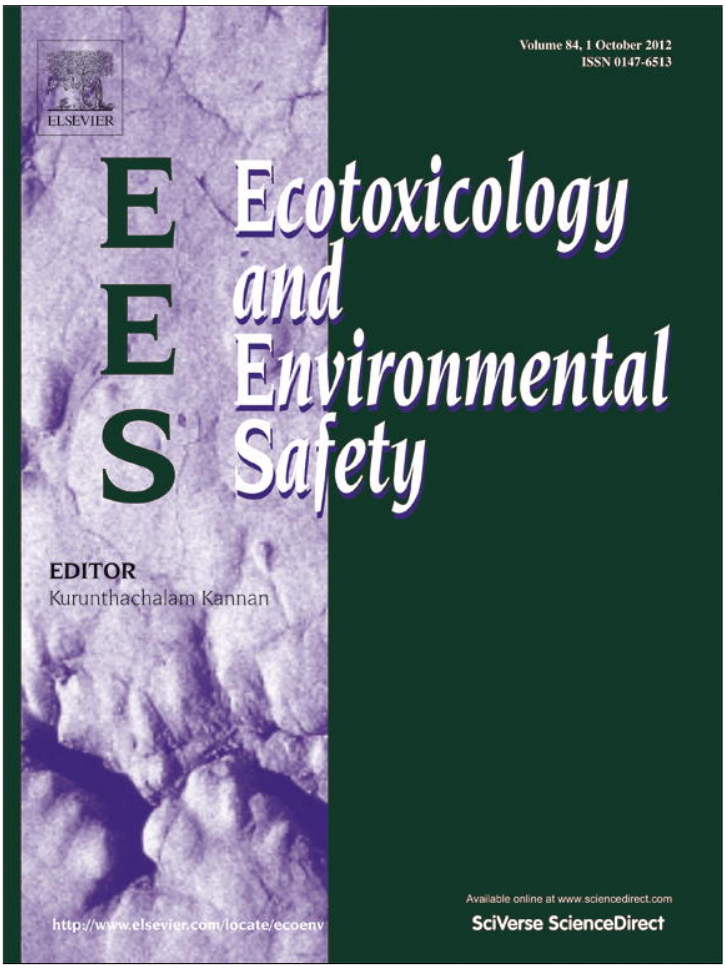

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Chromium induced stress conditions in heterotrophic and auxotrophic strains of *Euglena gracilis*

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ABSTRACT

Oxidative stress parameter and antioxidant defense compound as well as enzyme activity were studied in relation to different Cr(VI) concentrations (0, 10, 20, 40 μM) in two strains of *Euglena gracilis*, one isolated from a polluted river (MAT) and the other acquired from a culture collection (UTEX). Chromium toxicity was measured in the auxotrophic and obligated heterotrophic variants of the two strains. Chromium uptake was higher in auxotrophic cultures, reflected by their higher cell proliferation inhibition and lower IC_{50} levels compared to heterotrophic ones. In the Cr(VI) treatments a reduction of chlorophyll *a* and *b* ratio (Chl *a*/Chl *b*) was observed, the ratio of protein to paramylon content was augmented, and total lipid content increased, having the auxotrophic strains the highest values. TBARS content increased significantly only at 40 μM Cr(VI) treatment. Unsaturated fatty acids also increased in the Cr(VI) treatments, with the higher storage lipid (saturated acids) content in the heterotrophic cells. The antioxidant response, such as SOD activity and GSH content, increased with chromium concentration, showing the highest GSH values in the heterotrophic cultures and the SOD enzyme participation in chromium toxicity. The MAT strain had higher IC_{50} values, higher carbohydrate and saturated acid content, and better response of the antioxidant system than the UTEX one. This strain isolated from the polluted place also showed higher GSH content and SOD activity in control cells and in almost all treated cultures. SOD activity reached a 9-fold increase in both MAT strains. These results suggest that tolerance of MAT strain against Cr(VI) stress is not only related to GSH level and/or biosynthesis capacity but is also related to the participation of the SOD antioxidant enzyme.

1. Introduction

It is known that chromium can be found in several oxidation states that have different chemical properties. The most stable and common forms are trivalent [Cr(III)] and hexavalent [Cr(VI)] chromium (Bagchi et al., 2002). The hexavalent form is the most toxic; it usually associates with oxygen to form chromate (CrO_4^{2-}). This molecule can easily go through cell membranes as an alternative substrate on the sulfate transport system (Cieslak-Golonka, 1996; García-García et al., 2009).

Effects of heavy metals in algae have been subjected to extensive research for many years (Küpper et al., 2002; Mendoza-Cozatl and Moreno-Sánchez, 2005; Pinto et al., 2003; Sabatini et al., 2009). Most of the damages produced by these metals are caused by the strong inhibition of photosynthesis (Küpper and Kroneck, 2005; Rocchetta and Küpper, 2009).

It has been postulated that an important contribution to heavy metal stress is the increased production of reactive oxygen species (ROS) which consequently results in occurrence of oxidative stress (e.g., Pinto et al., 2003). Increased ROS levels produce oxidative damage to macromolecules such as proteins, nucleic acids, and lipids, leading to damage of different cellular organelles. Algae chloroplasts are formed by a complex system of membranes rich in polyunsaturated fatty acids, which are potential targets for peroxidation (Halliwell and Gutteridge, 1999). Increased ROS levels can also cause changes in lipid composition, damage to DNA, and ultra-structural disorganization (Watanabe and Suzuki, 2002; Pinto et al., 2003; Watanabe et al., 2003; Rocchetta et al., 2007).

Metal toxicity depends on the organism, the nature and concentration of the metal, and culture conditions (Küpper and Kroneck, 2005). In many cases metal resistance results from a

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very efficient defense/detoxification system, usually the system producing chelating compounds that reduce or inactivate the metal (Cobbett and Goldsbrough, 2000; Küpper and Kroneck, 2005), or metal exclusion and efflux mechanisms to prevent their accumulation inside the cell (von Hoof et al., 2001).

Euglena gracilis is a useful model to study cell damage caused by toxic compounds such as heavy metals. It has been used as a test organism to determine pollutant tolerance in urban aquatic ecosystems (Johnstone et al., 2006; Ahmed and Häder, 2010). This microorganism can grow in heterotrophic or auxotrophic conditions (with light, vitamins and exogenous organic matter or carbon source) depending on the culture medium and light conditions (Barsanti et al., 2000a), making them an interesting model to study different metabolic pathways. *E. gracilis* cells grown under constant darkness can lose their chloroplast, but this condition can be reversed (Schiff and Schwartzbach, 1982). Streptomycin (Sm) is an antibiotic that does not inhibit cell division or viability of *E. gracilis*, but 'bleaches' the cells by causing the permanent loss of plastids and plastid DNA in dividing photosynthetic cells and by blocking the development of chloroplasts in nondividing cells (Schwartzbach and Schiff, 1974).

Previous work revealed that the cytotoxic effect of hexavalent chromium in photosynthetic *E. gracilis* cells produces changes in cellular growth, proteins, and lipids (Rocchetta et al., 2006a; Rodríguez-Zavala et al., 2007; García-García et al., 2009). Recently, some studies have analyzed the effect of this metal on *E. gracilis* grown in heterotrophic conditions (Jasso-Chávez et al., 2010; Lira-Silva et al., 2011). Ultra-structural studies showed damages in the nucleus, mitochondria, and chloroplasts, the last being the most affected (Rocchetta et al., 2007). Changes of fatty acids related with thylakoids and chloroplast membranes were also observed (Rocchetta et al., 2006b). These previous results could be related with oxidative stress processes produced by chromium reacting with molecular oxygen (O_2), producing ROS via non-enzymatic or enzymatic reactions (Bagchi et al., 2002; Rai et al., 2004).

In this study, we propose to analyze oxidative stress parameters and antioxidant defense compounds in relation to different chromium concentrations in *E. gracilis*, and the modulation of their toxicity in auxotrophic and obligated heterotrophic cultures. Two strains have been used for this purpose, one isolated from a highly polluted river and the other obtained from a culture collection. Previous work demonstrated a natural resistance to Cr(VI) by the strain isolated from the polluted river (Rocchetta et al., 2003). We want to compare the effect and response of these strains to Cr exposure. For that, cell density, chlorophyll, carbohydrate and lipid contents were measured as proxies for metabolic damage. Fatty acid composition, lipid peroxidation and intracellular Cr content were analyzed and antioxidant responses as superoxide dismutase (SOD) activity and reduced glutathione (GSH) were measured.

2. Materials and methods

2.1. Micro-organism, culture conditions and metal toxicity assays

We used two auxotrophic strains of *E. gracilis*; one from the Culture Collection of Algae of Texas University, USA (UTEX 753), and the other isolated from a highly polluted river from Matanza River, Buenos Aires, Argentina (MAT) (Ruiz et al., 2004). This type of growth, auxotrophic, leads to live using the energy by the photosynthetic activity and the organic matter incorporation. Their obligated heterotrophic variants (MAT and UTEX) were obtained by bleaching with streptomycin (100 μg/ml during 7 day; Ruiz et al., 2004).

Master cultures were grown on static conditions in 500 ml glass flasks, with manual mixing twice per day, containing 250 ml of *E. gracilis* medium, characterized by being rich in organic matter (EGM; CCAP, 1988), pH 7, at 24 ± 1 °C with cool-white fluorescent continuous light at 150 μE m^{-2} s^{-1} irradiance. On this hand, *E. gracilis* have a good growth condition in both photosynthetic and heterotrophic way. Axenicity was monitored plating cultures in bacteria medium. A new culture was initiated six days before each experiment in order to obtain an inoculum in the exponential growth phase. For the toxicity assays, aliquots of the master cultures were used to inoculate each flask to a final concentration of 1×10^5 cells ml^{-1}. The assays conditions are the same as outlined above. $K_2Cr_2O_7$ was added from a 0.1 M stock solution until reaching total Cr concentrations. Several concentrations were used (0, 5, 10, 20, 40, 100 μM) to find out the 50% growth concentration ($IC_{50,96h}$, assays lasted 96 h; U.S. Environmental Protection Agency (1985)) using the Probit Algae Program (Walsh et al., 1987). Once we obtained the $IC_{50,\ 96h}$ values, which are presented in Results (Section 3.1), we used Cr concentrations (0, 10, 20, 40 μM) above and below the obtained $IC_{50,96h}$ for the biochemical determinations. Cellular density was determined with a Neubauer chamber, with less than 10% error, α 0.05 (Venrick, 1978). The percentages of growth inhibition were calculated according to Wong et al. (1983) on the basis of cell density, which was determined every day with a Neubauer chamber, with less than 10% error, α 0.05 (Venrick, 1978).

2.2. Chromium determination

Cultures were centrifuged (20 ml), the pellet was washed three times with distilled water, and the cells were digested with concentrated nitric acid. Total chromium concentration was measured in the cells and in the culture medium in the controls and treated cultures, 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 1000 $mg.ml^{-1}$ Cr (trace to SRM from NIST) from Merck Chemical. These solutions were used as standards for obtaining the calibration curve. Appropriate blank controls were conducted during all analytical methodology. The detection limit of the blank, based on the mean of three times the standard deviation, was estimated to be 0.020 $mg.ml^{-1}$.

2.3. Antioxidants determination

Cells were harvested by centrifugation for 15 min at 7400 × *g*, and washed three times with 0.154 M phosphate buffer, pH 7.0. The pellet was sonicated with 3% 5-sulfosalicylic acid and centrifuged 15 min at 7000 × *g*. The resulting supernatant was used for the assays. Reduced glutathione (GSH) levels were measured following the Anderson (1985) procedure. Absorbance at 412 nm was measured after 30 min incubation at room temperature. Results were expressed as nmol mg $proteins^{-1}$.

Superoxide dismutase (SOD) activity was determined by measuring the inhibition of the reduction of nitroblue tetrazolium (NBT) by the light activated generation of O_2^- by riboflavin in the presence of methionine. The rate of reduction of NBT was measured at 560 nm (Fridovich, 1997). Cells were harvested by centrifugation for 15 min at 7400 × *g*, and washed three times with 0.154 M phosphate buffer (pH 7). The pellet was sonicated and the resulting supernatant was used for the assays. Protein concentration was determined by the Bradford (1976) method using bovine serum albumin as standard.

2.4. Chlorophyll determination

Chlorophyll content was determined by following Wellburn (1994) procedure. Cells were harvested by filtering 5 ml sample using Whatman GF/C filter papers. The pigments (chlorophyll *a* and *b*) were extracted with 80% acetone solution (v/v) for 24 h at 4 °C, and measured spectrophotometrically. Results are expressed as Chlorophyll *a* and *b* ratio (Chl *a*/Chl *b*)

2.5. Carbohydrate determination

Total sugars were measured spectrophotometrically using the Dubois et al. (1956) procedure standardized with glucose. Cells were harvested by centrifugation of 5 ml culture for 15 min at 7400 × *g*. Dubois reagents were used and optical densities were analyzed at 490 nm.

Paramylon (β-1,3-glucans, storage carbohydrates in Euglenida) was extracted and purified following Barsanti et al. (2000b) specifications. To verify effectiveness of the procedure, paramylon granules in the supernatant were observed and measured with an Olympus BX 50 optical microscope. Before extraction, cells were washed three times. Then, 100 ml of the culture medium was centrifuged at 3700 × *g*. Pellets were frozen at −21 °C overnight and then resuspended in 1% SDS (w/v) and 5% Na_2 EDTA (w/v), shaken for mixing, and incubated for 60 min at 37 °C. Paramylon granules were recovered by centrifugation for 60 min at 3700 × g. The SDS-Na_2 EDTA treatment was repeated until a translucent supernatant was obtained. Paramylon granules were then washed twice with hot glass-distilled water (70 °C). After the second wash, granules were located in glass fiber filters (APFC type, Millipore) and dried overnight at 90 °C for weight determination. Results were expressed as μg paramylon 10^6 $cells^{-1}$. The quality of the grains

and the pellet obtained was determined measuring the total amount of proteins that remained in the pellet after the treatment (Bradford, 1976).

2.6. Lipid peroxidation evaluation

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 7.0. Total lipids were extracted with chloroform:methanol (2:1 v/v) and quantified according to the Bligh and Dyer (1959) method.

The level of lipid peroxidation was measured in terms of thiobarbituric acid reactive substances (TBARS) using the Vavilin et al. (1998) method, with the equations of Hodges (1999). Cells (125 ml of culture) were harvested by centrifugation and the pellet was washed three times.

2.7. Fatty acid determination

Dried biomass (15 mg) was exposed to direct transesterification with 1 ml acetyl chloride in methanol 1:20 (v/v), according to Lepage and Roy (1984). After tubes were cooled in water (25 °C), the reaction mixture was diluted with 1 ml water, and extracted three times with 1 ml hexane. The hexane phase was dried under gentle nitrogen stream, at atmospheric pressure and room temperature. Then, fatty acid methyl esters were resuspended in 100 µl hexane and injected into the chromatograph (Hewlett Packard GC5890) with a flame ionization detector, on an Innowax capillary column (30 m, 0.32 mm ID, 1 µm film thickness). Helium was used as carrier gas. The column was held at 150 °C for 3 min, and then temperature was increased 5 °C/min until 280 °C (held for 15 min). Injection port and detector temperatures were 250 °C and 280 °C, respectively. Determinations were carried out in triplicate. A standard of fatty acid methyl ester mixture (Supelco Inc., Supelco Park, Bellfonte) was run under identical conditions to identify compounds on the basis of their retention times.

Fatty acid quantitation was performed using heptadecanoic acid (C17:0) as internal standard. Thus, an aliquot of 125 µg heptadecanoic acid dissolved in 5 µl toluene was added to the biological samples before transesterification.

2.8. Statistical analysis

Quantitative data for fatty acids content represent the mean of three independent measurements. The statistical significance of differences between controls and treated cultures, photosynthetic and non-photosynthetic strains, and MAT and UTEX strains, was determined by an analysis of variance (ANOVA). For the other data analysis, mean and standard deviation were obtained from the duplicates of each concentration. Each treatment was performed in duplicate and each assay was repeated three times. Data were evaluated by an analysis of variance (ANOVA) using STATISTICA 8.0 program. Values of $P < 0.005$ were considered significant.

3. Results

3.1. Cell growth and chromium uptake

Auxotrophic and heterotrophic control cells showed very similar growth curves, having the bleached ones lower cellular density (Fig. 1) and lower growth rate (Table 1). The chromium treatment produced changes in culture growth depending on the strain and the metal concentrations employed (Fig. 1). Both bleached strains presented higher IC_{50} values (25.37 ± 1.20 µM Cr(VI) for the MAT strain and 17.18 ± 0.9 µM Cr(VI) for the UTEX one) than the green ones, having the MAT strain the highest value. IC_{50} values of MAT and UTEX photosynthetic strains were also significantly different (21.10 ± 1.50 µM Cr(VI) and 14.02 ± 1.20 µM Cr(VI) respectively). Growth inhibition was detected in all treated strains. Independently of the culture condition, the major differences were observed between MAT and UTEX strains in the presence of 20 µM Cr(VI). The photosynthetic and bleached counterpart for MAT showed a reduced growth rate of 49% and 48% respectively, while the UTEX ones showed a reduced growth rate of 74% and 77% respectively.

Fig. 2 shows the total chromium uptake by all treated cells. Both bleached strains showed a significant lower metal incorporation than their green counterparts, being more remarkable in the MAT strain. UTEX photosynthetic cultures had the highest metal incorporation at the 80 µM Cr(VI) treatment.

3.2. Determination of antioxidants

Fig. 3 shows the reduced glutathione (GSH) amount and SOD activity of both strains cultures in all conditions. All treated cells show

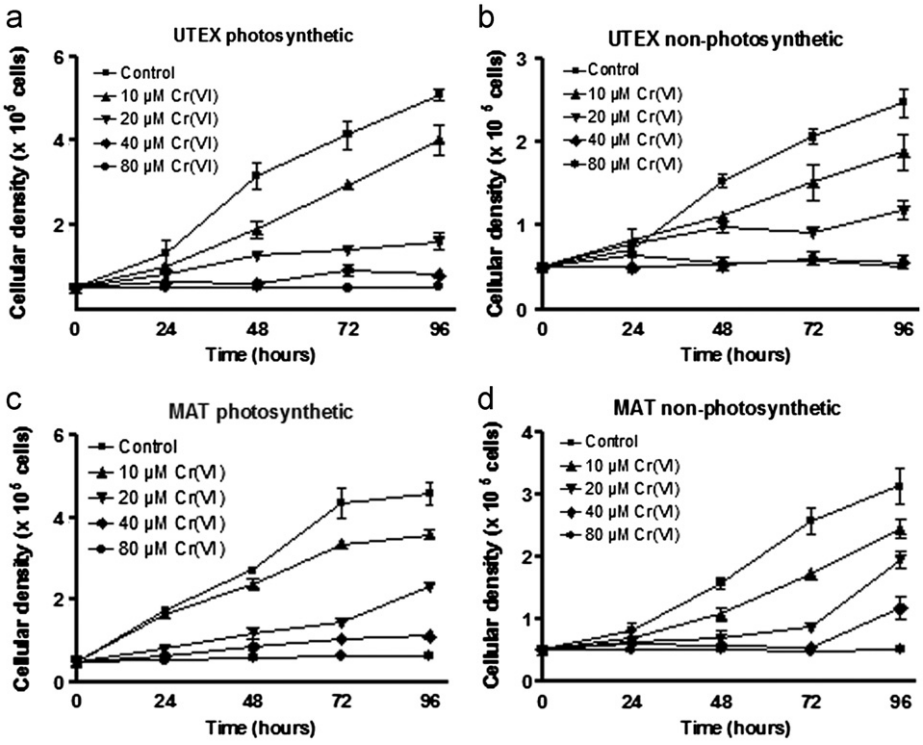

Fig. 1. The effect of different Cr(VI) concentrations on the growth of two strains of photosynthetic *E. gracilis* and their heterotrophic (non-photosynthetic) counterparts after 24, 48, 72 and 96 h of exposure. (a) Photosynthetic UTEX strain, (b) heterotrophic UTEX strain, (c) photosynthetic MAT strain, (d) heterotrophic MAT strain. Results are expressed as mean ± SD of three different experiments.

Table 1
Growth rate and % of growth inhibition of *E. gracilis* MAT and UTEX cultured in autotrophic (photosynthetic) and heterotrophic (non-photosynthetic) conditions during 4 day, in presence of different chromium concentrations.

Strain	Cr(VI) concentration (μM)	Growth rate (day^{-1})	Growth inhibition (%) after 4 day of treatment
UTEX photosynthetic	Control	0.581	–
	10	0.507	28
	20	0.306	74
	40	0.121	93
	80	0.000	100
UTEX non-photosynthetic	Control	0.410	–
	10	0.351	30
	20	0.163	77
	40	0.030	97
	80	0.000	100
MAT photosynthetic	Control	0.544	–
	10	0.486	23
	20	0.239	49
	40	0.173	79
	80	0.046	97
MAT non-photosynthetic	Control	0.459	–
	10	0.371	35
	20	0.328	48
	40	0.173	81
	80	0.050	96

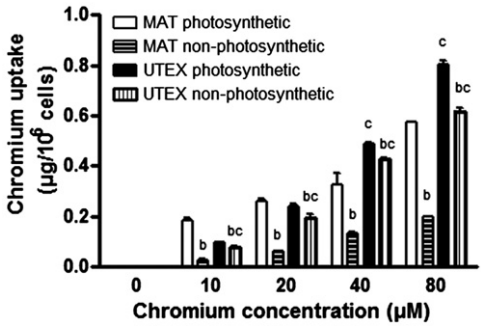

Fig. 2. Cr intracellular content determined in UTEX and MAT photosynthetic and heterotrophic (non-photosynthetic) strains of *E. gracilis* with different Cr concentrations: a, significant differences ($p<0.05$) between control and treated cells; b, significant differences ($p<0.05$) between photosynthetic cultures and their heterotrophic (non-photosynthetic) ones; c, significant differences ($p<0.05$) between both strains, UTEX and MAT.

a significant increase in GSH, with the MAT strain reaching the highest values at the highest chromium concentration. Both bleached strains showed higher values with this metal concentration than their green counterparts. In the 20 μM Cr(VI) treatment both MAT cultures, green and bleached, had significant higher GSH levels than the control cells. It is remarkable that both MAT strains had higher GSH basal levels than the UTEX ones (Fig. 3a).

SOD enzymatic activity also significantly increased in both photosynthetic and bleached MAT strains in the 20 μM chromium treatment, whereas the UTEX ones showed a significant increase only in the highest metal concentration (40 μM). It is remarkable that the enzyme activity in MAT cultures reached an increase of up to 9-fold with respect to control cells (Fig. 3b). Only photosynthetic MAT strain showed a significant increase with the 10 μM concentration.

3.3. Chlorophyll content

No changes in the chlorophyll ratio were observed in the bleached treated cells. MAT photosynthetic control cells showed a

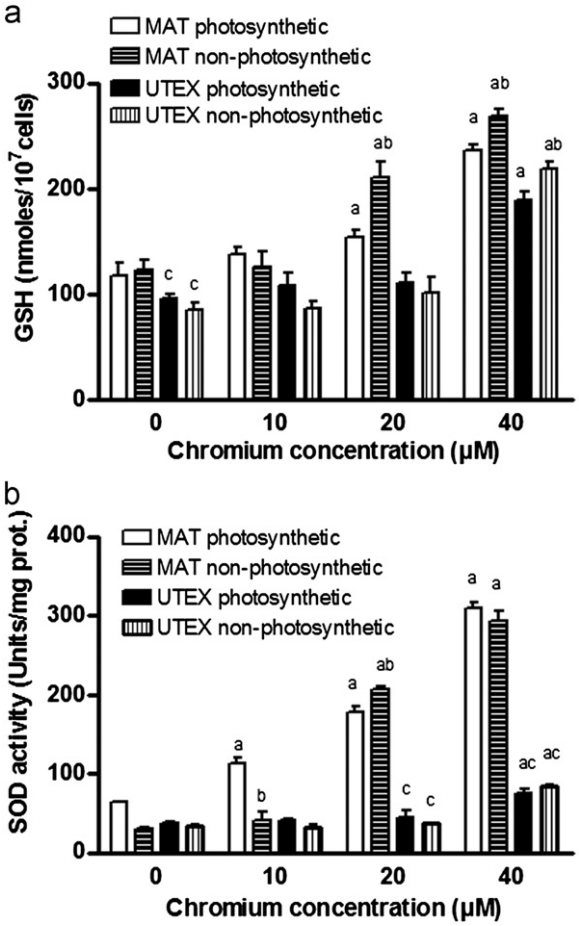

Fig. 3. Antioxidant response determined in UTEX and MAT strains of *E. gracilis* auxotrophic (photosynthetic) and heterotrophic (non-photosynthetic) cultures with different Cr concentrations. (a) Reduced glutathione (GSH) levels. (b) Superoxide dismutase (SOD) activity: a, significant differences ($p<0.001$) between control and treated cells; b, significant differences ($p<0.05$) between photosynthetic cultures and their heterotrophic (non-photosynthetic) ones; c, significant differences ($p<0.05$) between both strains, UTEX and MAT.

significant lower value for Chl *a*/Chl *b* (6.63 ± 0.14) respect to the UTEX ones (4.25 ± 0.20) ($p<0.001$). Chl *a*/Chl *b* decreased significantly in all treatments in both strains (MAT: 3.53 ± 0.21 and 2.34 ± 0.11, UTEX: $5.32\pm$ 0.21 and 3.90 ± 0.16, for 10 and 20 μM Cr(VI) respectively) ($p<0.05$); it was almost 50% less in the highest chromium concentration (2.12 ± 0.13 for MAT and 3.2 ± 0.17 for UTEX) compare to the control.

3.4. Carbohydrate determination

Carbohydrate content changed with the metal treatment. All treated cells with concentrations above 20 μM Cr(VI) showed a significant increase with respect to the control cultures (Fig. 4a). Both MAT strains showed the highest values with this metal concentration. Both bleached cultures showed higher values compared with the green ones. Paramylon (the major carbohydrate in Euglenoids) levels followed the same pattern than total sugar content, which increased in all treated cells above 20 μM concentration (data not shown). In this study we present the ratio between total protein and paramylon content as metabolic index (Fig. 4b). Both green strains showed a significant increase of the ratio in the 20 μM metal treatment due to an increase in the total protein content, whereas bleached cells presented a ratio increase only in 40 μM compared to the controls. The highest

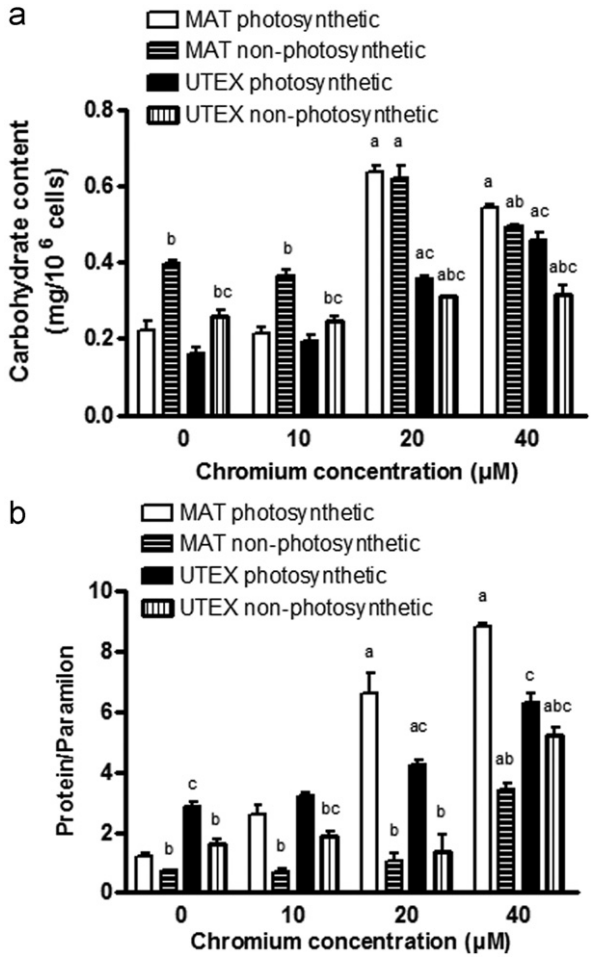

Fig. 4. Carbohydrate content and the ratio protein and carbohydrate content determined in UTEX and MAT strains of *E. gracilis* auxotrophic (photosynthetic) and heterotrophic (non-photosynthetic) cultures with different Cr concentrations. (a) Total carbohydrate levels. (b) Ratio protein and carbohydrate content: a, significant differences ($p<0.05$) between control and treated cells; b, significant differences ($p<0.05$) between photosynthetic cultures and their heterotrophic (non-photosynthetic) ones; c, significant differences ($p<0.05$) between both strains, UTEX and MAT.

metal concentration assayed lead to an 8-fold increase of the ratio in MAT green cells.

3.5. Lipid peroxidation evaluation

The increment in TBARS content was evaluated in both control and treated cells (Fig. 5). Control of both strains showed similar levels. All treated cells in the 40 μM concentration showed a significant TBARS content increase with respect to controls, the UTEX photosynthetic strain reaching the highest values. Only UTEX bleached cells showed a significant increase in 20 μM Cr(VI).

Total lipid content increased significantly in all strains in the highest Cr concentration, MAT strains both showing the lowest levels (for the MAT green cells 0.20 ± 0.01 μg 10^6 cells^{-1} and for the UTEX ones 0.32 ± 0.02 μg 10^6 cells^{-1}, whereas for the bleached cells 0.14 ± 0.02 μg 10^6 cells^{-1} for MAT and 0.26 ± 0.01 μg 10^6 cells^{-1} for UTEX). Both photosynthetic treated and control strains showed significant higher amounts compared to their bleached counterparts. The values for photosynthetic control cultures were 0.13 ± 0.01 μg 10^6 cells^{-1} for MAT and 0.18 ± 0.01 μg 10^6 cells^{-1} for UTEX, and for the bleached ones, and 0.08 ± 0.01 μg 10^{6-1} cells and 0.09 ± 0.02 μg 10^6 cells^{-1}, for MAT and UTEX respectively.

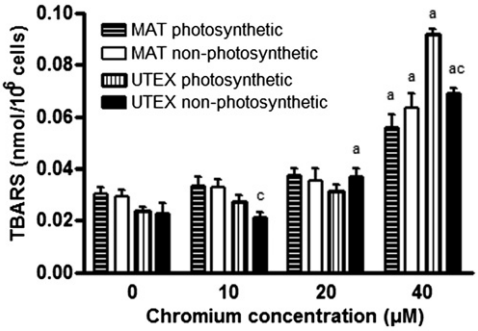

Fig. 5. TBARS content determined in UTEX and MAT strains of *E. gracilis* auxotrophic (photosynthetic) and heterotrophic (non-photosynthetic) cultures with different Cr concentrations: a, significant differences ($p<0.05$) between control and treated cells; c, significant differences ($p<0.05$) between both strains, UTEX and MAT.

3.6. Fatty acid determination

Table 2 describes the variation in fatty acid content due to the different conditions assayed. MAT control cells showed significant ($P<0.005$) higher saturated fatty acids' (SAFAs) values than UTEX ones, being this last one richer in ω3 than MAT. Fatty acids such as 16:2ω4, 16:3ω4, 18:3ω4, 18:4ω3, 20:1ω9, 22:5ω3 and 22:6ω3 were not detected in any culture (data not shown in Table 2). Under Cr exposure both auxotrophic and heterotrophic strains showed changes in fatty acid composition. When cultured with 20 μM Cr(VI), an increase in SAFAs content was observed in all strains (specially C16:0 for the green strains and C14:0 for the bleached ones). In the 20 μM Cr(VI) treatment depending on the culture type different responses were obtained for the polyunsaturated fatty acids (PUFAs) content. Both green strains increased their mono-unsaturated fatty acids (MUFAs) and PUFAs content (ω3 and ω6 types) in a significant way, whereas the bleached ones showed a decrease in these fatty acids, especially ω3 type (C20:5ω3). In contrast, on the highest Cr concentration treatment the most significant increment ($P<0.005$) in the PUFAs content was in the bleached cells, due to an increase of C16:1ω7, and specially ω3 and ω6 types (C18:2ω6, C18:3ω3, C20:4ω6 and C20:5ω3). SAFAs content significantly decreased in both MAT strains in 40 μM Cr(VI). The UTEX green strain was the only one which showed a significant negative relation ($P<0.005$) of linolenic acid (18:3ω3) with Cr concentrations.

The variation of the ratio between saturated and unsaturated fatty acids as a function of chromium is shown in Table 2. No significant difference was observed between the ratios of MAT and UTEX photosynthetic strains, for both control and treated cells. However, bleached cells showed an increase in the ratio at 20 μM concentration and a decrease with 40 μM, due to changes, specially, in the unsaturated fatty acids.

4. Discussion

4.1. Chromium toxicity in auxotrophic and heterotrophic cultures

Cell proliferation inhibition was time and Cr dose dependent in both cultures, which was reflected on the IC_{50} levels. This result might relate to the different Cr uptake levels of the cells (Fig. 2) and with some differences in the defense system observed. In almost all treatments both bleached strains had lower Cr accumulated compared to their green counterparts. These would indicate different detoxification mechanisms depending on the culture type, suggesting a more efficient system to avoid the metal intake in the bleached ones and/or a more active metal extrusion. It has been

Table 2
Fatty acid composition (% total fatty acids) of *E. gracilis* MAT and UTEX cultured in autotrophic (photosynthetic) and heterotrophic (non-photosynthetic) conditions, in presence of two different chromium concentrations (n.d.=not detectable).

Fatty acids	UTEX photosynthetic strain			UTEX non-photosynthetic strain		
	Control	20 μM Cr(VI)	40 μM Cr(VI)	Control	20 μM Cr(VI)	40 μM Cr(VI)
C14:0	4.1 ± 0.2	6.3 ± 0.7	5.0 ± 0.5	13.6 ± 1.5[b]	19.2 ± 0.6[a]	12.2 ± 0.1
C16:0	7.2 ± 0.3	9.6 ± 1.9[a]	7.3 ± 0.7	8.8 ± 1.0	9.9 ± 0.3	9.6 ± 0.9
C16:1ω7	2.1 ± 0.4	3.5 ± 1.2	2.2 ± 0.3	3.1 ± 0.3	3.5 ± 0.2	4.8 ± 0.1[a]
C18:0	2.3 ± 0.5	2.0 ± 0.3	2.6 ± 1.0	0.7 ± 0.1[b]	0.8 ± 0.1	0.6 ± 0.1
C18:1ω9	2.5 ± 0.6	4.1 ± 0.4[a]	3.6 ± 0.5	2.0 ± 0.2	2.1 ± 0.2	3.4 ± 0.2
C18:1ω7	n.d.	n.d.	n.d.	2.1 ± 0.3[b]	2.0 ± 0.1	2.4 ± 0.2
C18:2ω6	2.3 ± 0.3	4.8 ± 1.3[a]	3.1 ± 0.9	0.7 ± 0.2[b]	1.1 ± 0.1	2.5 ± 0.4[a]
C18:3ω3	12.6 ± 0.3	10.3 ± 1.0[a]	8.2 ± 0.6[a]	0.5 ± 0.0[b]	0.5 ± 0.0	1.0 ± 0.1[a]
C20:4ω6	2.5 ± 0.1	4.0 ± 0.6[a]	3.2 ± 0.5[a]	12.7 ± 1.4[b]	11.6 ± 0.4	14.7 ± 1.2[a]
C20:4ω3	1.4 ± 0.3	2.1 ± 0.2[a]	1.8 ± 0.2	1.9 ± 0.2	1.0 ± 0.1	1.7 ± 0.2
C20:5ω3	4.3 ± 0.1	6.9 ± 0.7[a]	5.9 ± 0.9	7.9 ± 0.8[b]	4.4 ± 0.2[a]	9.3 ± 0.7[a]
ω3	18.2	19.4	15.9[a]	10.3[b]	5.9[a]	12.0[a]
ω6	4.8	8.8[a]	6.3[a]	13.4[b]	12.6	17.23[a]
∑ SAFA	13.7	17.9[a]	14.9	23.1[b]	29.9[a]	22.4
∑ MUFA	4.6	9.5[a]	7.1[a]	7.2[b]	7.5	10.7[a]
∑ PUFA	23.1	28.2[a]	22.1	23.7[b]	18.5	29.2[a]
∑ SAFA/ (∑ MUFA+∑ PUFA)	0.5	0.5	0.5	0.8	1.2[a]	0.6
	MAT photosynthetic strain			MAT non-photosynthetic strain		
C14:0	3.4 ± 0.8	4.7 ± 0.7	3.6 ± 0.5	18.7 ± 1.4[bc]	21.5 ± 1.3[a]	14.6 ± 1.1[a]
C16:0	12.0 ± 1.5[c]	14.8 ± 1.4	12.1 ± 2.2	7.6 ± 1.1[b]	7.3 ± 0.7	8.0 ± 0.1
C16: ω17	1.4 ± 0.0	2.0 ± 1.5	1.9 ± 0.1	3.3 ± 0.2[b]	2.8 ± 0.2	5.8 ± 0.3[a]
C18:0	3.0 ± 0.7	0.6 ± 0.0[a]	1.0 ± 0.0	1.0 ± 0.1[b]	0.7 ± 0.2	1.3 ± 0.5
C18:1ω9	3.9 ± 1.0[c]	4.4 ± 0.9	4.6 ± 0.8	1.7 ± 0.3[b]	1.5 ± 0.2	2.5 ± 0.4
C18:1ω7	n.d.	n.d.	n.d.	2.1 ± 0.5[b]	1.4 ± 0.2	2.0 ± 0.4
C18:2ω6	3.2 ± 0.2[c]	4.3 ± 0.2[a]	3.4 ± 0.6	1.7 ± 0.2[bc]	1.9 ± 0.2	3.8 ± 0.2[a]
C18:3ω3	9.3 ± 0.9[c]	11.5 ± 0.3[a]	8.0 ± 1.4	0.6 ± 0.0[b]	0.7 ± 0.1	1.3 ± 0.4[a]
C20:4ω6	3.4 ± 0.3	5.1 ± 0.2[a]	4.4 ± 0.6	11.0 ± 1.1[b]	9.5 ± 0.8	15.5 ± 0.3[a]
C20:4ω3	1.0 ± 0.1	0.9 ± 0.0	1.0 ± 0.0	1.2 ± 0.1	0.8 ± 0.1	1.3 ± 0.1
C20:5ω3	5.7 ± 0.5[c]	9.2 ± 0.2[a]	7.6 ± 1.2	4.3 ± 0.5[c]	2.7 ± 0.2[a]	5.7 ± 0.9[a]
ω3	15.9[c]	21.6[a]	16.6	6.0[bc]	4.3[a]	8.0[a]
ω6	6.5	9.3[a]	7.8	12.6[b]	11.4	19.3[a]
∑ SAFA	18.40[c]	20.1	16.8[a]	27.3[bc]	29.5	23.9[a]
∑ MUFA	5.3	7.8[a]	7.0[a]	7.1[b]	5.7	10.3[a]
∑ PUFA	22.4	32.2[a]	24.5	18.6[bc]	15.7	27.5[a]
∑ SAFA/ (∑ MUFA+∑ PUFA)	0.7	0.5	0.5	1.1	1.4	0.6[a]

Data are mean ± SD of three independent experiments.

[a] Significant differences ($p < 0.05$) between control and treated cells.

[b] Significant differences ($p < 0.05$) between photosynthetic cultures and their non-photosynthetic ones.

[c] Significant differences ($p < 0.05$) between both strains, UTEX and MAT.

previously described for heterotrophic *E. gracilis* a higher resistance to heavy metal exposure (Navarro et al., 1997; Cervantes et al., 2001). In a recent work, Lira-Silva et al. (2011) suggested that several simultaneous mechanisms are turned on to inactivate chromium species and their toxic effects on heterotrophic *E. gracilis*. Mechanisms as the malate extrusion for reducing extracellular Cr(VI) to avoid the intake into the cell, and the increase of intracellular content of thiols to form chromium-complex, have been postulated. Several studies remark the important role that thiol compounds (Cys, GSH, phytochelatins) play in different microalgae treated with chromium (Gorbi et al., 2006; Rodríguez-Zavala et al., 2007; García-García et al., 2009). In our study, we observed an increase in GSH content in the highest chromium concentration, which was higher in bleached cultures in relation to the green ones. Heavy metal toxicity has also been reported to evoke increased activity of antioxidant enzymes such as SOD (Pinto et al., 2003). An enzymatic antioxidant response was observed in this study in all strains with the highest chromium concentration, but no significant difference was observed between bleached and photosynthetic cultures. The protective role of SOD against Cr(VI) on heterotrophic *E. gracilis* cultures was recently postulated (Jasso-Chávez et al., 2010). This would be indicating an increase in the anion superoxide (O^{-1}) generation in the toxicity process by Cr.

Chloroplasts have been reported as a primary target for metal toxicity in algae (Küpper et al., 2002; Mendoza-Cozatl and Moreno-Sánchez, 2005; Rodríguez et al., 2007). In our study, the increase of Cr concentration reduced the chlorophyll *a*/chlorophyll *b* ratio in both photosynthetic strains. Cr inhibits photosynthesis by disrupting the electron transport chain damaging the photosystem II and the antenna complex (Rocchetta and Küpper, 2009).

All treated cells showed with the highest metal concentration an increase in carbohydrate content, indicating that the gluconeogenic pathway was active in presence of chromium, as described by Jasso-Chávez et al. (2010). Hayashi et al. (1994) suggested that, depending on the culture conditions, paramylon could be used as energy source to synthesize proteins. Despite of the increase in carbohydrate in the Cr treatments, total protein and paramylon content ratio showed that protein content was also higher, reaching the highest values in the photosynthetic cultures (Fig. 4). These results could be indicating the existence of a mechanism to reduce the metal effect through the induction of protective protein synthesis. This could be transport proteins, phytochelatins, metal-binding compounds (malate, glutathione), and different enzymes (malate synthetase, chromate reductase, ribulose biphosphate, SOD), which have been shown to increase in algae cultures under Cr exposure (dos Santos et al., 2007;

García-García et al., 2009; Jasso-Chávez et al., 2010; Lira-Silva et al., 2011).

4.1.1. Lipid peroxidation

Total lipid content increased with the Cr treatment and was higher in both green strains. There are many lipids related to the chloroplast structure. Einicker-Lamas et al. (1996) reported a total lipid increase in Cd^{2+} *E. gracilis* treated cells due to a higher concentration of cholesterol and the phosphatidylglycerol (chloroplast membrane lipid).

Lipid oxidation was observed on the highest Cr concentration, as the increase of TBARS content, resulting probably from lipid membrane damage and cell lysis. These results would indicate the effect of ROS generated by chromium as was described previously by other authors (Bagchi et al., 2002; Cervantes et al., 2001; Gorbi et al., 2006). The increment of the antioxidant defenses observed in the *E. gracilis* treated cultures was not enough to prevent oxidative damage, especially with the highest chromium treatment, as it was evident in our results.

No differences were observed in TBARS values between control strains. Watanabe and Suzuki (2002) postulated that photosynthetic strains should have greater TBARS levels because unsaturated acids are the major fatty acids in these cells. In Table 2 we could observe that all control strains have similar amount of total unsaturated fatty acids (MUFAs +PUFAs). There are clear differences in the type of PUFAs between green cells, as C18:3ω3 (fatty acid (FA) related with the photosynthesis activity), and bleached cells, as C20:4ω6 (FA related with the respiration activity). That is related with the metabolic versatility of *E. gracilis*, which can follow a photosynthetic or non-photosynthetic synthetic pathway depending on the culture type (Barsanti et al., 2000a). A culture media enriched in organic matter as was used in our study (EGM) provides an important nutrient source for carbohydrate production. Paramylon is usually used to generate storage lipids, enriched in C14:0 and C16:0, which will be used in the PUFA synthesis (Regnault et al., 1995). This could be related with the higher SAFA levels in both control bleached strains cells compared with the photosynthetic counterparts. The saturated/unsaturated acids ratio was 0.5 for the green cultures whereas for the bleached ones was almost 1.

Mono- and polyunsaturated fatty acids, C18 and C20 (especially type ω6, C20:4ω6 and C18:2ω6) were significantly increased in all photosynthetic treated cells, and in the maximal exposure of chromium in the bleached cells (Table 2). Previous works relate the arachidonic acid (20:4ω6) increment with the activation of a membrane oxidase, generating in this way reactive oxygen species (Jia-Tsrong et al., 2000). Higher plant studies showed some lipases acting on galactolipids for the aldehyde production, releasing C18 fatty acids (Blee, 1998). Similar process could be happening in our treated cells, as a type of galactolipid synthetase showed changes in mRNA expression in both Cr treated strains cultured in EGM medium (dos Santos et al., 2007).

On the other hand, toxicological studies in diatoms revealed a phospholipase activity responsible for the release of free mono- and polyunsaturated fatty acids, mostly C20 fatty acids to form reactive aldehydes harmful to the cell (Pohnert, 2002). This would in agreement with the C20 fatty acid increase suffered by treated cells.

A decrease in type ω3 PUFAs was observed, affecting in a significant way the eicosapentaenoic acid (C20:5ω3) for the bleached cells treated with 20 μM Cr(VI). This large chain fatty acid is commonly used for other PUFA generation, necessary in the membrane restoration (Harwood, 1995).

Only UTEX green strain presented a significant decrease in FA type ω3 (C18:3ω3). More remarkable effects were observed in *E. gracilis* photosynthetic cultures grown in mineral medium in presence of chromium, suffering major damage at the ultrastructural level as well as in the fatty acid composition, especially a decrease in polyunsaturated fatty acids related to photosynthetic activities (Rocchetta et al., 2007). Assays carried out in *E. gracilis* cultures with Cd and Hg revealed different sensibilities depending on the culture medium (Navarro et al., 1997). A culture media enriched with organic matter provides an important nutrient source for carbohydrate production, an alternative energy source besides the photosynthetic one. Therefore, the auxotrophic *E. gracilis* strain in EGM medium would have a higher capacity of modulating the metal toxicity response on the fatty acid composition.

4.2. Metal toxicity in two E. gracilis strains

Some differences were observed between the MAT and UTEX strains growth in EGM with different Cr concentrations.

UTEX green cultures showed the highest total lipid content and TBARS values with the highest Cr concentration (40 μM), which could be related to the fact that this strain had also the highest metal incorporation in this treatment (Fig. 2). This last result is in agreement with the significant decrease of the fatty acid C18:3ω3 only observed in UTEX green cells. This result suggests damage to photosynthetic structures produced by Cr(VI), already reported by other studies (Ali et al., 2006; Rocchetta et al., 2007; Rodríguez et al., 2007). MAT green cells reached a 2-fold increase of the ratio protein/paramylon content compare to UTEX cells, due to a high protein content increase. The higher levels of storage lipids (SAFAs) found in MAT control and treated strains compared to UTEX ones (Table 2), could be related to the high carbohydrate values found in MAT cells (Fig. 3). A major capacity to accumulate storage compounds correlates to a better response to environment stress conditions.

MAT bleached cells had the highest IC_{50} and the lowest chromium amount incorporated. MAT photosynthetic strain also had a higher IC_{50} than the UTEX one, but the metal amount incorporated by the 10 μM treated cells was twice to the one incorporated by UTEX green cells (Fig. 2).

Research studies with a Cr-tolerant strain of *Scenedesmus acutus* suggested that tolerance to chromium could depend on pathways related to GSH synthesis (Gorbi et al., 2006). The MAT green strain showed the highest GSH content and SOD activity not only in control cells but also in almost all treated cultures compared to the UTEX one. SOD activity levels reached a 9-fold increase in both green and bleached MAT treated cells. The over-expression of this enzyme was observed in MAT strain exposed to chromium in previous molecular studies (dos Santos et al., 2007). The antioxidant defense system of *E. gracilis* observed in this study would suggest that tolerance to Cr(VI) in treated MAT strains not only is related to GSH levels and/or biosynthesis capacity, as was observed by other authors, but also to the participation of the SOD antioxidant enzyme. These results would suggest that the SOD participation would be related mainly with the MAT strain, isolated from the highly polluted river, more than the tolerance to chromium. Further assays would be carried out with different heavy metals or under different stress conditions in order to probe it.

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