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# Removal, accumulation and resistance to chromium in heterotrophic *Euglena gracilis*

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ABSTRACT

The removal, uptake and toxicity of chromium in *Euglena gracilis* cultured in absence and presence of malate with Cr(VI) or Cr(III) was evaluated. The malate extrusion and the extra- and intracellular Cr(VI) reduction capacity were determined and the contents of molecules with thiol group and ascorbate were also evaluated. Absence of malate in the medium decreased cell growth, increased Cr(III) toxicity, induced faster Cr(VI) disappearance from medium, and increased intracellular and intramitochondrial chromium accumulation. Both chromium species induced soluble and particulate ascorbate-dependent chromate reductase activities. Cells also secreted large amounts of malate and increased intracellular contents of thiol-molecules to bind extracellular and intracellular Cr(III), respectively. The former process was supported by significant increase in malate-producing enzyme activities and the assessment of the Cr-complexes indicated the *in situ* formation with thiol-molecules. The present results establish new paradigms regarding chromium stress on algae-like microorganisms: (i) Cr(III) may be more toxic than Cr(VI), depending on the culture (or environmental) conditions; (ii) several simultaneous mechanisms are turned on to inactivate chromium species and their toxic effects. These mechanisms, now well understood may further optimize, by genetically modifying *E. gracilis*, and facilitate the development of strategies for using this protist as potential bio-remediator of chromium-polluted water systems.

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## 1. Introduction

It is well documented that most organisms are susceptible to heavy metals exposure, affecting their growth, development, and morphology [1]. However, some plants, bacteria and microalgae species are able to survive in heavy metal polluted environments by means of internal and/or external detoxification mechanisms such as: (1) diminished uptake; (2) internal binding; (3) biotransformation; (4) compartmentalization; and (5) external chelation [2,3]. Nevertheless, during acute insult or long time exposure, these mechanisms may not suffice to avoid or neutralize the toxic effects of heavy metals.

Cr(III) and Cr(VI) discharges in wastewaters mainly originates from metal and tanning/painting industries, respectively [1,4]. In Mexico, the total chromium concentration reported in waste and ground water bodies near to industrial, mining and tannery industrial activities ranges from 1–5 μM up to 1 mM [5,6], values that are much higher than permissible. In consequence, at these elevated chromium levels, high toxicity in algae [7] and zooplankton [8] is observed.

Conventional methods for treatment of toxic chromium waste require large amounts of chemicals and energy and are unsuitable for small-scale leather, dye, and electroplating units [9]. Therefore, biotransformation of Cr(VI) to the putative less-toxic Cr(III) by biological agents offers a viable alternative, in particular for developing processes for Cr(VI) polluted water detoxification with bacterial strains [10,11], which has been considered economical, safe and sustainable [12].

The free-living flagellated protist *Euglena gracilis* belongs to a select group of organisms with a proven capacity to resist and accumulate heavy metals [3]. Indeed, *E. gracilis* is able to tolerate high concentrations of cadmium, zinc, lead, mercury and chromium. Cadmium is accumulated and compartmentalized into chloroplasts and mitochondria [13–16]. The increased synthesis of heavy metal-chelating molecules with thiol groups such as Cys, γEC, GSH and phytochelatins, together with the metal compartmentalization into

*Abbreviations:* AAS, atomic absorption spectrometry; BSA, bovine serum albumin; Cys, cysteine; DFC, diphenyl-carbazide; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid; γEC, γ-glutamyl-cysteine; GSH, glutathione; $NAD^+$-LDH, soluble lactate dehydrogenase; MDH, $NAD^+$-malate dehydrogenase; ME, $NADP^+$-malic enzyme; MS, malate synthase; PCA, perchloric acid; $Trp(SH_2)$, reduced trypanothione.

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organelles, seem to be the main resistance mechanisms developed by *Euglena* to contend against cadmium, mercury, zinc and chromium toxicity [14,16–18].

Remarkably, these two resistance mechanisms bring about intracellular accumulation of heavy metals. Indeed, photosynthetic *E. gracilis* is able to accumulate chromium when cells are incubated with either Cr(VI) or Cr(III), although levels of thiol-molecules only increase with Cr(VI) but not with Cr(III) [19], suggesting that other mechanisms may also be involved in the resistance to, and accumulation of chromium. For instance, the fraction of accumulated chromium in photosynthetic *Euglena* only accounted for a small percentage of the total Cr(VI) disappearance from the culture medium, suggesting an efficient Cr(VI) reduction [19]. Moreover, a mercury-pre-exposed *E. gracilis* strain cultured with cadmium, exhibited an enhanced content of heavy-metal chelating molecules with carboxylate group such as citrate and malate [20].

In a previous work, we determined that heterotrophic *E. gracilis*, cultured at pH 7.0 with glutamate + malate + ethanol as carbon source, did not consume malate if cells were cultured in presence of 250 μM Cr(III) or >50 μM Cr(VI) [18]. Malate might function as an external chelating agent as reported for plants [23]. Therefore, to gain a better understanding of the mechanisms of resistance to chromium in *E. gracilis*, in the present work cells were grown in absence or presence of malate and the toxic effect of Cr(VI) and Cr(III) on growth was determined. The results revealed the effective onset of several resistance mechanisms to chromium in *E. gracilis* including synthesis of heavy metal-chelating molecules with thiol- and carboxylate-groups, sub-cellular compartmentalization, metal chemical reduction and secretion of chelating molecules.

## 2. Materials and methods

### 2.1. Chemicals

Ascorbate oxidase, sodium borohydride ($NaBH_4$), ascorbic acid, trypan blue, and triton X-100 were from Sigma (St. Louis, MO, USA). Dichromate ammonium used as standard for determination of total chromium by AAS was from Fluka (Switzerland). $NAD^+$-MDH and $NADP^+$-glutamate dehydrogenase were from MP biomedicals (Ohio, USA).

### 2.2. Culture medium and growth conditions

*Euglena gracilis* was cultured axenically in 250 mL Erlenmeyer flasks containing 100 mL of culture medium under complete darkness with 34 mM glutamate + 15 mM malate + 177 mM ethanol (GME medium), or without malate (GE medium) as carbon sources at pH 7.0 [18]. The different concentrations of Cr(VI) and Cr(III) used in this work depended on the solubility and toxicity of both chromium species in GME or GE media. Because chromate solubility and toxicity did not change on GME and GE media the same concentrations were used in both conditions. As 100 μM Cr(III) in GME medium inhibited growth by only 15% and the total chromium accumulated intracellularly was near to the limit of detection (data not shown), only 250 μM Cr(III) was used for further comparisons (see section 3). In GE medium Cr(III) was less soluble and more toxic, that was the reason why in this condition, concentrations as low as 1 μM were used, and 100 μM was the highest concentration which Cr(III) was soluble as judged by changes in the transmittance at 540 nm.

Cultures with 5–250 μM Cr(VI) or 1–100 μM Cr(III) in the GE medium were prepared from 100 mM $K_2CrO_4$ or $CrCl_3$ stocks. Cultures were started by adding 2–5 mL of cellular inoculums ($0.6 \times 10^6$ cells/mL) from pre-activated culture, and grown in darkness at 25 °C under orbital shaking at 125 rpm. Cell counting and viability were carried out by means of a Neubauer chamber and by using 0.5% (w/v) vital dye trypan blue. Cells were harvested after 6–7 days of culture (stationary growth phase) and washed twice by centrifugation with a buffer containing 250 mM Sucrose, 10 mM Hepes and 1 mM EGTA (SHE buffer) at pH 7.2.

### 2.3. Analytical methods for chromium analysis

Cr(VI) content and total chromium accumulation were determined spectrophotometrically (UV-1800, Shimadzu) and by AAS (AA-99 Varian), respectively as described in [18]. The Cr(VI) accumulation was reported in terms of total chromium because an active Cr(VI) reduction process is undertaken by cells and hence chromium detected by AAS is in fact a mixture of Cr(VI) and Cr(III) species. In cultures with Cr(III), the concentration of total chromium determined by AAS in both the culture medium and cells was assumed to be exclusively Cr(III), as re-oxidation to Cr(VI) is not an efficient process under physiological conditions (and absence of potent oxidant agents).

### 2.4. Isolation of sub-cellular fractions

Sub-cellular fractionation was carried out by following the protocols previously reported [18,21]. The mitochondrial, cytosolic and plasma membrane fractions were re-suspended in a solution containing 50 mM Hepes/10 mM $MgCl_2$ and used freshly for enzyme assays.

### 2.5. Enzyme assays

The chromate reductase activity was determined by incubating the cytosolic or plasma membrane fraction (0.5–2 mg protein) in KME buffer (120 mM KCl, 20 mM Mops and 1 mM EGTA at pH 7.2) *plus* 10 mM $MgCl_2$, and 50 μM ascorbate under shaking (125 rpm) at 25 °C. The reaction was initiated by adding 100 μM Cr(VI) and stopped by adding 0.5 mL DFC dissolved in sulfuric acid. To correct for the small spurious reduction of Cr(VI) by ascorbate, parallel experiments without protein were carried out and values subtracted from those obtained with protein. The concentration of remaining Cr(VI) was determined. The kinetic parameters were estimated in both fractions incubated for 0.5 min with 5, 10 and 25 μM Cr(VI) or for 10 min with 50, 100, 250, 500 and 1000 μM Cr(VI).

$NAD^+$-LDH, $NAD^+$-MDH, $NADP^+$-ME and MS activities were determined by measuring either the production of NAD(P)H or oxidation of NADH at 340 nm, or the CoA released with DTNB (in the MS reaction) at 412 nm in a UV/Vis spectrophotometer as described elsewhere [18,25].

### 2.6. Cell breakdown and metabolite determination

Control and chromium-exposed cells were harvested, washed and re-suspended in 1 mL KME buffer. For ascorbate determination $50 \times 10^6$ cells were broken by mixing with 3% (v/v) PCA and stirring for 1 min. After centrifugation at 4 °C and 20,000 × *g* for 2 min, the supernatant was adjusted at pH ~6 with 3 M KOH/0.1 M Tris and centrifuged again. The second supernatant (cellular extracts) was incubated with an excess of $NaBH_4$ to ensure the complete reduction of ascorbate and used immediately to determine total ascorbate content by measuring the oxygen consumption with commercial ascorbate oxidase. Oxygen consumed was estimated by using a Clark-type electrode and an oxygen solubility of 420 nanoatoms gram oxygen per mL at 2240 m altitude and 25 °C [21].

Malate (and glutamate) quantification was made under various experimental settings. For malate secretion, culture media aliquots taken at different days of growth were centrifuged to discard cells

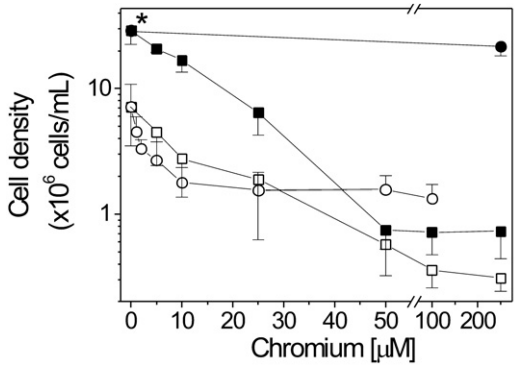


**Fig. 1.** Effect of Cr(VI) and Cr(III) on the growth of heterotrophic *E. gracilis*. Cells were cultured in GME with Cr(VI) (■) or Cr(III) (●) or in GE medium with Cr(VI) (□) or Cr(III) (○) at the indicated concentrations. Values are the mean ± SD of >4 independent experiments. * $P < 0.05$ *vs* control cells grown in GE media.

and supernatant was frozen until use. For secretion at shorter times, control cells were incubated in a buffer containing 50 mM Hepes/10 mM $MgCl_2$ at pH 7.0 in absence or presence of 100 μM of: Cr(VI), Cr(III), $Cd^{2+}$, $Hg^{2+}$, $Zn^{2+}$, $Fe^{3+}$ or $Pb^{2+}$; aliquots were withdrawn at different times, centrifuged and the supernatant was used for malate and glutamate quantification. The intracellular malate content was determined using $50 \times 10^6$ cells subjected to the procedure described above for cell rupture. Malate content was determined enzymatically [18] with commercial $NAD^+$-MDH; this enzyme showed <5% activity of that attained with malate with other four-carbon molecules such as β-hydroxybutyrate, asparagine or aspartate (data not shown).

For determination of thiol-molecules, 0.1% (w/v) Triton X-100 was added to $\sim 1 \times 10^7$ cells and the mixture incubated with 0.5 mM dithiotreitol and an excess of $NaBH_4$. Then, PCA was added. Lysates were centrifuged as above. An aliquot of filtered supernatant was injected to the HPLC apparatus and the different thiol-molecules were resolved as described in [13].

The protein content in mitochondria and cytosolic and membrane fractions were determined using the Biuret method with BSA as standard [24].

## 3. Results

### 3.1. Effect of malate on growth and susceptibility to chromium

*E. gracilis* cultured for 6 days reached a density of $29 \pm 6.6 \times 10^6$ cells/mL in GME medium, however, in GE medium a 4-fold decreased density was reached ($7.2 \pm 3.7 \times 10^6$ cells/mL; $n = 13$). The $IC_{50}$ values for growth in GME medium were $17 \pm 0.9$ μM Cr(VI) and >250 μM Cr(III), whereas in GE medium were $13 \pm 2.4$ μM Cr(VI) and surprisingly $1.8 \pm 0.3$ μM Cr(III) (Fig. 1). In cultures with ethanol + malate but without glutamate, the cell density was $4.2 \pm 0.6 \times 10^6$ cells/mL and, in presence of 100 μM Cr(VI) or Cr(III), cell densities were $1.6 \pm 0.08$ and $4.75 \pm 0.64 \times 10^6$ cells/mL, respectively (mean ± SD, $n = 3$). In GME- and GE-control grown cells the viability was >95%, whereas with Cr(III) or Cr(VI) the viability was >80%.

### 3.2. Effect of malate on external Cr(VI) stability and intracellular chromium accumulation and compartmentalization

Cr(VI) added to the GME media disappeared completely after 6-7 days of cell culture with 5 and 10 μM Cr(VI); at 100 μM, >50% of Cr(VI) remained in the medium [18]. The Cr(VI) disappearance was higher in GE medium with 25 and 50 μM (Fig. 2A) in comparison with GME medium. The Cr(VI) disappearance correlated

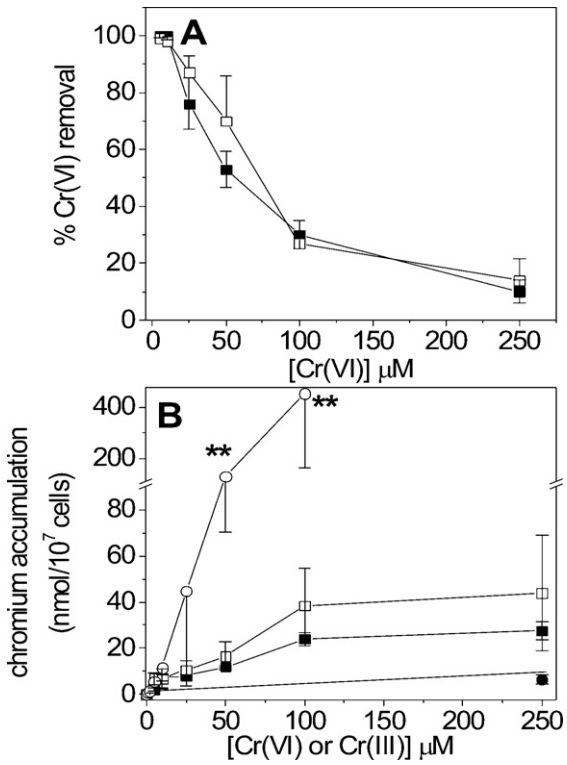


**Fig. 2.** Cr(VI) consumption and total chromium accumulation in *E. gracilis*. External Cr(VI) (A) and intracellular total chromium accumulation (B) were determined in GME with Cr(VI) (■) or Cr(III) (●) or GE medium with Cr(VI) (□) or Cr(III) (○) at the indicated chromium concentrations. Values are the mean ± SD of >4 independent experiments. Student's *t*-test for non-paired samples: ** $P < 0.05$ for accumulation in GE-grown cells with Cr(III) *vs*. accumulation in GE-grown cells with Cr (VI).

with a higher Cr(VI) uptake by cells cultured in GE medium. In GE media with 100 μM Cr(III), the accumulated chromium was 70-fold higher than in GME media with 250 μM Cr(VI) (Fig. 2B), whilst with 100 μM Cr(VI), no accumulation of chromium was detected (data not shown). No intracellular chromium was detected in cultures with cells but no chromium added [18].

In mitochondria isolated from cells cultured in GE with 25 μM Cr(VI), compartmentalization was 3 nmol total chromium/mg protein ($n = 2$). In mitochondria, isolated from 5 days old GE-grown cells and further exposed to 100 μM Cr(VI) (GE-CrVI-exposed cells) or 100 μM Cr(III) (GE-CrIII-exposed cells) for 48 h, total chromium content was $14.4 \pm 3.2$ or $41 \pm 16$ nmol/mg protein ($n = 5$), respectively. These values were significantly higher than those found in mitochondria from cells cultured in GME medium [18]. In mitochondria from GE-CrVI-exposed cells, 1.5 nmol Cr(VI)/mg protein was also determined ($n = 2$).

Growth was more sensitive to Cr(III) in GE than in GME media, particularly in the 0–10 μM range (see Fig. 1). However, cell density was always higher than $1.4 \times 10^6$ cells/mL under all Cr(III) concentrations tested, leading to an elevated percentage of Cr(III) removed by accumulation, which was 12–25 fold higher *versus* Cr(VI) at >50 μM (Table 1). Despite the higher intracellular accumulation *per* cell of chromium in GE medium (Fig. 2B), the total chromium accumulation by Cr(VI)-grown cells at 50 μM or higher concentrations reflected poor chromium removal from the culture brought about by cellular accumulation (<5%; Table 1), owing to the lower cell density obtained under these conditions. However, Cr(VI) disappeared from the culture medium at an extent which could not be accounted for by intracellular accumulation, mainly in cell cultures with 50 μM or higher concentrations (Fig. 2A). For instance, at 50 μM, Cr(VI) cellular removal was 20 fold higher than intracellular

**Table 1**
Total chromium accumulation in heterotrophic *E. gracilis*.

| Chromium (μM) | % of chromium accumulated | | | | | |
|---|---|---|---|---|---|---|
| | GME medium | | | GE medium | | |
| | Cr(VI) | Cr(VI) removal/accumulation | Cr(III) | Cr(VI) | Cr(VI) removal/accumulation | Cr(III) |
| 1 | ND | ND | ND | ND | ND | 68 (2) |
| 2 | ND | ND | ND | ND | ND | 24 ± 6 |
| 5 | 100 ± 34 | 1.0 | ND | 88 ± 6 | 1.0 | 31 ± 7 |
| 10 | 100 ± 20[a] | 1.0 | ND | 57 ± 11 | 1.7 | 22 ± 8 |
| 25 | 25 ± 5 | 3.04 | ND | 15 ± 5 | 5.8 | 30 ± 13 |
| 50 | 2.5 ± 0.8 | 21.2 | ND | 3.5 ± 0.4 | 20 | 43 ± 19[b] |
| 100 | 2.1 ± 0.2 | 14.3 | ND | 2.6 ± 0.3 | 10.4 | 64 ± 20[b] |
| 250 | 1 ± 0.1 | 10 | 7.7 ± 1.1 | 1.1 ± 0.1 | 14 | ND |

Values are the mean ± SD of 4 independent experiments, except where otherwise indicated in parenthesis. Removal values were taken from Fig. 2A.
ND: not determined.
[a] Student's *t*-test for non-paired samples; $P < 0.05$ *vs*. GE.
[b] Student's *t*-test for non-paired samples; $P < 0.01$ *vs*. Cr(VI).

accumulation (Table 1). Because the culture medium at pH 7 does not spontaneously reduce Cr(VI), and the removal/accumulation ratios for Cr(VI) were >1 at 25 μM or higher concentrations in GME cultures and 10 μM or higher concentrations in GE cultures (Table 1), a cell-dependent efficient extracellular reduction of Cr(VI) was suggested.

### 3.3. Induction of chromate reductase activity and ascorbate content

In control GE grown-cells a small rate of chromate reduction was determined in both, the cytosolic and plasma membrane fractions. No Cr(VI) reduction was observed in cultures without cells. Electron donors such as 0.25 mM NADH or 5 mM cysteine produced significant non-enzymatic chromate reduction (data not shown). Ascorbate was selected as electron donor because reduced Cr(VI) only at <25 μM Cr(VI). To assess whether Cr(VI) reduction was due to an enzyme activity, the cytosolic fraction was incubated for 10 min in either acid, at 80 °C, or with 1.8 M urea or proteinase in ice (Nagarse, 0.1 mg/mL); the inhibiton of Cr(VI) reduction activity by any of these protocols was >80% ($n = 2$, not shown), suggesting that this reaction was indeed due to an enzyme. In GE-CrVI-exposed cells as well as in GE-CrIII-exposed cells, a significant increase in ascorbate-dependent Cr(VI) reduction activity in both cytosolic and membrane fractions was detected (Fig. 3A). On the other hand, total content of ascorbate in control cells did not change in the absence or presence of malate (24.3 ± 9 nmol/$10^7$ cells; $n = 4$). The growth in presence of 50 μM Cr(VI) increased the ascorbate content by 3-fold in both media, whereas 50 μM Cr(III) decreased the ascorbate content by 75% (Fig. 3B).

The kinetic parameters of chromate reductase were determined in both cellular fractions. The GE-CrVI-exposed cells cytosolic fraction showed 23-times higher *Vmax* than that from control cells (see Fig. 3A); in addition, the activity was higher in the cytosolic fraction than in the membrane fraction. A similar pattern was observed in GE-CrIII-exposed cells. The $K_{0.5}$ values were also different among the cytosolic fractions from control, Cr(VI) and Cr(III)-exposed cells (Table 2), suggesting expression of different isoforms. As judged by the $NAD^+$-LDH activity, the percentage of contamination by cytosol in the plasma membrane fractions from both, Cr(VI) and Cr(III)-exposed cells was constant and low, suggesting that indeed a particulate enzyme was induced by exposure to both, Cr(VI) or Cr(III) (Fig. 3A).

### 3.4. Content of molecules with thiol group

The unexpected increased accumulation of Cr(III) in GE-grown cells suggested that increased synthesis of heavy metal-chelating molecules was triggered. Indeed, an increased content of molecules with thiol groups was determined in cells grown in presence of Cr(VI) or Cr(III) in both GME and GE media. Inside the cells, this enhanced thiol-molecules pool was able to form complexes with Cr(III) (see supplementary material and Fig. 2S).

Increase in Cys of 10-times was attained with 25 μM Cr(VI) in GME medium and decreased below control values at 250 μM

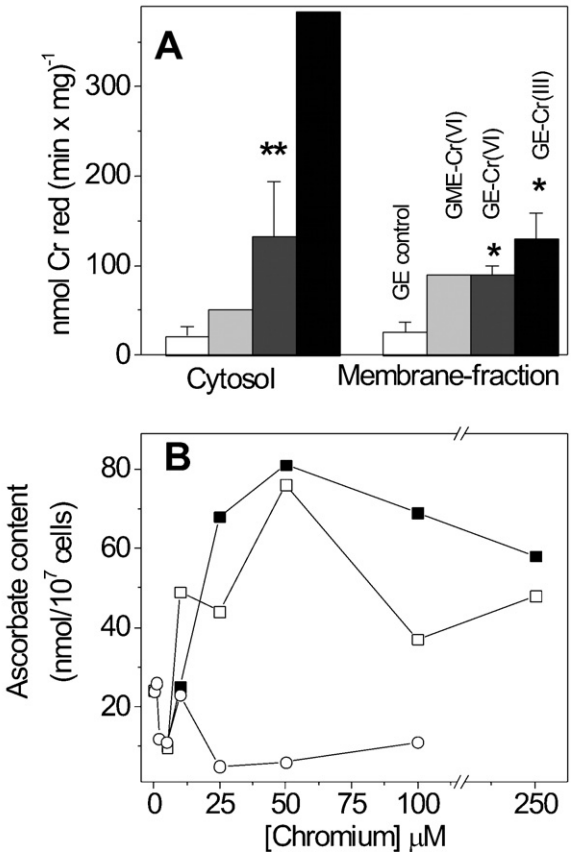


**Fig. 3.** Chromate reductase activity and ascorbate content. (A) Activity was determined in the cytosolic and plasma membrane fractions from GE-control-grown cells, GME-CrVI-, GE-CrVI-, and GE-CrIII-exposed cells using 1 mM Cr(VI) as substrate. Values are the mean ± SD of >3 independent experiments. *$P < 0.05$ for the cytosolic activity in GE-CrVI exposed cells *vs*. the cytosolic activity in GE-grown control cells. Student's *t*-test for non-paired samples: **$P < 0.05$ for the membrane-fraction activity in GE-CrVI- or GE-CrIII- exposed cells *vs* the membrane-fraction activity in GE-grown control cells. (B) The ascorbate content was determined in Cr(VI)-grown cells in GME (■) or GE (□) media as well as in Cr(III)-grown cells in GE medium (○). Values are the mean of two independent experiments.

**Table 2**
Kinetic constants of the chromate reductase from *E. gracilis* cultured in GE medium. Data are the mean ± SD of 3 independent experiments.

| | Cytosolic fraction | | | Plasma membrane fraction | | |
|---|---|---|---|---|---|---|
| | Control cells | CrVI-exposed cells | CrIII-exposed cells | control | CrVI-exposed cells | CrIII-exposed cells[b] |
| *Vmax* (nmol/min × mg protein) | 7 ± 0.1 | 162 ± 35[a] | 162 | 6.5 ± 0.1 | 79 ± 48[*] | 110 |
| $K_{05}$ (μM) | 70 ± 3 | 200 ± 90[*] | 92 | 67 ± 2 | 230 ± 306 | Not determined |
| Hill number | 5 ± 0.5 | 1.7 ± 0.8 | 3.2 | ND | 1.3 ± 1.3 | Not determined |

[a] In this fraction, saturation was not reached up to 2 mM Cr(VI); hence, the value was fixed in order to obtain $K_{0.5}$ value.
[b] Parameters determined from one experiment.
[*] Student's *t*-test for non-paired samples $P < 0.05$ *vs*. the value obtained for control condition.

**Table 3**
Thiol-molecules content in *E. gracilis* cultured with Cr (VI).

| | GME medium (nmol/$10^7$ cells) | | | GE medium (nmol/$10^7$ cells) | | |
|---|---|---|---|---|---|---|
| Cr(VI) μM | Cys | GSH + γEC | $Trp(SH)_2$ | Cys | GSH + γEC | $Trp(SH)_2$ |
| 0 | 12.5 ± 2 | 3.8 ± 0.5 | 0.84 ± 0.1 | 17 ± 12 | 9.3 ± 2.8 | 0.5 ± 0.14 |
| 5 | 34 ± 3.4[a] | 4.6 | 1.6 | 21 ± 19 | 17 ± 15 | 0.50 ± 0.4 |
| 10 | 87 ± 21[a] | 4.3 ± 1.1 | 0.6 ± 0.1 | 21.5 ± 10 | 11 ± 4.5 | 2.4 ± 0.69[c] |
| 25 | 118 ± 22[a] | 7.8 ± 1.8[b] | 0.5 ± 0.1 | 23 ± 14 | 14 ± 8 | 1.8 ± 1.1[c] |
| 50 | 43 ± 22 | 6.8 ± 1.4 | 0.5 ± 0.1 | 10.5 ± 8 | 40 ± 20[b] | 3.2 ± 2.8 |
| 100 | 17 ± 4 | 7.0 ± 0.6 | 0.6 | 13 ± 10 | 8 ± 6 | 2.8 ± 1.9[c] |
| 250 | 1.3 ± 0.3 | 5.7 ± 0.4 | 0.65 ± 0.2 | 4 ± 3 | 4 ± 1 | 3.3 ± 2.9 |

Values are the mean ± SD of 6 different cultures.
[a] Student's *t*-test for non-paired samples; $P < 0.05$ for Cys in control cells *vs* Cys in cells with Cr(VI) added in GME medium.
[b] Student's *t*-test for non-paired samples; $P < 0.01$ for GSH + γEC in control cells *vs* GSH + γEC in cells with Cr(VI) added in GME or GE medium.
[c] Student's *t*-test for non-paired samples; $P < 0.05$ for$Trp(SH)_2$ in control cells *vs* $Trp(SH)_2$ in cells with Cr(VI) added in GE medium.

Cr(VI); γ-EC + GSH content varied less drastically and $TrpSH_2$ did not change by exposure to Cr(VI). In GE medium with 50 μM Cr(VI), γ-EC + GSH and $TrpSH_2$ increased by 4 and 6 fold, respectively, *versus* control (Table 3), suggesting that there was an stimulated flux to γ-EC + GSH and $TrpSH_2$ synthesis from Cys. At higher Cr(VI) concentrations, Cys and γ-EC + GSH contents decreased below control values but $TrpSH_2$ remained higher than control. In contrast to GME, in GE medium with Cr(III), increased contents of Cys (10–100 μM Cr-III) and $TrpSH_2$ (1–50 μM Cr-III) were determined (Table 4).

### 3.5. Malate content and secretion, and malate-dependent enzymes

To determine whether the content of heavy metal chelating molecules with carboxylate group was also elevated, the intracellular content of malate was examined. However, neither Cr(VI) nor Cr(III) induced a significant change: values ranged from 140 to 170 nmol malate $(10^7 \text{cells})^{-1}$ in both, control and chromium-exposed cells (data not shown).

To evaluate whether to contend with the heavy metal stress, *E. gracilis* may have properties like those of C4 plants in which malate is synthesized and exported, the extracellular malate was determined.

Surprisingly, *E. gracilis* cells secreted large amounts of malate, in cultures in GE medium with either Cr(III) or Cr(VI). Cr(III) was a more potent inducer of malate secretion than Cr(VI) (Fig. 4A). This secretion reached up to 0.26 ± 0.09 or 0.09 ± 0.02 mmol malate/100 mL in the presence of Cr(III) or Cr(VI), respectively (Fig. 4A, inset). As the malate secretion was mainly produced in the first 3 days of culture (Fig. 4B), the process was further evaluated at shorter times in GE-control cells incubated with 100 μM Cr(VI) or Cr(III) for 2 h. The malate secretion was similar between them, but 3-fold higher (200 nmol $h^{-1}$ $10^7$ $cells^{-1}$) than in cells incubated without chromium (70 nmol $h^{-1}$ $10^7$ $cells^{-1}$; Fig. 4B, inset). Addition of Fe, Zn, Cd or Hg also stimulated the malate secretion by 4.3–5.4 times, respect to control. Under all these conditions, cells did not secrete glutamate (data not shown).

In turn, three different enzyme activities that may be producing malate were determined. $NAD^+$-MDH and $NADP^+$-ME activities

**Table 4**
Thiol-molecules content in *E. gracilis* cultured with Cr (III).

| | GME medium (nmol/$10^7$ cells) | | | GE medium (nmol/$10^7$ cells) | | |
|---|---|---|---|---|---|---|
| Cr(III) μM | Cys | GSH + γEC | $Trp(SH)_2$ | Cys | GSH + γEC | $Trp(SH)_2$ |
| 0 | 12.5 ± 5 | 3.8 ± 1.1 | 0.84 ± 0.3 | 19 ± 7 | 4.2 ± 1.8 | 0.34 ± 0.15 |
| 1 | ND | ND | ND | 31 ± 20 | 5 ± 2 | 1.5 ± 0.5[***] |
| 2 | ND | ND | ND | 31 ± 4 | 13 ± 10 | 1.9 ± 0.85[***] |
| 5 | ND | ND | ND | 24 ± 7 | 18 ± 8[**] | 1.2 ± 0.7[***] |
| 10 | ND | ND | ND | 36 ± 10[*] | 12 ± 6[**] | 2.0 ± 1.9 |
| 25 | ND | ND | ND | 41 ± 17[*] | 13 ± 8 | 3.1 ± 2.8 |
| 50 | ND | ND | ND | 67 ± 23[*] | 14 ± 11 | 2 ± 1.3[***] |
| 100 | ND | ND | ND | 68 ± 22[*] | 13 ± 6[**] | 0.5 ± 0.3 |
| 250 | 17 ± 4[a] | 5.7 ± 2.6[a] | 1.2 | ND | ND | ND |

Values are the mean ± SD of 6 different cultures.
ND; not determined.
[a] Data taken from [18].
[*] Student's *t*-test for non-paired samples; $P < 0.05$ for Cys in control cells *vs* Cys in cells with Cr(III) added in GE medium.
[**] Student's *t*-test for non-paired samples; $P < 0.01$ for GSH + γEC in control cells *vs* GSH + γEC in cells with Cr(III) added in GE or GE medium.
[***] Student's *t*-test for non-paired samples; $P < 0.01$ for $Trp(SH)_2$in control cells *vs* $Trp(SH)_2$ in cells with Cr(III) added in GE medium.

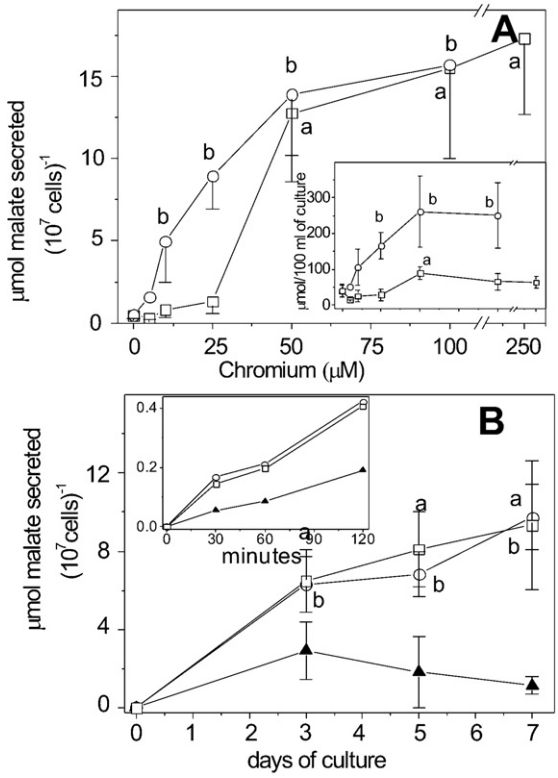


**Fig. 4.** Malate secretion by *E. gracilis* exposed to chromium. (A) Malate content in the GE medium with the indicated Cr(VI) or Cr(III) concentrations was determined. Inset: values of malate secreted were re-plotted as μmol malate/100 mL culture; inset x-axis is identical to that shown in (A). (B) Malate content was determined at different days of culture in the absence (▲) or in the presence of 100 μM Cr(VI) (□) or Cr(III) (○). Values are the mean ± SD of >3 independent experiments. Inset: malate secretion determined at shorter incubation times in control cells without (▲) or with 100 μM Cr(VI) or Cr(III); inset y-axis is identical to that shown in (B). Values are the mean of two independent experiments. Student's *t*-test for non-paired samples: $^{a,b}P < 0.05$ *vs.* cultures without chromium.

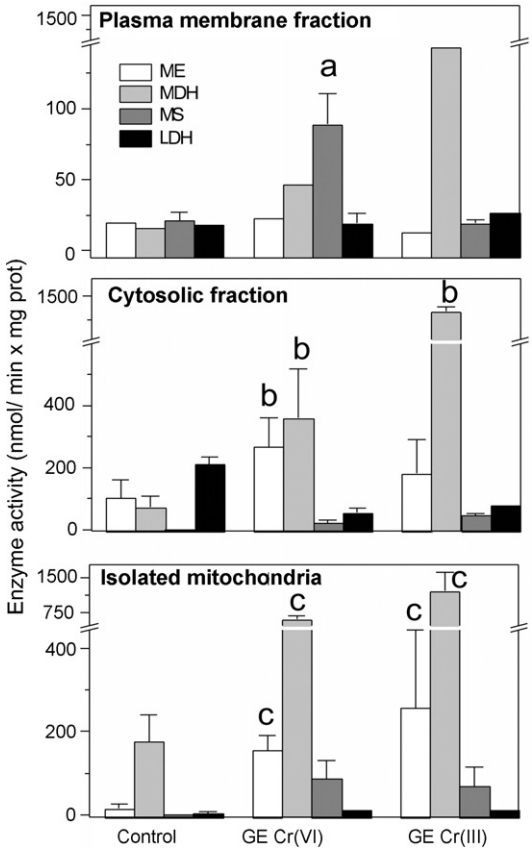


**Fig. 5.** Specific activities of malate-dependent enzymes in chromium-exposed cells. $NADP^+$-ME, $NAD^+$-MDH, MS and $NAD^+$-LDH activities were evaluated in the plasma membrane, cytosolic and mitochondrial fractions from GE-control-grown cells (control), GE-CrVI- (GECrVI) and GE-CrIII-exposed cells (GECrIII). Values are the mean ± SD of >3 independent experiments. Student's *t*-test for non-paired samples: $^{a,b,c}P < 0.05$ *vs.* control condition.

were mainly detected in the mitochondrial and cytosolic fractions; lower activities determined in the particulate fraction were due to cross contamination as judged by LDH activity. It has been documented that the glyoxylate-cycle MS is present in the particulate sub-cellular and mainly in mitochondrial fractions [25,26]. In contrast, MS activity in control and chromium-treated cells was mainly localized in the plasma membrane fraction. The MDH, ME and MS activities increased significantly in GE-CrVI-exposed cells and more importantly in the GE-CrIII-exposed cells (Fig. 5).

## 4. Discussion

Malate is the preferred substrate for *E. gracilis* growth at pH 3.5 [21,22] whereas at pH 7, ethanol and glutamate are preferred over malate [18]. However, the absence of malate in the culture medium at pH 7 decreased cell growth by 75%, indicating that malate is necessary for cell duplication (see Fig. 1). The presence of Cr(VI) diminished malate utilization and at the highest Cr(VI) concentrations assayed, malate content seemed to raise in the culture medium [18], suggesting that malate might be involved in extracellular chromium binding.

### 4.1. Chromium uptake and accumulation

In *E. gracilis* cultured in GE medium with 100 μM Cr(VI) or Cr(III), accumulation values of 40 and 400 nmol/$10^7$ cells equivalent to 10.8 and 108 nmol/mg DW were achieved (*cf.* Fig. 2A). As in *E. gracilis* $10^7$ cells are equal to 3.7 mg dry weight [30], the maximum accumulation in cells grown in GE medium with 250 μM Cr(VI) for 6 days was 12 nmol Cr (mg DW)$^{-1}$, which is certainly comparable with the 57 nmol Cr (mg DW)$^{-1}$ reported in leaves from the proposed chromium bio-remediator *Salsola kali* exposed to 384 μM Cr(VI) for 15 days [29]. Similarly, in celery 15-days old seedlings roots and kiwifruit pollen, the Cr(III) accumulation [27] and uptake rate [28] are higher than with Cr(VI), whereas in 15 days-old tumbleweed (*S. kali*) roots incubated with 385 μM Cr(VI), metal accumulation is 10–20 fold higher than that with Cr(III); however, Cr(III) exhibits higher toxicity [29].

Previous, indirect evidence has shown that the respiratory chain is deeply affected in Cr(VI)-grown cells, suggesting that Cr(VI) is entering into mitochondria [18]. Moreover, the sulfate assimilation pathway in *E. gracilis* is partially located in mitochondria [31], implying that sulfate (and chromate) transport is active [19]. Direct evidence provided in the present study showed that mitochondria isolated from cells exposed for 48 h to high concentrations of Cr(VI) or Cr(III) were indeed able to accumulate Cr(III) and Cr(VI).

### 4.2. Reduction of Cr (VI) to Cr(III)

*E. gracilis* grown in GE medium was able to reduce 14% of the original 250 μM Cr(VI) present in the medium to Cr(III) (*cf.* Fig. 2A), *e.g.*, 440 nmol Cr(VI) reduced/mg dry weight or 35 nmol/mL of culture after 6 days incubation, indicating that in *E. gracilis* the rate of Cr(VI) reduction to Cr(III) was several-fold faster than the Cr(VI) uptake rate. The green algae *Chlorella sp.* obtained from ponds

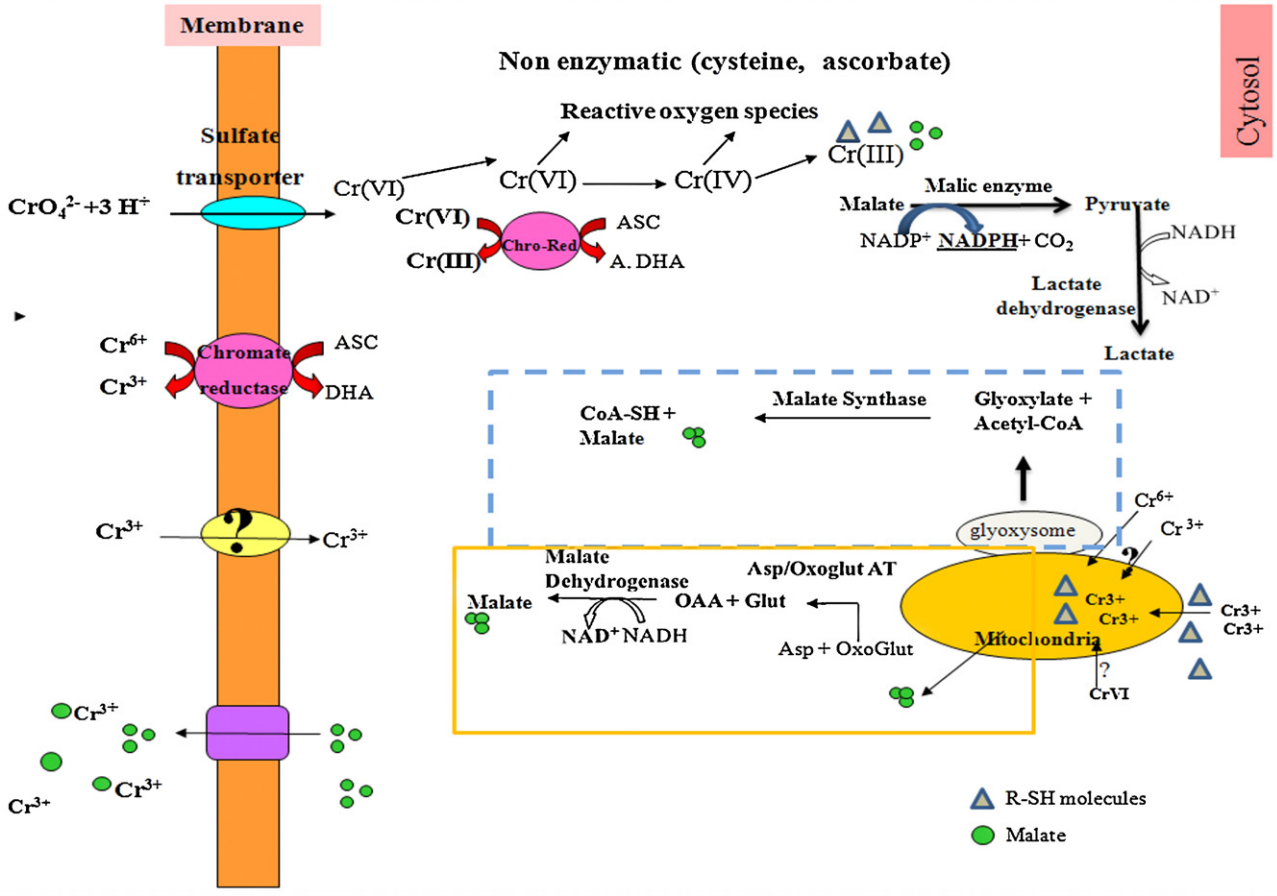


**Fig. 6.** Resistance mechanisms in heterotrophic *Euglena gracilis* developed against chromium stress. Subcellular enzyme localization: $NADP^+$-ME (cytosol, and mitochondria), $NAD^+$-MDH (cytosol and mitochondria), MS (glyoxysome and mitochondria) and $NAD^+$-LDH (cytosol).

receiving tannery effluents, is able to reduce 97% of Cr(VI) after 7 days when cultured with 192 μM Cr(VI) [32], suggesting that bio-reduction and accumulation are common responses against chromium stress in autotrophic-plankton species.

Reduction of Cr(VI) to Cr(III) is an essential step in the detoxification of Cr(VI)-polluted environments [1]. Bacterial species show resistance to chromium due to their capacity for reducing Cr(VI) by soluble or membrane-bound NAD(P)H-dependent proteins in *Pseudomonas putida* [33] and *Enterobacter cloacae* [34], respectively. *Pseudomonas* spp. shows aerobic Cr(VI) reduction mediated by cytosolic soluble enzyme and anaerobic reduction by membrane protein [35]. Cr(VI) reduction in whole cells was found in *Chlorella sp*. isolated from disposal sites of the paper-pulp and electroplating industries; this activity was stimulated by light, malate and acetate and inhibited by cyanide [36]. In the present work, cell exposure to Cr(VI) or Cr(III) under aerobiosis induced a >10 fold activity increase in both cytosolic and membrane fractions, suggesting that expression of chromate reductase did not depend on anaerobiosis as in bacteria. The bacterial chromate reductase may exhibit other enzyme activities [37,38]. Likewise, the increased chromate reductase activity in GECrIII-exposed cells might not be specific for Cr(VI).

Preliminary data indicated that cytosolic fraction from cells exposed to 100 μM $H_2O_2$ for 48 h, increased the chromate reductase activity up to 2600 nmol Cr(VI) reduced $(\text{min} \times \text{mg protein})^{-1}$, suggesting that the enzyme involved in the Cr(VI) reduction in *E. gracilis* is an ascorbate peroxidase. It seemed that the oxidative stress generated by Cr(VI) increased the content of anti-oxidant metabolites such as ascorbate as part of the cellular response against heavy metal stress; Cr(VI) reduction involves the generation of reactive oxygen species and hence oxidative stress. In turn, the decreased ascorbate content induced by Cr(III) was probably one of the reasons why Cr(III) was so much toxic for cells grown in GE medium.

The kinetic analysis of the chromate reductase activity revealed that $K_{0.5}$ values for chromate changed among the different cellular preparations, whereas *Vm* values markedly increased (Table 2), suggesting enzyme over-expression induced by chromium stress of more than one enzyme.

### 4.3. Malate secretion as a novel heavy metal-resistance mechanism

It is well documented the capacity of plants to exude organic acids as a response to environmental stress such as nutrient deficiency, anoxia and mainly exposure to toxic metal ions [39]. It has been proposed that the transport of malate outside the cell is the rate-limiting step in plants for the aluminum tolerance in barley (*Hordeum vulgare*) and hence malate transporter over expression increases metal resistance [40]. In the present study, it is shown for the first time that a heterotrophic protist is able to secrete malate in response not only to Cr(III) but also to Cr(VI) stress (see Fig. 4). The fast Cr(VI) reduction to Cr(III) in the cytosol (Fig. 3A) and the most potent Cr(III) effect suggested that Cr(III) was the most probable signal for the induction of malate secretion (Fig. 6). In turn, the fast consumption of malate by *E. gracilis* in cultures at pH 3.5 (*e.g.*, inward transport) and the fast malate secretion (*e.g.*, outward trans-

port; Fig. 4A) indicated the presence of efficient malate transport in these cellular processes (Fig. 4B, inset).

The $\Delta G^{\circ}$ values for the reactions catalyzed by MS (−11.3 kcal/mol; calculated from [41]), $NAD^+$-MDH (+ 6.7 kcal/mol; calculated from [42]) and ME (−7.1 kcal/mol; [43]) allow for visualizing that MS and MDH are physiologically producing malate, whereas ME is decarboxylating malate to produce the NADPH necessary to contend against oxidative stress induced by chromium (see Fig. 6). Indeed, the ME physiological role usually involves malate decarboxylation for NADPH production, as demonstrated in *P. fluorescens* exposed to $Al^{3+}$ [44]. However, in *Corynebacterium glutamicum*, when lactate metabolism is present and high pyruvate concentration is reached, the ME reverses reaction is thermodynamically favored [45]. Similarly, in *E. gracilis* the pyruvate concentration may be relatively high at 1 mM [21]. Thus, ME might be able to join with $NAD^+$-MDH and MS for active malate production for secretion. Moreover, the activities of these enzymes were higher in chromium-exposed cells (Fig. 5).

Cr(III) may readily form organo-Cr(III) complexes with organic acids, amino acids, proteins and synthetic nutrient media [46]. Therefore, similar to the complexes formed with $Al^{3+}$ [47], malate may also form stable complexes with Cr(III). On this regard, the dissociation constants of heavy metal-malate complexes are relatively low, indicating low affinity; for instance, the dissociation constant of the malate-zinc complex is 2–6 orders of magnitude ($pK = 2.8$) lower than those for Cys-Zn, GSH-Zn or Zn-glutamate complexes ($pK = 9.86$, 8.3 and 5.45, respectively) [48]. This is most likely the reason why amounts of malate up to 2.5 mM have to be released to the medium in an attempt to efficiently neutralize Cr(III), before they gain access to the intracellular targets. However, malate secretion reached saturation at approximately 50 μM external Cr(III) or Cr(VI) (see Fig. 4A), suggesting this resistance mechanism was able to contend against chromium stress only at external (environmental) concentrations below this value.

### 4.4. Heavy metal chelating thiol-molecules

Marked increase in Cys, γ-EC + GSH and $TrpSH_2$ contents was attained when cells were cultured with chromium in GE medium (Table 4), correlating with an enhanced metal accumulation (Fig. 2B). This variation in thiol-molecules is a common response against heavy metals stress in *E. gracilis* [14–16,20,49,50], and indicates that metal ion accumulation is mediated by binding with thiol–molecules [3] (see also Fig. S1). In contrast to cadmium, zinc and mercury, chromium did not increase the content on phytochelatins [52].

## 5. Concluding remarks

Fig. 6 shows the new insights found in heterotrophic *E. gracilis* when contending against chromium stress: (i) depending on the culture medium composition (mainly absence of organic acids), Cr(III) can be actively taken up, retained, accumulated and mitochondrially compartmentalized, and as a consequence, exert more potent toxic effects than Cr(VI); (ii) there is enhanced expression of ascorbate-dependent chromate reductase activities in the cytosol and plasma membrane; (iii) active malate secretion to bind Cr(III) extra-cellularly develops; and (iv) increased synthesis of thiol-molecules to bind Cr(III) intra-cellularly, and ascorbate, is also simultaneously triggered. Therefore, the findings of the present study establish new paradigms regarding chromium stress on algae-like microorganisms:

(i) Cr(III) can be more toxic than Cr(VI), depending on the culture (or environmental) conditions. Higher toxicity of Cr(III) than Cr(VI) was also recently reported in two freshwater algae [51]. However, the present study clearly establishes the reasons and conditions for this higher Cr(III) toxicity. (ii) Simultaneous and diverse molecular and cellular mechanisms (*i.e.*, intracellular binding by thiol-molecules, extracellular binding by organic acids, intra- and extracellular Cr(VI) reduction, sub-cellular compartmentalization) are turned on to inactivate chromium species and their toxic effects. Moreover, the present results unveil efficient mechanisms in *E. gracilis* to contend against chromium, which once optimized, may facilitate the development of strategies for using this protist as potential bio-remediator of chromium-polluted water systems.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jhazmat.2011.07.056.

## References

[1] C. Cervantes, J. Campos-Garcia, S. Devars, F. Gutiérrez-Corona, H. Loza-Tavera, J.C. Torres-Guzman, R. Moreno-Sánchez, Interactions of chromium with microorganisms and plants, FEMS Microbiol. Rev. 25 (2001) 335–347.
[2] X. Yang, Y. Feng, Z. He, P.J. Stoffella, Molecular mechanisms of heavy metal hyperaccumulation and phytoremediation, J. Trace Elem. Med. Biol. 18 (2005) 339–353.
[3] J.S. Rodríguez-Zavala, J.D. García-García, M.A. Ortiz-Cruz, R. Moreno-Sánchez, Molecular mechanisms of resistance to heavy metals in the protist *Euglena gracilis*, J. Environ. Sci. Health part A. Toxicol. Hazard. Subst. Environ. Eng. 42 (2007) 1365–1378.
[4] S. Rodgher, E.L. Espíndola, The influence of algal densities on the toxicity of chromium for *Ceriodaphnia dubia* Richard (*Cladocera, Crustacea*), Braz. J. Biol. 71 (2008) 341–348.
[5] M.A. Armienta, R. Rodríguez, A. Queré, F. Juárez, N. Ceniceros, A. Aguayo, Ground water pollution with chromium in Leon Valley, Mexico, Int. J. Environ. Anal. Chem. 54 (1993) 1–13.
[6] M.E. Gutiérrez-Ruíz, M. Villalobos-Peñaloza, J.A. Miranda, Chromium pollution in groundwaters in north of Mexico City, Rev. Int. Contam. Amb. 6 (1990) 5–18 (Web page: http://redalyc.uaemex.mx/redalyc/src/inicio/ArtPdfRed.jsp?iCve=37060101).
[7] G. Sudhakar, B. Jyothi, V. Venkateswarlu, Metal pollution and its impact on algae in flowing waters in India, Arch. Environ. Contam. Toxicol. 21 (1991) 556–566.
[8] S. Rodgher, E.L. Espindola, The influence of algal densities on the toxicity of chromium for *Ceriodaphnia dubia* Richard (Cladocera, Crustacea), Braz. J. Biol. 68 (2008) 341–348.
[9] A. Ganguli, A.K. Tripathi, Bioremediation of toxic chromium from electroplating effluent by chromate-reducing *Pseudomonas aeruginosa* A2Chr in two bioreactors, Appl. Microbiol. Biotechnol. 58 (2002) 416–420.
[10] H. Ohtake, C. Cervantes, S. Silver, Decreased chromate uptake in *Pseudomonas fluorescens* carrying a chromate resistance plasmid, J. Bacteriol. 169 (1987) 3853–3856.
[11] A.R. Shakoori, M. Makhdoom, R.U. Haq, Hexavalent chromium reduction by a dichromate-resistant gram-positive bacterium isolated from effluents of tanneries, Appl. Microbiol. Biotechnol. 53 (2000) 348–351.
[12] H. Eccles, Treatment of metal-contaminated wastes: why select a biological process? Trends Biotechnol. 17 (1999) 462–465.
[13] D. Mendoza-Cózatl, S. Devars, H. Loza-Tavera, R. Moreno-Sánchez, Cadmium accumulation in the chloroplast of *Euglena gracilis*, Physiol. Plant. 115 (2002) 276–283.
[14] D. Mendoza-Cózatl, R. Moreno-Sánchez, $Cd^{2+}$ transport and storage in the chloroplast of *Euglena gracilis*, Biochim. Biophys. Acta 1706 (2005) 88–97.
[15] D. Mendoza-Cózatl, E. Rangel-González, R. Moreno-Sánchez, Simultaneous $Cd^{2+}$, $Zn^{2+}$ and $Pb^{2+}$ uptake and accumulation by photosynthetic *Euglena gracilis*, Arch. Environ. Contam. Toxicol. 51 (2006) 521–528.
[16] D.G. Mendoza-Cózatl, J.S. Rodríguez-Zavala, S. Rodríguez-Enríquez, G. Mendoza-Hernández, R. Briones-Gallardo, R. Moreno-Sánchez, Phytochelatin-

cadmium-sulfide high-molecular-mass complex of *Euglena gracilis*, FEBS J. 273 (2006) 5703–5713.
[17] C. Avilés, M.E. Torres-Márquez, D. Mendoza-Cózatl, R. Moreno-Sánchez, Time-course development of the $Cd^{2+}$ hyper-accumulating phenotype in *Euglena gracilis*, Arch. Microbiol. 184 (2005) 83–92.
[18] R. Jasso-Chávez, A. Pacheco-Rosales, E. Lira-Silva, J.C. Gallardo-Pérez, N. García, R. Moreno-Sánchez, Toxic effects of Cr(VI) and Cr(III) on energy metabolism of heterotrophic *Euglena gracilis*, Aquat. Toxicol. 100 (2010) 329–338.
[19] J.D. García-García, J.S. Rodríguez-Zavala, R. Jasso-Chávez, D. Mendoza-Cózatl, R. Moreno-Sánchez, Chromium uptake, retention and reduction in photosynthetic *Euglena gracilis*, Arch. Microbiol. 191 (2009) 431–440.
[20] C. Avilés, H. Loza-Tavera, N. Terry, R. Moreno-Sánchez, Mercury pretreatment selects an enhanced cadmium-accumulating phenotype in *Euglena gracilis*, Arch. Microbiol. 180 (2003) 1–10.
[21] R. Jasso-Chávez, R. Moreno-Sánchez, Cytosol-mitochondria transfer of reducing equivalents by a lactate shuttle in heterotrophic *Euglena*, J. Eur. Biochem. 270 (2003) 4942–4951.
[22] J.S. Rodríguez-Zavala, M.A. Ortiz-Cruz, R. Moreno-Sanchez, Characterization of an aldehyde dehydrogenase from *Euglena gracilis*, J. Eukaryot. Microbiol. 53 (2006) 36–42.
[23] J.F. Ma, P.R. Ryan, E. Delhaize, Aluminium tolerance in plants and the complexing role of organic acids, Trends Plant Sci. 6 (2001) 273–278.
[24] R. Jasso-Chávez, M.E. Torres Márquez, R. Moreno-Sánchez, The membrane-bound L- and D-lactate dehydrogenase activities in mitochondria from *Euglena gracilis*, Arch. Biochem. Biophys. 390 (2001) 295–303.
[25] N. Bégin-Heick, The localization of enzymes of intermediary metabolism in *Astasia* and *Euglena*, The biochem. J. 134 (1972) 607–616.
[26] K. Ono, M. Kondo, T. Osafune, K. Miyatake, H. Inui, S. Kitaoka, M. Nishimura, Y. Nakano, Presence of glyoxylate cycle enzymes in the mitochondria of *Euglena gracilis*, J. Eukaryot. Microbiol. 50 (2003) 92–96.
[27] V. Scoccianti, R. Crinelli, B. Tirillini, V. Mancinelli, A. Speranza, Uptake and toxicity of Cr(III) in celery seedlings, Chemosphere 64 (2006) 1695–1703.
[28] V. Scoccianti, M. Iacobucci, M.F. Paoletti, A. Fraternale, A. Speranza, Species-dependent chromium accumulation, lipid peroxidation, and glutathione levels in germinating kiwifruit pollen under Cr(III) and Cr(VI) stress, Chemosphere 73 (2008) 1042–1048.
[29] J.L Gardea-Torresdey, G. de la Rosa, J.R. Peralta-Videa, M. Montes, G. Cruz-Jimenez, I. Cano-Aguilera, Differential uptake and transport of trivalent and hexavalent chromium by tumbleweed (*Salsola kali*), Arch. Environ. Contam. Toxicol. 48 (2005) 225–232.
[30] J.S. Rodríguez-Zavala, M.A. Ortiz-Cruz, R. Moreno-Sánchez, Increase synthesis of alpha-tocopherol, paramylon and tyrosine by *E. gracilis* under conditions of high biomass production, J. Appl. Microbiol. 109 (2010) 2160–2172.
[31] N.J. Patron, D.G. Durnford, S. Kopriva, Sulfate assimilation in eukaryotes: fusions, relocations and lateral transfers, BMC Evol. Biol. 4 (2008) 8–39.
[32] A. Rehman, A.R. Shakoori, Heavy metal resistance *Chlorella* spp., isolated from tannery effluents, and their role in remediation of hexavalent chromium in industrial waste water, Bull. Environ. Contam. Toxicol. 66 (2001) 542–547.
[33] C.H. Park, M. Keyhan, B. Wielinga, S. Fendorf, A. Martin, Purification to homogeneity and characterization of a novel *Pseudomonas putida* chromate reductase, Appl. Environ. Microbiol. 66 (2000) 1788–1795.
[34] P.C. Wang, T. Mori, K. Toda, H. Ohtake, Membrane-associated chromate reductase activity from *Enterobacter cloacae*, J. Bacteriol. 172 (1990) 1670–1672.
[35] J. McLean, T.J. Beveridge, Chromate reduction by a pseudomonad isolated from a site contaminated with chromated copper arsenate, Appl. Environ. Microbiol. 67 (2001) 1076–1084.
[36] S.N. Yewalkar, K.N. Dhumal, J.K. Sainis, Chromium (VI)-reducing *Chlorella* spp. isolated from disposal sites of paper-pulp and electroplating industry, J. Appl. Phycol. 19 (2007) 459–465.
[37] J. Mazoch, R. Tesarík, V. Sedlácek, I. Kucera, J. Turánek, Isolation and biochemical characterization of two soluble iron(III) reductases from *Paracoccus denitrificans*, Eur. J. Biochem. 271 (2004) 553–562.
[38] C.F. Gonzalez, D.F. Ackerley, S.V. Lynch, A. Martin, ChrR, a soluble quinone reductase of *Pseudomonas putida* that defends against $H_2O_2$, J. Biol. Chem. 280 (2005) 22590–22595.
[39] P.R. Ryan, E. Delhaize, D.L. Jones, Function and mechanism of organic anion exudation from plan roots, Ann. Rev. Plant Physiol. Plant Mol. Biol. 52 (2001) 527–560.
[40] E. Delhaize, P.R. Ryan, D.M. Hebb, Y. Yamamoto, T. Sasaki, H. Matsumoto, Engineering high-level aluminum tolerance in barley with the ALMT1 gene, Proc.Natl. Acad. Sci. USA 101 (2004) 15249–15254.
[41] R.A. Alberty, Thermodynamics and kinetics of the glyoxylate cycle, Biochemistry 26 (2006) 15838–15843.
[42] R.W. Guynn, H.J. Gelberg, R.L. Veech, Equilibrium constants of the malate dehydrogenase, citrate synthase, citrate lyase, and acetyl coenzyme a hydrolysis reactions under physiological conditions, J. Biol. Chem. 248 (1973) 6957–6965.
[43] G.J. Pilone, R.E. Kunkee, Carbonic acid from decarboxylation by "malic" enzyme in lactic acid bacteria, J. Bacteriol. 103 (1970) 404–409.
[44] J. Lemire, P. Kumar, R. Mailloux, K. Cossar, V.D. Appanna, Metabolic adaptation and oxaloacetate homeostasis in *P. fluorescens* exposed to aluminum toxicity, J. Basic Microbiol. 48 (2008) 252–259.
[45] P. Gourdon, M.F. Baucher, N.D. Lindley, A. Guyonvarch, Cloning of the malic enzyme gene from *Corynebacterium glutamicum* and role of the enzyme in lactate metabolism, Appl. Environ. Microbiol. 66 (2000) 2981–2987.
[46] E. Remoundaki, A. Hatzikioseyian, M. Tsezos, A systematic study of chromium solubility in the presence of organic matter: consequences for treatment of chromium-containing waste water, J. Chem. Technol. Biotechnol. 82 (2007) 802–808.
[47] M. Tashiro, K. Furihata, T. Fujimoto, T. Machinami, E. Yoshimura, Characterization of the malate-aluminum(III) complex using $^{1}H$ and $^{27}Al$ NMR spectroscopy, Magn. Reson. Chem. 45 (2007) 518–521.
[48] L.G. Sillen, A.E. Martell, Stability Constants of Metal-ion Complexes, Spec. Publ. No. 25, The Chemical Society of London, 1971, ISBN 85186-019-2.
[49] S. Devars, R. Hernández, R. Moreno-Sánchez, Enhanced heavy metal tolerance in *Euglena gracilis* by preexposure to mercury or cadmium, Arch. Environ. Contam. Toxicol. 34 (1998) 128–135.
[50] S. Devars, C. Avilés, C. Cervantes, R. Moreno-Sánchez, Mercury uptake and removal by *Euglena gracilis*, Arch. Microbiol 174 (2000) 175–180.
[51] D.A.L. Vignati, J. Dominik, M.L. Beye, M. Pettine, B.J.D. Ferrari, Chromium (VI) is more toxic than chromium (III) to freshwater algae: A paradigm to revise? Ecotoxicol. Environ. Saf. 73 (2010) 743–749.
[52] E. Grill, E.-L. Winnacker, M.H. Zenk, Phytochelatins, a class of heavy-metal-binding peptides from plants, are functionally analogous to metallothioneins, Proc. Natl. Acad. Sci. U.S.A. 84 (1987) 439–443.