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# Increase of chromium tolerance in *Scenedesmus acutus* after sulfur starvation: Chromium uptake and compartmentalization in two strains with different sensitivities to Cr(VI)

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ABSTRACT

In photosynthetic organisms sulfate constitutes the main sulfur source for the biosynthesis of GSH and its precursor Cys. Hence, sulfur availability can modulate the capacity to cope with environmental stresses, a phenomenon known as SIR/SED (Sulfur Induced Resistance or Sulfur Enhanced Defence). Since chromate may compete for sulfate transport into the cells, in this study chromium accumulation and tolerance were investigated in relation to sulfur availability in two strains of the unicellular green alga *Scenedesmus acutus* with different Cr-sensitivities. Paradoxically, sulfur deprivation has been demonstrated to induce a transient increase of Cr-tolerance in both strains. Sulfur deprivation is known to enhance the sulfate uptake/assimilation pathway leading to important consequences on Cr-tolerance: (i) reduced chromate uptake due to the induction of high affinity sulfate transporters (ii) higher production of cysteine and GSH which can play a role both through the formation of unsoluble complexes and their sequestration in inert compartments. To investigate the role of the above mentioned mechanisms, Cr accumulation in total cells and in different cell compartments (cell wall, membranes, soluble and miscellaneous fractions) was analyzed in both sulfur-starved and unstarved cells. Both strains mainly accumulated chromium in the soluble fraction, but the uptake was higher in the wild-type. In this type a short period of sulfur starvation before Cr(VI) treatment lowered chromium accumulation to the level observed in the unstarved Cr-tolerant strain, in which Cr uptake seems instead less influenced by S-starvation, since no significant decrease was observed. The increase in Cr-tolerance following S-starvation seems thus to rely on different mechanisms in the two strains, suggesting the induction of a mechanism constitutively active in the Cr-tolerant strain, maybe a high affinity sulfate transporter also in the wild-type. Changes observed in the cell wall and membrane fractions suggest a strong involvement of these compartments in Cr-tolerance increase following S-starvation.



## 1. Introduction

In the last decades microalgae have received particular attention due to their potential application in different biotechnology fields. In environmental studies, some microalgal species can be used as biosensors able to provide important information in predicting the impact of metal pollution (Brayner et al., 2011). On the other hand, studies on the mechanisms involved in tolerance to different toxicants can provide important information for the use of tolerant species or ecotypes in bioremediation processes (Priyadarshani et al., 2011).

Chromium, a non-essential metal highly toxic for microorganisms and plants, represents a main environmental concern due to the Cr-containing effluents discharged by a number of industrial activities. Whereas in natural freshwater chromium concentration ranges between 0 and 2 μg/l, in effluents of leather tanning, dye, wood preservation and electroplating industries amounts ranging from ten to hundred micrograms per liter have been reported (Han et al., 2006). Cr(VI) is rapidly reduced to chromium III in presence of organic matter and acidic environment. Nevertheless, high levels of Cr(VI) could overcome the reducing capacity of the environment and persist as a great concern pollutant (Cervantes et al., 2001). In plants, hexavalent chromium is known to induce oxidative stress, inhibition of cell division, reduction of photosynthesis and imbalance in cell nutrition due to the competition with essential ions for uptake and translocation (Cervantes et al., 2001; Shanker et al., 2005). Although natural tolerance to Cr(VI)

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has been reported in plants (Arora et al., 2006; Cervantes et al., 2001; Choo et al., 2006; Citterio et al., 2003; Kidd et al., 2004; Mei et al., 2002; Qiu et al., 2010) and algae (Corradi et al., 1995; Yewalkar et al., 2007), the underlying mechanisms are still poorly understood. Photoreduction of Cr(VI) has been reported in the unicellular alga *Chlorella* spp. (Han et al., 2006; Yewalkar et al., 2007), while a reduced metal uptake or more efficient detoxification processes inside the cells have been hypothesized as mechanisms of chromium tolerance in some green plants and in the freshwater unicellular alga *Scenedesmus acutus*. Both latter mechanisms seem strictly dependent on sulfur availability and connected to its uptake/assimilation pathway (Appenroth et al., 2008; Gorbi et al., 2006; Pereira et al., 2008; Schiavon et al., 2007, 2008).

Besides being a component of polysaccharides and proteins with essential structural and metabolic role (Giordano et al., 2005; Mera et al., 2014; Nocito et al., 2006), sulfur is essential in the synthesis of important defence compounds such as the small cysteine-rich peptides, phytochelatins (PCs) and metallotioneins (MTs) (Cobbett, 2000; Cobbett and Goldsbrough, 2002), and reduced glutathione (GSH) involved in both metal chelation and in scavenging potential oxidative stress as a result of ROS production induced by Cr exposure (Grill et al., 2004; Panda, 2007; Upadhyay and Panda, 2010; Volland et al., 2011). Hence sulfur availability can lead to an increased capacity to cope with biotic and abiotic stresses, a phenomenon known as SIR/SED (Sulfur Induced Resistance or Sulfur Enhanced Defence) (Bloem et al., 2015; Ernst et al., 2008; Höller et al., 2010; Nazar et al., 2011; Nocito et al., 2006). Although a few reports suggest the involvement of PCs or MTs in Cr detoxification (Diwan et al., 2010; Shanker et al., 2004), two direct products of sulfur assimilation pathway other than PCs or MTs can be involved in chromium detoxification, since the metal can be sequestered through the formation of thiolate and cysteinthiolate complexes with glutathione and various derivatives of cysteine (Brauer et al., 1996; Brauer and Wetterhahn, 1991). It also has to be stressed that chromate enters the cells through the sulfate uptake system. Many researches on plants, algae, yeast and fungi report a possible competitive interaction for the uptake between chromate ($CrO_4^{2-}$) and sulfate ($SO_4^{2-}$) which have similar size (Kaszycki et al., 2005; Marzluf, 1970; Pereira et al., 2008; Pèrez-Castìneira et al., 1998; Riedel, 1985). A chromate/sulfate interaction is strengthened by the observation that chromium exposure induces the same effects as sulfur starvation through competition both at the transporter level and at some step of the subsequent assimilation pathway (Pereira et al., 2008). In prokaryotic organisms $SO_4^{2-}$ is transported into the cells by a single transporter system with the features of an ABC transporter: in many algae and some basal land plants, this kind of transporter has been found in the chloroplast envelope and represents the entrance of sulfate in the organelle in which reductive assimilation occurs. Eukaryotes have instead multiple $SO_4^{2-}$ transporters with different affinities for the substrate. Beyond the chloroplast ABC transporter (Lindberg and Melis, 2008; Melis and Chen, 2005), six further plasma membrane sulfate transporters with different substrate affinities, have been recently identified in the green alga *Chlamydomonas reinhardtii*. (Pothakam et al., 2010). Three of them (SULTR1 to SULTR3) belong to the $H^+/SO_4^{2-}$ transporter family, characteristic of vascular plants, whereas the other three (SLT1 to SLT3, Sac1 like transporters) belong to the $Na^+/SO_4^{2-}$ transporter family, found in bacteria, nonvascular plants and mammals. Members of both groups show different substrate affinities and are inducible by S-starvation.

Although sulfur availability is at the basis of resistance to many biotic and abiotic stresses, a previous study by Gorbi et al. (2007) indicates that a period of S-deprivation induces a transient increase in chromium tolerance in the wild-type and a Cr-tolerant strain of *S. acutus*. After S-starvation indeed both strains are able to divide and increase cell density at Cr(VI) concentrations normally inhibiting their growth. During recovery in standard medium, an elevated S uptake and a consequent increase in cysteine biosynthesis have been observed. The up-regulation of the sulfur uptake/assimilation pathway was hypothesized to explain this transient tolerance increase which was lost after two days of recovery in standard medium. This is in agreement with recent studies on *C. reinhardtii* acclimation to sulfur deprivation, indicating that arylsulfatase, high affinity sulfate transporters, as well as many enzymes of the S assimilation pathway are specifically induced by S-deprivation (Aksoy et al., 2013; González-Ballester et al., 2010; Zhang et al., 2004). A strong increase in sulfate uptake has also been described in different prokaryotic and eukaryotic organisms after a period of sulfur starvation (Clarkson and Saker, 1989; Gyaneshwar et al., 2005; Shibagaki et al., 2002; Yildiz et al., 1994).

The up-regulation of the sulfur uptake/assimilation pathway, following S starvation, may have two direct consequences which can strongly enhance Cr-tolerance: (i) decrease in Cr uptake due to the induction of high affinity sulfate transporters; (ii) a greater sulfate uptake and sulfur availability inside the cells for the synthesis of sulfur-containing molecules with the consequent increased capacity to cope with intracellular chromium, either through chelation and compartmentalization or through an enhanced antioxidant response (utilizing GSH). Q2

The aim of the present study is to investigate the mechanisms underlying the transient increase in Cr-tolerance observed after S-starvation in two strains of *S. acutus* through the analysis of chromium uptake and compartmentalization after exposure to a metal concentration which in standard conditions discriminates the two strains on the basis of their different sensitivities. To achieve this aim the cells of the two strains, were first pre-cultured in standard and sulfur-deprived medium for 3 days. Thereafter exposure to hexavalent chromium in standard medium followed, and the sampled cells were fractionated into cell walls, membranes, soluble and miscellaneous fractions to evaluate differences in chromium distribution. This information could enable elucidating if Cr-tolerance increase after S-starvation occurs through the same mechanism in the two strains and if the same mechanism is responsible of the constitutive difference in chromium sensitivity between the two strains.

## 2. Materials and methods

### 2.1. *In vitro culture of* S. acutus

Synchronized axenic cultures of the wild-type and Cr-tolerant strains (Corradi et al., 1995) of the green unicellular alga *S. acutus* were maintained in liquid culture medium (US EPA, 1978; pH $7.7 \pm 0.1$, modified by dissolving both micro- and macronutrients in distilled water to obtain a final concentration double of that indicated), in a climate-controlled chamber ($23 \pm 1\,^{\circ}C$, $230\ \mu mol\ m^{-2}\ s^{-1}$ light intensity irradiance, white-cool fluorescent lamps, 16:8 h photoperiod). No organic matter was present in the medium at the beginning of the culture. The cultures were continuously aerated (sterile filtered air). To perform the experiments with algae in exponential growth phase, culture medium was always renewed 3 days before starting each experiment by adding 900 ml of fresh medium to 100 ml of algal suspension in 1 l Erlenmeyer flasks (Stock Culture). Medium quality parameters were evaluated in the medium before and at the end of the 4 day treatments. Measures at the end of the experiments were carried out on the supernatant of culture media after algal removing by centrifugation.



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### 2.2. Sulfur starvation and Cr-tolerance

Aliquots of the stock cultures, in exponential growth phase, were centrifuged for 10 min at 2200 × *g* and washed with distilled water. The pellets were suspended at $3 \times 10^6$ cells/ml density in 200 ml of standard culture medium (+S) containing $MgSO_4$ (14.36 mg/l), or in sulfate-deprived medium (−S). Since $MgSO_4$ is the only source of sulfur in the standard medium, the amount of $MgCl_2$ was simultaneously increased to restore standard magnesium concentration in sulfate-deprived medium. After a 3-day pre-culture in +S (unstarved cells) or in −S medium (S-starved cells) at the same conditions as above, cells of both strains were collected by centrifugation, washed and treated with Cr(VI) supplied as potassium dichromate ($K_2Cr_2O_7$) in standard culture medium. Unstarved cells were exposed to Cr(VI) at the Lowest Observed Effect Concentration (LOEC), as established in previous experiments (Gorbi et al., 2007), namely 1 mg/l for the wild-type and 2 mg/l for the Cr-tolerant strain. S-starved cells were exposed to 1, 2, 3, 4 mg Cr(VI)/l. Culture medium and metal solutions were sterilized through 0.2 μm nitrocellulose membranes. Treatments and controls had three replicates. The initial cell density ($3 \times 10^6$ cells/ml) was chosen according to previous studies (Corradi et al., 1995; Gorbi and Corradi, 1993; Gorbi et al., 2007; Torricelli et al., 2004) to ensure comparison of the response to metal poisoning.

After 1-, 2-, 4- and 7-day treatment, the cell density was determined by means of a Neubauer hemocytometer to check growth rate.

### 2.3. Dry weight, cell fractionation and determination of chromium content

Aliquots of S-starved and unstarved cultures of both strains were collected by centrifugation and re-suspended at $3 \times 10^6$ cells/ml density in standard medium (control) or in standard medium containing 1 mg Cr(VI)/l and cultured for 4 days at the same conditions as above. From now on, the term "S-replete cells" will be used to indicate S-starved cells which were transferred to standard medium, while the term "S-sufficient cells" will be used to indicate unstarved cells transferred to and maintained in standard medium.

For dry weight determination, aliquots (100 ml) of the cultures were centrifuged, washed three times with doubly distilled water and filtered on pre-weighed mixed cellulose ester filters with pore size of 0.45 μm. The filters were dried at 95 °C for at least 3 h and weighed. Dry weight was determined in S-replete and S-sufficient cells in the beginning (time 0) of the experiments and after 4 days in standard medium with or without chromium. Other aliquots of the same cultures were used to determine cell density by cell counting.

For chromium determination in the whole cells (Cr in), 100 ml of each culture were centrifuged and washed two times in Tris-HCl buffer (50 mM, pH 7.5). The obtained pellets were disrupted using "One Shot" Cell Disrupter (Constant Systems Ltd., England) at 2700 bars three times. Ten ml of the supernatants of each culture were used to evaluate residual chromium in the culture medium (Cr out). Cell homogenates, obtained from 100 ml of each culture disrupted as described above, were fractioned according to the procedure described by Okamura and Aoyama (1994) to determine chromium content in 4 cell fractions: soluble, membrane, cell wall and miscellaneous.

Homogenates of whole cells and cell fractions were completely digested with $HNO_3$:$H_2O_2$ (3:1) in a microwave mineralizator and the amounts of chromium in each cell fraction and in the medium were determined using a flameless atomic absorption spectrophotometer Zeeman/3030 (Perkin Elmer, Waltham, MA, USA) at λ 357.9 nm. Calibration curve was obtained by diluting the Cr reference standard solution for AAS (purchased by ISO certified companies) in the digestion solution at the final concentrations of 0, 20, 60 and 100 ng/ml. The digestion solution was used as blank. The detection limit of the method was 2 ng/ml.

### 2.4. Protein extraction and 1D PAGE analysis

Protein extraction from each cell fraction, obtained as described above, was performed according to Hawkesford and Belcher (1991). Proteins were separated on 12% acrylamide gels and silver stained.

### 2.5. Statistical analysis

All experiments were repeated at least three times. The significance of the differences in growth between the wild-type and the Cr-tolerant strains subjected to Cr treatments after S-starvation, was tested by one-way ANOVA after verification of variance homogeneity (Levene test). Growth parameters (dry weight, cell density and biomass/ml) were subjected to the same analyses to test the significance of the differences among treatments (S-sufficient, S-replete, in the beginning and at the end of 4-day culture with or without 1 mg Cr(VI)/l) and between strains. Tukey's test was applied when appropriate. Cell densities in the beginning of the experiment were excluded from the statistical analysis.

The significance of the differences in chromium uptake in S-sufficient and S-replete conditions for each strain and chromium content in the various fractions was analyzed by one-tail Student *t*-test. The comparisons were made between strains subjected to the same treatment and within strain between S-sufficient and S-replete conditions.

Before statistical analysis, angular transformation was conducted on data expressed as percentage.

## 3. Results

### 3.1. Sulfur starvation and Cr-tolerance

As shown in Fig. 1, Cr tolerance increased after 3 days of S-starvation since both S-replete wild-type and Cr-tolerant strains (panel A and B, respectively) were able to grow and divide in the presence of Cr(VI) concentrations which normally inhibit cell division in S-sufficient cells. Actually in S-sufficient conditions (on the left-shaded background in Fig. 1) cells, exposed to the LOEC (1 and 2 mg Cr(VI)/l for the wild-type and the Cr-tolerant strains, respectively), did not divide and their number did not increase above the initial cell density ($3 \times 10^6$ cell/ml indicated by the horizontal line in Fig. 1). In S-replete conditions, the Cr-tolerant strain "shifts" its tolerance from 1 to 2 mg Cr(VI)/l, while the wild-type strain appears to get more advantage by gaining tolerance to 1 and 2 mg Cr(VI)/l. Only a slight and not significant increase in cell density (to about $4 \times 10^6$ cell/ml for both strains) was instead observed in the presence of 3 mg Cr(VI)/l, which becomes the new LOEC for S-replete cells of both strains.

### 3.2. Biomass variation

In both strains, the initial cell weight was nearly 15 μg/$10^6$ cells (Fig. 2, stock culture). During the 3-day pre-culture in standard medium, cells of both strains divided and, in the same time, increased their mass to similar values (26.01 and 28.89 μg/$10^6$ cells for the wild-type and the Cr-tolerant strain, respectively) (Fig. 2, unstarved). In S-deprived medium cell division was inhibited and the cells of both strains enlarged becoming nearly three fold heavier than the unstarved ones (Figs. 2 and 3), the mass increase being significantly more pronounced in the Cr-tolerant strain (86.40 μg/$10^6$ cells) than in the wild-type (66.52 μg/$10^6$ cells) (Fig. 2, S-starved).



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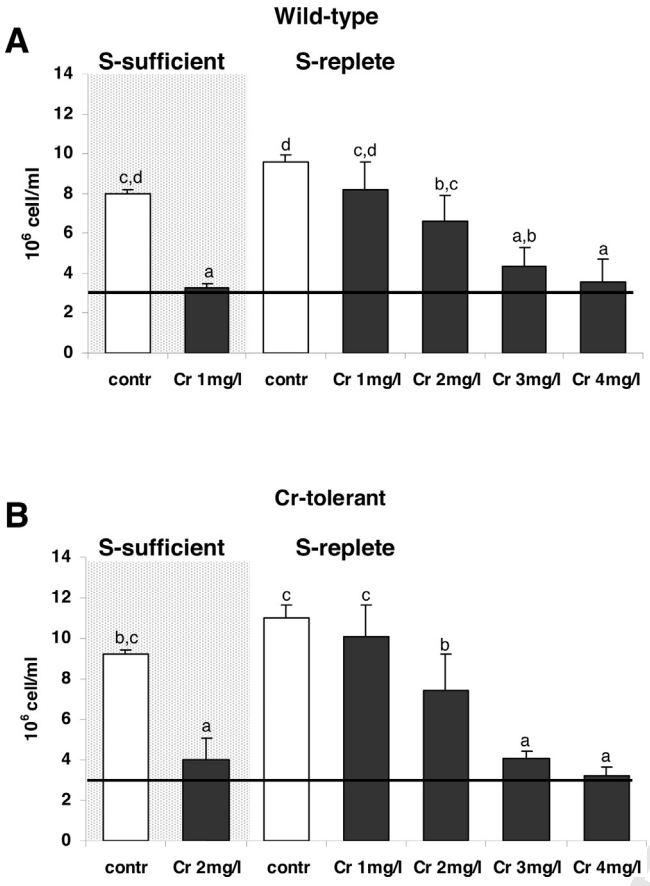


**Fig. 1.** Growth of the wild-type (A) and Cr-tolerant (B) strains of *Scenedesmus acutus* exposed to various Cr(VI) concentrations in Standard Medium for 4 days. Algae were pre-cultured for 3 days in standard (S-sufficient, shaded panel on the left) or sulfur-deprived medium (S-replete, on the right) before Cr(VI) treatment. S-sufficient cells were exposed to 1 or 2Cr(VI) mg/l, i.e., the LOEC for wild-type and Cr-tolerant strain, respectively. Horizontal line indicates the initial cell density. Error bars: standard deviation. Different letters label significantly different values ($p < 0.05$).

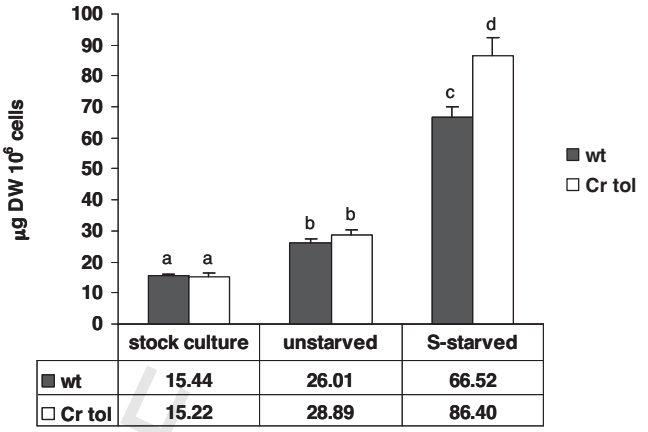


**Fig. 2.** Dry weight of the wild-type (wt) and Cr-tolerant (Cr-tol) strains in the beginning (t0) and the end of the 3-day pre-culture in standard (unstarved) or in S-deprived (S-starved) medium. Error bars: standard deviation. Different letters label significantly different values ($p < 0.05$).

After re-suspension in standard medium ($3 \times 10^6$ cells/ml) supplemented or not with 1 mg Cr(VI)/l, S-sufficient and S-replete cells of both strains nearly tripled their number in 4-day culture in all conditions except for the S-sufficient wild-type strain exposed to Cr(VI). In this case, as reported above, Cr-tolerance did not increase and cell density remained at $3.37 \times 10^6$/ml. In S-replete condition, both strains, even when treated with chromium, reached cell densities significantly higher than the respective controls in S-sufficient condition (Table 1). Despite their different weight in the beginning of the culture, due to the diverse sulfur availability during the pre-culture, cells which were able to divide showed a similar weight (µg DW per $10^6$ cells) at the end of the 4-day culture, differences between strains or among treatments not being significant. Also in this case, the only exception was represented by the S-sufficient wild-type strain treated with Cr(VI): in this condition cells did not divide, but increased their mass reaching a weight nearly double that of the Cr-tolerant strain (both S-sufficient and S-replete) and of the wild-type cells in S-replete conditions. The cell density of S-sufficient wild-type strain at the end of the treatment with 1 mg Cr(VI)/l was the lowest observed ($3.37 \times 10^6$ cells/ml Table 1), while the weight per $10^6$ cells (71.89 µg) was the highest. This different behaviour could lead to opposite data interpretation when these parameters (cell density and µg DW per $10^6$ cells) are used as a reference for chromium uptake. A third growth parameter was thus considered to avoid data misinterpretation, i.e., the dry weight of total biomass in 100 ml culture (mg DW/100 ml). This parameter takes into account the contribution of both cell number and cellular weight increase of the biomass of the whole culture, thus allowing a better comparison between treatments. Due to the difference in weight between unstarved and S-starved cells at the end of the 3-day pre-culture, the dry weight of 100 ml cultures at time 0 was significantly lower in S-sufficient (7.8 mg in the wild-type and 8.67 mg in the Cr-tolerant strain) than

**Table 1**
Growth parameters of the wild-type and Cr-tolerant strains at the beginning and the end of the 4-day culture following 3-day pre-culture on standard (*S-sufficient*) or S-deprived medium (*S-replete*). Data are reported as mean value ± standard deviation.

| Time (day) | Cr (VI) mg/l | Wild-type | | Cr-tolerant | |
|---|---|---|---|---|---|
| | | *S-sufficient* | *S-replete* | *S-sufficient* | *S-replete* |
| Cell density ($\times 10^6$ cells/ml) | | | | | |
| 0 | | 3 | 3 | 3 | 3 |
| 4 | 0 | $7.96 \pm 0.24^{b}$ | $9.56 \pm 0.36^{d}$ | $9.19 \pm 0.21^{c,d}$ | $11.02 \pm 0.65^{f}$ |
| 4 | 1 | $3.37 \pm 0.06^{a}$ | $9.91 \pm 0.59^{d,e}$ | $8.47 \pm 0.58^{b,c}$ | $10.66 \pm 0.84^{e,f}$ |
| Dry weight of $10^6$ cells (µg) | | | | | |
| 0 | | $26.01 \pm 1.58^{a}$ | $66.52 \pm 3.47^{d}$ | $28.89 \pm 1.46^{a,b}$ | $86.40 \pm 6.03^{e}$ |
| 4 | 0 | $37.41 \pm 3.33^{a,b,c}$ | $42.84 \pm 5.68^{c}$ | $35.77 \pm 2.12^{a,b,c}$ | $37.83 \pm 4.91^{b,c}$ |
| 4 | 1 | $71.89 \pm 6.80^{d}$ | $38.91 \pm 4.56^{b,c}$ | $46.45 \pm 4.07^{c}$ | $39.43 \pm 6.60^{b,c}$ |
| Dry weight of 100 ml culture (mg) | | | | | |
| 0 | | $7.80 \pm 0.47^{a}$ | $19.96 \pm 1.04^{b}$ | $8.67 \pm 0.44^{a}$ | $25.92 \pm 1.81^{c}$ |
| 4 | 0 | $30.00 \pm 3.74^{c,d}$ | $41.07 \pm 3.78^{f}$ | $33.07 \pm 2.83^{d,e}$ | $41.83 \pm 2.84^{f}$ |
| 4 | 1 | $24.20 \pm 2.22^{b,c}$ | $38.40 \pm 2.55^{e,f}$ | $39.20 \pm 0.80^{f}$ | $41.63 \pm 3.82^{f}$ |

Different letters labels significantly different values ($p < 0.05$) in the blocks describing each parameter.



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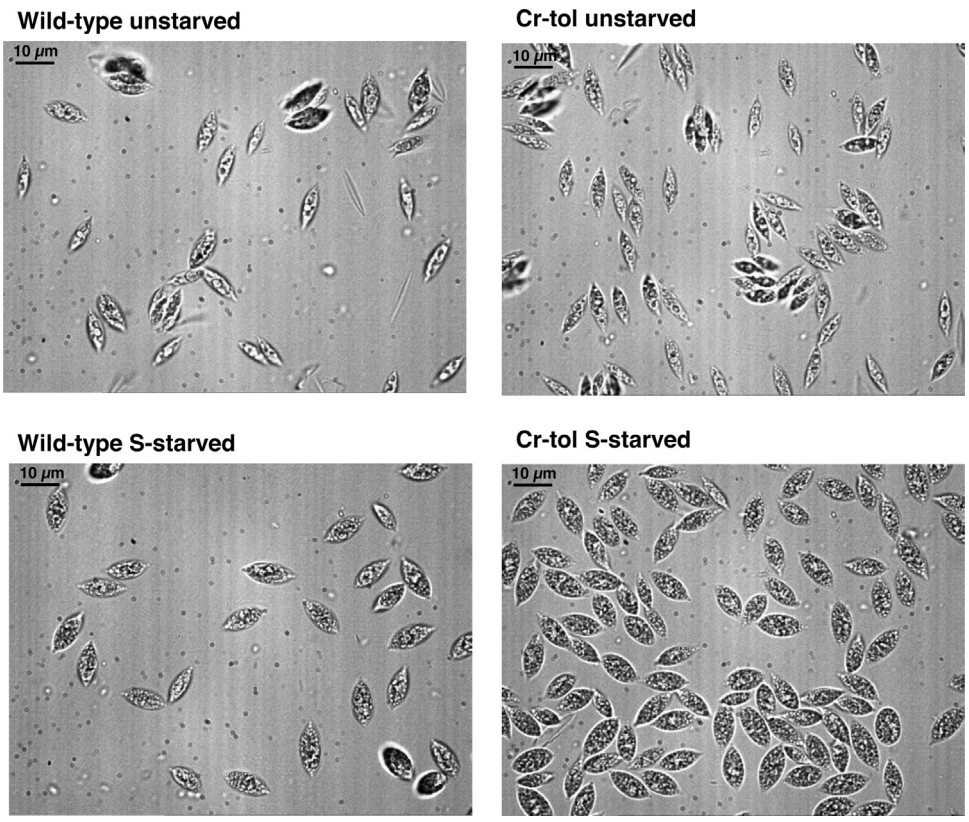


**Fig. 3.** Cells of *S. acutus* (wild-type on the left, Cr-tolerant strain on the right) at the end of 3-day pre-culture in standard (unstarved) or in S-deprived (S-starved) medium. Enlargement 1000×.

in S-replete condition (19.96 mg in the wild-type and 25.92 mg in the Cr tolerant strain) and lower in S-replete wild-type than in the Cr-tolerant strain (Table 1). During the 4-day culture, the biomass significantly increased in all treatments and, despite a more pronounced growth rate in S-sufficient than in S-replete condition (a fourfold versus twofold increase), the final biomass of untreated S-sufficient cells (30 and 33 mg for wild-type and Cr-tolerant strain, respectively) was significantly lower than that of S-replete algae (41.07 and 41.83 mg for wild-type and Cr-tolerant strain, respectively). The fluctuation of this third parameter was less extreme and allowed an interpretation of the behavior of chromium-treated S-sufficient wild-type not biased by the divergent trend of cell number/milliliter and DW/$10^6$ cells.

### 3.3. Chromium uptake and compartmentalization

The quality parameters of the culture medium in the beginning and at the end of the culture period were measured and are summarized in Table 2. No organic matter was present in the medium in the beginning of the experiment. The pH of the standard medium was 7.7 and it was lowered to 7.5 following $K_2Cr_2O_7$ addition. This pH value was in the range of the pH found in freshwater systems (pH 6.5–8.5). Global hardness of the culture medium before and in the end of the culture period was stable at around 3.5 °f. During the 4-day culture an increase in pH (ranging between 8.08 and 9.4) and in the Dissolved Inorganic Carbon (DIC) content, and a decrease in conductivity were observed.

At the end of the culture in S-sufficient condition and in presence of 1 mg Cr(VI)/l, nearly 25% of the available chromium (namely 1920 Cr(VI) nanomoles in 100 ml culture) was taken up by the cells (516 and 502 nanomoles in the wild-type and the Cr-tolerant strain, respectively): the residual 75 % was found in the culture medium ("Cr out"). In S-replete condition, a reduced chromium uptake was

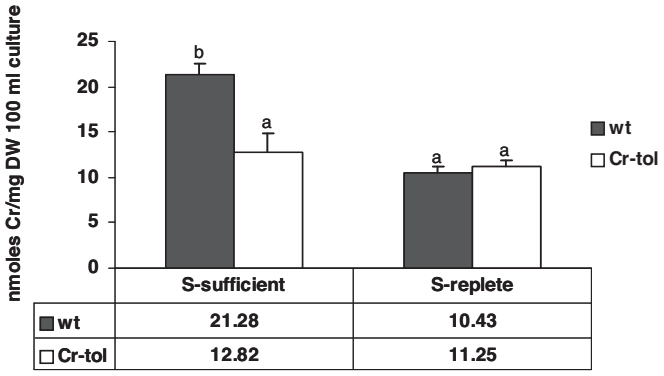


**Fig. 4.** Total chromium content in the wild-type (wt) and Cr-tolerant (Cr-tol) strains at the end of the 4-day culture in standard medium supplemented with 1 mg Cr(VI)/l. Algae were pre-cultured for 3 days in standard (S-sufficient), or sulfur-deprived medium (S-replete) before exposure to Cr(VI). Error bars: standard deviation. Different letters label significantly different values ($p < 0.05$).

observed in both strains since only 20% of the total available Cr was found inside the cells. (402 and 391 nmol in the wild-type and in the Cr- tolerant strain, respectively).

These data became more meaningful when referred to the dry weight of the final total biomass in 100 ml of culture (nanomoles Cr/mg DW in 100 ml). In S-sufficient conditions, the wild-type strain accumulated a significantly higher chromium amount (nearly twice) than that observed in the Cr-tolerant strain (21.28 versus 12.82 nmol). In S-replete conditions, chromium uptake was nearly halved in the wild-type strain (10.43 nmol) whereas it remained nearly unchanged in the Cr-tolerant strain (11.25 nmol) (Fig. 4).

Table 3 summarizes the results of the experiments carried out to evaluate if the increase in Cr-tolerance was due to a different

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**Table 2**
Culture medium quality parameters.

| | | | pH | Conductivity (μS/cm) | Alcalinity (DIC* mM) |
|---|---|---|---|---|---|
| | Standard medium | Complete | 7.76 ± 0.12 | 154.5 ± 2.12 | 0.38 ± 0.02 |
| | | −S | 7.69 ± 0.15 | 145.5 ± 4.95 | 0.41 ± 0.11 |
| | | +1 mg Cr(VI)/l | 7.51 ± 0.06 | 160 ± 1.73 | 0.38 ± 0.04 |
| 4-day culture | | | | | |
| Wild type | *S-sufficient* | Control | 8.82 ± 0.31 | 144.4 ± 2.12 | 1.05 ± 0.04 |
| | | +1 mg Cr(VI)/l | 8.08 ± 0.06 | 145.5 ± 3.54 | 1.02 ± 0.08 |
| | *S-replete* | Control | 9.1 ± 0.08 | 141 ± 1.41 | 0.97 ± 0.01 |
| | | +1 mg Cr(VI)/l | 9.4 ± 0.43 | 151 ± 5.66 | 0.98 ± 0.09 |
| Cr-tolerant | *S-sufficient* | Control | 8.83 ± 0.44 | 150 ± 1.41 | 1.0 ± 0.08 |
| | | +1 mg Cr(VI)/l | 9.16 ± 0.02 | 146 ± 2.83 | 1.04 ± 0.06 |
| | *S-replete* | Control | 8.66 ± 0.52 | 136.6 ± 9.19 | 0.98 ± 0.01 |
| | | +1 mg Cr(VI)/l | 9.21 ± 0.06 | 139 ± 8.49 | 0.98 ± 0.04 |

**Table 3**
Total chromium content (percentage) in cell fractions of the wild-type and Cr-tolerant strains exposure to 1 mg Cr(VI)/l in standard medium following a 3-day pre-culture in standard (*S-sufficient*) or S-deprived medium (*S-replete*). Chromium content in each fraction was reported as percentage of total chromium nanomoles, calculated as the sum of metal found in the different fractions (medium + cell wall + membrane + soluble + miscellaneous) for each experiment. Before statistical analysis (one tail Student *t* test), angular transformation was conducted on data expressed as percentage. In brackets the chromium nanomoles observed in each fraction. Data are reported as mean value ± standard deviation.

| | | Wild-type | | Cr-tolerant | |
|---|---|---|---|---|---|
| | Cell fraction | *S-sufficient* | *S-replete* | *S-sufficient* | *S-replete* |
| Cr out (medium) | | 71.0 ± 2.11° (1322.5 ± 60.0) | 79.4 ± 2.96** (1429.6 ± 29.4) | 76.8 ± 2.55 (1490.5 ± 149.7) | 79.6 ± 4.05 (1423.2 ± 76.9) |
| Cr in | Cell wall | 4.71 ± 1.80° (86.9 ± 29.9) | 2.60 ± 0.06* (46.8 ± 2.2) | 2.55 ± 0.29 (49.3 ± 3.4) | 2.17 ± 0.62 (38.8 ± 11.5) |
| | Membrane | 1.006 ± 0.26°° (18.6 ± 4.1) | 0.282 ± 0.11** (5.1 ± 2.2) | 0.391 ± 0.02 (7.6 ± 1.2) | 0.251 ± 0.06* (4.5 ± 1.1) |
| | Soluble | 21.8 ± 3.92 (407.2 ± 85.5) | 16.8 ± 2.64 (303.2 ± 58.9) | 19.2 ± 2.83 (379.2 ± 100.9) | 17.1 ± 3.6 (305.1 ± 63.6) |
| | Miscellaneous | 0.955 ± 0.24* (17.3 ± 5.1) | 1.03 ± 0.14 (19.9 ± 3.3) | 0.897 ± 0.06 (16.0 ± 1.1) | |
| "Cr in" sum | | 29.4 ± 1.94° (541.2 ± 72.0) | 20.3 ± 3.14** (372.5 ± 67.3) | 22.7 ± 2.91 (456.1 ± 105.9) | 20.1 ± 4.11 (364.4 ± 72.0) |

Asterisk labels significantly different values in % chromium between S-replete and S-sufficient cells within strain. Empty circle labels significantly different values in % chromium between the two strains in similar nutrient conditions, * or °($p < 0.05$), ** or °°($p < 0.01$).

metal compartmentalization after S-starvation. Data are reported as nanomoles of chromium found in each fraction (in brackets) and as mean percentage of the total available chromium in each experiment ("Cr in", i.e. the sum of the chromium amount in the cell fractions, plus "Cr out", i.e., residual chromium in the medium). As reported above, nearly 20–30% of the available chromium was found inside the cells. In the wild-type strain, the Cr uptake was significantly lowered from 29.4% in S-sufficient to 20.3% in S-replete condition, while in the Cr-tolerant strain it was nearly the same in both conditions (22.7% and 20.1%). This seems, however, not to be due to a weaker effect of S-starvation in this strain but rather to its constitutively lower Cr uptake, regardless of sulfur availability.

The decrease in chromium uptake after S-starvation involved a lower accumulation in all the considered cell fractions. Chromium accumulation occurred in both strains mainly in the soluble fraction (21.8% and 19.2% in the wild-type and Cr-tolerant strain, respectively) and decreased in S-replete cells (16.8% and 17.1%), even if the differences between strains or growth conditions were not significant for this fraction. In all the remaining cell fractions, Cr accumulation in S-sufficient conditions was significantly higher in the wild-type than in the Cr-tolerant strain and in S-sufficient than in S-replete conditions. The strongest variation was observed in the membrane fraction, where the decrease from S-sufficient (1.006%) to S-replete (0.282%) conditions was highly significant ($p < 0.01$) in the wild-type, as well as that of the difference between strains in S-sufficient conditions (1.006 % in the wild-type vs 0.251% in the Cr-tolerant strain). Moreover, this was the only fraction in which chromium accumulation after S-starvation was significantly lowered also in the Cr-tolerant strain.

### 3.4. Protein variations in cell fractions

The preliminary analysis, by 1-Dimensional PAGE, highlighted variations occurring in the protein patterns of cell wall, soluble, and membrane fractions (indicated by arrows in Fig. 5). A restructuring in cell wall took place during starvation (−S) as evidenced by the synthesis of a new 56 kDa protein and the disappearance of a 190 kDa peptide in both strains. A peptide of the apparent mass of 23 kDa, not present in the Cr-tolerant strain, was detected in wild-type in S-sufficient condition (+S) and disappeared after S-starvation. In addition, S-starvation induced the over-expression of two proteins (52 and 60 kDa) and the decrease of a 43 kDa peptide in the membrane fraction and the appearance of a 40 kDa protein in the soluble fraction of both strains. The presence of 52 kDa protein was observed in standard medium (+S) in the Cr-tolerant strain, whereas it was detected in the wild-type only after S-starvation.

## 4. Discussion

Although sulfur availability is at the basis of resistance to many biotic and abiotic stresses (Bloem et al., 2015; Ernst et al. 2008; Höller et al. 2010; Nazar et al. 2011; Nocito et al. 2006), previous studies paradoxically indicated that sulfur starvation (7 days) induces a transient increase in Cr(VI) tolerance in two strains of the unicellular green alga *S. acutus* (Gorbi et al. 2007). This behavior was believed to be compatible with the upregulation of mechanisms which are downregulated once S-availability is restored. Changes in the expression of genes involved in the acclimation to S-starvation can thus putatively be associated to the transient Cr-tolerance increase observed in the previous work. Also the shortened (3-day) culture in S-deprived medium, used in the present study,



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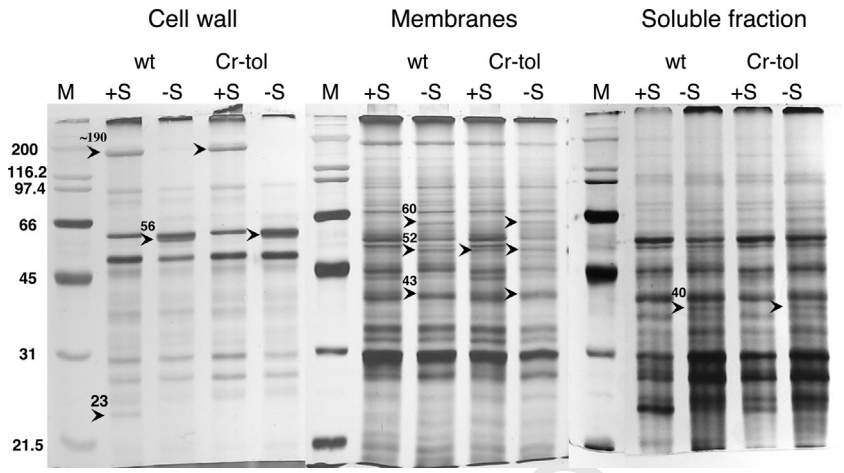


**Fig. 5.** Electrophoretic patterns of the cell wall, membrane and soluble proteins of the wild-type (wt) and Cr-tolerant (Cr-tol) strains of *S. acutus* grown for 3 days in standard (+S) or S-deprived medium (−S). M: molecular weight marker. Arrows indicate peptides which show level variations after S-starvation.

induced an increase in Cr-tolerance in both strains, which, during S-starvation, not only cope with the nutritional stress but also activate mechanisms allowing them to increase their chromium tolerance and to improve their fitness once restored the standard culture conditions. During the pre-culture in S-deprived medium, even if cell division was inhibited, the cell biomass increased in both strains becoming nearly threefold that of the cells grown in sulfur-containing medium. The biomass increase was however significantly higher in the Cr-tolerant strain. Although the two strains showed similar sulfur content both in standard and in S-deprived conditions (Gorbi et al., 2007), the constitutively higher content in free cysteine in the Cr-tolerant strain (Torricelli et al., 2004) could account for its prompter and more efficient acclimation to S deprivation ending in a better management of inner sulfur reserves or a better exploitation of external nutrients with respect to the wild-type. The induction of several genes encoding for proteins involved in acquisition and reductive assimilation of sulfate was observed in *C. reinhardtii* over the course of sulfur starvation, i.e., genes encoding for arylsulfatases (Davies et al., 1994; Ravina et al., 2002; Zhang et al., 2004), sulfate transporters (Chen and Melis, 2004; Chen et al., 2003; Takahashi et al., 2001; Yildiz et al., 1996; Zhang et al., 2004) and enzymes involved in the reductive assimilation pathway of sulfate (Allmer et al., 2006; Ravina et al., 1999, 2002; Yildiz et al. 1996; Zhang et al., 2004). The overexpression of arylsulfatases during S-starvation (Aksoy et al., 2013; González-Ballester et al., 2010; Sugimoto et al., 2007; Takahashi et al., 2001; Zhang et al., 2004) would facilitate the access to the limited amount of sulfate in the environment and contribute to reallocating sulfur present either in structural/functional components (like membrane sulfolipids or methionine/cysteine-containing proteins) of the living cells or in other organic compounds dispersed in the medium by dead cells or mother cells at the release of daughter colonies. A different regulation of arylsulfatases in the two strains could account for the higher biomass increase in the Cr-tolerant strain during the culture in S-deprived medium. The induction of high affinity sulfate transporters and enzymes of the sulfate assimilation pathway could instead give a further advantage once starved cells are transferred to standard medium. Actually, S-replete cells of both strains, even when treated with chromium, reached biomasses (mg DW/100 ml culture) significantly higher than those of the S-sufficient control cultures.

S-starvation caused the disappearance of a 23 kDa protein in the cell wall of the wild-type and, in both strains, the disappearance of a peptide with an apparent mass of 190 kDa, the synthesis of a new 56 kDa protein and a reduction (data not shown) in the transcript of the protein $S_2$TA63, a putative cell wall polypeptide rich in cysteine residues (Torelli et al., 2008). These observations are in agreement with the results reported by Takahashi et al. (2001), who observed an overall metabolic rearrangement during the acclimation of *C. reinhardtii* to S-starvation, involving a cell wall reorganization with the substitution of a polypeptide with another one containing fewer or no cysteine residues, aimed to economize intracellular sulfur resources. Cysteine-rich cell wall proteins dispersed in the medium may become substrate for arylsulfatases and the failure of their synthesis during starvation might be also involved in the block of cell division and colony formation.

Interestingly, S-starvation induced more profound changes in the wild-type than Cr-tolerant algae making their behavior similar to that observed in the Cr-tolerant strain. After starvation the wild-type algae indeed significantly decreased chromium uptake (both when referring to the mg DW/100 ml culture and when evaluated as percentage of the total available Cr(VI)) reaching the levels observed in the Cr-tolerant strain. In the latter, chromium uptake after S-starvation remained nearly unchanged.

Which changes did S-starvation induce leading the behaviour of the wild-type to fit to that of the Cr-tolerant strain? It is well known that Cr(VI) enters the cells mainly through sulfate transporters and competitively inhibits sulfate uptake. Competition between chromate and sulfate takes place, however, not only at the transporter level but also at some step of the sulfur assimilation pathway and is at the basis of a chromate-induced sulfur depletion (Pereira et al., 2008). Appenroth et al. (2008) reported the increase of chromium toxicity in two *Lemnaceae* species after S-starvation and hypothesized that it was due to the increase in the number of sulfate transporters without a change in their affinity for the substrate. This would increase the competition between chromate and sulfate, leading to a greater uptake of chromium. Furthermore, an enhanced chromium accumulation in the roots of maize and *Brassica juncea* after S-starvation, coupled with the repression of a low affinity (maize) and a high affinity (*B. juncea*) sulfate transporters, was reported by Schiavon et al. (2007, 2008). In the unicellular green alga *S. acutus*, S-starvation instead enhances tolerance in both strains, but significantly decreases chromium uptake only in the wild-type. The activation of high affinity sulfate transporters rather than the increase in the number of the low affinity ones may be thus hypothesized in this strain in agreement with the responses observed in *C. reinhardtii* by Yildiz et al. (1996) and Zhang et al. (2004). The virtually unvaried Cr uptake observed in the Cr-tolerant strain suggests that high affinity sulfate transporters are instead constitutively expressed in the Cr-tolerant strain. The low affin-



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ity sulfate transporter system would, however, remain active in S-replete cells allowing chromium entrance in an amount that the cells can cope with.

As a cause of a change in Cr uptake a reduction of chromium dissolved in the medium in the form of Cr(VI) and an increase of the less mobile form Cr(III) should also be considered. This reduction should reduce the bioavailability of chromium and should involve a change from an energy driven active transport through the sulfate transporter system to a passive diffusion driven by chromium concentration.

However, this is probably not be the case, since in our experimental conditions (no organic matter, pH above 7.5, and oxygen continuously produced by algae in active growth and photosynthesis) the Cr(VI) dissolved in the medium is hardly reduced to the less mobile form Cr(III). We can thus reasonably exclude that the decrease in chromium uptake is due to a reduced bioavailability of the metal.

Tolerance could be achieved by maintaining a low chromium concentration in the cytosol. This could happen through sequestration in "inert" cell compartment like vacuoles (Cervantes et al., 2001; Gharieb and Gadd, 1998; Shanker et al., 2004, 2005) or cell wall (Volland et al., 2012). In our studies, despite significant differences in chromium tolerance, no significant difference in the percentage of uptaken Cr in the soluble fraction was observed between the two strains, either S-sufficient or S-replete. Furthermore, despite the lower tolerance, a higher percentage of chromium was observed in the wild-type cell wall than in the cell wall of the Cr-tolerant strain, and the percentage decreased after S-starvation. The observed decrease in chromium percentage in the cell wall may be due to changes occurring during starvation, as suggested by the modifications observed in the peptide composition of this cell fraction. We can thus exclude that the different sensitivity of the two strains and the increased tolerance after S-deprivation rely on chromium localization in "inert" compartments, since a decrease of Cr accumulation in all the considered cell fractions was observed. Besides, this decrease was more evident in the wild-type than in the Cr-tolerant strain. After S-starvation the wild-type algae accumulated Cr amounts similar to those found in the Cr-tolerant strain in S-sufficient conditions. This behavior further suggests that S-starvation induces mechanisms already active in the Cr-tolerant strain also in the wild-type.

In the wild-type the decrease of Cr in the membrane fraction as a consequence of S-starvation was highly significant, which, on the other hand, was the only significant decrease induced by S-starvation also in the Cr-tolerant strain. This suggests that, in starved cells, lower quantities of chromium can be retained and bind to the cell membrane thus strengthening the hypothesis of the involvement of transporter systems, with higher sulfate affinity, in acquiring tolerance to Cr(VI). The modification in the membrane activity can be inferred by the variations observed in the membrane electrophoretic pattern.

The outermost compartment (cell wall) and membranes seem to play an important role in Cr-tolerance. Thus, elucidating their role deserves further investigation.

As a consequence of a change in the transporter affinity, chromate vs sulfate competition can be reduced and two important mechanisms of tolerance could be the result: (i) decrease in chromium uptake (present study) (ii) increase in sulfur uptake (Gorbi et al., 2007) and cell detoxifying potential which can account also for the co-tolerance to Cu and Cd observed in the Cr-tolerant strain (Morsi Abd-El-Monem et al., 1998; Torricelli et al., 2004). These two mechanisms can, however, overlap. After starvation the first seems to prevail in the wild-type and the second in the Cr-tolerant strain. Besides having a higher constitutive level of cysteine in standard condition (Torricelli et al., 2004), the Cr-tolerant strain showed a nearly double sulfur uptake during recovery after S-starvation with elevated cysteine production even when the wild-type restored the basal rate of biosynthesis (Gorbi et al., 2007). These observations, as well as the greater mass increase during S-starvation, suggest that the tolerant strain is less sensitive to negative feedback regulation exerted by Cys and GSH on the synthesis of arylsulfatases, sulfate transporters and key enzymes of the sulfate uptake/assimilation pathway. In *C. reinhardtii* this pathway is regulated through control of the genes *SAC1*, *SAC3* and *SNRK2.1*. This pathway controls S-deprivation responses (Gonzàlez-Ballester and Grossman, 2009). The difference observed between the two strains could be due to mutations in one of the genes involved in the regulation of this chromium-induced pathway. Alternatively, the gene isoform can pre-exist in the original population, and be selected during the isolation processes that gave rise to the Cr-tolerant strain (Corradi et al., 1995).

Cr(VI) photoreduction has been observed in the green alga *Chlorella* spp. and found to be associated with tolerance of Cr(VI) (Han et al., 2006; Yewalkar et al., 2007). Although we can exclude a chromium reduction occurring in the medium with a certain confidence, we cannot exclude that this process takes place in the cells. The method we used for the determination of cell chromium content was based on the complete oxidation of the organic material, and thus did not allow us to verify if the metal detoxification after S-starvation also relies on the reduction from Cr(VI) to Cr(III) as a consequence of the increased synthesis of reducing molecules.

## 5. Conclusions

Sulfur starvation induces an increase in chromium tolerance both in the wild-type and in the Cr-tolerant strain of *S. acutus*, even after a 3-day sulfur deprivation. The increase in tolerance cannot be ascribed to a change in chromium compartmentalization following S-starvation. It was rather caused by an overall decrease in Cr uptake, which was, however, significant only in the wild-type.

Tolerance increase seems thus to rely on overlapping mechanisms both linked to enhancement of the sulfur uptake/assimilation pathway after S-starvation. These mechanisms appear to prevail differently in the two strains: (i) In the wild-type the reduction in chromium uptake may occur through the induction of high affinity sulfate transporters, which presumably are constitutively active in the Cr-tolerant strain and can account for the different Cr(VI) sensitivities of the two strains; (ii) A defective feedback regulation of the sulfate uptake/assimilation pathway in the Cr-tolerant strain can be followed by more efficient detoxifying processes than in the wild-type.

The strict relation between chromium tolerance and sulfur uptake/assimilation pathway gives a reason for further research on genes coding for transporters and enzymes of this pathway and their expression. The comparison between these genes and their expression in the two strains will give interesting insights both on the regulation of the sulfate pathway and its involvement in conferring metal tolerance.

## Uncited references

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Gonzàlez-Ballester et al. (2010) and Pootakham et al. (2010).

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