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Toxic effects and specific chromium acquired resistance in selected strains of *Dyctiosphaerium chlorelloides*

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ABSTRACT

Due to its various uses, chromium contamination has become widespread in a diverse array of environments. The present study was carried out to investigate the toxic effect of chromium exposures on sensitive and resistant strains of the green algae *Dyctiosphaerium chlorelloides*, and to determine the nature and mechanism of chromium-resistant cells that arise. The toxic effect on the photosynthetic performance of chromium exposures in both cell populations, and the sensitive differences due to chromium oxidation state, were estimated, and the results indicate that although the photosynthetic performance in both strains were inhibited, there are not significant differences among $IC_{50(72)}$ values obtained in toxicity assays with both chromium oxidation states in wild-type cells, and however these differences are very significant when the assays were performed with Cr(VI) resistant cells. The 72-h 50% inhibitory concentration values obtained with Cr(III) exposures were similar for both strains. Additionally, by means of the SEM/EDX and TEM microscopic techniques, the occurrence of rapid morphological evolution in the microalgal cells and the possible detoxificant mechanisms was observed after exposure of the wild strain to chromium hexavalent. Moreover, the different response in photosynthetic activity observed between sensitive and resistant cells of *D. chlorelloides* in the presence of Cr(VI) and Cr(III) could be used to obtain a chromium-specific eukaryotic microalgal biosensor.

1. Introduction

Concentrations of heavy metals in natural waters may vary widely with location and time. Anthropogenic sources contribute to such variations but natural fluctuations also often predominate. The main source of heavy metals in natural waters is pollution from mining areas. Another source of major heavy metal pollution problems in many areas is wastewater from industry and municipal sewage treatment facilities (Vymazal, 1984).

Chromium is a naturally occurring heavy metal found in small quantities associated with other metals, particularly iron. It is commonly used for making steel and other alloys, bricks in furnaces, dyes and pigments, chrome plating, leather tanning, and wood preserving. Due to its extensive use in industrial processes, large quantities of chromium compounds are discharged into the environment (Kotas and Stasicka, 2000). Although chromium can exist in all oxidation states from 0 to VI, chromium trivalent [Cr(III)] and hexavalent [Cr(VI)] are the most prevalent (Murray et al., 1983).

The use of microorganisms for decontaminating areas with heavy metals has attracted growing attention because there are several problems associated with pollutant removal using conventional methods. Bioremediation strategies have been proposed as an attractive alternative owing to their low cost and high efficiency (Mejare and Bülow, 2001). Many studies on heavy metals polluted waters have revealed that metal pollution decreases algal diversity, productivity and alters algal species composition. However, there are also many reports concerning occurrence of several algal (Takamura et al., 1989) and cyanobacterial species (Takamura et al., 1990) which are tolerant or resistant to selected heavy metals.

The algal species occurring in polluted sites could become useful ecological indicators, if their occurrence corresponds to the concentration of a polluting metal, or is specific to a polluting metal species. It is therefore important to know the range of tolerance of these species to each metal. However, there have been only a few investigations on green algae, which are easy to cultivate (Pawlik-Skowronska, 2001).

Algae appearing in polluted sites are considered to be either metal-tolerant or metal-resistant species (Stokes, 1983). The former species have metal-sensitive populations in the absence of metal, and the latter species are those in which all populations can tolerate metals.

The main aims of this work were to estimate the toxic effect of chromium exposures on Cr(VI)-sensitive and Cr(VI)-resistant strains of the green algae *Dyctiosphaerium chlorelloides*, and to

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determine the nature and mechanism of chromium-resistant cells that arise.

2. Material and methods

2.1. Test chemicals

Chromium(VI) oxide (CrO_3), potassium chromate (K_2CrO_4), potassium dichromate ($K_2Cr_2O_7$), and chromium(III) chloride ($CrCl_3$) were purchased from Sigma–Aldrich Chemical Co. (St. Louis, MO, USA). All drugs were dissolved in distilled water. In all assays, the working solutions were freshly prepared each time.

2.2. Experimental organisms

Hexavalent chromium sensitive (DcCrS) and resistant (DcCrR25) strains of *D. chlorelloides*, from the algal culture collection of the Environmental Toxicology Laboratory (Toxicology and Pharmacology Department, UCM, Madrid, Spain), were used in these assays. DcCrS strain (wild-type Dc1M) grew axenically in culture flasks (Greiner, Bio-One, Longwood, NJ, USA) with 20 mL of BG-11 medium (Sigma–Aldrich Chemie, Taufkirchen, Germany), while DcCrR25 strain was cultured in BG-11 medium supplied with 25 ppm CrO_3. Both strains were maintained at 20 °C under continuous light of 60 μmol m^{-2} s^{-1} over the 400–700 nm waveband. Cells were maintained in mid-log exponential growth by serial transfers of one-cell inoculums to fresh medium once a fortnight. Prior to the experiments, the cultures were re-cloned (by isolating a single cell) to avoid including any previous spontaneous mutants accumulated in the cultures.

2.3. Effect of chromium on photosynthetic performance

The toxic effect of selected Cr(III) and Cr(VI) compounds, on the photosynthetic performance of both *D. chlorelloides* strains, was previously tested using 13 mL polystyrene sterile tubes (Sarstedt, Nümbrecht, Germany). Prior studies determined the suitability in the use of polystyrene sterile tubes for these toxicity assays, assuring that neither chemicals nor microalgal cells adhered to the tube walls (Costas et al., 2001; García-Villada et al., 2004). Preliminary toxicity tests were performed to define the range of concentrations that included 0% and 100% photosynthetic quantum yield (Φ_{PSII}) inhibition. Test concentrations, chosen on the basis of preliminary range finding tests, covered the range of 5–25 mg L^{-1} for all assays performed with DcCrS strain, while the high differences appeared in the preliminary assays with DcCrR25 strain determined ranges of 5–25, 50–500, and 500–5000 mg L^{-1} for $CrCl_3$, $K_2Cr_2O_7$, and K_2CrO_4 toxicity exposures, respectively.

With the results obtained by means these previous assays, serial dilutions in BG-11 medium were prepared to obtain specific concentrations for each chromium compound tested. For each dose, eight replicates were inoculated with 10^4 cells mL^{-1} from mid-log exponentially growing cell cultures.

The effect of Cr(III) and Cr(VI) was estimated in both strains (sensitive and resistant Cr(VI) cells) after 72 h exposures, by Φ_{PSII}. This photosynthetic response was measured using a ToxY-PAM fluorimeter (Walz, Effeltrich, Germany). Effective quantum yields were calculated as follows:

$$\Phi_{PSII} = (F'_m - F_t)/F'_m, \quad (1)$$

where F'_m and F_t are the maximum and the steady-state fluorescence of light-adapted cells, respectively (Schreiber et al., 1986).

The concentration causing 50% of Φ_{PSII} inhibition in algae was evaluated according to the *area under the curve* method prescribed by International Organization for Standardization (ISO, 1982). The IC_{50}-values were determined by nonlinear regression analysis, and all the results are expressed as mean ± standard deviation. Data were presented as inhibition percentage of Φ_{PSII} with regard to controls. Statistical analysis was performed using the computer software package GraphPad Prism version 4.0 (Graph-Pad Software, La Jolla, CA, USA).

2.4. Scanning electron microscopy (SEM) processing

Samples were fixed with 1% paraformaldehyde/0.5% glutaraldehyde solution in PBS 0.1 M, pH 7.2–7.4, for 1 h at room temperature. The field fixed samples were washed in the laboratory with PBS and postfixed with 1% osmium tetroxide in distilled water for a minimum of 8 h. Samples were dehydrated using an ascending series of acetone (25%, 50%, 75%, 85%, 95% and 100%); they were also critical point dried (CPD) prior to gold coating, and examined using a JEOL JSM6400 SEM at 80 kV.

With the aim to study the cell morphology and determine the mean cellular size, different samples were taken and 20 randomly-selected cells from DcCrS and DcCrR25 cultures were digitally photographed. For each cell, the diameter was measured directly.

2.5. Chromium analysis by scanning electron microscope/energy dispersive X-ray analyzer (SEM/EDX)

The chromium analysis was analyzed by an Oxford Link Penta-FET energy dispersive X-ray (EDX) microanalyzer, with a resolution of 133 eV at 5.39 keV. In this technique, specific X-rays spectrum, whose energy (or) wavelength depends on the nature of the atoms, can be obtained by bombardment of a material with high-energy electrons.

For each sample plot 40 points were analyzed on the surface of twenty different microalgal cells, corresponding to Cr(VI)-sensitive and resistant groups. Although the total element contents of the whole concerned surface could be measured by means of this technique, only chromium has been studied. Therefore, the results of this study are given only for chromium.

2.6. Transmission electron microscopy (TEM) processing

The samples fixed previously were postfixed with 1% osmium tetroxide/1% potassium ferrocianate solution in distilled water. Samples were dehydrated using an ascending series of acetone (30%, 50%, 70%, 80%, 95% and 100%). Afterwards, samples were embedded for 24 h in SPURR resin and polymerized at 60 °C for 24 h. Finally, ultrathin section were mounted on cupper grids and examined in a JEOL JEM1010 TEM at 100 kV.

2.7. Data analysis

The photosynthetic response was monitored on the software package ToxyWin v1.14 (Heinz Walz GmbH, Germany). All experiments were performed at least eight times and each algal sample was analyzed in triplicates. Standard deviation and means were calculated for each treatment, and 72-h 50% inhibitory concentration values ($IC_{50(72)}$) were calculated according to the data obtained in these experiments. The significance of differences between samples was determined by using Student's *t* test, where *p* value less than 0.05 was considered to be significant. A logistic response curve as the trend of the data was determined for correlation analysis by using the computer software package GraphPad Prism v4.0 (Graph-Pad Software Inc., USA).

To determine the cell sphericity mathematically, a coefficient of form (CF), modified from Renau-Piqueras et al. (1985), was used. This CF was calculated by using the expression CF = Pm/Pr, where

Pm is the perimeter of the largest circumference that can be included within the limits of each scanned picture and Pr is the real perimeter of such a picture.

3. Results

3.1. Inhibitory effect on photosynthetic yield

The results corresponding to the effect of chromium on photosynthetic performance are summarized in Table 1, and showed that there are not significant differences among $IC_{50(72)}$ values obtained in these toxicity assays with both chromium oxidation states in DcCrS cells, with values of 8.87-, 12.36-, and 17.34-mg L^{-1} for $K_2Cr_2O_7$, K_2CrO_4, and $CrCl_3$, respectively. However, these differences are very significant when the assays were performed with DcCrR25 cells, obtaining values of 161.32-, 2573.59-, and 13.06-mg L^{-1} for $K_2Cr_2O_7$, K_2CrO_4, and $CrCl_3$, respectively.

Comparisons among assayed compounds showed that DcCrR25 cells are capable to tolerate higher concentrations of $K_2Cr_2O_7$ and K_2CrO_4 (oxidation state of chromium VI compounds) than DcCrS cells (Figs. 1 and 2), and however the $IC_{50(72)}$ values obtained with $CrCl_3$ (oxidation state of chromium III compound) were similar for both strains of *D. chlorelloides* studied (Fig. 3).

3.2. Morphological changes and SEM–EDX chromium analysis

Under SEM technique, the wild-type cells showed the typical spherical–ellipsoidal shape of this specie (CF = 0.90 ± 0.02, n = 20), with a rough surface (Fig. 4), whereas the DcCrR25 variant showed similar geometric shape (CF = 0.85 ± 0.03, n = 20) but an unusual smooth surface (Fig. 5). The size range obtained in both strains showed that DcCrR25 cells, with mean diameter values of 3.01 ± 0.38 μm, were significantly smaller in comparison with DcCrS cells (4.80 ± 0.32 μm). Additionally, in all DcCrR25 cell images analyzed, a significant cell population exhibited removed cellular walls, while these specific forms did not appear in the images corresponding to the wild-type cells.

Additionally, the use the SEM–EDX technique showed that small amounts of chromium (0.27 ± 0.06 atomic%) were concentrated in the surface of these removed walls, while this analysis was negative when the SEM–EDX technique was applied in the surface of DcCrS cells (Fig. 5).

Comparative study among images obtained under TEM technique evidence structural differences between both strains. TEM observation of DcCrR25 cells revealed the presence of high density of 45–85 nm diameter precipitates. These structures were observed inside the cytoplasm, with variable disposition between isolated or grouped in clusters of 3–5 of them. However, these precipitate did not appear inside the DcCrS cells, or their number

Table 1
Comparison of 72-h IC_{50}-values and associated 95% confidence limits (CL), expressed in mg L^{-1}, correspondent to photosynthetic quantum yield (Φ_{PSII}) inhibition with sensitive (DcCrS) and resistant (DcCrR25) strains of *Dyctiosphaerium chlorelloides* exposed to trivalent ($CrCl_3$) and hexavalent ($K_2Cr_2O_7$ and K_2CrO_4) chromium compounds.

Compound	n	IC_{50} (CL 95%)	
		DcCrS	DcCrR25
$CrCl_3$	8	17.34 (15.71–18.39)	13.06 (12.09–13.88)
$K_2Cr_2O_7$	8	8.87 (8.53–9.09)	161.32[*,a] (143.32–172.82)
K_2CrO_4	8	12.36 (10.62–22.49)	2573.59[*,a,b] (2157.25–3694.03)

[*] Significant differences ($p < 0.05$) with respect to values obtained for sensitive populations.
[a] Significant differences ($p < 0.05$) with respect to values obtained for $CrCl_3$.
[b] Significant differences ($p < 0.05$) with respect to values obtained for $K_2Cr_2O_7$.

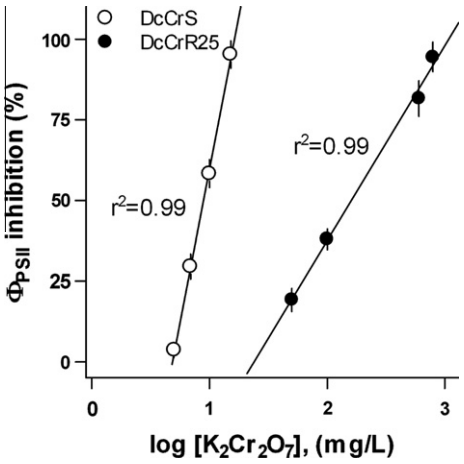

Fig. 1. $\Phi_{(PSII)}$ inhibition response induced by potassium dichromate ($K_2Cr_2O_7$) exposure in sensitive (○) and resistant (●) *D. chlorelloides* populations. Points represent means with vertical lines showing standard deviation (n = 8).

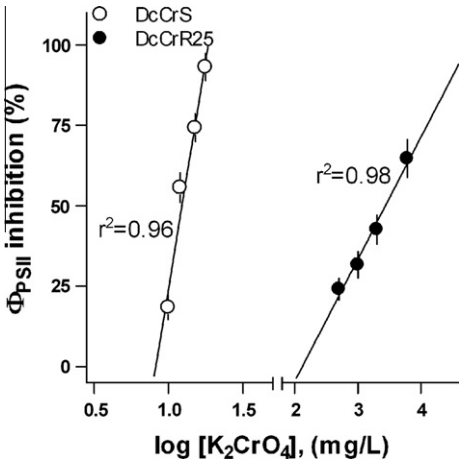

Fig. 2. $\Phi_{(PSII)}$ inhibition response induced by potassium chromate (K_2CrO_4) exposure in sensitive (○) and resistant (●) *D. chlorelloides* populations. Points represent means with vertical lines showing standard deviation (n = 8).

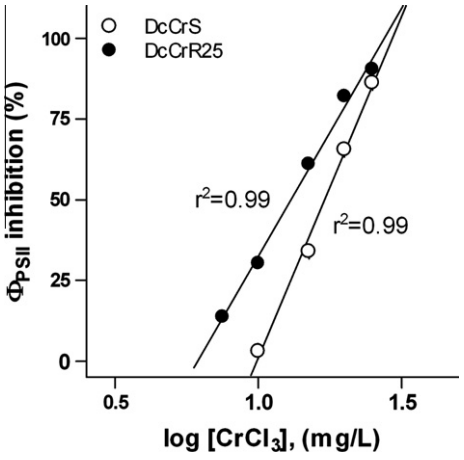

Fig. 3. $\Phi_{(PSII)}$ inhibition response induced by chromium(III) chloride ($CrCl_3$) exposure in sensitive (○) and resistant (●) *D. chlorelloides* populations. Points represent means with vertical lines showing standard deviation (n = 8).

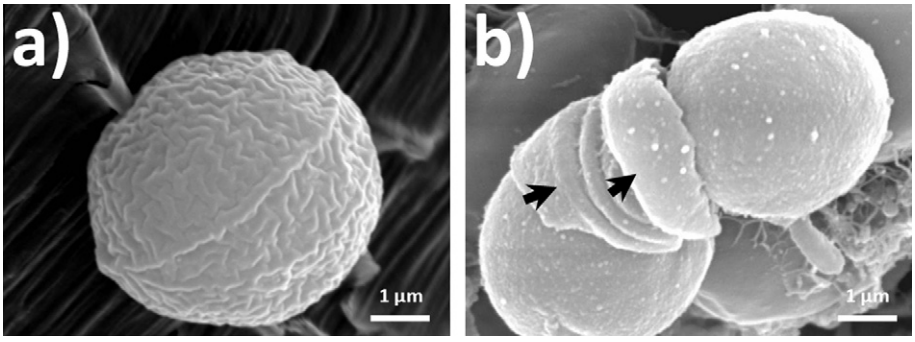

Fig. 4. SEM micrographs of: (a) Cr(VI)-sensitive, and (b) Cr(VI)-resistant *D. chlorelloides* cells. The black arrows are pointing to removed cellular wall from Cr(VI)-resistant cell. Magnification: 15 000×.

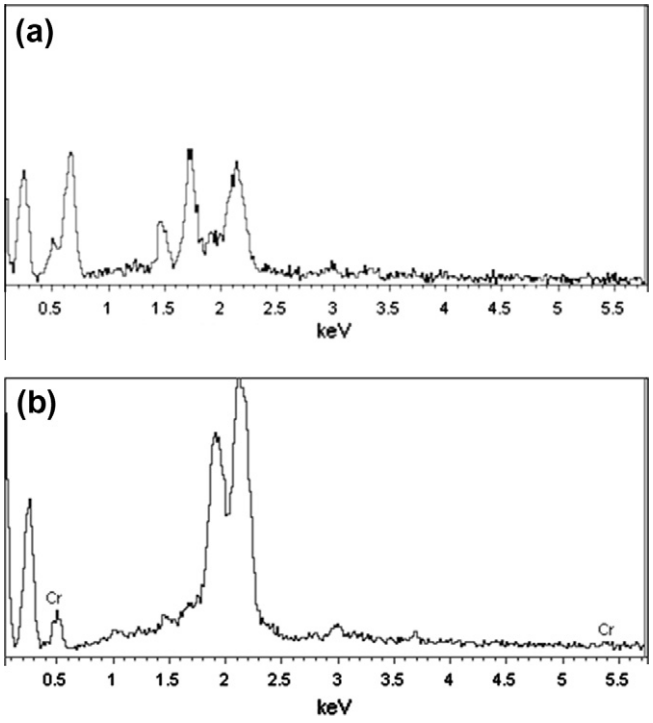

Fig. 5. X-ray photoelectron spectroscopy analysis of chromium from surface of: (a) Cr(VI)-sensitive, and (b) Cr(VI)-resistant *D. chlorelloides* cells.

was very limited (Fig. 6). Comparison between the ultrastructure of the cell walls from both strains evidence an irregular form in wild-type cells, but it looks more regular and denser in the resistant cells, fact that can be in agreement with the differences observed in SEM micrographs. Another difference in morphology seems to be the presence of pyrenoids. Additionally, significant accumulation of vacuoles on the bottom of DcCrR25 strain cells were found, and they could be correlated with an increment of the cellular metabolism induced by chromium exposure.

4. Discussion

Although all chromium compounds included in this work induced acute toxicity in both strains of *D. chlorelloides* studied, there are significant differences depending of the oxidation state of chromium compound and the Cr(VI)-sensitive or Cr(VI)-resistant character of the strain exposed. These results are in agreement with those obtained by other authors using toxicity test assays with marine (Kusk and Nyholm, 1992) and freshwater (Rojíckova and Marsálek, 1999) algal species, although there are evidences in the literature that demonstrate the existence of some kind of algal interspecies variability for chromium sensitivity (Stauber, 1995; Peterson and Stauber, 1996).

It was verified that after destruction of most algae following a chromium exposure, some rare, resistant algal variants are able to grow in an environment with chromium levels approximately 26-fold higher than those that destroy the sensitive, wild-type algae (Corradi et al., 1995). Because only algae previously exposed to chromium seem to be resistant to treatments with this metal, it has been proposed that the reduction of toxic effects of chromium results from interaction among chromium and the algal cell surface (Campbell et al., 1997).

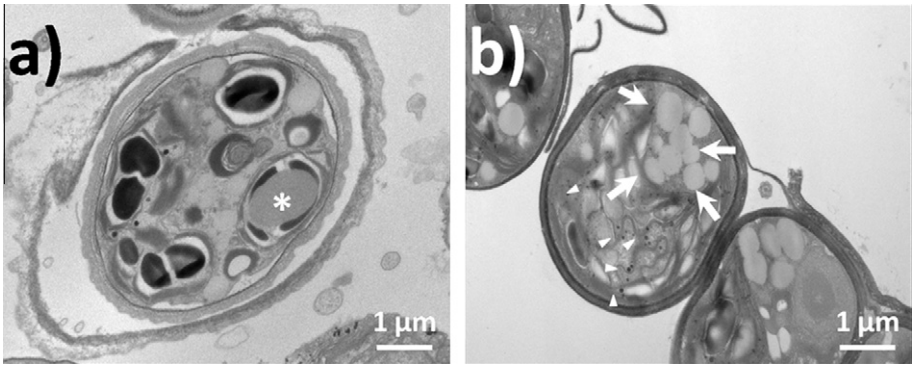

Fig. 6. TEM micrographs of: (a) Cr(VI)-sensitive, and (b) Cr(VI)-resistant *D. chlorelloides* cells. The white arrows are pointing to significant accumulation of vacuoles on the bottom of resistant strain cells. The white head arrows are pointing to dense precipitate isolated or grouped in clusters. The asterisk (*) indicates the pyrenoid localization in sensitive cell. Magnification: 15 000×.

Morphological differences between wild-type population of *D. chlorelloides* and the variant that was resistant to the effect of Cr(VI) were noticeable under SEM and TEM techniques. These differences could be explained by means of the results obtained by other authors, which indicate that the cellular size evolves quickly due to successive beneficial mutations rapidly spreading in the population by natural selection (Lenski and Travisano, 1994; Elena et al., 1996).

However both strains showed similar geometric shape, and although in previous studies have been described CF variations in microalgal populations exposed to pollutants (López-Rodas et al., 2006), the results obtained in the present study are in agreement with those that did not found significant CF variations (Baos et al., 2002; García-Villada et al., 2004).

The presence of chromium in the surface of removed walls from resistant cells, together with the absence of these deposits in the surface of DcCrS cells could indicate a mechanism of detoxification that would justify, at least partially, the chromium tolerance developed by these cells. Further studies have demonstrated that the application of SEM–EDX technique in biological samples provides a new suitable tool for investigating both intracellular structural interactions and physiological processes, such as mineral uptake and storage (Richmond and Sussman, 2003), heavy metal tolerance (Heumann, 2002) detoxification (Nassiri et al., 1997) or effects of environmental pollution (Liu and Kottke, 2003, 2004).

The presence of precipitates inside the DcCrR25 cells is compatible with the theory exposed by Gadd (1988) which suggests that, once metals are inside the cell, they may bind to intracellular components or precipitate. Biological macromolecules and enzymes with appropriate functional groups or metal co-factors are impacted by metal activity. Zhang and Majidi (1994) indicated that metals may be detoxified by accumulation in polyphosphate bodies and in intracellular metal-binding proteins of cyanobacteria and eukaryotic algae, and other authors have demonstrated that heavy metals may be detoxified within the vacuoles of some eukaryotic algae (Gadd, 1988; Garnham et al., 1992). These studies could explain the presence of precipitates and the significant accumulation of vacuoles on the bottom of DcCrR25 strain cells. Furthermore, the presence of pyrenoids in wild-type chloroplasts and its absence in resistant cells, observed in this work, is in agreement with the, modification reported for metal-stressed algal cells by Barceló and Poschenrieder (1999).

The results obtained in this study suggest that chromium exposure induce to the appearance of these morphological and structural changes. However, previous studies unequivocally demonstrated that chromium-resistant algal variants also occur within clonal cultures that have not been exposed previously to chromium (Sánchez-Fortún et al., 2009). Consequently, chromium resistance may be explained in terms of physiological adaptation, mainly as a consequence of changes in gene expression (Belfiore and Anderson, 2001) or genetic adaptation as a result of rare mutations that occurred within the clonal cultures before chromium exposure (Sniegowski, 2005).

5. Conclusions

In summary, wild-type *D. chlorelloides* populations previously exposed to Cr(VI) causes the appearance of resistant cells by means rare spontaneous mutation to this specific chromium oxidation state, because they continue being sensitive to chromium trivalent. Additionally, the present results tentatively indicate that, taking advantage of the morphological and structural changes in these cells, the different response in photosynthetic activity observed between sensitive and resistant cells of *D. chlorelloides* in presence of Cr(VI) and Cr(III) could be used to obtain a specific microalgal biosensor according to oxidation state of chromium.

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