Multi-approach microscopy techniques to evaluate the cytotoxic effect of chromium (III) on the cyanobacterium Chroococcus sp. PCC 9106

Z. M. Puyen, E. Villagrasa, L. Millach, I. Esteve, J. Maldonado and A. Solé
Departament de Genètica i Microbiologia, Facultat de Biociències, Universitat Autònoma de Barcelona, Bellaterra, Cerdanyola del Vallès, 08193 Barcelona, Spain.

Different high-resolution microscopy techniques were used to determine the in vivo effect of different metals on phototrophic microorganisms by Confocal Laser Scanning Microscopy, and their capacity to remove them by Scanning and Transmission Electron Microscopy, both coupled to an Electron Dispersive X-ray detector [1,2]. The chromium (III) repair capacity of Chroococcus sp. PCC 9106 has been studied in this paper. Hence, the in vivo effect of Cr (III) in individual cells and the changes in the total biomass and viability of Chroococcus sp. PCC 9106 were investigated by λ-scan function of CLSM and a modification of the fluorochrome-CLSM-Image Analysis (FLU-CLSM-IA) method [3], respectively. Also, the capacity of this microorganism to remove chromium (III) from laboratory cultures was determined by SEM-EDX and TEM-EDX, chemical and kinetics studies. The results indicated that the minimum dose of Cr (III) that was capable of significantly affecting chlorophyll a fluorescence intensity was 0.26mM. On the other hand, Chroococcus sp. PCC 9106 maintained high levels of biomass and viability (72.92 % and 70.28 %, respectively) at 1mM Cr (III). Furthermore, this cyanobacterium had a great ability to remove Cr (III) extra- and intracellularly and a maximum specific metal removal of 54.29 mg/g at a sorption time of 72 h at 0.75mM (metal initial concentration). Considering these results, it could be concluded that Chroococcus sp. PCC 9106 holds great potential in helping to detoxify environments polluted by this metal. On the other hand, these methods can be easily applied as in oxygenic as in anoxygenic phototrophic microorganisms, in axenic cultures or forming consortia.

Keywords: Chromium (III); Chroococcus sp. PCC 9106; CLSM; TEM; SEM; EDX; removal efficiency.

1. Introduction
In natural environments, chromium has increased as a result of human activities such as electroplating, textile production, mining, wood preservation, animal husbandry and used as biocide in water-cooling for industrial and manufacturing plants. The breakdown and by-products generated as part of the production process of these activities end up in natural aquatic environments, leading to serious environmental and health problems [4, 5, 6 and 7]. The main forms of chromium that can be found in the aquatic environments are Cr (VI) and Cr (III) [8, 9 and 10]. On the one hand, chromium (VI) is the more toxic form of the metal [11, 12 and 13], due to its association with oxygen as chromate (CrO₄²⁻) ions. However, in the presence of organic matter it is reduced to chromium (III), which occurs more rapidly in acidic environments. On the other hand, chromium (III) is considered an essential trace element with known biological functions in all microorganisms. At high concentrations, however, it can be an important pollutant with toxic effects on both terrestrial and aquatic habitats [14]. In previous works, in order to determine the in vivo effect of different heavy metals on phototrophic microorganisms and their capacity to remove them, our research group optimized different high-resolution microscopy techniques. Confocal Laser Scanning Microscopy (CLSM), Scanning Electron Microscopy (SEM) and Transmission Electron Microscopy (TEM) both coupled to an Electron Dispersive X-ray detector (EDX) were employed for this purpose [1, 2, 15 and 16]. In a previous study, Chroococcus sp. PCC 9106 showed a great tolerance to Pb (II) and Cu (II) and its ability to accumulate these heavy metals extra- and intracellularly [17]. Nevertheless, very little is known about the effect of Cr (III) on this microorganism. With this in mind, the aim of this study is to evaluate the cytotoxic effect and the capacity of Chroococcus sp. PCC 9106 remove chromium (III) in laboratory cultures. The first objective of this study is to evaluate the in vivo effect of Cr (III) on the fluorescence intensity of chlorophyll a in individual cells; and the changes in the total biomass and viability using optical microscopic techniques (CLSM). The second objective is to analyze the capacity of this microorganism to accumulate Cr (III) extra- and/or intracellularly and determine its Cr (III) uptake efficiency combining high-resolution electronic microscopic techniques with chemical analysis and kinetics studies.

2. Material and methods

2.1 Microorganism and culture conditions
In this paper, Chroococcus sp. PCC 9106, a coccoid cyanobacterium obtained of Pasteur Culture Collection of Cyanobacteria (PCC), was used [18]. Cultures of this microorganism were grown in plates with a 1:1 (v/v) BG11 and ASN III mixture medium [19] and were maintained at 27 °C in a growth chamber (Climas Grow 180, ClimasLab, Barcelona) under continuous illumination with a light intensity of 15 µEm⁻²s⁻¹. Chromium stock solution was prepared
as Cr(NO$_3$)$_3$ (Sigma-Aldrich, Bellefonte, PA, US). The 5 mM Cr (III) stock solution was made dissolving the exact quantities of the Cr(NO$_3$)$_3$ in Milli-Q water and sterilized by filtration through a 0.2 µm filter (Millipore, USA). Working concentrations of Cr (III) were obtained by serial dilution of the stock solution. Heavy metal stock solution was stored in the dark at 4 ºC. To analyze the cytotoxic effect of Cr (III) on *Chroococcus* sp. PCC 9106, cultures of this microorganism were supplemented with different Cr(NO$_3$)$_3$ concentrations for 9 days. Unpolluted culture was used as a control experiment. Triplicate samples were grown for each heavy metal concentration.

### 2.2 Confocal Laser Scanning Microscope

The *in vivo* effect of Cr (III) on cultures of *Chroococcus* sp. PCC 9106 was determined by λscan function of CLSM (CLSM Leica TCS SP5; Leica Heidelberg, Germany). Furthermore, to evaluate the changes in the total biomass and viability of *Chroococcus* sp. PCC 9106 a modification of the Fluorochrome – CLSM – Image Analysis (FLU-CLSM-IA) method was used [3].

#### 2.2.1. λscan function

In order to evaluate the *in vivo* effect of Cr (III), cultures of *Chroococcus* sp. PCC 9106 polluted at different metal concentrations, ranging between 0 (unpolluted culture) and 1.5 mM, were analyzed by λscan function of CLSM. This function provides information on the state of the photosynthetic pigments of phototrophic microorganisms on the basis of the emission wavelength region and the fluorescence intensity emitted (autofluorescence). Each image sequence was obtained by scanning the same $xy$ optical section throughout the visible spectrum. Images were acquired at the $z$ position at which the fluorescence was maximal, and acquisition settings were constant throughout the experiment. The sample excitation was carried out with an Argon Laser at 488 nm ($\lambda_{ex} 488$ nm) with a $\lambda_{step}$ size of 3 nm for an emission wavelength between 550 and 750 nm. So that to measure the Mean Fluorescence Intensity (MFI) of the $x\ y\ z$ data sets, the Leica Confocal LAS AF Software (Leica Microsystems CMS GmbH) was applied. The regions-of-interest (ROIs) function of the software was used to measure the spectral signature of the samples. 70 ROIs of 1µm$^2$ taken from *Chroococcus* sp. PCC 9106 cells were analyzed for every culture tested. Finally, the minimum metal dose capable of significantly altering the fluorescence intensity of chlorophyll $a$ (Chl $a$) (utilized as a biomarker) of *Chroococcus* sp. PCC 9106 was determined.

#### 2.2.2. FLU-CLSM-IA modified method

To estimate the changes in total biomass and cellular viability of *Chroococcus* sp. PCC 9106, cultures of this cyanobacterium, exposed to different Cr (III) concentrations: 0 (unpolluted culture), 0.05, 0.1, 0.2, 0.25, 0.5 and 1 mM (polluted cultures), were performed following a modification of the FLU-CLSM-IA method [3]. This method combines the use of: specific fluorochromes, the CLSM microscope and the ImageJ v1.48s software. In this study, *Chroococcus* sp. PCC 9106 autofluorescence (emission at 615-695 nm) and SYTOX Green Nucleic Acid Stain fluorescence (emission at 520-580 nm; Invitrogen, Life Technologies) were used simultaneously as markers for live and dead cells from each same $xyz$ optical section, respectively, in a simple dual-fluorescence viability assay [20]. In order to differentiate between living and dead cells, red (live cells) and green (dead cells) pseudocolors were used. 20 red and green confocal images were acquired from every culture to calculate the total biomass and cellular viability at each Cr (III) concentration. These CLSM images were transformed to binary images (black/white) applying fluorescence thresholds values of 50 (red pixels) and 80 (green pixels) by means of the ImageJ v1.48s software. Every pair of images was filtered to minimize the background (smoothing median filter with a radius of 3.0 pixels). To obtain biovolume values, the Voxel Counter plug-in was applied to these filtered images [21]. This specific application calculates the ratio between the thresholded voxels (red and green fluorescent voxel counts) to all voxels from every binary image analyzed. The biovolume value (Volume Fraction) was finally multiplied by a conversion factor of 310 fgC µm$^3$ to convert it to biomass [22].

### 2.3 Scanning and Transmission Electron Microscopy coupled an Energy Dispersive X-ray

To determine structural characteristics of *Chroococcus* sp. PCC 9106 cells and to assess whether this cyanobacterium was able to capture Cr (III) extracellularly, SEM and SEM coupled to EDX (SEM-EDX) were respectively utilized. For SEM analysis, samples of *Chroococcus* sp. PCC 9106 cultures were fixed in 2.5 % glutaraldehyde in Millonig buffer phosphate [23] for 2 h, washed four times in the same buffer, dehydrated in increasing concentrations of ethanol (30, 50, 70, 90, and 100 %) and dried by critical-point drying. Finally, all samples were mounted on metal stubs and coated with gold. A Jeol JSM-6300 SEM (Jeol, Tokyo, Japan) was employed to view the images. To analyze the samples with SEM-EDX, an EDX Link Isis-200 (Oxford Instruments, Bucks, England) coupled to a Zeiss EVO® MA 10 scanning electron microscope (Carl Zeiss NTS GmbH, Oberkochen, Germany) operated at 20 kV was used. To determine the ultrastructure of *Chroococcus* sp. PCC 9106 and evaluate whether this cyanobacterium strain was able to bioaccumulate intracellularly the chromium (III), TEM and TEM coupled to EDX (TEM-EDX) were respectively utilized. For TEM analysis, samples were fixed in 2.5 % glutaraldehyde in Millonig buffer phosphate [23] for 2 h, washed in the same
buffer, post-fixed in 1 % OsO₂ at 4 °C for 2 h, and washed again. They were then dehydrated in a graded series of acetone (30, 50, 70, 90, and 100 %) and embedded in Spurr resin. Ultrathin sections of 70 nm were mounted on carbon coated titanium grids and stained with acetate and lead citrate. Samples were viewed in a Hitachi H-7000 electron microscope (Hitachi Ltd., Tokyo, Japan). To analyze the samples with TEM-EDX, sections of 200 nm thick mounted on carbon-coated titanium grids were employed. Samples were analyzed with an EDX Link Isis-200 (Oxford Instruments, Bucks, England) coupled to a Jeol Jem-2011 (Jeol Ltd., Tokyo, Japan) operated at 20 kV. In all cases, unpolluted (0 mM Cr (III)) and polluted cultures (0.05, 0.1, 0.2, 0.25, 0.5 and 1 mM Cr (III)) were incubated under the same conditions described in 2.1 paragraph.

2.4 Inductively Coupled Plasma Optical Emission Spectrometer

For chemical studies, *Chroococcus* sp. PCC 9106 was grown in solid BG11 and ASN III mixture medium plates incubated at 27 °C and 15 µEm⁻²s⁻¹ for 7 days. 10 ml of the cells suspension were centrifuged and subsequently the mg metal removed / g dry weight and calculated as:

\[ q = \frac{V(C_i - C_f)}{m} \]

biosorption capacity and the biosorption yield. The first one is given as the amount of adsorbed metal (\(q\) mg/g) expressed as:

\[ q = \frac{V}{m} (C_i - C_f) \]

the residual Cr (III) concentrations in the filtrate were analyzed by a Perkin-Elmer OPTIMA-3200RL Inductively Coupled Plasma Optical Emission Spectrometer (ICP-OES). The analytical wavelength was set at 267.716 nm for Cr detection. All experiments were done in quadruplicates, yielding an experimental error of less than 5 %. Experimental control samples with no biomass added were treated identically as blanks to ensure the metal uptake was carried out by the cyanobacterium in the sorption samples. Efficiency of Cr (III) uptake was calculated by the

\[ \text{Efficiency} = \frac{C_i - C_f}{C_i} \times 100\% \]

compared with the control (F = 245.67) (< 0.05). Using the Tukey and Bonferroni comparison tests, the minimum metal dose capable of significantly altering the fluorescence intensity of Chl a (used as a biomarker) of *Chroococcus* sp. PCC 9106 was 0.26 mM Cr (III). At higher concentrations, the changes in the Mean Fluorescence Intensity (MFI) could be due to the degradation of photosynthetic pigments. Other authors demonstrated the diminishing of fluorescence in cyanobacteria as a consequence of formation of phaeophytins and the magnesium-free derivative of chlorophylls, and reported an adverse effect of heavy metals including chromium on chloroplasts and chlorophyll biosynthesis of some microalgae and higher plants [25].
3.2 Changes in the total biomass and viability

The total biomass expressed as mg Carbon/cm³ of Chroococcus sp. PCC 9106 varied significantly depending on the metal concentration. A decreasing value from 44.57 mgC/cm³ in the control experiment, to 32.5 mgC/cm³ at 1.0 mM Cr (III), corresponding to a decrease in biomass of 27.08 %, was observed (Fig. 2a). Statistically significant differences were observed between all the conditions tested ($F = 5.387$) ($p < 0.05$). Using the Tukey and Bonferroni comparison tests, the minimum metal concentration (when compared with the control) that affected the total biomass was 1.0 mM Cr. These results are in accordance with other authors to demonstrate that both biomass and chlorophyll content decreases with increasing concentration of chromium in the growth media [26]. In addition, the effect of Cr (III) on the viability of Chroococcus sp. PCC 9106 cells was evaluated (Fig. 2b). A decreasing value from 90.08 % in the control experiment to 70.28 % at 1.0 mM Cr (III) corresponding to a reduction in viability of 19.80 %, was observed. Statistically significant differences were found between all the conditions tested ($F = 20.876$) ($p < 0.05$). Using the Tukey and Bonferroni comparison tests, the minimum metal concentration (when compared with the control) that affected viability was 0.1 mM Cr (III). In other study, the authors demonstrate that Cr concentrations above 0.1 mM lead to a cessation in growth of Synechococcus sp. (unicellular cyanobacterium) and Nostoc sp. (filamentous cyanobacterium) [27], while Chroococcus sp. PCC 9106, in this study, is still growing even at higher Cr (III) concentrations. Although at this chromium concentration (1 mM Cr (III)) a reduction of biomass in living cells of 41.84 % respect to the control (0 mM Cr (III)) was observed.

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**Fig. 1** CLSM image and λscan plot of Chroococcus sp. PCC 9106. (a) CLSM image from a non-Cr treated culture (b) λscan plot of cultures treated with different Cr (III) concentrations.

**Fig. 2** Changes in total biomass and viability of Chroococcus sp. PCC 9106 at different Cr (III) concentrations. (a) Total Biomass (mgC / cm³) and (b) percentages of live and dead cells. The bars indicate the Standard Error of the Means (S.E.M.)
In the second objective, to determine the capacity of to capture Cr (III) and to obtain its adsorption efficiency and removal capacity of Cr (III) of \textit{Chroococcus} sp. PCC 9106 SEM and TEM with coupled to EDX and ICP-OES were used, respectively.

### 3.3 Chromium sequestration

The capacity of \textit{Chroococcus} sp. PCC 9106 to sequestrate Cr (III) was determined at two levels: outside or inside the cells from cultures growing with or without the tested metal. In control cultures, Cr (III) neither was detected externally (Fig. 3a and 3b) nor internally (Fig. 4a and 4b). In cultures exposed to Cr (III), the difference in the surface morphology after the metal uptake was evident in the cyanobacterium (Fig. 3c). The surface of the cell became rough after metal uptakes; this was probably due to an increase in the production of exopolymeric substances (EPS). Other authors have demonstrated that the exposure to elevated concentrations of Cr affected the composition of EPS produced by \textit{Synechocystis} sp. BASO671\cite{28}. Moreover, it has been proved that different microorganisms have an EPS matrix which can protect cells against toxic compounds such as metals and that its presence can overproduce exopolymer secretion \cite{29}. Additionally, the SEM-EDX spectrum indicated that \textit{Chroococcus} sp. PCC 9106 accumulated Cr (III) in the EPS (Fig. 3d). Different parts of the filter were also tested, as a control that Cr (III) was retained only in cells, being the results negatives in all cases. The ultrathin sections of this microorganism growing in polluted cultures showed abundant high electrondense inclusions of different sizes in its cytoplasm (Fig. 4c). EDX analysis of these inclusions demonstrates the presence of polyphosphate inclusions (PP) which retain Cr (III) (Fig. 4d). These results indicated that \textit{Chroococcus} sp. PCC 9106 accumulated Cr (III) inside the cells in PP inclusions. The presence of these inclusions indicate that once metals are inside the cell, they may bind to intracellular components. Further, similar inclusions have been found when cyanobacteria are grown in adverse culture conditions, including metal pollution \cite{2, 15, 17}, suggesting that these kind of inclusions had a detoxifying effect by sequestering heavy metals \cite{14}. Chromium (III) was not detected or the filter (SEM-EDX) or Spurr resin (TEM-EDX).

![SEM image and EDX spectra of Chroococcus sp. PCC 9106. (a) Unpolluted culture (0 mM Cr) and (b) its EDX spectrum. (c) Polluted culture (0.75 mM Cr) and (d) its EDX spectrum. In SEM images, the different analysis points are indicated by arrows. The scale bar represent (a) 10 µm and (c) 2 µm. In EDX spectra from polluted cell, arrow indicates the main Cr peak at 5.4 KeV.](image-url)
3.4 Cr (III) uptake efficiency

In this study, the metal removal efficiency of Cr (III) on *Chroococcus* sp. PCC 9106 from aqueous solutions was determined taking into account the initial metal concentration of the solution and contact time. The sorption uptake versus the sorption time at different initial metal concentrations is shown in Fig. 5. The Cr (III) uptake kinetic can be mainly divided into two stages: a rapid increase at the beginning followed by a slow uptake. The amount of metal removed up while the time increased until reach the equilibrium (state considered as the inflection point between the two stages and the best to evaluate the biosorption process). The equilibrium time required for the sorption of Cr (III) on *Chroococcus* sp. PCC 9106 was 72 h. According to the removal capacity of Cr (III) at this time (Table 1), when the initial metal concentration ranged from 13 to 39 mg/L (equivalent to 0.25 – 0.75 mM) the specific metal removal ($q_e$) increased from 17.56 to the maximum 54.29 mg of metal removed/g dry biomass with a biosorption yield of 61.26 %. However, the $q_e$ lightly decreases from 52 mg/L Cr (III) (1.0 mM). Therefore, the specific metal removal at 72 h was 39 mg/L (0.75 mM), when equilibrium was attained.
Fig. 5 Sorption kinetics of Cr (III) at different initial metal concentrations by Chroococcus sp. PCC 9106 (pH=6.5; T=27ºC). Arrow indicates the equilibrium time reached at 72 h

Table 1. Removal capacity of Cr (III) at equilibrium by Chroococcus sp. PCC 9106

<table>
<thead>
<tr>
<th>Heavy metal</th>
<th>Initial metal concentration (mg/L)</th>
<th>Equilibrium metal concentration (mg/L)</th>
<th>Metal adsorbed (mg/L)</th>
<th>Specific metal removal at equilibrium (qₑ)ᵃ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cr (III)</td>
<td>13</td>
<td>5.27</td>
<td>7.7</td>
<td>17.56</td>
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<tr>
<td></td>
<td>26</td>
<td>5.88</td>
<td>20.1</td>
<td>45.72</td>
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<td>39</td>
<td>15.11</td>
<td>23.9</td>
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<td></td>
<td>52</td>
<td>29.82</td>
<td>22.2</td>
<td>50.40</td>
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<td></td>
<td>78</td>
<td>56.31</td>
<td>21.7</td>
<td>49.30</td>
</tr>
</tbody>
</table>

ᵃSpecific metal removal at equilibrium (qₑ) expressed as (mg metal removed)*(g dry biomass)⁻¹

On comparing these results with those obtained by other authors, the maximum chrome bio-uptake capacity (𝑞ₑ) obtained by Chroococcus sp PCC 9106 was higher and more efficient than that obtained for this metal by other phototrophic microorganisms, for example the 𝑞ₑ for Spirulina sp. [30], for Nostoc muscorum [31] and for Spirogyra sp. [32] were respectively 35, 22.92 and 30.21 mg of metal removed/g dry biomass. Furthermore, according to the results obtained, there is a correlation between the specific removal capacity and the ability of Chroococcus sp. PCC 9106 to sequester Cr (III) extra and intracellularly.

4. Conclusions

The cyanobacterium Chroococcus sp. PCC 9106: I) is resistant to Cr (III) and maintains a high level of biomass and viability, even at the highest concentration of Cr (III) tested (1 mM). II) This microorganism has the ability to sequester this metal extra and intracellularly, and also a maximum specific metal removal of 54.29 mg/g at a sorption time of 72 h at 0.75 mM (metal initial concentration). III) It holds great potential in helping to detoxify environments polluted by this metal. IV) CLSM, SEM and TEM both coupled to an EDX detector and ICP-OES can be easily applied as in oxygenic as in anoxygenic phototrophic microorganisms, in axenic cultures or forming consortia.
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