

# Using extracellular polymeric substances (EPS)-producing cyanobacteria for the bioremediation of heavy metals: do cations compete for the EPS functional groups and also accumulate inside the cell?

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Many cyanobacteria produce extracellular polymeric substances (EPS) mainly of polysaccharidic nature. These EPS can remain associated to the cell surface as sheaths, capsules and/or slimes, or be liberated into the surrounding environment as released polysaccharides (RPS). The ability of EPS-producing cyanobacteria to remove heavy metals from aqueous solutions has been widely reported in the literature, focusing mainly on the biotechnological potential. However, the knowledge of the effects of the metals in the cell's survival/growth is still scarce, particularly when they are simultaneously exposed to more than one metal. This work evaluated the effects of different concentrations of Cu<sup>2+</sup> and/or Pb<sup>2+</sup> in the growth/survival of *Gloeothece* sp. PCC 6909 and its sheathless mutant *Gloeothece* sp. CCY 9612. The results obtained clearly showed that both phenotypes are more severely affected by Cu<sup>2+</sup> than Pb<sup>2+</sup>, and that the mutant is more sensitive to the former metal than the wild-type. Evident ultrastructural changes were also observed in the wild-type and mutant cells exposed to high levels (10 mg l<sup>-1</sup>) of Cu<sup>2+</sup>. Moreover, in bi-metal systems, Pb<sup>2+</sup> was preferentially removed compared with Cu<sup>2+</sup>, being the RPS of the mutant that is the most efficient polysaccharide fraction in metal removal. In these systems, the simultaneous presence of Cu<sup>2+</sup> and Pb<sup>2+</sup> caused a mutual inhibition in the adsorption of each metal.

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## INTRODUCTION

Cyanobacteria can be found in almost any ecological niche from fresh and salt water to terrestrial and extreme environments, including metal-contaminated habitats (Burnat *et al.*, 2009; Kiran *et al.*, 2008). Most of the strains are able to produce extracellular polymeric substances (EPS) mainly of polysaccharidic nature. The EPS can

remain associated to the cell surface as sheaths, capsules and/or slimes, or be liberated into the surrounding environment as released polysaccharides (RPS) (De Philippis & Vincenzini, 2003; Pereira *et al.*, 2009). The EPS serve as a boundary between the cyanobacterial cell and its immediate environment. Many putative roles have been proposed for these polymers, such as protection against dehydration or UV radiation, biomineralization and phagocytosis. It has also been postulated that EPS confer on the cyanobacteria the ability to adhere to solid substrates, as well as facilitate the locomotion of gliding strains. Moreover, the overall negative charge of cyanobacterial EPS may be essential for sequestering metal cations that are essential for cell growth but present at low

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**Abbreviations:** EPS, extracellular polymeric substances; mt, mutant; RPS, released polysaccharides; TEM, transmission electron microscopy; wt, wild-type.

A supplementary figure is available with the online version of this paper.

concentrations in their surroundings, and/or preventing the direct contact between the cells and toxic heavy metals dispersed in the environment (De Philippis & Vincenzini, 2003; Pereira *et al.*, 2009). The ability of EPS-producing cyanobacteria to remove heavy metals from aqueous solutions has been widely reported in the literature, focusing mainly on its biotechnological potential (De Philippis *et al.*, 2001, 2007; De Philippis & Micheletti, 2009; De Philippis & Vincenzini, 2003; Li *et al.*, 2001). On the other hand, information about the effects of the metals on the physiology of cyanobacteria is still scarce particularly for situations of simultaneous exposure to more than one metal, which frequently occur in polluted environments (Baptista & Vasconcelos, 2006; Burnat *et al.*, 2009; Fiore & Trevors, 1994; Heng *et al.*, 2004). From the physiological point of view, metals can be grouped into two main categories: (i) essential (e.g. Ca, Mg, Fe, Zn and Cu) and (ii) non-essential to life, i.e. without any known biological function (e.g. Hg, Cd and Pb) (Ishaque *et al.*, 2006). However, when in excess, even the essential ones may become toxic to the cells (Lewinson *et al.*, 2009; Valls & de Lorenzo, 2002). Cyanobacteria can decrease metal uptake by sequestering the metals ions in their extracellular surfaces and/or by releasing ligands to the surrounding environment. Once the metals ions enter the cell, their toxicity can be avoided by their reduction to a less toxic oxidation state, intracellular complexing (e.g. metallothioneins) or metal efflux by export systems, e.g. P-type ATPases (Baptista & Vasconcelos, 2006; Nies, 1999; Roy *et al.*, 2008). When these protection mechanisms fail, the cell experiences the toxic effects of high metal concentration that can cause the disintegration and disorganization of thylakoid membranes, large intrathylakoidal spaces, increase of polyphosphate bodies and even cell death (Baptista & Vasconcelos, 2006; Nies, 1999).

A previous study tested the capability of the unicellular cyanobacterium *Gloeothece* sp. PCC 6909 and its sheathless mutant *Gloeothece* sp. CCY 9612 (obtained by random chemical mutagenesis and still largely uncharacterized) to remove copper ions from aqueous solutions, and demonstrated that specific binding sites for  $\text{Cu}^{2+}$  were present in the sheath of the wild-type and in the RPS of both the wild-type and the mutant, with the RPS of the mutant being the more effective polymer (Micheletti *et al.*, 2008b). The aim of this work was to evaluate the effects of different concentrations of  $\text{Cu}^{2+}$  and/or  $\text{Pb}^{2+}$  in the growth/survival of the two phenotypes, as well as changes in their ultrastructure. In addition, their capability to remove  $\text{Cu}^{2+}$  and/or  $\text{Pb}^{2+}$  from bi-metal systems, and the affinity of the various culture fractions for each metal ion were assessed.

## METHODS

**Organisms and growth conditions.** *Gloeothece* sp. PCC 6909 wild-type (Pasteur Culture Collection) and its sheathless mutant *Gloeothece* sp. CCY 9612 (obtained by chemical mutagenesis using

nitrosoguanidine, S. Shestakov, Moscow, Russia, and provided by L. Stal, CYY – Culture Collection of Yerseke, The Netherlands) were grown in BG11<sub>0</sub> (Rippka *et al.*, 1979) supplemented with  $0.375 \text{ g NaNO}_3 \text{ l}^{-1}$ , in an orbital shaker at 80 r.p.m. at 25 °C, under a 16 h light [ $10 \mu\text{mol photons (photosynthetic active radiation; PAR) m}^{-2} \text{ s}^{-1}$ ] / 8 h dark regimen, unless stated otherwise.

**Growth curves.** *Gloeothece* wild-type (wt) and sheathless mutant (mt) were grown in the medium mentioned above (control) or in the same medium supplemented with different concentrations of copper ( $\text{Cu}^{2+}$ ) and/or lead ( $\text{Pb}^{2+}$ ). For mono-metal solutions, media were supplemented with 0.05, 0.10, 0.30, 0.50, 0.70 and 1.0 mg  $\text{Cu}^{2+} \text{ l}^{-1}$  (0.8, 1.6, 4.7, 7.9, 11.0 and 15.7  $\mu\text{M}$ , respectively) or 10, 20, 30, 40 and 50 mg  $\text{Pb}^{2+} \text{ l}^{-1}$  (48.3, 96.5, 144.8, 193.1 and 241.3  $\mu\text{M}$ , respectively) (stock solutions  $10\,000 \mu\text{g ml}^{-1}$  in 2%  $\text{HNO}_3$ , Sigma-Aldrich). For bi-metal solutions, the medium was supplemented with 0.10 mg  $\text{Cu}^{2+} \text{ l}^{-1}$  and 10 mg  $\text{Pb}^{2+} \text{ l}^{-1}$ , 0.10 mg  $\text{Cu}^{2+} \text{ l}^{-1}$  and 20 mg  $\text{Pb}^{2+} \text{ l}^{-1}$ , or 0.30 mg  $\text{Cu}^{2+} \text{ l}^{-1}$  and 20 mg  $\text{Pb}^{2+} \text{ l}^{-1}$ . Growth measurements were performed by monitoring  $\text{OD}_{730}$  with a SmartSpec3000 spectrophotometer (Bio-Rad). The correlation between optical density and protein concentration was  $\text{OD}_{730} 0.5:170 \mu\text{g protein ml}^{-1}$  for the wild-type, and  $\text{OD}_{730} 0.5:112 \mu\text{g protein ml}^{-1}$  for the mutant. Protein extraction and quantification, using the BCA protein assay, were performed as described previously (Leitão *et al.*, 2005). The total carbohydrate content was measured by using the phenol-sulphuric acid method (Dubois *et al.*, 1956) in samples withdrawn from 10- and 30-day-old cultures.

**Transmission electron microscopy (TEM).** *Gloeothece* wt and mt cells were collected by centrifugation and processed as described by Seabra *et al.* (2009). Sections were examined using a Zeiss EM C10 electron microscope operating at 80 kV, and unstained sections (without uranyl acetate and lead citrate) were used as controls. In the controls, the post-fixation with osmium tetroxide was kept in order to preserve and contrast the membranes.

### Removal assays in mono-metal and bi-metal systems.

*Gloeothece* wt and mt cultures grown at 30 °C and under continuous illumination provided by cool white fluorescent tubes [ $100 \mu\text{mol photons (PAR) m}^{-2} \text{ s}^{-1}$ ] were placed in dialysis tubes (12–14 kDa of molecular weight cut-off; Medicell International) and dialysed before and after a pre-treatment with 0.1 M HCl as described previously (Micheletti *et al.*, 2008b). Subsequently, 30 ml confined dialysed cultures were incubated in 80 ml aqueous solutions containing 10 mg  $\text{l}^{-1} \text{ Cu}^{2+}$  or  $\text{Pb}^{2+}$  (mono-metal systems) or 10 mg  $\text{l}^{-1}$  each metal (bi-metal system) for at least 24 h. The pH of the system (biosorbent + metal solution) was adjusted to 4.5–5.5. The final metal content was determined with an atomic absorption spectrophotometer (SpectrAA 10 plus, Varian) operating at wavelengths of 232 and 283.3 nm for  $\text{Cu}^{2+}$  and  $\text{Pb}^{2+}$  quantification, respectively. The amount of metal removed from the aqueous solution was calculated as the difference in the metal concentration before and after contact with the cultures, using a blank obtained by adding 30 ml confined distilled water (pH 4.5–5.5) to 80 ml metal solution. Specific metal removal ( $q$ ), expressed as (mg metal removed) (g dry weight) $^{-1}$  was calculated as:  $q \text{ (mg g}^{-1}\text{)} = V(C_i - C_f)m^{-1}$ , where  $V$  is the sample volume (l),  $C_i$  and  $C_f$  are the initial and final metal concentrations (mg  $\text{l}^{-1}$ ), respectively, and  $m$  is the amount (g) of dry biomass (Volesky & May-Phillips, 1995). The dry weight (g  $\text{l}^{-1}$ ) was determined by vacuum filtration of 10 ml of the dialysed cultures, followed by drying the filter at 50 °C until a constant weight was reached, and the amount of carbohydrates was determined as described above. In order to determine the contribution of each fraction of the culture to the removal of  $\text{Cu}^{2+}$  and  $\text{Pb}^{2+}$  ions, metal removal assays were performed in parallel with dialysed whole cultures (biomass + RPS), isolated biomass or isolated RPS suspended in deionized water at pH 4.5–5.5. For this purpose, the carbohydrate content of each fraction was determined as described above, and the specific metal removal

efficiencies were expressed as (mg metal removed) (g carbohydrates present in the biosorbent)<sup>-1</sup>.

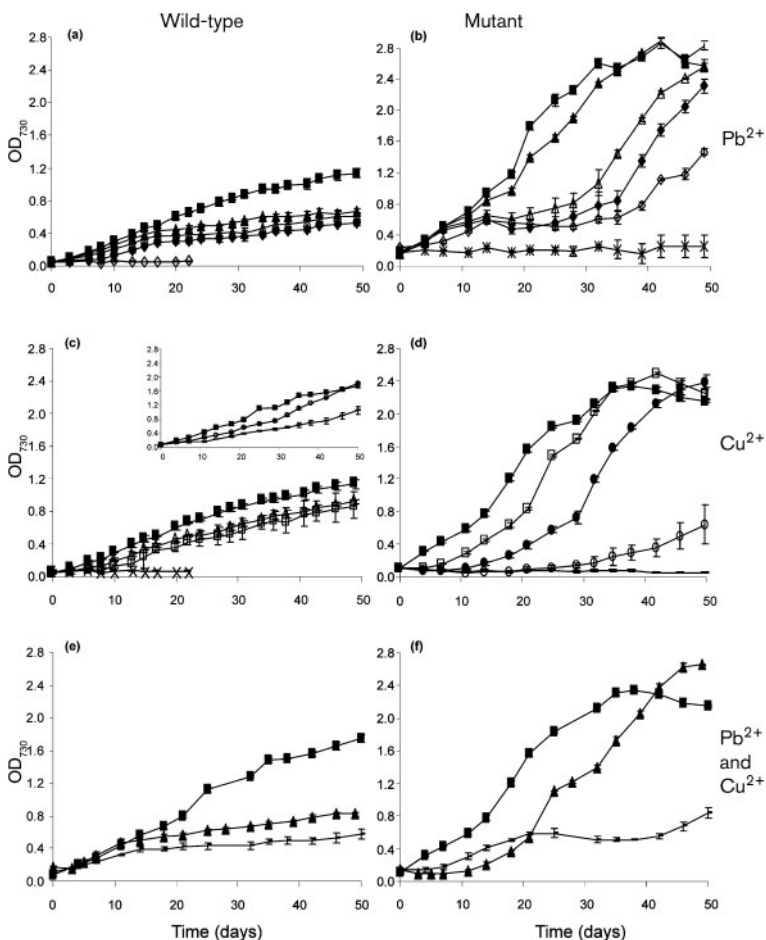
**Sequential metal removal assays.** To assess the efficiency of the isolated biomass in the removal of Cu<sup>2+</sup> after a previous exposure to Pb<sup>2+</sup> and vice versa, removal assays were performed as described above, with the following modifications: after the incubation of the confined biomass in a solution containing the first metal, the biosorbent was dialysed against deionized water at pH 4.5–5.5 for at least 16 h, and transferred to the solution containing the second metal. The amount of metal released into the second metal solution was also determined by atomic absorption spectroscopy.

## RESULTS

### Effects of Cu<sup>2+</sup> and Pb<sup>2+</sup> on the growth/survival of *Gloeothece* cells

Cultures of *Gloeothece* wt and mt were grown in BG11<sub>0</sub> supplemented with 0.375 g NaNO<sub>3</sub> l<sup>-1</sup> (control) or medium supplemented with different concentrations of Cu<sup>2+</sup> and/or Pb<sup>2+</sup>. The data presented in Fig. 1 show that generally the mutant grows faster than the wild-type. For both organisms, the growth was negatively affected by the presence of the metal(s), with increasing concentrations leading to a gradual

decrease in growth (Fig. 1). Both the wild-type and mutant were considerably more susceptible to Cu<sup>2+</sup> than to Pb<sup>2+</sup>. The presence of 0.5 (mutant) or 1.0 (wild-type) mg Cu<sup>2+</sup> l<sup>-1</sup> in the culture medium led to apparent cell death, whereas for Pb<sup>2+</sup>, similar effects were only observed for metal concentrations of 40 or 50 mg l<sup>-1</sup> (Fig. 1a–d). The wild-type grew to a similar extent with 0.05, 0.1 and 0.3 mg Cu<sup>2+</sup> l<sup>-1</sup>; therefore, this last growth curve was omitted from the graph. The growth curves with 0.5 and 0.7 mg Cu<sup>2+</sup> l<sup>-1</sup>, and the respective control, are shown as an insert since these data are from a separate experiment. Interestingly, the growth of the mutant was transiently arrested for lower concentrations of copper [0.05 mg l<sup>-1</sup> (data not shown) 0.1 and 0.3 mg l<sup>-1</sup>], resuming after approximately 10 days (Fig. 1d), while such an effect was not observed for the wild-type. When the cells were exposed to Pb<sup>2+</sup>, the decrease in the growth rate was higher for the wild-type. When the wild-type and mutant were exposed to the simultaneous presence of both metals (0.1 mg Cu<sup>2+</sup> l<sup>-1</sup> + 10 mg Pb<sup>2+</sup> l<sup>-1</sup> and 0.1 mg Cu<sup>2+</sup> l<sup>-1</sup> + 20 mg Pb<sup>2+</sup> l<sup>-1</sup>) their growth decreased compared with growth in cultures supplemented with one of the metals at the same concentration; this effect was more noticeable for the mutant (Fig. 1c–f). When the cells were exposed to 0.3 mg Cu<sup>2+</sup> l<sup>-1</sup> + 20 mg Pb<sup>2+</sup> l<sup>-1</sup>, their growth followed a



**Fig. 1.** Growth curves of *Gloeothece* sp. PCC 6909 wild-type (a, c, e) and its sheathless mutant (b, d, f) *Gloeothece* sp. CCY 9612 in BG11<sub>0</sub> medium + 0.375 g NaNO<sub>3</sub> l<sup>-1</sup> (■), with different concentrations of Pb<sup>2+</sup> (a, b) or Cu<sup>2+</sup> (c, d), and with Pb<sup>2+</sup> plus Cu<sup>2+</sup> (e, f). Media were supplemented with 10 (▲), 20 (△), 30 (◆), 40 (◇) or 50 (\*) mg Pb<sup>2+</sup> l<sup>-1</sup> (a, b), 0.05 (▽), 0.10 (□), 0.30 (●), 0.50 (○), 0.70 (–) or 1.00 (×) mg Cu<sup>2+</sup> l<sup>-1</sup> (c, d), 10 mg Pb<sup>2+</sup> l<sup>-1</sup> plus 0.1 mg Cu<sup>2+</sup> l<sup>-1</sup> (▲) or 20 mg Pb<sup>2+</sup> l<sup>-1</sup> plus 0.1 mg Cu<sup>2+</sup> l<sup>-1</sup> (–) (e, f). Data are means ± SD (n=3). Inset in (c) shows the growth curves with 0.5 and 0.7 mg Cu<sup>2+</sup> l<sup>-1</sup>, and the respective control, since these data are from a separate experiment.

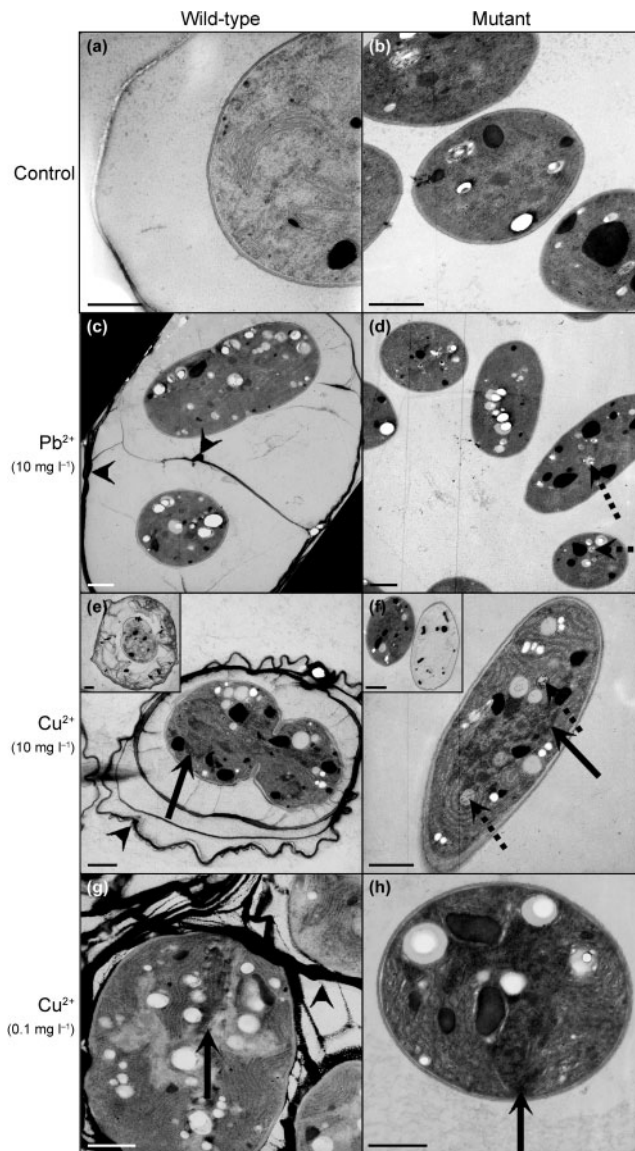
similar pattern to that observed for  $0.1 \text{ mg Cu}^{2+} \text{ l}^{-1} + 20 \text{ mg Pb}^{2+} \text{ l}^{-1}$  (data not shown). The total carbohydrate content was measured for 10- and 30-day-old cultures, and the values obtained follow the pattern of the growth curves (data not shown).

### Ultrastructural changes and sites of metal accumulation

TEM studies were performed in order to investigate the sites of metal accumulation, as well as possible structural changes occurring in *Gloeotheca* wild-type and mutant cells (Fig. 2). When the wild-type and mutant were exposed to sublethal metal concentrations (Fig. 2c, d, g, h), no major ultrastructural changes were observed, including in the arrangement of the thylakoid membranes. However, regardless of the metal, the number of intracellular inclusions generally increased compared with the controls (Fig. 2a, b). When the wild-type and mutant cells were exposed to  $10 \text{ mg Cu}^{2+} \text{ l}^{-1}$ , a large number of cells were severely damaged and apparently dead (Fig. 2e, f inserts). As expected, large amounts of metal ions, either  $\text{Pb}^{2+}$  or  $\text{Cu}^{2+}$ , were adsorbed to the sheath of the wild-type (Fig. 2c, e, g, arrowheads). In contrast, for the cells of the mutant, and since the RPS were removed during the sample processing for TEM, only intracellular accumulations can be perceived. In fact, electron-dense deposits can be observed in vacuolar structures of the mutant cells exposed to either  $\text{Pb}^{2+}$  or  $\text{Cu}^{2+}$  (Fig. 2d, f, dashed arrows). Moreover, cytoplasmic electron-dense deposits could be discerned in the cells of the wild-type and mutant exposed to  $\text{Cu}^{2+}$  (Fig. 2e–h, full arrows). Controls without uranyl acetate and lead citrate (unstained sections) are shown in Supplementary Fig. S1 (available with the online version of this paper).

### Affinity of culture fractions for $\text{Cu}^{2+}$ and $\text{Pb}^{2+}$

The specific metal removal  $q$  [expressed as  $(\text{mg metal removed}) (\text{g dry biomass})^{-1}$ ] was determined in mono- and bi-metal systems. When whole cultures (biomass + RPS) of the wild-type and mutant were in contact with  $\text{Cu}^{2+}$  and/or  $\text{Pb}^{2+}$ , the specific metal removal was generally higher for the mutant. Both the wild-type and the mutant showed higher  $q$  values for  $\text{Pb}^{2+}$  than  $\text{Cu}^{2+}$  (Table 1). The simultaneous presence of the two cations caused a decrease in the removal of each metal.  $\text{Cu}^{2+}$  removal decreased by 47.2% for the wild-type and 65.4% for the mutant, whereas the  $\text{Pb}^{2+}$  removal decreased by 13.8 and 39.8% respectively. The contribution of each culture fraction to metal removal was also evaluated. In both the mono- and bi-metal systems, the isolated biomass of *Gloeotheca* wild-type was more effective in metal removal than the biomass of the mutant, particularly for  $\text{Cu}^{2+}$ , whereas the isolated RPS of the mutant consistently removed larger amounts of metals than those of the wild-type (Table 1). Mutual inhibition of metal binding, which was invariably more accentuated for  $\text{Cu}^{2+}$ , was also observed for the isolated biomass and RPS of both organisms, and was particularly evident for the biomass of



**Fig. 2.** TEM of *Gloeotheca* sp. PCC 6909 wt (a, c, e, g) and *Gloeotheca* sp. CCY 9612 (b, d, f, h) in BG11<sub>0</sub> medium +  $0.375 \text{ g NaNO}_3 \text{ l}^{-1}$  (a, b), or in the same medium supplemented with  $10 \text{ mg Pb}^{2+} \text{ l}^{-1}$  (c, d),  $10 \text{ mg Cu}^{2+} \text{ l}^{-1}$  (e, f) or  $0.1 \text{ mg Cu}^{2+} \text{ l}^{-1}$  (g, h). Samples were withdrawn from cultures after 24 h (a–f) or 10 days (g, h). Inserts show severely damaged and/or dead cells. Arrowheads, metal accumulation in the EPS; full arrows, metal accumulation in the cytoplasm; dashed arrows, accumulation in vesicular structures. Bars,  $1 \mu\text{m}$ .

the mutant where the inhibition of  $\text{Cu}^{2+}$  removal was complete. In addition, the inhibition of  $\text{Pb}^{2+}$  or  $\text{Cu}^{2+}$  adsorption in the bi-metal system was more accentuated for the mutant compared with the wild-type.

In order to further evaluate the different affinities of the isolated biomasses of the wild-type and mutant for  $\text{Cu}^{2+}$  and  $\text{Pb}^{2+}$ , sequential metal removal assays were performed (Table 2). The results obtained revealed that, regardless of

**Table 1.** Specific metal removal ( $q$ ) and inhibition of metal sorption from mono-metal ( $\text{Cu}^{2+}$  or  $\text{Pb}^{2+}$ ) or bi-metal ( $\text{Cu}^{2+} + \text{Pb}^{2+}$ ) solutions using whole cultures, isolated biomass or isolated RPS of *Gloeotheca* sp. PCC 6909 (wt) or *Gloeotheca* sp. CCY 9612 (mt)

Data are means  $\pm$  SD ( $n=3$ ). Both the wild-type and the mutant showed higher  $q$  values for  $\text{Pb}^{2+}$  than  $\text{Cu}^{2+}$  (bold type).

Phenotype	Culture fraction	$q \text{ Cu}^{2+}$		$q \text{ Pb}^{2+}$		Inhibition of metal sorption (%) <sup>*</sup>	
		Mono-metal	Bi-metal	Mono-metal	Bi-metal	$\text{Cu}^{2+}$	$\text{Pb}^{2+}$
Wild-type†	Whole culture	25.76 $\pm$ 1.44	13.60 $\pm$ 0.61	<b>44.03 <math>\pm</math> 0.72</b>	<b>37.95 <math>\pm</math> 3.10</b>	47.2	13.8
Mutant†	Whole culture	43.80 $\pm$ 1.70	15.15 $\pm$ 1.81	<b>108.21 <math>\pm</math> 1.10</b>	<b>65.10 <math>\pm</math> 8.36</b>	65.4	39.8
Wild-type‡	Whole culture	76.54 $\pm$ 11.30	41.65 $\pm$ 2.85	131.41 $\pm$ 14.00	110.66 $\pm$ 3.11	47.2	13.8
	Isolated biomass	83.80 $\pm$ 6.93	47.82 $\pm$ 4.75	157.77 $\pm$ 5.86	114.99 $\pm$ 6.21	42.9	27.1
	Isolated RPS	68.32 $\pm$ 3.91	32.94 $\pm$ 14.48	185.74 $\pm$ 12.87	113.58 $\pm$ 1.85	51.8	39.5
Mutant‡	Whole culture	88.88 $\pm$ 5.93	34.46 $\pm$ 6.88	225.63 $\pm$ 17.11	134.66 $\pm$ 25.46	65.4	39.8
	Isolated biomass	43.71 $\pm$ 3.58	<0.005§	147.70 $\pm$ 53.18	29.90 $\pm$ 13.99	100.0	79.8
	Isolated RPS	92.38 $\pm$ 26.16	26.50 $\pm$ 5.60	237.05 $\pm$ 47.28	138.33 $\pm$ 24.96	71.3	41.6

<sup>\*</sup>Inhibition in bi-metal solutions compared with the  $q$  value obtained in mono-metal solutions.

†Specific metal removal expressed as (mg metal removed) (g dry weight)<sup>-1</sup>.

‡Specific metal removal expressed as (mg metal removed) (g total carbohydrates)<sup>-1</sup>.

§Detection limit of the instrument.

the order in which the biomasses were exposed to the metals, aliquots of the first metal were always released into the second solution, and part of the second metal was bound to the biosorbent. However, when the biomasses were transferred from the  $\text{Pb}^{2+}$  to the  $\text{Cu}^{2+}$  solution, 37.3 and 18.6 % of the already adsorbed  $\text{Pb}^{2+}$  was released into the second solution by the wild-type and mutant, respectively. In contrast, in the reverse situation, the values of  $\text{Cu}^{2+}$  desorption were 38.9 and 48.0 % for the wild-type and mutant, respectively.

## DISCUSSION

The data obtained revealed significant differences in the growth of *Gloeotheca* wild-type and its sheathless mutant. While the mutant shows a typical bacterial growth curve, the wild-type does not exhibit a clear exponential phase.

This type of linear growth is not unknown for cyanobacteria, particularly for strains that produce large amounts of structured extracellular polysaccharides, and may be related to the considerable amount of energy spent in the production of EPS and the physical constraints caused by these extracellular complex structures (Yu *et al.*, 2009, 2010).

The higher tolerance exhibited by *Gloeotheca* wild-type and mutant for  $\text{Pb}^{2+}$  strongly indicates that these organisms are more severely affected by  $\text{Cu}^{2+}$  than by  $\text{Pb}^{2+}$ . The differences observed are probably related to the specific nutritional requirements and mechanisms of metal uptake and accumulation in the cells (Nies, 2003; Rosen, 2002; Rensing *et al.*, 1999). Copper is an essential micronutrient for all organisms, functioning as a cofactor and being required for structural and catalytic properties of enzymes such as the copper/zinc superoxide dismutase (Burnat *et al.*,

**Table 2.** Specific metal removal ( $q$ ) and metal desorption from mono-metal solutions using isolated biomass cultures of *Gloeotheca* sp. PCC 6909 (wild-type) or *Gloeotheca* sp. CCY 9612 (sheathless mutant)

Data are means  $\pm$  SD ( $n=3$ ).

Phenotype	$\text{Cu}^{2+}$		$\text{Pb}^{2+}$	
	$q^*$	Desorption†	$q^*$	Desorption†
Wild-type	90.61 $\pm$ 9.69	35.25 $\pm$ 4.37 (38.9)	144.59 $\pm$ 7.26	53.90 $\pm$ 4.06 (37.3)
Mutant	44.38 $\pm$ 6.99	21.09 $\pm$ 0.67 (48.0)	139.38 $\pm$ 2.72	25.91 $\pm$ 4.10 (18.6)

<sup>\*</sup> $q$ , specific metal removal expressed as (mg metal removed) (g total carbohydrates)<sup>-1</sup>.

†Values in parentheses indicate the percentage of metal removed from the first metal solution and subsequently desorbed into the second solution (containing the other metal).

2009). In cyanobacteria, copper is also a cofactor of the electron transport protein plastocyanin and other proteins associated with thylakoids, which imposes an extra complexity to copper homeostasis in these organisms compared with non-photosynthetic prokaryotes (Cavet *et al.*, 2003; De la Cerda *et al.*, 2007; Shcolnick & Keren, 2006). However, excessive amounts of copper have been reported to cause serious damage to photosynthetic cells (Stuart *et al.*, 2009) due to (i) the formation of reactive oxygen species, mainly hydroxyl radicals, which can damage several cellular components (Gaetke & Chow, 2003), (ii) competitive binding to metal transporters inhibiting the uptake of other essential metals (Sunda & Huntsman, 1998) and (iii) the inhibition of photosynthetic electron transport especially in PSII (Baron *et al.*, 1995). On the other hand, lead is a non-redox-active metal with no known biological function, and its toxicity has been associated with indirect effects such as the ability to mimic essential metals, the inhibition of the photosynthetic electron transport, mainly at the PSII reaction centre, and reduction of the cellular antioxidants pool (Burnat *et al.*, 2009; Chaloub *et al.*, 2005; Pinchasov *et al.*, 2006; Pinto *et al.*, 2003; Xiong, 1998).

Although both phenotypes were more severely affected by  $\text{Cu}^{2+}$  than by  $\text{Pb}^{2+}$ , the mutant was shown to be even more sensitive. Moreover, the extended lag phase and the time needed for recovery observed for the mutant in  $\text{Cu}^{2+}$ -supplemented medium is consistent with the activation of a specific detoxification mechanism(s). The absence of this phenomenon in the wild-type and the difference in the growth rates observed for the two phenotypes highlight major differences in central metabolic processes rather than only the presence/absence of the structure sheath. This could be expected for a mutant obtained by random mutagenesis and it has been confirmed by proteomic studies (S. Pereira and others, unpublished). The lower sensitivity to  $\text{Cu}^{2+}$  and the higher metal removal capability exhibited by the isolated biomass of the wild-type compared with the mutant, together with the accumulation of  $\text{Cu}^{2+}$  on the sheath, suggest that the presence of a structured envelope prevents, at least partially, the entry of this metal into the cell. In all situations, the total carbohydrate content of the cultures varied in parallel with the OD values, indicating that the presence of a metal(s) did not inhibit or stimulate the production of EPS.

TEM studies confirmed the higher toxicity of  $\text{Cu}^{2+}$ , and the ultrastructural damage observed when the cells are exposed to high metal concentrations ( $10 \text{ mg l}^{-1}$ ) is in agreement with previous studies that reported membrane disorganization as a common cyanobacterial response to metal toxicity (Arunakumara *et al.*, 2008). The high number of intracellular inclusions observed in cells exposed to sublethal concentrations of  $\text{Pb}^{2+}$  or  $\text{Cu}^{2+}$ , as well as their characteristic sublimation under the electron beam, strongly suggest that they are polyphosphate bodies (PPB) (Jensen, 1968; Jensen *et al.*, 1977). In fact, it has been

previously suggested that PPB, as well as functioning as phosphorus storage and an energy source, could also be involved in the sequestration of metal ions and their detoxification (Allen, 1984; Jensen *et al.*, 1982; Rachlin *et al.*, 1982; Surosz & Palinska, 2005). The high amount of PPB in the cells could explain the growth recovery of the mutant after the extended lag phase that occurs in the presence of high concentrations of  $\text{Cu}^{2+}$ . The presence of electron-dense deposits in the cytoplasm of both the wild-type and mutant cells exposed to  $\text{Cu}^{2+}$  is probably related to the existence of a specific transport system related to the demand of this element for proteins located at/in the thylakoids. It is known that cyanobacteria possess two different P-type ATPases, CtaA and PacS, which transport copper across the plasma and thylakoidal membranes, respectively (Baptista & Vasconcelos, 2006; De la Cerda *et al.*, 2007).

Since the binding of metal ions to EPS can prevent, up to a point, the entrance of the metals into the cells, it will also affect the metal homeostasis in these organisms (Baptista & Vasconcelos, 2006; Nies, 1999). In all experiments,  $\text{Pb}^{2+}$  was preferentially removed compared with  $\text{Cu}^{2+}$ , in agreement with observations from previous work using different biological materials such as cyanobacteria, algae, fungi and plants (Can & Jianlong, 2007; Hawari & Mulligan, 2007; Singh *et al.*, 2000, 2007; Zhang & Banks, 2006). This phenomenon can be explained by the Pearson's hard-soft acid base theory, in which the biosorbent acts as a Lewis base and the metal acts as a Lewis acid. According to this theory, metals ions are classified as class A (hard acids), class B (soft acids) or borderline, according to their affinity for different ligands (Pearson, 1963, 1968). Both  $\text{Pb}^{2+}$  and  $\text{Cu}^{2+}$  are borderline (Nieboer & Richardson, 1980; Pearson, 1968). However, due to the higher values of electronegativity and radius of  $\text{Pb}^{2+}$  (2.33 and 1.18 Å, respectively, compared with 1.90 and 0.73 Å for  $\text{Cu}^{2+}$ ), its covalent index, calculated as  $X_m^2 r$  (in which  $X_m$  is the electronegativity and  $r$  is the radius of the metal ion), is higher than that of  $\text{Cu}^{2+}$ , which increases its affinity for the EPS binding sites (Can & Jianlong, 2007; Hawari & Mulligan, 2007; Nieboer & Richardson, 1980).

The larger amounts of  $\text{Pb}^{2+}$  that remain sequestered in the EPS of *Gloeotheca* may contribute to the higher tolerance observed for this metal, by decreasing the amount that enters the cells. However, in the case of the wild-type, the binding of large amounts of  $\text{Pb}^{2+}$  to its sheath may also affect the transit of nutrients into the cell, causing a more significant decrease in the growth rate compared with the mutant. Although  $\text{Pb}^{2+}$  is a non-essential metal, it seems that it can accumulate in vacuolar structures inside the cells, as observed in TEM studies. The mechanisms by which  $\text{Pb}^{2+}$  enters the cyanobacterial cells are not yet fully elucidated, although P-type ATPases involved in the transmembrane transport (uptake and/or efflux) of  $\text{Zn}^{2+}$ ,  $\text{Cd}^{2+}$  and  $\text{Pb}^{2+}$  have been identified in some strains (Baptista & Vasconcelos, 2006).

The higher efficiency of metal removal observed for the whole cultures and isolated RPS of the mutant compared with the wild-type shows that the role of the sheath in metal binding is limited, and that the increased metal binding efficiency of the mutant can mainly be ascribed to its RPS. In fact, the RPS of the mutant contain higher amounts of acidic sugars, which increases their affinity towards metal cations, and possess a smaller amount of deoxysugars, which increases their solubility and, thus, their accessibility to the metal cations. Furthermore, the mutant's RPS were shown to possess a higher number and/or more accessible carboxyl and amide functional groups, which are the most important metal binding sites in *Gloeotheca*, as demonstrated by Micheletti *et al.* (2008b).

In the presence of more than one metal, the process of metal binding to the cyanobacterial EPS can be non-interactive, synergistic or competitive, depending on the metal ions and characteristics of the polysaccharide (Micheletti *et al.*, 2008a). In the present study, the results obtained in the bi-metal systems unveiled a mutual inhibition in the adsorption of  $\text{Cu}^{2+}$  and  $\text{Pb}^{2+}$ , as the presence of one metal inhibited, at least partially, the adsorption of the other. This mutual inhibition can result from (i) direct competition between the two metals for the EPS binding sites and/or (ii) modification of the EPS conformation by the already adsorbed metal ions, which will hinder the access of other ions to adjacent adsorption sites (Micheletti *et al.*, 2008a; Parker *et al.*, 2000). The higher inhibition of  $\text{Cu}^{2+}$  adsorption in the presence of  $\text{Pb}^{2+}$  supports the higher affinity of these EPS for  $\text{Pb}^{2+}$ . This hypothesis is further confirmed by the lower values of  $\text{Pb}^{2+}$  desorption compared with  $\text{Cu}^{2+}$  in bi-metal systems, especially considering that both solutions contained 10 mg metal  $\text{l}^{-1}$  and thus, due to their atomic weights, the molarity is 3.25 times higher for  $\text{Cu}^{2+}$ . Furthermore, in the presence of both metals, the higher affinity towards  $\text{Pb}^{2+}$  probably reduced the amount of  $\text{Cu}^{2+}$  entering the cells and partially protected them from the harmful effects of this metal.

In conclusion, this study confirms that the simultaneous presence of two metals in aqueous solution may cause a mutual inhibition in the adsorption of each metal to the EPS, emphasizing the need to select a specific cyanobacterial strain/EPS for a given metal-containing effluent. Moreover, depending on the metal(s) concentration(s) and its toxic effects, it may also be necessary to assess the advantages/disadvantages of using whole cultures versus isolated EPS. In this particular case, RPS, which are the most efficient fraction in the metal adsorption, can be easily separated from the cultures and immobilized or confined for biotechnological applications.

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