Appl Microbiol Biotechnol (2016) 100:7765–7775
DOI 10.1007/s00253-016-7602-9

ENVIRONMENTAL BIOTECHNOLOGY

Released polysaccharides (RPS) from *Cyanothece* sp. CCY 0110 as biosorbent for heavy metals bioremediation: interactions between metals and RPS binding sites

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Received: 12 January 2016 / Revised: 28 April 2016 / Accepted: 2 May 2016 / Published online: 18 May 2016

Abstract Bioremediation of heavy metals using microorganisms can be advantageous compared to conventional physicochemical methods due to the use of renewable resources and efficiencies of removal particularly cations at low concentrations. In this context, cyanobacteria/cyanobacterial extracellular polymeric substances (EPS) emerge as a valid alternative due to the anionic nature and particular composition of these polymers. In this work, various culture fractions of the unicellular cyanobacterium *Cyanothece* sp. CCY 0110 were employed in bioremoval assays using three of the most common heavy metal pollutants in water bodies—copper, cadmium, and lead—separately or in combined systems. Our study showed that the released polysaccharides (RPS) were the most efficient fraction, removing the metal(s) by biosorption. Therefore, this polymer was subsequently used to evaluate the interactions between the metals/RPS binding sites using SEM-EDX, ICP-OES, and FTIR. Acid and basic pretreatments applied to the polymer further improve the process efficiency, and the exposure to an alkaline solution seems to alter the RPS conformation. The differences observed in the specific metal bioremoval seem to be mainly due to the RPS organic functional groups available, mainly carboxyl and hydroxyl, than to an ion exchange mechanism. Considering that *Cyanothece* is a highly efficient RPS-producer and that RPS can be easily separated from the culture, immobilized or confined, this polymer can be advantageous for the establishment/improvement of heavy metal removal systems.

Keywords Bioremediation · Cyanobacteria · *Cyanothece* · Extracellular polymeric substances (EPS) · Heavy metals · Released polysaccharides (RPS)

Introduction

The increasing industrial activity in processes such as metallurgical work and mining is leading to an accumulation of heavy metals, causing significant problems for the environment and human health (Jarup 2003; Lesmana et al. 2009). The conventional physicochemical methods used nowadays to remove these elements from polluted waters present some disadvantages such as considerable costs, low efficiency when contaminants are present in low concentrations, and difficulties in the recovery of the removed metal (De Philippis and Micheletti 2009; Volesky 2001).

Bioremediation by microorganisms is the result of two processes: bioaccumulation and biosorption (De Philippis et al. 2011; Volesky and Holan 1995). Bioaccumulation is

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an active process where the metal is transported through the cell wall and the plasma membrane resulting in intracellular metal accumulation mediated by cell metabolism, while biosorption is a passive process generally involving several complex physicochemical mechanisms, such as ion exchange, complexation, adsorption, precipitation, etc. Ion exchange is the most common process and occurs between light metal ions initially bound to the biosorbent's binding sites and the heavy metal ions present in the aqueous solution (Sulaymon et al. 2013). This biosorption process can be influenced by several factors, such as type and concentration of the biosorbent and the metal, the presence of other competing ions and, in particular, by the pH, which will determine the protonation or deprotonation of the metal ions binding sites (Chojnacka 2010). The binding of different metals to the biomaterials will depend on the properties of the metal ions such as electronegativity and ionic radius, and on the characteristics of the biosorbent, such as surface area and content/availability of the functional groups (De Philippis and Micheletti 2009).

Cyanobacteria and their extracellular polymeric substances (EPS), mainly of polysaccharidic nature and that can remain attached to the cell surface as sheaths, capsules, and slimes or be released into the surrounding environment (RPS), have been shown to be efficient chelating agents for heavy metal ions in aqueous solutions (De Philippis et al. 2011; Pereira et al. 2009). The overall anionic nature and different possible conformations of these polymers (mainly attributed to the presence of uronic acids and sulfate groups and to the high number of different monosaccharides, respectively) make them promising for biotechnological applications (De Philippis et al. 2011; Pereira et al. 2009).

Recently, the RPS produced by *Cyanothece* sp. CCY 0110, a marine N_2-fixing unicellular cyanobacterium isolated from coastal waters of Zanzibar, were extensively characterized (Mota et al. 2013). The results obtained by these authors showed that, in agreement to what was previously described for other cyanobacterial polymers, *Cyanothece*'s RPS are complex macromolecules, composed by nine different monosaccharides including two uronic acids and contain peptides and sulfate groups. Moreover, it was also shown that these RPS are remarkably thermostable and mainly of amorphous nature and that this particular cyanobacterium strain is among the most efficient RPS producers (Mota et al. 2013).

In this work, we aimed at (i) understanding the role of various culture fractions of *Cyanothece* sp. CCY 0110 on the bioremoval of three of the most common heavy metal pollutants in water bodies: copper, cadmium, and lead, in mono- and multi-metal systems, (ii) identifying the major RPS functional groups responsible for metal binding, and (iii) evaluating the interactions between the metals/RPS binding sites.

Material and methods

Organism and culture conditions

The unicellular cyanobacterium *Cyanothece* sp. CCY 0110 (Culture Collection of Yerseke, The Netherlands, https://ccy.nioz.nl/) was grown in 1-L Erlenmeyer flasks with ASNIII medium (Rippka et al. 1979), at 30 °C under a 12 h light (50 μE m^{-2} s^{-1})/12 h dark regimen with orbital stirring (150 rpm) (Mota et al. 2013).

Metal bioremoval assays

Cyanothece cultures were grown until an optical density at 730 nm of approximately 1.0 and were placed in dialysis membranes (12–14 kDa of molecular weight cut-off; Medicell International Ltd., London, UK) and dialyzed against deionized water (pH 5.0) for 24 h. To determine the contribution of each fraction of the culture to the bioremoval of heavy metals, metal removal assays were performed using living cells + RPS, dead cells + RPS, or isolated RPS. For dead cells + RPS and isolated RPS, the cells were removed by centrifugation at 3400 *g* for 15 min. Additionally, for dead cells + RPS, the cells were autoclaved and then resuspended in the RPS. Subsequently, 30 mL of each culture fraction were confined in dialysis membranes and incubated in 80 mL of aqueous solutions containing 10 mg L^{-1} of Cu^{2+}, Cd^{2+}, or Pb^{2+} (Sigma-Aldrich Co., St. Luis, MO, USA) for 24 h with orbital stirring (85 rpm). The pH of all systems (biosorbent + metal solution) was adjusted to 5.0, using HCl or NaOH solutions when necessary. The final metal content of the solution was determined with an atomic absorption spectrometer (AAnalyst 400, Perkin Elmer Inc., Waltham, MA, USA) operating at wavelengths of 232.0 nm for Cu^{2+}, 228.8 nm for Cd^{2+}, and 283.3 nm for Pb^{2+} quantification. The amount of metal removed from the aqueous solution was calculated as the difference in the metal concentration before and after contact with the biosorbent, using ASNIII medium as blank. Specific metal bioremoval (q), expressed as mmol metal removed per g dry weight was calculated as $q = V\,(Ci - Cf)/m \times M$, where V is the sample volume (L), Ci and Cf are the initial and final metal concentrations (mg L^{-1}), respectively, m is the amount (g) of dry biomass, and M is the relative molecular mass of the metal (Volesky and May-Phillips 1995). The dry weight was determined by vacuum filtration of 5 mL of the dialyzed cultures, followed by drying of the filter at 60 °C until a constant weight was reached. In addition, the RPS content was measured using the phenol–sulfuric acid assay (Dubois et al. 1956). All measurements were performed in triplicate and results are expressed as mean values ± standard deviation.

In order to further investigate the biosorption process, cultures were dialyzed before and after a pretreatment

with 0.1 M HCl or NaOH as described previously (Micheletti et al. 2008b). Subsequently, 30 mL of confined isolated RPS were incubated in 80 mL of aqueous solutions containing 10 mg L^{-1} of Cu^{2+}, Cd^{2+}, or Pb^{2+} (mono-metal systems) or 10 mg L^{-1} of each metal (multi-metal system) with or without a pretreatment, for 24 h with orbital stirring. The final metal content was determined as described above.

In order to study the ion-exchange mechanism, metal bioremoval assays were prepared as described above, using isolated RPS, with or without a pretreatment, and metal solutions of 10 mg L^{-1} of Cu^{2+}, Cd^{2+}, or Pb^{2+} (mono-metal systems). Two different blanks were performed: one consisting of each initial metal solution at pH 5.0 and other using deionized water instead of each metal solution in the bioremoval assay. After the assays, 5 mL of HNO_3 (67 % *v*/*v*) were added to 5 mL of each biosorbent in Teflon reaction vessels to perform the mineralization in a microwave oven (Mars 5, CEM Corp., Matthews, NC, USA), using the program 1600 W, 100 % power, at 210 °C for 20 min, while the metal solutions were measured directly. The concentrations of Na^+, Mg^{2+}, Ca^{2+}, Cu^{2+}, Cd^{2+}, and Pb^{2+} in the RPS and metal solutions were determined using an inductively coupled argon plasma optical emission spectrometer (IRIS Intrepid II ICP-OES, XPS Radial, Thermo Electron Corp., Franklin; MA, USA). A standard method for 24 different elements was chosen using the TEVA/CID Analyst (Thermo Electron Corp., Franklin; MA, USA) and the wavelengths selected were 589.5 nm for Na^+, 285.2 nm for Mg^{2+}, 315.8 nm for Ca^{2+}, 327.3 nm for Cu^{2+}, 214.4 nm for Cd^{2+}, and 216.9 nm for Pb^{2+} quantification. The calibration was performed with several dilutions of the multi-element standard Astasol®-Mix (ANALYTIKA®, spol. s r.o., Prague, Czech Republic) in 1 % HNO_3 (*v*/*v*).

In order to further investigate the competition between the cations for the RPS, metal bioremoval assays were performed using 15 mL of isolated RPS incubated in 40 mL of aqueous solutions containing 10 mg L^{-1} of Cu^{2+} or Cd^{2+}. After this 24-h dialysis, 40 mL of deionized water (blank) or a solution containing 10 mg L^{-1} of Cd^{2+} or Cu^{2+}, respectively, were added for another 24 h. After each dialysis, the concentration of Cu^{2+} and/or Cd^{2+} in solution was determined using an ICP-OES as described above.

In order to confirm the entrapment of the metals and to determine the interaction of the cations within the different functional groups of the RPS, metal bioremoval assays were performed with isolated RPS, with or without a pretreatment as described above, with the exceptions that deionized water was used as blank instead of metal solution and all incubated RPS were lyophilized after the 24-h dialysis.

Scanning electron microscopy–energy dispersive X-ray spectroscopy

The lyophilized RPS samples were mounted on metal stubs using double-sided carbon tape and coated with a gold/palladium thin film, for 100 s and a 15 mA current by sputtering, using the SPI module sputter coater equipment (Structure Probe Inc., West Chester, PA, USA). The scanning electron microscopy–energy dispersive X-ray (SEM-EDX) analysis was performed using a high resolution scanning electron microscope (JSM 6301F, Jeol Ltd., Tokyo, Japan) incorporated with an X-ray elemental microanalyzer detection system (INCA Energy 350, Oxford Instruments, Abingdon, UK), operating at 15 kV.

Potentiometric titration

RPS solutions were titrated by adding 0.1 M HCl or NaOH, and the pH was measured with a pH meter (FE20 Five Easy™ pH, Mettler-Toledo AG, Urdorf, Switzerland).

Fourier transform infrared spectroscopy

Two milligrams of lyophilized RPS were grinded with 100 mg dry KBr and pressed into a mold in a uniaxial hydraulic press under a load of 0.9 MPa to obtain IR-transparent pellets. The infrared spectra were recorded with a FTIR system 2000 (Perkin Elmer Inc., Waltham, MA, USA), in absorbance mode in the region of 4000–400 cm^{-1} and a resolution of 4 cm^{-1}.

Data analysis

Data obtained in the metal bioremoval assays were statistically analyzed in GraphPad Prism v6 (GraphPad Software Inc., San Diego, CA, USA) using unpaired *t* test with a confidence level of 95 % ($P < 0.05$).

Results

Bioremoval of Cu^{2+}, Cd^{2+}, and Pb^{2+} in mono- and multi-metal systems using different culture fractions

In order to study the affinity of various *Cyanothece* sp. CCY 0110 culture fractions, namely (i) living cells + RPS, (ii) dead cells + RPS, or (iii) isolated RPS, bioremoval assays of Cu^{2+}, Cd^{2+}, and Pb^{2+} were performed. The results obtained revealed that the metals studied are removed by all fractions (Fig. 1). The bioremoval of Cu^{2+} was more efficient in the presence of living cells + RPS (0.29 ± 0.02 mmol metal removed per g of dry weight), followed by dead cells + RPS and isolated RPS, with 25 and 38 % decrease, respectively

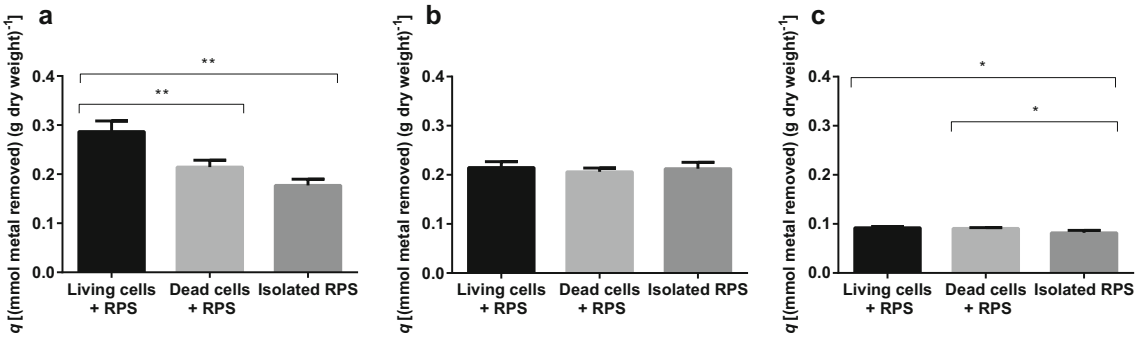

Fig. 1 Specific metal removal (q) from mono-metal solutions containing 10 mg L^{-1} of **a** copper, **b** cadmium, or **c** lead, expressed as mmol of metal removed per gram of biomass (dry weight) used as biosorbent living cells + RPS, dead cells + RPS, or isolated RPS from *Cyanothece* sp. CCY 0110. Data are means ± standard deviations (n = 3). Statistically significant differences are identified: *$P < 0.05$ and **$P < 0.01$

(Fig. 1a). On the other hand, when the three types of biosorbents were exposed to Cd^{2+}, no significant differences were observed, with approximately 0.21 ± 0.005 mmol metal removed per g of dry weight (Fig. 1b). Regarding Pb^{2+}, the specific metal bioremoval was lower (0.09 ± 0.00 mmol g^{-1} dry weight using living cells + RPS or dead cells + RPS and 0.08 ± 0.01 mmol g^{-1} dry weight using isolated RPS; Fig. 1c).

Since RPS were shown to be the most efficient fraction in metal bioremoval, the subsequent studies were performed using isolated RPS only. In order to improve the RPS performances, the polymer was subjected to different pretreatments prior to the bioremoval assays (Fig. 2). The acid pretreatment was shown to improve the metal-binding efficiency by 86 % for Cu^{2+}, 43 % for Cd^{2+}, and 22 % for Pb^{2+} (Fig. 2a). The basic pretreatment increased even further the specific metal bioremoval of Cu^{2+} in 318 % and Pb^{2+} in 92 % but decreased the bioremoval of Cd^{2+} in 26 % (Fig. 2a). Similar to what was observed in mono-metal systems, in the presence of the three metals, the acid and the basic pretreatments were shown to improve the metal-binding efficiency of the RPS, in particular for Cu^{2+} removal (Fig. 2b). The comparison between the total number of mmol of the three metals adsorbed per gram of dry biomass in multi-metal solutions and the number of mmol of metal removed with the highest q value in mono-metal solutions, usually Cu^{2+} in this study (Fig. 3 arrows), pointed out to a general antagonistic behavior (Fig. 3). In order to compare the RPS affinity for a given metal in the presence of a putative competitor, competition experiments between Cu^{2+} and Cd^{2+} were performed with sequential addition of the metals. It is clear that even with one metal already bound (either Cu^{2+} or Cd^{2+}), the RPS maintain the ability to bind the other metal (Fig. 4). While the displacement of Cu^{2+} by Cd^{2+} is small but significant, the displacement of Cd^{2+} by Cu^{2+} is quite noticeable. Moreover, when the first metal provided to the RPS is Cd^{2+} the amount of Cu^{2+} removed is higher compared to that observed with the empty polymer (Fig. 4).

Heavy metals biosorption mechanism by RPS

SEM-EDX analyses were performed to compare the concentration of elements present on the surface of RPS after dialysis (control) with the concentration on RPS after the bioremoval assays in mono- and multi-metal systems. These analyses clearly showed the presence of the metal used in each assay on the RPS surface and its complete absence in the controls of mono-metal systems (Table 1). Furthermore, the micro-elemental analysis showed the presence of several other elements, such as Ca, Mg, Na, and Cl. In general, an increase in C concentration and a decrease in O, Mg, S, and Ca concentrations were observed on the surface of the RPS after the contact with the heavy metals.

ICP-OES was performed to detect the release of the light metals Ca^{2+}, Mg^{2+}, and Na^{+} in the presence of Cu^{2+}, Cd^{2+}, or Pb^{2+} in solution. The results obtained for the biosorption of the three heavy metals were in agreement with the specific bioremoval values detected by atomic absorption spectroscopy (data not shown). In the RPS without pretreatment, only a fraction of the light metals was released to the solution after the contact of RPS with the heavy metals (Table 2). The light metal released in higher percentage was Mg^{2+} (up to 82.1 % after the contact of the RPS with Pb^{2+}), while the release of Na^{+} was limited. After acid or basic pretreatments, the percentage of light metals released to the solution was even lower in the presence of any of the heavy metal tested.

Detection of the main functional groups involved in the metal-binding process

To evaluate the reactivity of the major functional groups, potentiometric titrations of aqueous solutions of the RPS, after an acid or basic pretreatment, were performed. The potentiometric curve of RPS subjected to an acid pretreatment was characterized by the presence of one inflection point at pH 6.0 (Fig. 5). On the other hand, the curve of RPS subjected

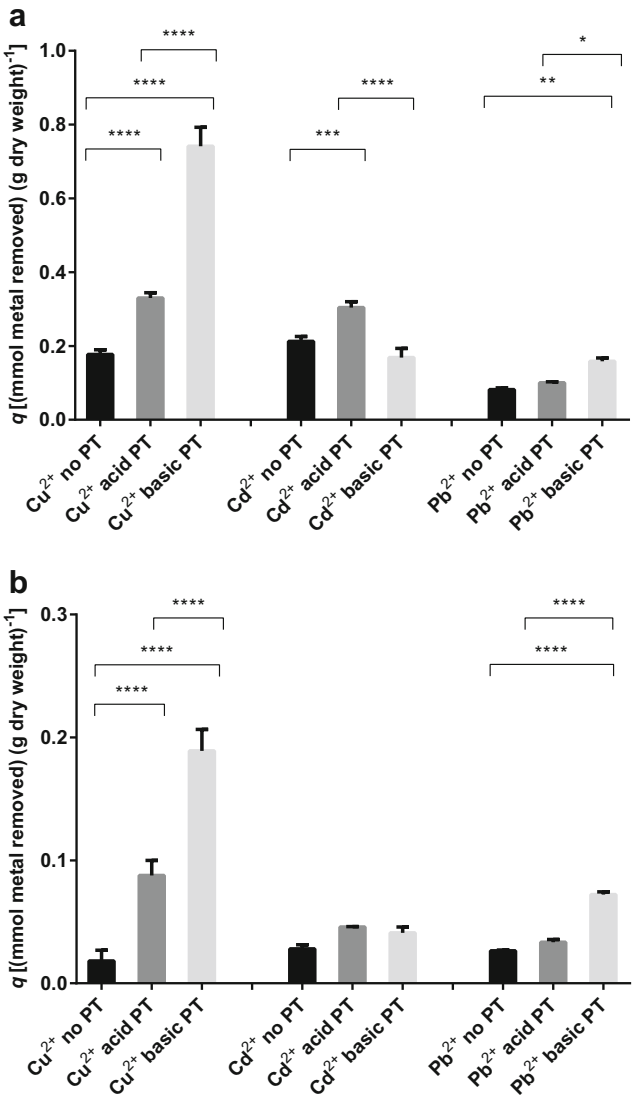

Fig. 2 Specific metal removal (q) from **a** mono-metal and **b** multi-metal solutions containing 10 mg L^{-1} of copper, cadmium, or/and lead, expressed as mmol of metal removed per gram of biomass (dry weight) used as biosorbent isolated RPS from *Cyanothece* sp. CCY 0110 without pretreatment (no PT), with 0.1 M HCl pretreatment (acid PT) or 0.1 M NaOH pretreatment (basic PT). Data are means ± standard deviations (n = 3). Statistically significant differences are identified: *P < 0.05, **P < 0.01, ***P < 0.005, and ****P < 0.001

to a basic pretreatment showed no inflection point (data not shown).

FTIR spectra of lyophilized RPS were performed to confirm and detect possible additional functional groups involved in the metal-binding process. Several characteristic absorption bands of carbohydrates could be observed (Fig. 6) (Comte et al. 2006; Stuart 2004). Two main differences were detected between RPS dialyzed with deionized water (control) or exposed to the mono-metal solutions: one in the bands around 1923–1868 cm^{-1}, characteristic of the O–H stretching vibration of the hydroxyl groups, and the other in the absorbance

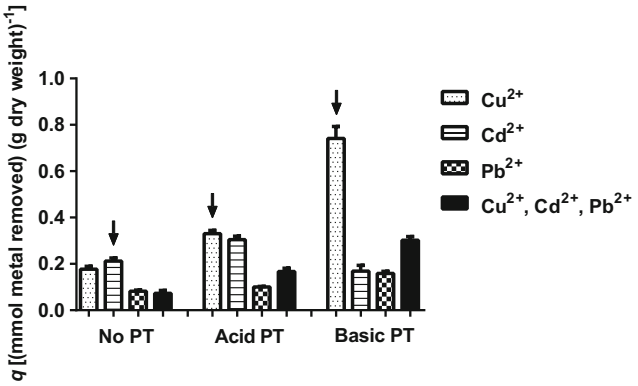

Fig. 3 Specific metal removal (q) from the mono-metal solutions and the multi-metal solution containing 10 mg L^{-1} of copper, cadmium, or/and lead, expressed as mmol of metal removed per gram of biomass (dry weight) used as biosorbent isolated RPS from *Cyanothece* sp. CCY 0110 without pretreatment (no PT), with 0.1 M HCl pretreatment (acid PT) or 0.1 M NaOH pretreatment (basic PT). Data are means ± standard deviations (n = 3). *Arrows* indicate the mono-metal solutions with higher q values

band characteristic of the C–H deformation of the carboxyl groups, which is shifted from around 1420 cm^{-1} (control) to about 1385 cm^{-1} in the samples exposed to metals (Fig. 6a). In RPS subjected to an acid pretreatment, these shifts were also detected (Fig. 6b). In the RPS subjected to a basic pretreatment, the absorbance band around 3350 cm^{-1} detected in the untreated or acid-treated RPS and characteristic of the O–H stretching vibration of the hydroxyl groups was clearly shifted to about 3450 cm^{-1} (Fig. 6c). Moreover, in the spectra region between 1900 and 1500 cm^{-1} characteristic of hydroxyl, carboxyl, and amino groups, the differences due to the basic pretreatment were even more accentuated. Within all spectra, the RPS subjected to a basic pretreatment and exposed to Cu^{2+}

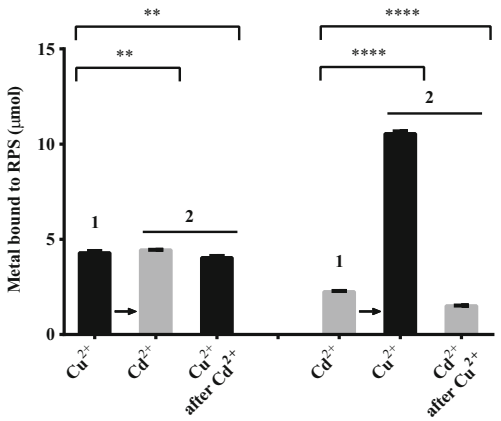

Fig. 4 Amount of metal bound used as biosorbent RPS from *Cyanothece* sp. CCY 0110 (expressed as μmol). The metal solutions (10 mg L^{-1} of copper or cadmium) were sequentially added to the biosorbent as indicated by the *arrows*. (*1*) metal bound to the RPS in the solutions containing only one metal and (*2*) metals bound to the RPS after the addition of the second metal. Data are means ± standard deviations (n = 3). Statistically significant differences are identified: *P < 0.05, **P < 0.01, and ****P < 0.001

Table 1 Elements detected by SEM-EDX in the surface of isolated RPS of *Cyanothece* sp. CCY 0110, expressed as weight percentage, after dialysis against deionized water (control) or exposed to a solution containing 10 mg L^{-1} of each metal (Cu^{2+}, Cd^{2+}, or Pb^{2+}) or the three metals together (multi-metal), without pretreatment (no PT), with HCl pretreatment (acid PT) or NaOH pretreatment (basic PT)

Sample[a]	Elements (%)[b]									
	C	O	Na	Mg	S	Cl	Ca	Cu	Cd	Pb
Control—no PT	53.26	42.20	1.06	0.77	1.77	0.42	0.51	0.00	0.00	0.00
Cu^{2+}—no PT	57.52	38.06	0.57	0.38	1.32	0.55	0.28	1.30	0.00	0.00
Cd^{2+}—no PT	57.24	36.51	1.84	0.51	1.49	1.03	0.30	0.00	1.07	0.00
Pb^{2+}—no PT	54.87	39.68	1.04	0.59	1.77	0.37	0.36	0.00	0.00	1.28
Control—acid PT	56.88	37.14	2.40	0.30	1.23	1.74	0.28	0.00	0.00	0.00
Cu^{2+}—acid PT	61.31	31.07	2.50	0.18	1.46	1.92	0.15	1.38	0.00	0.00
Cd^{2+}—acid PT	52.02	41.32	2.43	0.27	1.59	1.22	0.24	0.00	0.76	0.00
Pb^{2+}—acid PT	56.60	35.25	2.57	0.29	1.69	1.63	0.24	0.00	0.00	1.70
Control—basic PT	58.76	38.14	0.58	0.65	1.22	0.34	0.31	0.00	0.00	0.00
Cu^{2+}—basic PT	64.69	32.11	0.26	0.46	0.71	0.21	0.17	1.39	0.00	0.00
Cd^{2+}—basic PT	65.64	31.00	0.78	0.53	0.75	0.60	0.13	0.00	0.58	0.00
Pb^{2+}—basic PT	63.14	31.57	0.84	0.74	0.93	0.47	0.18	0.00	0.00	2.13
Multi-metal—no PT	61.46	30.57	0.96	0.52	2.46	0.30	0.56	1.11	0.67	1.39
Multi-metal—acid PT	66.67	31.46	0.56	0.16	0.50	0.17	0.09	0.29	0.03	0.05
Multi-metal—basic PT	65.28	31.03	0.50	0.58	0.85	0.21	0.18	0.77	0.16	0.45

[a] $n = 8$

[b] Sums may not be exactly 100 % due to rounding

or Pb^{2+} showed more modifications in comparison with the control. The absorbance bands around 1250 and 820 cm^{-1} detected in all spectra are common in polysaccharides containing sulfate groups.

Discussion

The use of total cultures of microorganisms to remove heavy metal from polluted waters allows the exploitation of their full bioremediation capacity but can have several disadvantages such as the exposure of cells to the toxic effects of metals (Mota et al. 2015) and the slow rates of metal uptake (Chojnacka 2010). These drawbacks can be overcome by using dead cells (killed by physical or chemical methods) and/or extracellular polymeric substances in the metal bioremoval process (De Philippis and Micheletti 2009). The role of the bioaccumulation process in Cu^{2+} removal was expected since this metal is essential and it is known that cyanobacteria possess specific Cu^{2+} transporters, the P_1-type ATPases (Cavet et al. 2003; Lopéz-Maury et al. 2012). However, the majority of Cu^{2+} was removed by biosorption, mainly by the RPS but with a significant contribution of the cell surface. Similarly, Pb^{2+} was mainly removed by RPS biosorption while Cd^{2+} was totally removed by this process. In cyanobacteria, a family of CPx-type ATPases is also known

Table 2 Amount of light metals (Ca^{2+}, Mg^{2+}, and Na^{+}) detected by ICP-OES, expressed as percentage of metal in the biosorbent or in solution (total amount of the metal present in the system: biosorbent plus solution), after metal bioremoval assays using as biosorbent 30 mL of isolated RPS of *Cyanothece* sp. CCY 0110 and dialyzed against 80 mL of a solution containing 10 mg L^{-1} of each metal (Cu^{2+}, Cd^{2+}, or Pb^{2+}), without pretreatment (no PT), with 0.1 M HCl pretreatment (acid PT), or 0.1 M NaOH pretreatment (basic PT)

		No PT			Acid PT			Basic PT		
		Cu^{2+}	Cd^{2+}	Pb^{2+}	Cu^{2+}	Cd^{2+}	Pb^{2+}	Cu^{2+}	Cd^{2+}	Pb^{2+}
Ca^{2+}	Biosorbent	66.2	74.1	67.3	83.2	89.4	96.3	95.2	60.1	98.5
	Solution	33.8	25.9	32.7	16.8	10.6	3.7	4.8	39.9	1.5
Mg^{2+}	Biosorbent	34.0	20.2	17.9	71.4	78.9	89.1	63.7	69.2	79.2
	Solution	66.0	79.8	82.1	28.6	21.1	10.9	36.3	30.8	20.8
Na^{+}	Biosorbent	85.3	90.1	82.3	91.5	95.4	93.5	85.3	78.3	66.3
	Solution	14.7	9.9	17.7	8.5	4.6	6.5	14.7	21.7	33.7

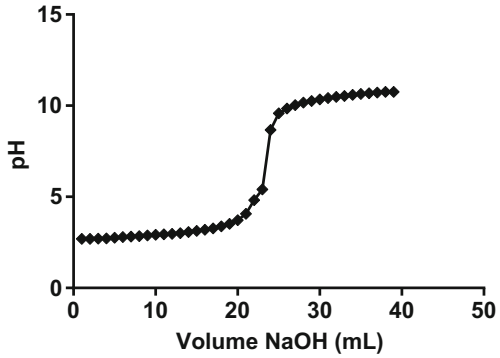

Fig. 5 Potentiometric titration of RPS solution of *Cyanothece* sp. CCY 0110 after 0.1 M HCl pretreatment

to be involved in the transport of other divalent cations like Cd^{2+}, Pb^{2+}, Zn^{2+}, and Co^{2+} (Nies 2003). Still, a previous study revealed that Cu^{2+} is more toxic to *Cyanothece* cells compared to Cd^{2+} and Pb^{2+} (Mota et al. 2015). This observation suggests that the import of the essential metal Cu^{2+} is more efficient than the import of the non-essential metals Cd^{2+} and Pb^{2+}. Altogether, our results point out to a minimal contribution of the metabolism-dependent metal uptake to the bioremoval process, revealing that biosorption is the main driving force, in agreement with previous reports (De Philippis et al. 2011; Volesky and Holan 1995). Likewise, the small difference between the amount of metals removed in the presence of dead cells + RPS compared to isolated RPS strongly indicates that the RPS play the predominant role in the bioremoval process. Previous works suggested that cyanobacterial EPS/RPS are responsible for the majority of the metal removed due to the presence of a high number of functional groups in these polymers (Anjana et al. 2007; Ozturk et al. 2014). However, only a few studies actually used cyanobacterial isolated EPS/RPS as biosorbents, and these ones suggested that their efficiency is related with the high number and availability of binding sites and the presence of negatively charged uronic acids (De Philippis et al. 2007; Parker et al. 2000; Pereira et al. 2011).

In general, the acid and basic pretreatments applied in this study improved the RPS biosorption capability with the exception of Cd^{2+} removal after a basic pretreatment. HCl and NaOH have been described as very effective desorbing agents, since, by an ion exchange mechanism, H^+ and Na^+ replace the cations derived from the culture medium that are bound to the negatively charged binding sites of the polymer. Subsequently, the H^+ and Na^+ are easily replaced by the metal ions. Accordingly, the majority of the studies have reported a significant increase in the specific metal removal after a pretreatment (Azizi et al. 2012; Micheletti et al. 2008b; Nagase et al. 2005). However, in some cases, the pretreatment was shown to be ineffective (Paperi et al. 2006; Sampedro et al. 1995), suggesting that this efficiency can depend on the metal, and on the type of biosorbent particularly due to the characteristics of its binding sites, as in the case of Cd^{2+} and the basic pretreatment.

The comparison of the metal removal values obtained for *Cyanothece*'s isolated RPS with other biosorbents of polysaccharidic nature is not straightforward, due to the lack of normalization of the data available in the literature. The maximum specific metal removal (q_{max}) expressed in mmol g^{-1} by biopolymers from different sources was compiled in Table 3. The isolated RPS of *Cyanothece* do not show the highest specific removal values; however, one should bear in mind that (i) cyanobacteria have minimal nutritional requests and low cultivation costs and (ii) this particular cyanobacterial strain is among the most efficient EPS producers releasing most of the polymer to the culture medium (~1.8 g L^{-1}), and the RPS can be harvested with a single centrifugation step (Mota et al. 2013).

As heavy metals are rarely present separately in waste waters, it is important to investigate the performances of a new biosorbent in multi-metal systems. In the presence of more than one metal, the process of metal binding to the cyanobacterial polymers can be non-interactive, synergistic, or antagonistic, depending on the number of metals competing for binding sites, the metal combinations, and the surface-specific properties of the biosorbent utilized (De Philippis and Micheletti 2009). The antagonistic interaction observed in this study was previously suggested to be the consequence of two possible mechanisms: (i) the generation of a competitive interaction between the metal ions when they are approaching the binding sites or (ii) a modification of the binding sites due to the quick sorption of the ions with higher affinity that modify the accessibility of the sites for the ions with lower affinity (Micheletti et al. 2008a; Pereira et al. 2011). In addition, the experiments of sequential addition of two metals (Cu^{2+} and Cd^{2+}) to the RPS revealed that the binding sites for these metals cannot be fully overlapping, since most of the first metal remains attached to the polymer after the addition of the second metal. Moreover, the removal of Cu^{2+} seems to increase when the RPS was previously exposed to Cd^{2+}, suggesting that the first metal changes the conformation of the polymer allowing more Cu^{2+} to bind (Parker et al. 2000).

The SEM-EDX analysis revealed that the elements present on the surface of the polymer before the contact with the metals remained partially bound to the RPS even after the contact with the metal solution(s). It was previously suggested that the cations, Mg^{2+}, Ca^{2+}, and Na^+, remain present on the surface of cyanobacterial biosorbents even after the dialysis and pretreatments, due to the high concentration of these ions in the culture medium (Paperi et al. 2006). Similar behavior was previously observed in *Enteromorpha* sp. and *Spirulina* sp. biomass exposed to various metals (Dmytryk et al. 2014; Michalak et al. 2011). The increase in the C

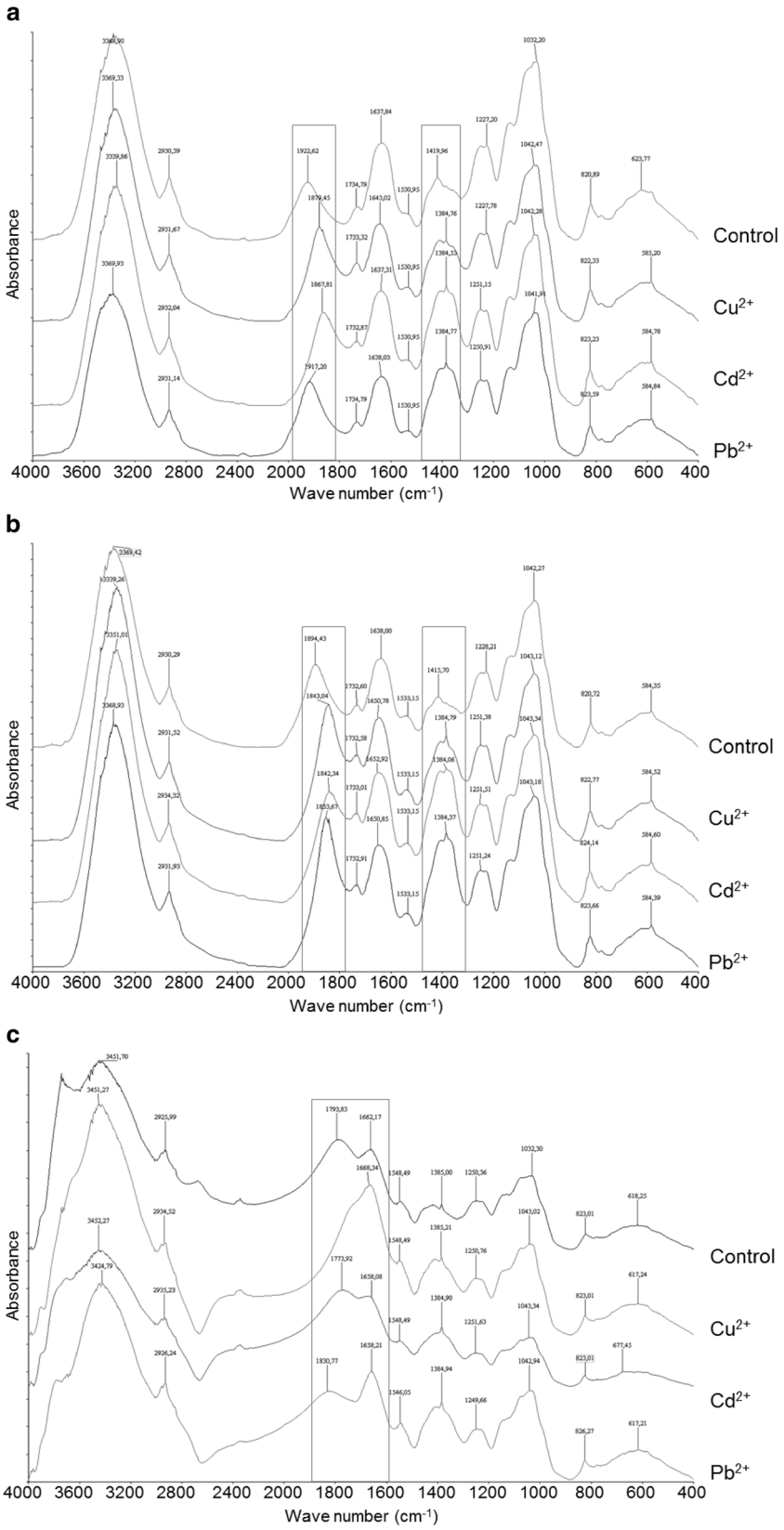

Fig. 6 Fourier transform infrared (FTIR) spectra of RPS from *Cyanothece* sp. CCY 0110 after dialysis against deionized water (control) or exposure to a solution containing 10 mg L^{-1} of copper, cadmium or lead, **a** without pretreatment (no PT), **b** with 0.1 M HCl pretreatment (acid PT), or **c** 0.1 M NaOH pretreatment (basic PT). The major absorption bands and the corresponding wave numbers are indicated. The regions with band shifts are highlighted in each spectrum

concentration after the pretreatments suggests that the inorganic fraction, mostly constituted by the ions Ca, Mg, and Na, was partially detached from the RPS during the pretreatments. Indeed, after both acid and basic

Table 3 Maximum specific metal removal (q_{max}), expressed as mmol of metal removed per gram of polysaccharides, using different biosorbents for Cu^{2+}, Cd^{2+}, or Pb^{2+} removal

Source	Biosorbent	Cu^{2+}	Cd^{2+}	Pb^{2+}	Reference
Cyanobacteria	*Cyanothece* sp. CCY 0110—RPS	0.17	0.22	0.15	This work
	Anabaena spiroides NIES-77—mucilage	0.04	0.18	0.3	(Tien 2002)
	Cyanospira capsulata—RPS	0.32	–	–	(De Philippis et al. 2007)
	Gloeothece sp. PCC 6909—RPS	1.08	–	0.16	(Pereira et al. 2011)
	Gloeothece sp. CCY 9612—RPS	1.45	–	0.13	(Pereira et al. 2011)
	Microcystis aeruginosa—capsule	4.10	1.23	1.50	(Parker et al. 2000)
	Nostoc PCC 7936—RPS	0.17	–	–	(De Philippis et al. 2007)
Algae	*Eudorina elegans* NIES-568—mucilage	0.09	0.11	0.30	(Tien 2002)
	Laminaria japonica—alginate	1.59	1.88	1.73	(Fourest and Volesky 1997)
	Sargassum fluitans—alginate	0.87	0.93	1.20	(Fourest and Volesky 1997)
Crustacean	Crab shell—chitosan	0.27	0.08	0.08	(Huang et al. 1996)
Plant	*Ceiba pentandra*—activated carbon	0.33	0.31	0.40	(Rao et al. 2006, 2008)

pretreatments, a significant reduction in the percentage of Mg and Ca ions was observed. In the case of Na, the use of NaOH both for the basic pretreatment and for adjusting the pH after the acid pretreatment made the results not utilizable for understanding this fact.

To further investigate the possible occurrence of an ion exchange mechanism in the metal binding on the RPS, the release of the light metals Ca^{2+}, Mg^{2+}, and Na^{+} in the presence of Cu^{2+}, Cd^{2+}, or Pb^{2+} in solution was investigated by ICP-OES, a technique more sensitive and accurate than SEM-EDX (Michalak et al. 2011). Most probably, some light metals are removed by the pretreatments remaining only the cations more tightly bound to the polymer due to the existence of sites with different binding strengths. Therefore, the biosorption of heavy metals by *Cyanothece*'s RPS thus not seem to be based only on an ion exchange with Ca^{2+}, Mg^{2+}, and Na^{+} but also on other mechanisms such as adsorption or ion exchange with H^{+} ions. Actually, only a few studies showed that ion exchange is definitely involved in biosorption (Kapoor and Viraraghavan 1997; Schiewer and Volesky 1995; Sulaymon et al. 2013). For example, Guo et al. demonstrated that ionic exchange interactions played only a minor role in the Cd^{2+} biosorption by *Pseudomonas plecoglossicida*, while the organic functional groups in the cell wall have a major role in adsorption (Guo et al. 2012). In addition, Dmytryk et al., using *Spirulina* sp. biomass as biosorbent, detected that Na^{+} and K^{+} were exchanged within all conditions tested, while Ca^{2+} was only exchanged by some of the heavy metals studied (Dmytryk et al. 2014). In any case, it was shown that an ion exchange mechanism could also occur between the H^{+} and the heavy metals due to a competition for the same binding sites (Schiewer 1999).

The RPS potentiometric titration after an acid pretreatment revealed a pKa mainly assigned to carboxyl groups that can also be ascribed to the presence of phosphate groups, both playing significant roles in biosorption by RPS (Chojnacka et al. 2005; Micheletti et al. 2008b). On the other hand, the RPS treated with an alkaline solution showed a lower ionic strength which may be due to a deprotonation of their binding sites. Moreover, the consequent increase in the number of free binding sites may explain the general increase in their availability to metal ions (Dmytryk et al. 2014; Schiewer and Volesky 1997).

The clear changes revealed by FTIR spectroscopy in the RPS functional groups may reflect an alteration(s) in polymer conformation, which may justify the significant increase in the bioremoval of Cu^{2+} or Pb^{2+} after the basic pretreatment. In contrast, this change seems to decrease the affinity for Cd^{2+}. Characteristic bands of sulfate groups were detected in the samples, as previously described in *Cyanothece* CCY 0110 (Mota et al. 2013). However, due to the lack of accentuated changes within the spectra after the contact with the metals, sulfhydryl functional groups do not seem to be actively involved in metal absorption. Carboxyl, hydroxyl, phosphoryl, sulfhydryl, and amino functional groups have been previously described as involved in the metal binding process, depending on the abundance, accessibility and chemical state of the sites, and on the affinity between the metal and adsorption site on each polymer/strain (De Philippis et al. 2011). Here, we demonstrated that both the carboxyl and the hydroxyl groups play an important role in the binding of positively charged metal ions to the RPS of *Cyanothece* CCY 0110, as it was expected for a cyanobacterial EPS characterized by the presence of significant amount of uronic acids (Mota et al. 2013), while apparently, the other functional groups gave only a marginal contribution, if any.

In conclusion, the RPS of *Cyanothece* sp. CCY 0110 are able to remove the heavy metals commonly found in polluted waters from aqueous solutions and the pretreatments applied to the polymer improved the removal efficiency. The mechanism of removal seems to be more related to the organic functional groups available than to ion exchange, indicating a path for the optimization/manipulation of this process. The low cost associated with the cultivation of phototrophic organisms, the high amounts of RPS produced by this particular cyanobacterial strain, and the easiness on isolating the polymer can be a valuable asset for its use in industrial settings.

Acknowledgments We thank Daniela Silva from Centro de Materiais da Universidade do Porto (CEMUP), Porto, Portugal, for technical assistance with SEM-EDX. We also thank Ricardo Silva from INEB—Instituto de Engenharia Biomédica, Universidade do Porto, Porto, Portugal, for technical assistance with FTIR. The financial support was by national funds through FCT—Fundação para a Ciência e a Tecnologia/MEC—Ministério da Educação e Ciência and when applicable co-funded by FEDER funds within the partnership agreement PT2020 related with the research unit number 4293, by the project FCOMP-01-0124-FEDER-028314 (PTDC/BIA-MIC/2889/2012) and by the scholarships SFRH/BD/84914/2012 and SFRH/BDP/72400/2010.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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