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Strategy of Cr detoxification by *Callitriche cophocarpa*

Research Article

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Abstract: The present work focused on the qualitative and quantitative analysis of Cr detoxification strategy of aquatic cosmopolitan plant *Callitriche cophocarpa*. This plant species has just been described in the context of its unusual accumulation potential of Cr. The emphasis of the work was placed on the redox reaction Cr(VI)→Cr(III) which is considered to be remediation mechanism of highly reactive and mobile Cr(VI) ions. Plants were immersed for 5 days in 1 mM of Cr(VI) (potassium dichromate) or 1 mM of Cr(III) (chromium sulphate) solutions in *semi*-natural conditions. Cr was effectively removed from the solution up to the extent of *ca.*58% or 35% of the starting amount, in the case of Cr(III) and Cr(VI), respectively. No plant-induced Cr(VI) reduction accompanying Cr accumulation was observed in Cr(VI) solutions except from the apparent one, noticed at the fourth day of incubation. On the contrary to these results, according to the method of electron paramagnetic resonance spectroscopy (*L*-band EPR), biphasic signal of Cr(V) attending Cr(VI) to Cr(III) reduction was detected inside the plant tissue every day of investigations. Our results show that phytoextraction but not phytostabilization is the main strategy of Cr detoxification by *C. cophocarpa* in aquatic systems.

Keywords: *Callitriche • Chromium • EPR • Phytoremediation • Aquatic macrophytes*

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1. Introduction

Heavy metal compounds are considered as main mobile environmental pollutants. Pollution with chromium is a serious environmental problem worldwide, *e.g.* it is regarded as a priority contaminant by the US EPA. Several areas of Poland are under the risk of Cr contamination which causes threat to agriculture, natural ecosystems and human population. The problem concerns industrialized regions of Silesia and southern Małopolska. The watershed of Dunajec river is especially exposed to severe contamination by Cr. It is charged with wastewater originating from numerous small leather tanneries of Nowy Targ region, that endanger the purity class I water of the Czorsztyńskie Lake [1,2]. The Directive 2000/60/EC of the European Parliament and of the Council of 23 October 2000 established a framework for European Community action in the field of water policy. According to the objectives of this Directive (Water Framework Directive) good ecological, *i.e.*, chemical as well as ecological standard of water must be achieved up to 2015 [3].

Several treatment technologies are applied to remove Cr from water, *e.g.*: chemical precipitation, ion exchange,

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membrane separation, flotation, electrochemical precipitation, electrokinetic extraction, reduction, chelation and adsorption on polymer resins. However, high operational costs, problems with sludge disposal or a requirement for a specific chemical modification (sorbents) make their use disadvantageous [4]. Biological treatment methods based on the remediation potential of living organisms, like microorganisms and plants, make alternative to these technologies. Phytoremediation, low-cost and eco-friendly technology, utilizes plants for cleanup of moderately contaminated areas. In the case of heavy-metallic elements phytoremediation relates mainly to accumulation or stabilization. Plants possess a plenty of mechanisms responsible for the removal and sequestration of an element in their bodies. They are also able to reduce mobility of elements, stabilizing them by root exudates [5].

Cr exists in two stable oxidation states in the environment: Cr(III) and Cr(VI). These two forms are distinct in their physicochemical properties, mobility, chemical behavior, bioavailability and toxicity. Cr(VI) originates mainly from anthropogenic sources. Cr(VI) compounds are mobile, easily soluble in a wide range of pH and their ionic forms are anions like CrO_4^{2-}, $HCrO_4^-$ or $Cr_2O_7^{2-}$. Hexavalent Cr compounds are also more bioavailabile. According to high redox potential and ability to penetrate biological membranes Cr(VI) compounds are toxic, mutagenic and carcinogenic chemicals. Cr(III) is a trace element for mammals; in aquatic environment it is present as complexed cation, frequently as hydroxo- and aquo- complex, which easily precipitates in neutral and slightly alkaline solutions [6]. According to the regulation of the Polish Ministry of the Environment [7] concentration of these two forms in river waters should not exceed 20 or 50 μg L^{-1} in the case of Cr(VI) and total Cr, respectively.

Investigation of the strategy for Cr phytoremediation by *Callitriche cophocarpa* was the objective of the present work. The outstanding ability of this aquatic higher plant to accumulate Cr from the Cr(VI) solution has been recently discovered and described [8]. Under the natural environmental conditions Cr(VI) toxicity is decreased by its reduction to Cr(III). This reduction can be executed by abiotic or biologically-controlled reactions. The experiments were carried out in order to find out whether the phytoremediation of Cr occurs only *via* the metal binding by the plant tissues (phytoextraction) or together with the biologically-controlled Cr(VI) reduction, followed by further Cr(III) precipitation in the solution (phytostabilisation). In the work reported here we used a unique custom-built low frequency electron paramagnetic resonance spectrometer (*L*-band EPR). This noninvasive, highly sensitive method, enables real time-detection of the biphasic signal of Cr(V), which is unstable oxidation state accompanying Cr(VI) to Cr(III) reduction in the solution or undamaged plant tissue. We believe that this work may have contribution firstly into the knowledge concerning mechanisms controlling heavy-metal-compounds bioremediation, and secondly into further potential utilization of *C. cophocrapa* in low-cost wastewater treatment phytotechnologies.

2. Experimental procedure

2.1. Plant collection and incubation in Cr solutions

Callitriche cophocarpa was collected from the Dłubnia river, southern Poland: 50º16' N/19º56' E, during vegetation season 2011. The mature shoots about 10 cm in length were several times rinsed with tap following distilled water and used for further experiments. Tests were performed for 5 days; measurements were prolonged up to 10 days in the case of EPR study. Plants were incubated in 50 mg L^{-1} (*ca.* 1 mM of Cr) of Cr(VI) (as $K_2Cr_2O_7$) or Cr(III) (as $Cr_2(SO_4)_3 \times 18H_2O$; POCh Gliwice, Poland) solution in water derived from the natural environment of the plant. Before experiment, to prevent growth of microorganisms, the water was filtered (Supelco filters, 0.2 μm pore size). Cr concentration was selected according to the amount of Cr in wastewater as well as to match the optimal sensitivity of the EPR spectrometer [9]. Ionic composition of water was determined according to the method of ICP-OES (Perkin Elmer Optima 7300 DV), TOC (analyzer of total organic carbon type 1200, Thermo Elektron) and titration. The spectrometer was calibrated, using the ICP multi-element standard (Merck). The average amounts of elements were (mg L^{-1}): inorganic (as HCO_3^-) C 279.33; organic C 4.65; N 6.70; P 0.06; S 27.55; K 2.19; Fe 0.11; Mg 5.62; Mn 0.01; Ca 86.41. The amount of heavy metals like Cr, Ni, Pb, Cd and Zn did not exceed relevant norms. Other water parameters were as follows: pH=7.6, electric conductivity = 0.65 mS cm^{-1} and Eh=-33,0 mV. Shoots in amount equal to 1.75 g or 3.5 g were submersed in 75 or 150 mL of the aforementioned Cr solution, respectively. The starting pH of water decreased after addition of Cr salts. Before application of Cr(III), to prevent precipitation of basic salts, the pH of the water was decreased to 5.5. Finally, the starting pH for Cr(VI) solution was 6.8 and the one for Cr(III) was 5.5. Plants were incubated in the fitotron under the 16 h of light intensity 35 μmol m^{-2} s^{-1} (LF 36W/54, Piła, Poland) and 8 h of darkness, 23°C. There were two types of control samples: the Cr solutions with no plants or the media with plants but without Cr addition.

2.2. Determination of Cr content

Concentration of total Cr, after acidification of the medium, was measured based on inductively coupled plasma optical emission spectrometry (ICP-OES Perkin Elmer Optima 7300 DV). In relation to colorimetric detection of Cr(VI) with diphenylcarbazide this technique has proved to be much easier and faster. The spectrometer was calibrated using the ICP single-element standard (Merck). Cr(VI) and Cr(III) contents were computed as a difference between the total Cr content in solution before and after Cr(III) precipitation. Cr(III) was precipitated and removed from the solution according to the analytical standard PN-77/C-04604/08 [10].

2.3. Electron paramagnetic resonance measurements

Reduction of Cr(VI) by the studied plants was measured *in vivo* using the *L*-band electron paramagnetic resonance (EPR) spectroscopy. This method enables to detect a signal of the highly unstable Cr(V) intermediate that is generated upon Cr(VI)→Cr(III) reduction. Prior to the experiments shoots of the plant were washed with distilled water twice and drained gently with the filter paper. The sample for EPR measurements was prepared by immersing of 0.4 g of the material into 1.5 mL of the dichromate solution, used for incubation. The EPR analyses were carried out using a custom-built low frequency (*L*-band, 1.2 GHz) spectrometer, with the typical settings: maximum microwave power of 16 mW and 33.8 kHz field modulation frequency. Every biological sample was prepared and measured up to seven independent experimental runs. Each run was obtained upon averaging of 20 individual 20-sec scans.

2.4. Electrolyte leakage

Measurements were performed according to the protocol given by Prášil and Zámečnik [11] and Kim *et al.* [12] with some modifications. This method enables to determine permeability of biological membranes based on the measurements of electrical conductivity. 0.2 g of the shoots were thoroughly washed in distilled water. Then the samples were carefully dried with the paper towel, submersed in 20 mL of deionized water (Milipore) and kept for 24 h in dark, at room temperature. After that, the electrical conductivity (pH/conductivity meter CPC-501, Elmetron, Poland) of solution was detected [μS cm^{-1}] and referred as A. Following these measurements the samples were deep-frozen at -80°C and kept at this temperature for the next 24 h. Finally, the samples were refrozen and warmed to room temperature, and the total conductivity [μS cm^{-1}] was measured and referred as B. The electrolyte leakage (EL) was calculated as: $EL=\frac{A}{B}100[\%]$.

2.5. Statistics

Two or three independent series of experiments with several independent replicates were performed. Results were statistically verified based on the software of STATISTICA 10. Since the data were not normally distributed a non parametric one-way analysis of variance (Kruskal-Wallis non-parametric ANOVA) test was applied to compare differences between groups [13]. Following rejection of null hypothesis non parametric multiple comparison test (Dunn's test) was performed to determine the statistical significance of results. Statistical differences between groups were highlighted by letters placed in graphs and table. All the analysis were done at the significance level α = 0.05. P-values for Kruskal-Wallis and Dunn's tests were calculated and provided in the legend of each graph and table. The same letters indicate no statistical differences (α ≥ p); different letters indicate significant differences (α < p).

3. Results

3.1. Influence of plants on Cr removal and reduction in solution

During 5-day incubation Cr was effectively removed from the solutions by plants (Fig. 1). However, Cr was more efficiently removed from sulphate (Cr(III)) than from dichromate (Cr(VI)) solutions starting at the first day of incubation. The maximal removal of Cr was achieved at the fifth day of experiment and this final value of Cr elimination reached *ca.*58% of Cr(III) in the case of $Cr_2(SO_4)_3$ or 35% of Cr(VI) in the case of $K_2Cr_2O_7$. Taking into consideration the biomass of plants (dry weight of plants was about 7 to 8% of fresh weight) and volume of the medium, both mentioned values correspond to *ca.* 16700 or 6860 mg kg^{-1} d.w. of Cr, respectively.

In the next step of experiment the level of Cr(VI) reduction in the solutions containing dichromate ions was investigated. Value of total Cr in control medium (without plants) stayed at the same level during the experiment on the contrary to the total level of Cr in medium containing plants (see above). The amount of Cr(III) formed by Cr(VI) reduction was expressed therefore as percentage of total amount of Cr in a solution (Table 1). It was found that the percentage of Cr(III) accompanying Cr(VI) reduction in control medium slightly decreased and at the fifth day of experiment the amount of Cr(III) turned out to be lower, not only in

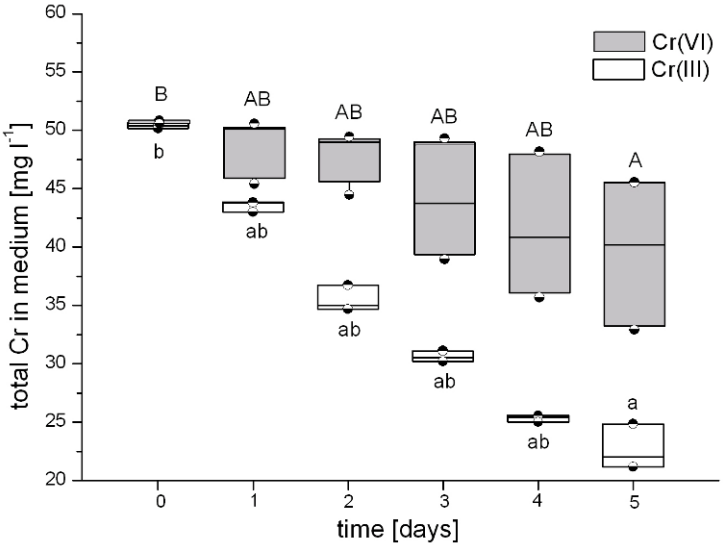

Figure 1. The loss of Cr in medium accompanying its removal by Callitriche shoots during 5-day incubation in 1 mM Cr solutions. Two independent series of experiments were performed with n = 6 replicates. Different letters indicate statistically significant differences for Cr(III) and Cr(VI) treatment separately according to Kruskal-Wallis non-parametric ANOVA (α = 0.05; Cr(VI) $p < 0.0086$, Cr(III) $p < 0.0391$) and Dunn's test (α = 0.05; Cr(VI) day 1st *vs* Cr(VI) day 5th $p < 0.0096$, Cr(III) day 1st *vs* Cr(III) day 5th $p < 0.0471$). Box – 25%, 75%; vertical line inside box – median; circles – min, max.

Table 1. The amount of Cr(III) accompanying Cr(VI) reduction in control medium as well as in medium containing plants during 5-day incubation. The amount of Cr(III) was normalized to total Cr in medium [%].Two independent series of experiments were performed with n = 6 replicates. Different letters indicate statistically significant differences for Cr(III) in medium with plants and control medium separately according to Kruskal-Wallis non-parametric ANOVA (α = 0.05; Cr(III) in medium with plants $p < 0.0028$, Cr(III) in control medium $p < 0.0032$) and Dunn's test (α = 0.05; group X4 *vs* X5 $p < 0.0006$, group Y1 *vs* Y5 $p < 0.0045$, group Y2 *vs* Y5 $p < 0.0316$, group Y3 *vs* Y5 $p < 0.0147$).

Cr (III) in external media [%]									
with plants					control (no plants)				
day	group name	median	min	max	day	group name	median	min	max
1	X1	27.60 (ab)	16.35	43.07	**1**	Y1	27.56 (b)	25.47	29.52
2	X2	27.40 (ab)	20.34	36.96	**2**	Y2	27.76 (b)	23.12	32.30
3	X3	22.90 (ab)	21.83	24.47	**3**	Y3	26.19 (b)	24.30	29.81
4	X4	52.02 (b)	40.22	63.13	**4**	Y4	25.30 (ab)	23.99	25.79
5	X5	17.75 (a)	16.30	22.88	**5**	Y5	18.26 (a)	14.56	20.27

comparison with the beginning, but also in comparison with the fourth day. No differences between control and plant-containing solutions were found except from the fourth day of incubation, when unexpectedly the level of Cr(III) was increased to the value of 51.88%. Cr in media supplemented with $Cr_2(SO_4)_3$ was totally in Cr(III) form (data not shown), what proved no oxidation of Cr(III) under the conditions of experiment.

3.2. EPR study of Cr reduction/oxidation in plant tissue

EPR signal, derived directly from Cr(V) involved in reduction of Cr(VI) to Cr(III), was recorded every day in plant tissue of all tested samples incubated in dichromate solutions (Fig. 2). Control plant samples, *i.e.*, immersed in the solution free of chromium, showed no paramagnetic activity during the whole experiment. No activity was observed either in the dichromate solution in which the plants were incubated (Fig. 2A). The tendency to increase of the amplitude of the signal [au] was measured starting at the fourth day of experiment (Fig. 2B). On the contrary to the above observation of Cr(VI) reduction in plant tissue, no signal of Cr(V) was found in Cr(III)-medium where the plants were incubated (Fig. 2A). It means no oxidation of Cr(III) to Cr(VI) inside *Callitriche* shoots.

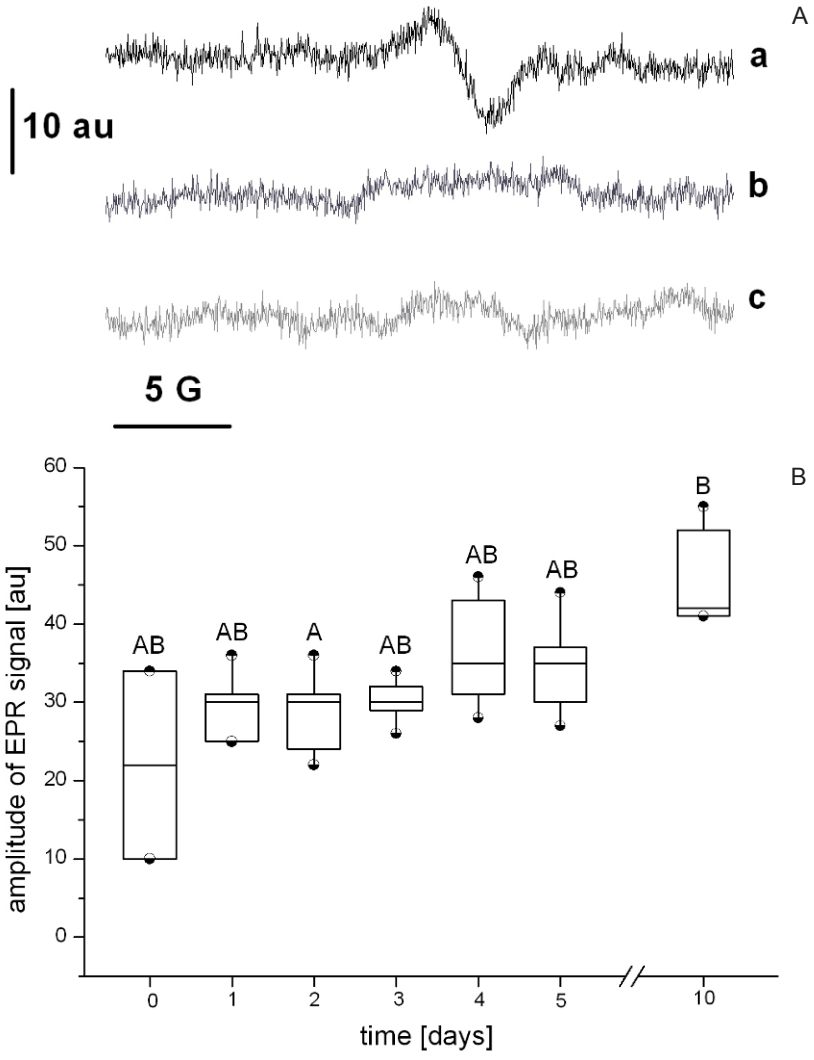

Figure 2. EPR signal of Cr(V). **A** – representative EPR spectra: **a** – EPR signal as a result of Cr(VI) reduction in plant tissue (black line), **b** – no signal detected in Cr(VI) media (dark gray line), **c** – no signal following Cr(III) to Cr(VI) oxidation either in plant or Cr(III) media (light gray line); **B** – average values of the amplitudes of Cr(V) signal following Cr(VI) reduction inside plant tissue during subsequent days of experiment. Three independent series of experiments were conducted. The values are the averages of n ∈ <5; 7> independent runs and each run of 20 individual 20-sec scans. Different letters indicate statistically significant differences according to Kruskal-Wallis non-parametric ANOVA (**α** = 0.05; $p < 0.0116$) and Dunn's test (**α** = 0.05; day 2^{nd} *vs* day 10^{th} $p < 0.0116$). Box – 25%, 75%; vertical line inside box – median; circles – min, max.

3.3. Electrolyte leakage accompanying Cr incubation

The electrical conductivity method is based on the measurements of the amount of the electrolyte leaked outside plasma membrane; the highest value of conductivity means the highest injury of the cell. According to statistical analysis, no significant cells injuries were observed in Cr(III)-treated material during the whole experiment in comparison to control plants. On the contrary to plants incubated in Cr(III) solution, Cr(VI)-influenced plants exhibited tendency toward increased electrolyte leakage and significant difference was observed at the fourth day of the experiment (Fig. 3).

4. Discussion

In relation to other heavy metal compounds like the ones of Cd, Pb, Ni, Zn, the literature data (*e.g.* according to Science Direct) concerning chromium phytoremediation is relatively scarce. Research on the Cr detoxification carried out by *Callitriche* is important due to some important issues. Firstly, this plant species is geographically cosmopolitan and centered in temperate zones of both hemispheres, which is an essential feature with respect to global science development. Secondly, there are only few examples of chromium plant hypperacummulators – by definition extracting

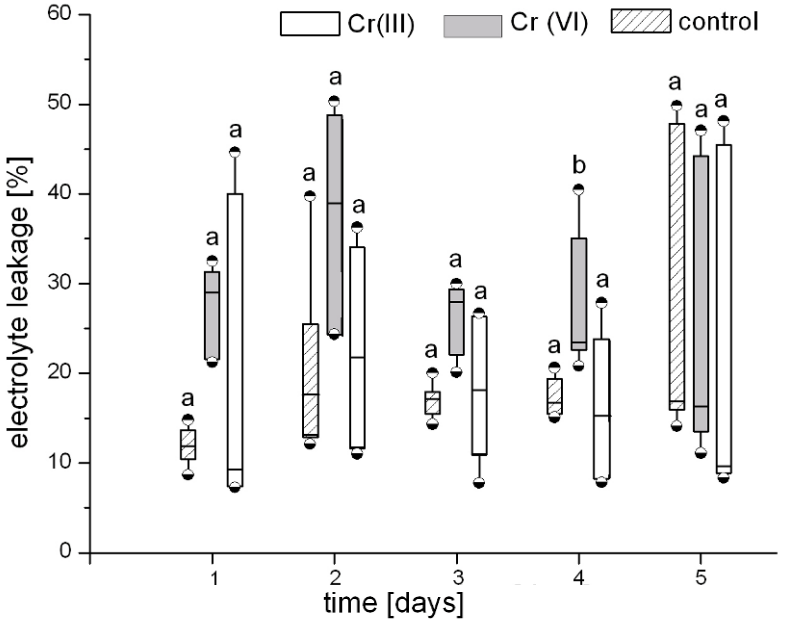

Figure 3. Electrolyte leakage out of *Callitriche* cells determined as percentage of total electrical conductivity of cell sap [μS cm^{-1}] under Cr(VI) or Cr(III) influence during subsequent days of experiment. Two independent series of experiments were performed with n = 6 replicates. Statistical differences are calculated for every day separately according to Kruskal-Wallis non-parametric ANOVA ($\alpha = 0.05$; day 1st $p < 0.1244$, day 2nd $p < 0.1199$, day 3rd $p < 0.0508$, day 4th $p < 0.0329$, day 5th $p < 0.3585$) and Dunn's test ($\alpha = 0.05$; Cr(VI) day 4th *vs* Cr(III) day 4th $p < 0.0480$, Cr(VI) day 4th *vs* control day 4th $p < 0.0490$). Box – 25%, 75%; vertical line inside box – median; circles – min, max.

more than 1000 mg kg^{-1} d.w. of Cr, but they are locally distributed or they originate from tropical or subtropical regions (Southern China, Zimbabwe, New Caledonia) [14,15].

The results reported in this study confirm unusual phytoremediation potential of *C.cophocarpa* to accumulate Cr in aquatic environment. Cr is more effectively removed from the Cr(III) solution than Cr(VI) one that can be explained by the higher Cr(VI) toxicity. The observation is consistent with reports of other researches [15,16]. The removal and accumulation capacities of Cr are comparable or higher to those described for other aquatic plant accumulators [17].

Since the toxicity and bioavailability is far more pronounced for Cr(VI) than Cr(III), remediation strategies focus on the reduction of Cr(VI) to Cr(III) [16]. Direction of the redox reaction Cr(VI)↔Cr(III) depends on the presence of electron donors/acceptors and pH. The redox potential of the couple Cr(VI)/Cr(III) is high in acidic media. Therefore there are only a few oxidants, like manganese dioxide, hydrogen peroxide, ozone, lead dioxide, which are able to mediate the oxidation of Cr(III) to Cr(VI) in natural environment [6,19]. Cr(VI) in water occurs in a very mobile anionic form within a wide range of pH. On the contrary to Cr(VI), Cr(III) is easily precipitated in neutral and alkaline solutions and it occurs as cation, complexed mainly by water molecules and hydroxyl anion together with other functional groups. The major abiotic reducing agents include dissolved Fe(II) compounds, sulfur(II) compounds and organic matter, like humic or fulvic acids [20]. Since the costs of technological operation of the abiotic reduction is high, its alternative is biological transformation of Cr(VI) to Cr(III). Organism-, especially microorganism-mediated reduction is well documented in literature [21]. Cr(VI) can be reduced directly or indirectly outside or/and inside their cells. Reduction may proceed spontaneously with low-molecular weight compounds like glutathione, cysteine, ascorbate (absent in bacterial cells), hydrogen peroxide, NAD(P)H and monosaccharides [22]. In the case of microorganisms this redox reaction can be also catalyzed by soluble or membrane-associated enzymes [21,23]. Plant-mediated response to elevated concentration of heavy metal compounds may be achieved either by accumulation of the element in a plant tissue or by its detoxification through the above-described external plant-based reduction/chelation, known as phytostabilization [14,16].

Our results showed that the studied plant is capable to efficiently remove Cr from Cr(VI) solution without its reduction in the external medium. The presence of Cr(III) in control media supplemented with Cr(VI) is a result of a reaction between Cr(VI) and naturally occurring abiotic reducing compounds. The Cr(V) species of chromium in aquatic solutions is very unstable [24,25]. Therefore reduction Cr(VI)→Cr(III) succeeds very

rapidly. This is the probable reason why intermediates of this reaction were not observed in control medium (without the plants) using the EPR technique. We measured slight decrease in Cr(III) amount during days of experiment in control medium. Thus it would seem that Cr(III) undergoes oxidation in the following days of experiment. Still we postulate that this effect is a result of Cr(VI) equilibrium changes during subsequent days of experiment. We might speculate that Cr(III) makes complexes with Cr(VI) like the mixed Cr(VI)-Cr(III) oxides described by Xia and coworkers [26]. Since pH of the Cr(VI)/(III) solutions slightly increased − this effect has been already described in the earlier work − the equilibrium of Cr(VI) concentration can also be altered as a result of hydrolysis of the described complexes [26].

We have found that phytoremediation of Cr(VI) by *C. cophocarpa* is carried out with no external Cr(VI) reduction. Only at the fourth day of experiment we measured higher amount of Cr(III), apparently resulting from Cr(VI) reduction, in comparison to control. Moreover, at the same day we observed elevated electrolyte leakage from the Cr(VI)-treated cells, too. Potassium ions are especially significant factors regulating water balance in plants and water balance changes are considered as one of the most sensitive indicators of the heavy-metal stress in plants [27]. Therefore, we might suppose that the ion leakage, probably mostly potassium ions, from the studied material can interfere with the adopted method of Cr determination. Applied analytical standard for a simultaneous determination of Cr(VI) and Cr(III) in water is based on measurement of total chromium as Cr(VI) after exhaustive oxidation. In the parallel run this standard is also based on removal of Cr(III) present in a water sample by coprecipitation together with Zn(II) in a form of a mixed hydroxide and determination of Cr(VI) in the filtrate. Amount of Cr(III) is calculated as a difference between total and hexavalent chromium present in the sample. Recently it has been found, however, that Zn^{2+} salts together with soluble Cr^{3+} compounds form layered double hydroxide (LDH) upon addition of alkali [28]. This very useful class of compounds can act as anion exchanger [29]. As a result of much earlier research works, ability of Cr(VI) to make hardly soluble basic zinc chromate of the formula $4Zn(OH)_2 \bullet ZnCrO_4$ and particularly basic zinc potassium chromates, like the one of the formula $(HO)ZnKCrO_4 \bullet ZnCrO_4$, has been discovered and practically used as corrosion-resistant yellow pigments [30]. Hence, it might be concluded that under certain circumstances potassium ions released from plant tissue to the incubating solution facilitate immobilization of chromates in the LDH structure in the course of analytical procedure applied. Further research work is necessary to elucidate convincingly observed apparent reduction of Cr(VI) at the fourth day of experiment and subsequent apparent oxidation of Cr(III) in the same solution at the fifth day. It is worth mentioning, however, that the membrane status of Cr(VI)-treated cells reflected by electrolyte leakage, returned to the level compared with control at the fifth day of experiment. A mechanism responsible for this return may be related to some unknown metabolic-adaptation factors. The nature of these factors has to be investigated in further, separate study.

In the work reported here we have shown that there is no plant-controlled Cr(VI) reduction outside the plant tissue accompanying Cr accumulation. The obtained result is different form the one presented by Fedorovych *et al*. [31] as well as Kostecka-Gugała and coworkers [32] in studies on different yeast strains of *Saccharomyces cerevisiae, Pichia guilliermondi* and *Phaffia rhodozyma*. The researches have measured significant continues Cr(VI) reduction under control of some water-soluble, low-molecular factors in external medium. Still, due to *L*-band EPR real-time measurements we have measured active Cr(VI) reduction inside plant shoots. Our results also pointed out that phytoremediation of Cr(III) is provided with no Cr(III) oxidation either in media or inside the plant material. The analysis of the amplitude of Cr(V) EPR signal showed that Cr(VI) reduction inside the plant tissue occurs more intensely during subsequent days of the study. This phenomenon can be explained on the basis of the pysiological status of the plant. As it has been already shown [33] and confirmed in this study *Callitriche* shoots are far more sensitive to Cr(VI) than Cr(III). The metabolic status of plants is highly altered by Cr(VI) influence which may result in synthesis of redox compounds, like some thiol S(II) compounds known as abiotic stress factors [34,35]. The ability of *C. cophocarpa* to internal reduction of Cr(VI) to Cr(III) has been already presented by the authors of the present work [36]. However, these *L*-band EPR analysis concerned only the first 5-hour kinetics. The present study was performed for 5 days according to the earlier observations dealing with physiological status of the plant [33]. Hexavalent Cr reduction measured either by *L*-band EPR or *X*-ray emission fluorescence spectrometers were earlier conducted also on other aquatic plants like *Spirodela polyrhiza*, *Lemna trisulca*, *Eicchornia crassipess*, *Salvinia auriculata* and *Pistia stratiotes* [9,36−38]. Though, these species undertake Cr(VI) reduction following further Cr(III) biosorption in the roots and fronds and thus roots in general are mainly responsible for the Cr and other metallic elements sequestration [5]. *C. cophocarpa* exploits its submersed

shoots to accumulate Cr ions with elevated surface area. Thus, utilization of this plant in contaminated aquatic system ensures more effective remediation.

5. Conclusions

Our results showed that phytoextraction but not phytostabilization is the main strategy of Cr phytoremediation by *C. cophocarpa* in aquatic systems. While extracting Cr *C. cophocarpa* is able to reduce Cr(VI) inside the tissues with no secretion of any reducing factors into surrounding medium. Therefore in the natural environment it provides safe extraction of the toxic element by the plant material with no further external Cr(III) precipitation and binding into a sediment. The presence of Cr(III) in a sediment might be risky when changing physicochemical parameters of water. The results obtained in the present studies may also be useful for further elucidation of molecular mechanisms of Cr(VI) detoxification in tissues of living organisms.

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References

[1] M. Pawlikowski, E. Szalińska, M.Wardas, J. Dominik, Pol. J. Environ. Stud. 15, 885 (2006)
[2] J. Dominik, D. A. L. Vignati, B. Koukal, M.-H. Pereira de Abreu, R. Kottelat, E. Szalińska, B. Bas, A. Bobrowski, Eng. Life Sci. 7, 1 (2007)
[3] The Water Framework Directive - Directive 2000/60/EC of the European Parliament and the Council of 23 October 2000 establishing a framework for Community action in the field of water policy (European Parliament and Council, 2000) http://ec.europa.eu/environment/water/water-framework/info/intro_en.htm
[4] D. Mohan, Ch.U. Pittman, J. Hazard. Mater. 137, 762 (2006)
[5] P.P. Padmavathiamma, L.Y. Li, Water Air Soil Poll. 184, 105 (2011)
[6] J. Kotaś, Z. Stasicka, Environ. Pollut. 107, 263 (2000)
[7] Rozporządzenie Ministra Środowiska z dn. 20.08.2008 w sprawie sposobu klasyfikacji stanu jednolitych wód powierzchniowych. Dziennik Ustaw nr 162, poz. 1008 (In Polish)
[8] J. Augustynowicz, M. Grosicki, E. Hanus-Fajerska, M. Lekka, A. Waloszek, H. Kołoczek, Chemosphere 79, 1077 (2010)
[9] P. Kaszycki, H. Gabryś, K.-J. Appenroth, A. Jaglarz, S. Sędziwy, T. Walczak, H. Koloczek, Plant Cell Environ. 28, 260 (2005)
[10] PN-77/C-04604/08 Water and waste water. Tests for chromium. Determination of the hexavalent chromium (Cr^{6+}) and trivalent chromium (Cr^{3+})
[11] I. Prášil, J. Zámečnik, Environ. Exp. Bot. 40, 1 (1998)
[12] J.G. Kim, Y. Luo, Y. Tao, R.A. Saftner, K.C. Gross, J. Sci. Food Agr. 85, 1622 (2005)
[13] Y. Dodge, The Concise Encyclopedia of Statistics (Springer, NY, 2008)
[14] A.M. Zayed, N. Terry, Plant Soil 249, 139 (2003)
[15] X.-H Zhang, J.Liu, H.-T. Huang, J. Chen, Y.-N. Zhu, D.-Q. Wang, Chemosphere 67, 1138 (2007)
[16] K.B. Santana, A-A.F. de Almeida, V.L. Souza, P.A.O. Mangaberia, D.C. Silva, F.P. Gomeas, L. Dutruch, L.L. Loguercio, Environ. Exp. Bot. 80, 35 (2012)
[17] A.K. Shanker, C. Cervantes, H. Loza-Tavera, S. Avudainayagam, Environ. Int. 31, 739 (2005)
[18] S.M. Williams, C.S. Criddle, M.J. Dybas, In: J. Guertin, J.A. Jacobs, C.P. Avakian (Eds.), Chromium(VI) Handbook (CRC Press, Boca Raton, 2004) 346
[19] F.T. Stanin, M. Pirnie, In: J. Guertin, J. A. Jacobs, C.P. Avakian (Eds.), Chromium(VI) Handbook (CRC Press, Boca Raton, 2004) 161
[20] L.A. Hellerich, M.A. Panciera, G.M. Dobbs, N.P. Nikolaidis, B.F. Smets, In: J. Guertin, J. A. Jacobs, C.P. Avakian (Eds.), Chromium(VI) Handbook (CRC Press, Boca Raton, 2004), 438
[21] P. Kanmani, J. Aravind, D. Preston, IJEST 9, 183 (2012)
[22] R. Saha, R. Nandi, B. Saha, J. Coord. Chem. 64, 1782 (2011)
[23] K.H. Cheung, J.-D. Gu, Int. Biodeter. Biodegr. 59, 8 (2007)
[24] M. Krumpolc, J. Rocek, J. Am. Chem. Soc. 98, 872 (1976)

[25] T. Ramasami, B.U. Nair, M. Kanthimathi, C.K. Ranganathan, J. Chem. Sci. 107, (1995)
[26] L. Xia, E. Akiyama, G. Frankel, R. McCreery, J. Electrochem. Soc. 147, 2556 (2000)
[27] K.-J. Appenroth, Acta Physiol. Plant 32, 615 (2010)
[28] J. Zhang, Y. Li, J. Zhou, D. Chen, G. Qian, J. Hazard. Mater. 29, 205 (2012)
[29] J.W. Boclair, P.S. Braterman, J. Jiang, S. Lou, F. Yarberry, Chem. Mater. 11(2), 303 (1999)
[30] E.R.A. Rutgers, In: M.R. van Vliet (Ed.), Coatings for the Aerospace Environment (McGregor & Werner, Inc., Dayton, 1961) 335
[31] D.V. Fedorovych, M.V. Gonchar, H.P. Ksheminska, T.M. Prokopie, H.I. Nechay, P. Kaszycki, H. Koloczek, A.A. Sibirny, Microbiology & Biotechnology 3, 15 (2009)
[32] A. Kostecka-Gugała, M. Gołda, P. Kaszycki, H. Kołoczek, M.V. Gonchar, M. Grządka-Osior, H.I. Nechay, Abstracts of the Central European Congress of Life Science EUROBIOTECH, Kraków, Poland, 22th-22nd SEP, Acta Bioch. Polonica 57 S2, 18 (2010)
[33] J. Augustynowicz, A. Kołton, A. Baran, A. Świderski, Environ. Prot. Nat. Resour. 50, 98 (2011) (in Polish)
[34] S. Dubey, P. Misra, S. Dwivedi, S. Chatterjee, S.K. Bag, S. Mantri, M.H. Asif, A. Rai, S. Kumar, M. Sari, P. Tripathi, R.D. Tripathi, P.K. Trivedi, D. Chakrabarty, R. Tuli, BMC Genomics 11, 648 (2010)
[35] M. Hellado, R.A. Contreras, A. González, G. Dennett, A. Moenne, Plant Physiol. Bioch. 51, 102 (2012)
[36] J. Augustynowicz, A. Kostecka-Gugała, H. Kołoczek, Environ. Prot. Nat. Resour. 41, 210 (2009) (in Polish)
[37] K.J. Liu., J. Jiang, X. Shi, H. Gabrys, T. Walczak, H. Swarz, Biochem. Bioph. Res. Co 206, (1995)
[38] F.R Espinoza-Quiñones, N. Martin, G. Stutz, G. Tirao, S.M. Palácio, M.A. Rizzutto, A.N. Módenes, F.G. Silva Jr., N. Szymansky, A.D. Kroumov, Water Res. 43, 4159 (2009)