Chemosphere 120 (2015) 23–30

Contents lists available at ScienceDirect

Chemosphere

journal homepage: www.elsevier.com/locate/chemosphere

Physiology and methodology of chromium toxicity using alga *Scenedesmus quadricauda* as model object

Jozef Kováčik [a,*], Petr Babula [b], Josef Hedbavny [a], Olga Kryštofová [a], Ivo Provaznik [c]

[a] Institute of Chemistry and Biochemistry, Faculty of Agronomy, Mendel University in Brno, Zemědělská 1, 613 00 Brno, Czech Republic
[b] Department of Natural Drugs, Faculty of Pharmacy, University of Veterinary and Pharmaceutical Sciences Brno, Palackého 1/3, 612 42 Brno, Czech Republic
[c] International Clinical Research Center, Center of Biomedical Engineering, St. Anne's University Hospital Brno, Pekařská 53, 656 91 Brno, Czech Republic

HIGHLIGHTS

- Uptake and toxicity of trivalent and hexavalent chromium in green alga was compared.
- Viability, chlorophyll and mitochondrial proteins were more depleted by Cr(VI).
- Cr(III) stimulated ROS and lipid peroxidation more than Cr(VI).
- Reduction of Cr(VI) to Cr(III) and higher Cr content in high Cr(VI) dose was found.
- pH affected Cr(VI) reduction and total Cr uptake.

ARTICLE INFO

Article history:
Received 24 March 2014
Received in revised form 15 May 2014
Accepted 23 May 2014

Handling Editor: A. Gies

Keywords:
Antioxidative enzymes
Bioaccumulation
Confocal/fluorescence microscopy
Heavy metals
Oxidative stress

ABSTRACT

Physiological responses of *Scenedesmus quadricauda* to Cr(VI) and Cr(III) excess were studied in buffer with circumneutral pH (6.5). Total Cr content was similar in low (1 μM of both oxidation states) but higher in 10 μM Cr(VI) treatment and high accumulation potential was detected (80–82% and 41–65% in 1 and 10 μM treatments, respectively). Specific fluorescence indicator (6-((anthracen-9-yl) methyleneamino)-2H-chromen-2-one) confirmed partial reduction of Cr(VI) to Cr(III) under exposure conditions. Viability and chlorophyll autofluorescence were more depleted by Cr(VI) while Cr(III) stimulated increase in ROS and lipid peroxidation. Antioxidative enzyme activities showed significantly higher values in 10 μM treatments of both Cr oxidation states. Depletion of mitochondrial proteins was not reflected in alteration of total soluble proteins indicating sensitivity of this organelle to Cr and TTC test showed no clear oxidation state-related effect. In this view, "Cr(VI) is not more toxic than Cr(III)" at least for some parameters. Subsequent study with the application of 10 μM Cr(VI) confirmed that HEPES buffer is more suitable exposure solution for toxicological studied than water or inorganic salts (higher chlorophyll autofluorescence was observed) and pH 6.5 is more suitable than low or high pH (4.5 or 8.5) in terms of Cr uptake. Another known Cr(III) fluorescence indicator (naphthalimide–rhodamine) also confirmed partial reduction of Cr(VI) to Cr(III) at acidic pH but only traces were seen at alkaline pH.

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1. Introduction

Chromium (Cr) is the seventh most abundant element in the earth's crust and non-essential for plant nutrition (Volland et al., 2012). It occurs in various oxidation states among which Cr(III) and Cr(VI) are stable enough in the environment (Yu and Gu, 2007). They differ considerably in toxicity and Cr(VI) typically shows more negative impact on plants (Gangwar et al., 2011; Santana et al., 2012). Depending on the conditions, reduction of Cr(VI) to Cr(III) is often favored (Deiana et al., 2007; Yu and Gu, 2007). Toxicity is also affected by concentration and exposure time as well as by other physicochemical factors such as pH (Vignati et al., 2010) and applied Cr form (Dazy et al., 2008).

Similarly to other metals, Cr stimulates reactive oxygen species (ROS) formation and alters activities of antioxidative enzymes in relation to oxidation state (Rai et al., 2004; Dazy et al., 2008). In terms of non-enzymatic antioxidants, thiol-containing metabolites (Gorbi et al., 2006; Dazy et al., 2008) and phenolic metabolites (Tripathi et al., 2012; Kováčik et al., 2013) are also variably affected by Cr oxidation state.

* Corresponding author. Tel.: +420 545 133281; fax: +420 545 212044.
E-mail address: jozkovacik@yahoo.com (J. Kováčik).

http://dx.doi.org/10.1016/j.chemosphere.2014.05.074
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Algae are potent accumulators of some metals (Štork et al., 2013). It was also found that they can effectively absorb trivalent Cr (Pakshirajan et al., 2013) whereas hexavalent Cr is toxic (Volland et al., 2012). Recently, higher toxicity of Cr(VI) in comparison with Cr(III) was questioned using standard algal test (Vignati et al., 2010). Besides, higher plants (Santana et al., 2012) and algae show strong reducing properties towards Cr(VI) that can be affected by exogenous factors (Deng et al., 2006; Yewalkar et al., 2007).

Despite numerous studies focused on Cr toxicity in terrestrial plants (Rai et al., 2004; Yu and Gu, 2007; Santana et al., 2012), little attention was paid to aquatic species. More recently, comparable toxicity of Cr(III) nitrate salt and Cr(VI) in aquatic moss *Fontinalis antipyretica* was reported (Dazy et al., 2008). Algae were mainly studied in terms of eventual absorption or photoreduction of hexavalent Cr (Deng et al., 2006; Pakshirajan et al., 2013). Deeper physiological, morphological and biochemical alterations are known in green alga *Micrasterias denticulata* but only Cr(VI) was tested (Volland et al., 2012, 2014).

We therefore compared Cr(VI) and Cr(III) toxicity using widely distributed freshwater alga *Scenedesmus quadricauda* exposed to Cr in HEPES buffer (4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid, Duchefa Biochemie B.V., The Netherlands) with circumneutral pH (6.5). This pH value is more probable in the nature and allows to verify recent discrepancy in terms of Cr(VI) and Cr(III) toxicity (Vignati et al., 2010). Additionally, we further tested the impact of other exposure solutions (water and liquid 'Milieu Bristol' medium) with various pH on Cr(VI) reduction, uptake and selected microscopic responses. We tested not only common biochemical parameters such as activities of some enzymes, basic metabolites and TTC (2,3,5-triphenyltetrazolium chloride) test but extensive fluorescence and confocal microscopy was also carried out. This allows comparison of some qualitative and quantitative data. Two specific fluorescent dyes were used to monitor reduction of Cr(VI) to Cr(III) under assay conditions. Data are explanatively compared with limited available literature.

2. Materials and methods

2.1. Cultivation, experimental design and statistics

S. quadricauda (Turp.) Bréb. (Chlorophyta, Chlorophyceae), strain UTEX 76 (originated from The University of Texas, Austin) was cultured under sterile conditions on Petri dishes in cultivation room (25/20 °C day/night) at PAR ~30 μmol m^{-2} s^{-1} in 'Milieu Bristol' medium containing inorganic salts (in mg L^{-1}: 750 $NaNO_3$, 175 KH_2PO_4, 75 K_2HPO_4, 75 $MgSO_4{\cdot}7H_2O$, 25 $CaCl_2{\cdot}2H_2O$, 20 Fe-EDTA, 20 NaCl, 2.86 H_3BO_3, 1.81 $MnCl_2{\cdot}4H_2O$, 0.22 $ZnSO_4{\cdot}7H_2O$, 0.1 $CoCl_2{\cdot}6H_2O$, 0.08 $CuSO_4{\cdot}5H_2O$ and 0.052 $Na_2MoO_4{\cdot}2H_2O$, pH corrected to 6.5), glucose (2%), casein hydrolysate (1%) and solidified with 1% agar. Algae were collected from the surface of cultivation medium 4–5 weeks after inoculation, weighted and resuspended in 5 mM HEPES buffer (pH 6.5) in order to achieve 0.2 g fresh weight (FW) algal biomass/50 mL of buffer. Exposure was realized using 50 mL volume of experimental solutions in screw-cap tubes (Sarstedt, Nümbrecht, Germany). Trivalent and hexavalent chromium was added in the form of $CrCl_3{\cdot}6H_2O$ and $K_2Cr_2O_7$ (Lachema Brno, Czech Republic) in the final concentrations 1 and 10 μM of Cr. After 24 h of exposure to these treatments, samples were centrifuged (5 min, 2000 rpm), washed twice with HEPES buffer and algal pellet was extracted with respective solvents mentioned below. Processing of samples for the estimation of enzymes, metabolites and TTC test involved cold mortar and pestle with the addition of inert sand (to achieve complete cell disruption). Subsequent experiment studied the impact of composition of exposure solution and its pH on algae exposed to 10 μM Cr(VI) over 24 h: distilled water, HEPES buffer and 'Milieu Bristol' medium mentioned above (only inorganic salts without addition of organic compounds) with various pH (4.5, 6.5 and 8.5) were used. Algae pre-cultured on solid medium as mentioned above were harvested and resuspended in these three treatment solutions (0.2 g FW per 50 mL) with the addition of 10 μM Cr(VI) and pH was corrected to desired value using HCl/NaOH stock solutions. Additional stress-related parameters, Cr uptake and Cr(VI) reduction were monitored. Further processing was done as mentioned below. One-way ANOVA followed by a Tukey's test (MINITAB Release 11, Minitab Inc., State College, Pennsylvania, USA) was used to evaluate the significance of differences ($P < 0.05$) between treatments. Student's *t*-test (Microsoft Excel) was also used to compare total and intracellular Cr amount in the given treatment. Three individual 50-mL tubes were assessed for each treatment and each parameter (then $n = 3$ in all figures), thus the whole experiment included 159 tubes and 31.8 g FW of algal biomass. Two independent repetitions of the whole experiment were performed in order to check reproducibility.

2.2. Cr quantification and staining

Algal cells were washed with deionised water (total Cr content) or with 5 mM Na_2-EDTA over 20 min (intracellular Cr content) and dried at 70 °C to constant weight. Samples were prepared by mineralization of dry material (~30 mg) in the mixture of concentrated HNO_3 and water (3 + 3 mL) using microwave decomposition (Ethos Sel Microwave Extraction Labstation, Milestone Inc.) at 200 °C over 1 h. Resulting clear solution was quantitatively placed in glass flasks and diluted to a final volume of 10 mL. All measurements were carried out using an atomic absorption spectrometer AA30 (Varian Ltd.; Mulgrave, Australia) and the air-acetylene flame. Measurements of total Cr and K contents were done as described previously (Kováčik et al., 2013). For specific fluorescence visualization of trivalent Cr, two specific recently reported dyes 6-((anthracen-9-yl) methyleneamino)-2H-chromen-2-one (Guha et al., 2012) and naphthalimide–rhodamine (Mahato et al., 2012) were used. Stock solution of 6-((anthracen-9-yl) methyleneamino)-2H-chromen-2-one (compound was synthesized as reported earlier, Guha et al., 2012) was prepared in DMSO and diluted to 10 μM with phosphate buffered saline (PBS, pH 6.8). Alternative Cr(III) staining was performed using specific naphthalimide–rhodamine derivative (kindly provided by Dr. Amitava Das) with 10 μM working solution prepared in PBS (pH 7.4) and excitation/emission according to first report of this reagent (Mahato et al., 2012). For all staining, cells were incubated for 30 min at room temperature (RT) followed by washing in respective buffer.

2.3. Fluorescence and confocal microscopy of viability and oxidative stress

The algal cell viability was assayed using fluorescein diacetate (FDA, $494_{Ex}/521_{Em}$, Sigma-Aldrich, USA) and propidium iodide (PI, $536_{Ex}/617_{Em}$, Sigma–Aldrich, USA). A 20 μL sample of the algal cell suspension culture was diluted to 50 μL by fresh PBS buffer and incubated for 30 min at 25 °C and dark with FDA (final concentration 2.4 μmol L^{-1}) and PI (30 μmol L^{-1}). PI, a nucleic acid stain, penetrates through damaged cell membranes and intercalates the DNA of the cell, so PI positive cells are dead or dying. Living cells metabolize FDA to fluorescein, which emits green light upon excitation. Texas Red emission filter was used to visualize autofluorescence of chlorophyll by fluorescence microscopy while this parameter by confocal microscopy was monitored at $450_{Ex}/630–680_{Em}$. Reactive oxygen species (ROS) were stained using 2′,7′-dichlorodihydrofluorescein diacetate (DCF-DA, $502_{Ex}/526_{Em}$, Life Technologies,

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