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Phosphorus availability changes chromium toxicity in the freshwater alga *Chlorella vulgaris*

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HIGHLIGHTS

- Chromium (Cr) is one of the most serious pollutants in aquatic systems.
- Cr greatly inhibited *Chlorella vulgaris* growth in a dose-dependent manner.
- Cr exposure changed the metal ion and anion absorption profiles, and disrupted cell substructure.
- High P could alleviate the toxicity of Cr by decreasing Cr absorption.

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ABSTRACT

Chromium (Cr) is one of the most serious pollutants in aquatic systems. This study examined the relationship between the toxic effects of Cr on the freshwater alga *Chlorella vulgaris* and phosphorus (P) availability on the algal physiology and ultrastructure. Cr inhibited *C. vulgaris* growth in a concentration- and time-dependent manner, and its inhibitory effect was related to the P concentration. In a low-P medium, Cr showed approximately 2.2–3.7-fold stronger toxicity than in a high-P medium. Cr was absorbed into the algal body where it disrupted the chloroplast structure and decreased the chlorophyll content. However, Cr had a weaker chlorophyll inhibitory ability and destructive power against the chloroplasts in the high-P medium than in the low-P medium due to the partial blockage of Cr absorption in high P-medium. Cr exposure also changed the metal ion and anion absorption profiles, which was also closely related to the concentration of P. Cr treatment increased the volume of the vacuole, and the larger vacuole reduced the space available for chloroplasts, as based on optical and electron microscopy results, but a higher P availability could alleviate this damage. These results suggest that high P alleviated the toxicity of Cr by decreasing Cr absorption and increasing the absorption of beneficial ions. It is, therefore, necessary to consider the phosphorus availability when the toxicity of metal compounds is evaluated.

1. Introduction

The contamination of freshwater systems with trace metal compounds has increased worldwide because of increased anthropogenic activities, such as mining operations, metal-refining industries, boating activities, agricultural fungicide runoff and domestic garbage dumps (Schutzendubel and Polle, 2002; Ruangsomboon and Wongrat, 2006). In contrast to other environmental pollutants, metal compounds are not degraded in the ecosystem and accumulate in organisms, persistently affecting their growth and causing ecological disturbances (Scoccianti et al., 2008). Chromium (Cr) is the seventh most abundant metal in the Earth's crust (Katz and Salem, 1994), but it is not essential for any metabolic process and has no biological function in most organisms. Cr is commonly found in water, soil and air ecosystems due to its wide usage in the leather tanning, textile and pigment-electroplating industries (Zayed and Terry, 2003; Rai et al., 2004; U.S. EPA, 2010). With regard to non-polluted waters, the concentration of Cr was only 1–10 μM in freshwater and 0.03–1 μM in oceanic water, but its level increased up to 48 mM in wastewater (Cervantes et al., 2001). The increasing contamination of Cr in freshwater is a major environmental problem due to its impact on aquatic organisms and on human health through unsafe drinking water (Schwarzenbach et al., 2006).

Cr exerts phytotoxicity on multiple levels, from reduced biomass by affecting vegetative growth to the inhibition of enzymatic activities and inducement of mutagenesis. It has been reported that the mechanism of Cr phytotoxicity is through the decrease of photosynthesis efficiency and electron transfer rates, producing reactive oxygen species (ROS) to induce oxidative stress (Deng et al., 2006; Gorbi et al., 2006; Takami et al., 2012). Microalgae

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are organisms at the bottom of the food chain, are of vital importance in the primary production of aquatic ecosystems, and are regarded as model organisms that are sensitive to pollutants. Recent studies reported the toxicity of Cr to physiological and biochemical processes in algae (Hörcsik et al., 2007; Vignati et al., 2010). Volland et al. (2012) proposed that Cr could harm the metabolism and development of the unicellular alga *Micrasterias*. A few studies have demonstrated that metal compounds likely affect algal growth by altering the ionomic uptake (Webster et al., 2011; Volland et al., 2012), which is also involved in multiple biochemical and physiological processes (Baxter et al., 2008).

In this study, we analyzed the toxicity of Cr on the cellular substructure of *Chlorella vulgaris* after a short-term (4 d) exposure. The toxicity of Cr in the organism was determined by metal speciation, which is responsible for the mobilization, subsequent uptake and resulting toxicity of Cr (Shanker et al., 2005). In biological systems, the trivalent Cr (III) and the hexavalent Cr (VI) species are the most stable forms, whereas the other valences of Cr are unstable and short-lived. Some reports showed that Cr (VI) is the most toxic form of Cr and usually occurs in association with oxygen as chromate (CrO_4^{2-}) or dichromate ($Cr_2O_7^{2-}$) oxyanions (Shanker et al., 2005; Sinha et al., 2005). Therefore, we selected $Cr_2O_7^{2-}$ as the Cr reagent used to investigate its toxicity. *C. vulgaris* is a common freshwater unicellular green alga that exists ubiquitously throughout freshwater environments worldwide. As one of the primary producers of organic compounds, *C. vulgaris* plays crucial roles in aquatic and terrestrial ecosystems and is a representative aquatic phytoplankton species for environmental toxicology research (Qian et al., 2008b). To more closely reflect the true freshwater scenario, we analyzed the toxic effect of Cr in combination with external phosphorus (P), given that P is likely the main limiting nutrient in freshwater systems (Wetzel, 2001) and the amount available is dependent on the environment.

2. Materials and methods

2.1. Algal culture

The *C. vulgaris* strain used in the present study was purchased from the Institute of Hydrobiology, Chinese Academy of Sciences. The algae were grown photoheterotrophically in revised Shuisheng-4 medium, as defined by Zhou and Zhang (1989), which is mainly composed of the following components (mg L^{-1}): $(NH_4)_2SO_4$, 660; $MgSO_4{\cdot}7H_2O$, 82; $NaHCO_3$, 100; KCl, 25; $FeCl_3$, 15; $CaCl_2$, 8.58 and 1 mL L^{-1} A5. The A5 is composed of the following components (g L^{-1}): H_3BO_3, 2.86; $MnCl_2{\cdot}4H_2O$, 1.86; $ZnSO_4{\cdot}7H_2O$, 0.22; $Na_2MoO_4{\cdot}2H_2O$, 0.39; $CuSO_4{\cdot}5H_2O$, 0.08; $Co(NO_3)_2{\cdot}6H_2O$, 0.05. The inorganic phosphate (K_2HPO_4) in this medium was categorized into three concentrations, 10 μM, 100 μM and 1000 μM, defined as low, middle and high concentrations of P, according to Webster et al. (2011), and the medium of pH was adjusted to 7.4. The algae were cultured at 25 ± 0.5 °C, with a light illumination of approximately 2500 Lux under a 12:12-h light–dark cycle. Algal cells in the exponential growth phase were used for all of the experiments, and the initial cell density for each experiment was approximately 3.5×10^5 cells mL^{-1}. The algal cell culture density was monitored spectrophotometrically at 685 nm. The density of algal cells ($Y \times 10^5$ mL^{-1}) and OD_{685} (X) was established as $Y = 162.1X + 1.3463$ ($r^2 = 99.34\%$), which was defined in our previous report (Qian et al., 2008b).

2.2. Test chemicals and Cr concentration determination

Potassium dichromate ($K_2Cr_2O_7$, Wuxi Haishuo Biology Co., China, reagent grade, 99.8% purity) was prepared as an aqueous solution. The concentration–response curves for Cr are shown in Fig. S1 for the 100 μM P concentration. Based on these curves, we selected two concentrations of Cr (50 and 100 μM) to study Cr toxicity at different P concentrations, and the inhibitory ratio of *C. vulgaris* after 96 h of exposure to these two Cr concentrations was 22.8% and 40.9%, respectively. Four replicates were performed for each bioassay.

2.3. Pigment assays

A 10-mL sample of each culture was collected to analyze the chlorophyll content after 48 and 96 h of Cr exposure, and N,N-dimethylformamide was used to extract the chlorophyll from *C. vulgaris*. Spectrophotometry was used to measure the content of chlorophyll a (Chl a), chlorophyll b (Chl b) and total chlorophyll (total Chl) according to the method of Inskeep and Bloom (1985).

2.4. Cellular elemental absorption changes in response to Cr exposure

C. vulgaris was cultured in Shuisheng-4 medium with a high P concentration (1000 μM) to the exponential growth phase (OD_{685} = 0.08). All of the algae were washed with medium without P to remove the residual P and then cultured in medium at the different concentrations of P (10 μM, 100 μM and 1000 μM) to OD_{685} = 0.08. Cr was added to a final concentration of 100 μM in the cultural medium, and the cells were exposed for 48 and 96 h. Four replicates of each bioassay were performed. These samples were washed with EDTA and Milli-Q water to remove the externally bound metals and then were used to measure the metal ions and anions. We used Dionex ICS-2000 Reagent-Free™ Ion Chromatography (RFIC) to measure the level of anions in the medium supernatant with or without algae after culturing for 48 and 96 h. The sample D-value reflected the anions absorbed by the algal cells. The metal ions were measured using an inductively coupled plasma mass spectrometer (ICP-MS, Agilent 7500a, USA) after the algal cells were digested with HNO_3 at 110 °C for 1 d, as described in our previous report (Qian et al., 2011).

2.5. Electron microscopy analysis

Samples from the control (grew in 10 and 1000 μM P medium for 96 h without Cr treatment) and Cr-treated algae (exposed to 100 μM Cr for 96 h in 10 and 1000 μM P medium) were fixed and embedded as described in our previous reports (Qian et al., 2008a). The samples were then cut into ultra-thin sections (70–90 nm) using a Reichert Ultracuts ultramicrotome and stained with uranyl acetate, followed by lead citrate. Transmission electron microscopy (TEM) using a JEM-1230 microscope (JEOL Ltd., Tokyo, Japan) was used to observe the substructure of the algae.

2.6. Data analysis

The data are presented as the mean ± standard error of the mean (SEM). The intergroup differences were assessed using a one-way analysis of variance (ANOVA), followed by the LSD post hoc test. A two-way ANOVA was performed to assess the individual effects of P and Cr on ion absorption. All of the statistical analyses were performed using SPSS 13.0 (SPSS, Chicago, IL, USA) and Origin 7.0 (OriginLab, Northampton, MA, USA). Values were considered significantly different when p was less than 0.05.