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Title: Cyto-histological and morpho-physiological responses of common duckweed (Lemna minor L.) to chromium

Article Type: Research paper

Section/Category: Environmental Toxicology and Risk Assessment

Keywords: Chromium; growth inhibition test; Lemna minor; photosystem II; plastid; starch.

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Response to Reviewers: Reviewer #2:
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Basically they planned properly the experiment with different chromium treatments but there is no description whether they performed only a single experiment or the reported results came from a representative experiment.
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It is true, we corrected the mistake.
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* We reported the details required about the experimental set-up in the ‘Material and Methods’ section (Lines 129-145).
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extent to which Co2+ altered starch accumulation in L. minor clone 9441 and attempted to determine the underlying mechanism(s). Why Co2+ ?
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General remarks
The manuscript fits the aims and scope of the Environmental Toxicology and Risk Assessment section of Chemosphere. Its questions are clearly addressed and the applied methods are appropriate to answer them. The results are well documented and the conclusions are in line with them. The manuscript, however, needs thorough improvement from several aspects before its publication. The most basic weaknesses are as follows:
- The 'Materials and Methods' and 'Results' chapters suggest that the treatments were conducted only once with three parallel samples (see specific notes). Standardized laboratory experiments can be easily reproduced. The findings of the study would be better supported if the experiments were replicated and at least the growth rates were analyzed in larger sample numbers.
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- Line 64: Terminologically FM is the maximal fluorescence yield of a dark adapted plant sample measured during a single saturation pulse. According to the subchapter '2.1 Maximum quantum yield of photosystem II' ('Materials and Methods') this parameter was measured instead of the light adapted FM' as it was indicated in the 'Abbreviations'.
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Dear Editor

I would like to submit the revised manuscript "Effects of chromium on the growth of common duckweed (*Lemna minor* L.) " (Ref.: Ms. No. CHEM37218) for publication in "Chemosphere". We appreciated the constructive comments of the reviewers, which have greatly improved the article. We modified our paper according to their suggestions and now we hope that it is suitable for publication.

Our responses to the comments of the single reviewers are reported in the response to reviewers.

Best regards,

Lara Reale

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Cyto-histological and morpho-physiological responses of common duckweed (Lemna minor L.) to chromium~~Effects of chromium on the growth of common duckweed (Lemna minor L.)~~

L. Reale[1*], F. Ferranti[1], S. Mantilacci[2], M. Corboli[2], S. Aversa[2], F. Landucci[3], C. Baldisserotto[4], L. Ferroni[4], S. Pancaldi[4], R. Venanzoni[1]

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[2] Biotecnologie B.T. Srl, Frazione Pantalla di Todi, 06059 Perugia, Italy

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[4] Department of Life Sciences and Biotechnology~~ies~~, University of Ferrara, C.so Ercole I d'Este, 32, 44121 Ferrara, Italy

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Abstract

Along ~~Chromium represents~~ with cadmium, lead, mercury and other heavy metals, chromium is an important environmental pollutant, mainly concentrated in ~~the~~ areas of intense anthropogenic pressure. ~~Plant tolerance to heavy metals requires morpho-physiological mechanisms that are still poorly understood, especially in aquatic plant species. We verified t~~The effect of potassium dichromate on *Lemna minor* populations was tested using the growth inhibition test. ~~but also c~~Cyto-histological and physiological analys~~e~~is were also conducted to aid in~~, which enabled us to~~ understanding the strategies ~~utilized~~ used by ~~the~~ plants during ~~the~~ exposition~~ure~~ to ~~the~~ chromium. ~~The t~~Treatment with potassium dichromate ~~determined~~ caused a reduction in growth rate and frond size in all treated plants and especially at the highest concentrations ~~of the pollutant~~. ~~Significant variations in leaf size were observed only at the highest concentration of the potassium dichromate; a~~At th~~e~~cs~~e~~ concentrations the ~~alteration of the~~ photosynthetic pathway was also altered as shown by ~~demonstrated by~~ the decrease of maximum quantum yield of photosystem II and the chlorophyll *b* content and by the chloroplast ultrastructural modifications. ~~The s~~Starch storage was also investigated by microscopic~~al~~ observations~~,~~. ~~I~~It was the highest at the high concentrations of the pollutant. The data suggested a correlation between ~~the~~ starch storage and ~~the~~ reduced growth; there was greater inhibition of plant growth than inhibition of photosynthesis, resulting in a surplus of carbohydrates that may be stored as starch ~~Plants under pollution stress reduced respiration and consequently required less photosynthate. The lower request for triose determined the higher storage of starch, therefore the availability of triphosphate for the production of ATP decreased and the photosynthesis was further slowed. Our~~ The investigation helps to understand the mechanism related to heavy metal tolerance of *Lemna minor* and supplies information about the behavior of this species widely used as a biomarker.

Key words*:* Chromium; growth inhibition test; *Lemna minor*; photosystem II; plastid; starch.

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Abbreviations

Car	Carotenoids
Chl	Chlorophyll
Cr	Chromium
Cr^{III}	Chromium (III)
Cr^{VI}	Chromium (VI)
EDX	Energy dispersive X-ray analysis
FM	Maximum fluorescence in the dark-adapted state~~FM Maximum fluorescence in the light-adapted state~~
F0	Minimum fluorescence in the dark-adapted state
~~F_0 Minimum fluorescence~~	
F_V	Variable fluorescence
PAM	Pulse amplitude modulated fluorimetry
PSII	Photosystem II
SEM	Scanning electron microscopy
TEM	Transmission electron microscopy

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1. Introduction

~~Ions and n~~Non-essential heavy metals, such as cadmium (Cd), chromium (Cr), lead (Pb) and mercury (Hg) are very important environmental inorganic pollutants, concentrated in areas characterized by the presence of waste products of many industrial processes. ~~Heavy metals produce toxic effects for the plants due to interactions they establish with the cellular components via covalent and/or ionic bonds which are the result of the cellular metabolic activity alteration (Bruins et al, 2000).~~

Cr ~~is a widespread contaminant entering the air, water and soil environment through different industrial activities such as iron and steel manufacturing, chromium leather, chromium plating, wood preservation and other anthropogenic activities. It~~ exists in the environment in two stable forms: chromium (III) and chromium (VI) ~~originated~~ originating ~~through~~ from natural processes and human activities.

~~Cr^{VI} compounds are highly reactive, mobile and easily soluble in water. These properties cause several environmental health risks, because Cr^{VI} compounds are highly toxic for aquatic organisms and are accumulated by their bodies.~~

The phytotoxicity of both Cr^{III} and Cr^{VI} has been studied in many higher and lower plants. Cr^{VI} is more phytotoxic than Cr^{III} (Han et al., 2004) and retards growth, reduces the number of palisade and spongy parenchyma cells of leaves, and increases the number of vacuoles and electron dense material along the walls of xylem and phloem (Han et al., 2004). Cr phytotoxicity can also ~~result in inhibition~~inhibit ~~of~~ seed germination, degrad~~eation of~~e pigment status, alter ~~ration of~~ nutrient balance~~, and, modify~~modify antioxidant enzymes activity~~,~~ and induce ~~tion of~~ oxidative stress in plants (Poschenrieder et al., 1991, Barcelo and Poschenrieder, 1997, Panda and Choudhury, 2005). Apart from these effects, Cr can also alter the chloroplast and membrane ultrastructure in plants (Bassi et al., 1990, Panda and Choudhury, 2005). ~~More in~~In greater detail, Cr inhibits photosynthesis and the PSII is known to be the main target, also in relation to structural changes within the PSII complex (Fasulo et al., 1983; Bishnoi et al., 1993; Davies et al., 2002; Shanker et al., 2005; Ait Ali et al. 2006; Rocchetta et al., 2006; Olah et al., 2010). ~~The uptake of Cr^{VI} is thought to be an active mechanism performed by carriers for the uptake of essential elements such as sulphate (Kim et al., 2006; Cervantes et al., 2001). Cr competes with Fe, S, and P for carrier binding (Shanker et al., 2005). The h~~Heavy metals ~~cannot~~ must be extracted from polluted areas but they cannot be degraded in the environment like other organic xenobiotics ~~but they must be extracted from a polluted area~~ (Augustynowicz et al., 2010). ~~Methods u~~Using living plants to remove metal ions from a polluted area with organic and inorganic compounds ~~are~~ is commonly called phytoremediation.

One of the most important aquatic famil~~ies~~y in phytoremediation research is Lemnaceae. Members of Lemnaceae, especially *Lemna minor*, are now test organisms ~~also being commonly~~

~~used for routine ecotoxicological risk assessments~~extensively used ~~as~~for assessing the potential impact of environmental chemicals in ecotoxicology and plant physiology. The International Organization for Standardization (ISO) and the Organization for Economic Co-operation and Development (OECD) have developed standard growth inhibition tests using duckweed~~s~~, namely *L. minor* (clone St; clone no. 9441) and *L. gibba* (clone G3; clone no. 9260), respectively (ISO, 2004; OECD, 2004). Being important elements in primary production and in the food chain, sensitivity of such aquatic macrophytes to various toxic chemicals may impact the functioning of the whole aquatic ecosystem.

Unpredictable industrial accidents ~~could~~ can result in high loads of toxic chemicals entering ~~to~~ the environment within short time intervals as happened to River Tisza in Hungary in 2000 when heavy-metal and cyanide contamination entered the river ~~and~~ caus~~ed~~ing an ecological catastrophe (Lakatos *et al*. 2003). Hence, ~~For such considerations~~ it is essential to predict the possible effects of toxic substances on vital processes and species composition of aquatic biota.

~~Duckweed species are extensively used test organisms for assessment of potential impact of environmental chemicals in ecotoxicology and plant physiology (Environment Canada 1999).~~

Duckweed~~s~~ ~~are~~ is a freefloating plant~~s~~ ~~showing~~ with wide distribution in different types of aquatic ecosystems. In spite of their small size, they exhibit ~~large~~ great potential for vegetative reproduction and thereby rapid biomass growth, in fact they are known to be the fastest growing angiosperms (Ziegler *et al*., 2015). ~~Being important elements in primary production and food chain, sensitivity of such aquatic macrophytes to various toxic chemicals may impact the functioning of the whole aquatic ecosystem.~~

Olah *et al*., (2010) suggested that various duckweed species respond with different sensitivity to the same ~~ambient~~ environmental concentrations of $Cr^{(VI)}$~~)~~ in the growth medium, and presumably to other environmental stresses too~~.~~~~,~~ ~~which~~ This may have an influence on their competitive relations when heavy metal pollution occurs in an aquatic ecosystem.

In *L. minor* plants exposed to chromate (Appenroth *et al*., 2003) and nickel (Xyländer *et al*., 1993; Appenroth *et al*., 2010), one of the evident effects was starch accumulation in plastids ~~was one of the evident effects~~.

High biomass production, especially when rich in starch, is of immense biotechnological importance (Sree and Appenroth, 2014; Zhao *et al*., 2015; Ziegler *et al*., 2015). In the present paper the extent to which Cr^{VI} altered starch accumulation in *L. minor* ~~we~~ was investigated ~~the extent to which $Co2^{+}$ altered starch accumulation in *L. minor* clone 9441~~ and we attempted to determine the underlying mechanism(s).

We investigated the effects of $Cr^{(VI)}$~~)~~ treatments on *Lemna minor* plants taking into account ~~also~~ the photosynthetic and cyto-histological parameters, which ~~was~~ in the past had been studied independently~~,~~ and, for the first time to our knowledge, microanalysis of the frond sections was ~~carriyng~~ carried out ~~for the first time, at our knowledge, the~~ ~~microanalysis on the leaf sections~~.

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2. Materials and Methods

2.1 Plant material

The organisms were originally supplied by the "Friedrich-Shiller University of Jena - Botanic Institute" and the stock cultures were subcultured in the Test Facility BioTecnologie B.T.. The test was performed according to OECD Guideline for the testing of chemicals n° 221 ("Lemna sp. Growth Inhibition Test", adopted on 23 March 2006).

Duckweed colonies with total of 12 fronds were taken from the stock culture and placed in crystallizer glass dishes with 100 mL nutrient solution (SIS growth medium, pH 6.5±0.2), containing different concentrations of potassium dichromate [0.50 mg/L (C1); 0.93 mg/L (C2); 1.73 mg/L (C3); 3.22 mg/L (C4); 6.00 mg/L (C5)]; an untreated group was prepared using 100 mL of SIS growth medium.

Expressed as concentrations of chromium, the test concentrations were the following: 3.4 µM (C1); 6.3 µM (C2); 11.7 µM (C3); 21.9 µM mg/L (C4); 40.7 µM (C5).

Cultures were grown in an incubator chamber at 24±2°C and continuous illumination in the range 6500 - 10000 Lux.

The toxicity of potassium dichromate was assessed after seven days of exposure, under static conditions.

Three replicates (test vessels) were carried out for each untreated and treated group.

~~The plants were grown in the lab of "Biotecnologie B.T. Srl", Todi (Italy), in the Swedish Standard culture medium as required in the "OECD Guidelines for the testing of chemicals" (OECD 221). Temperature was maintained at 24 ± 2 °C and plants were exposed to a light intensity ranging from 6500 to 10000 Lux. Plants were treated with five different concentrations of potassium dichromate [0.5 mg/L (C1); 0.93 mg/L (C2); 1.73 mg/L (C3); 3.22 mg/L (C4); 6 mg/L (C5)] for seven days. A control was also prepared of untreated *Lemna minor* plants kept in the same conditions. Three replicates were carried out for each untreated and treated group.~~ The experiments were repeated three times and the results of one representative experiment are reported in this paper.

2.2 Calculation of growth rates

The average specific growth rate (µ) for a specific period (from time *i* to time *j*) was calculated as the slope of the logarithmic growth curve from the equation:

$$\mu = \frac{\ln N_{tj} - \ln N_{ti}}{t_j - t_i}$$

where:

- µ : average specific growth rate from time *i* to time *j*

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- N_{ti} : number of fronds observed in the test or control vessel at time *i*

- N_{tj} : number of fronds observed in the test or control vessel at time *j*

- *ti* : ~~moment~~ time at the start of the period

- *tj* : ~~moment~~ time at the end of the period

The "*i*" corresponds to the start of experiment while the time "*j*" corresponds to seven days of treatment with potassium dichromate. Growth rates used for the calculation of inhibition ~~were~~ are usually given as the average of 3 independent ~~experiments~~ replicates (*n*=3). ~~Errors given are standard errors of the mean or percentage of confidence interval (level 95%).~~

2.3 Determination of ~~leaf~~frond size

Images of the ~~leaves~~fronds were taken using a stereo-microscope and quantified by the Leica IM 1000 software.

2.4 Cyto-histological observations

To obtain semi-thin and ultra-thin sections, ~~leaf~~ portions of fronds were fixed in 3% (w/v) glutaraldehyde in 0.075 M phosphate buffer, pH 7.2, for 5h. The samples were then washed four times for 15 min in 0.075 M phosphate buffer, pH 7.2, post-fixed in 1% (w/v) OsO_4, dehydrated in increasing concentrations of ethanol and, finally, embedded in resin (Epon, 2-dodecenylsuccinic anhydride, and methylnadic anhydride mixture) (Reale et al. 2014). The semi-thin sections (1–2 µm) were cut with an ultramicrotome (OmU2, Reichert, Heidelberg) equipped with a glass blade, stained with 0.5% (w/v) toluidine blue in 0.2 % $NaHCO_3$ buffer and observed under a light microscope (DMLB, Leica, Wetzlar, Germany). Semi-thin sections were also stained by Periodic Acid Schiff's reaction (O'Brien and McCully, 1981). ~~Semi-thin sections were placed in 0.5% periodic acid for 30 min, rinsed with tap and demineralised water and placed in Schiff's reagent for 15 min; finally sections were washed with tap water, SO2 water and then with demineralized water.~~ Slides were observed under a light microscope; the presence of starch was indicated by a magenta colour, while proteins appeared blue.

2.5 Evaluation of starch content

To evaluate the starch content, semi-thin sections of ~~leaves~~fronds were treated with sodium metoxide and methanol to remove the resin. After deresination, sections were rehydrated and treated with an iodine-potassium iodide solution (Johansen 1940) and observed under a light microscope. Starch grains appeared ~~blue~~ dark blue ~~colored~~. To quantify starch content, the image of each section was analysed by the image processing program "ImageJ" (Abramoff *et*

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al., 2004). The amount of starch is reported as relative units (pixel μm^{-2}), calculated as the number of pixels, measured by the software, with respect to the surface observed (μm^2).

2.6 Photosynthetic pigment analysis

Samples were cut into small pieces and extracted with 80% acetone. Extracts were maintained at 4 °C until ~~analyses~~ analysis and all manipulations ~~had been~~were performed in dim green safe light to avoid photo-degradation.

Absorption spectra (400‑750 nm range) of extracts were recorded at room temperature (25 °C) by the spectrophotometer. For Chls and carotenoids determinations, the extracts were measured at 661 nm (Chl a), 644 nm (Chl b), and 470 nm (carotenoids) and pigment concentrations were evaluated as mg/g of fresh substance~~s~~ according to the equations proposed by Wellburn (1994).

2.7 Maximum quantum yield of photosystem II

A portable pulse amplitude modulated (PAM) fluorometer (ADC OS1-FL, ADC BioScientific Ltd., Hoddesdon, UK) was used for *in vivo* measurements of chlorophyll fluorescence. Fluorescence measurements were performed on ~~leaves~~fronds positioned onto a wet filter paper placed on ~~the floor of~~ the clip floor of the instrument. T~~In t~~he background fluorescence level F_0 was measured on the ~~leaves~~fronds adapted to darkness for at least 20 min~~, the background fluorescence level F_0 was measured~~. The maximum fluorescence level of PSII (F_M) was obtained by exposing the sample to a saturating pulse of white light (0.8 s). The PSII maximum photochemistry was measured using the variable fluorescence ratios $F_V/F_M = (F_M - F_0)/F_M$ and $F_V/F_0 = (F_M - F_0)/F_0$ (Lichtenthaler et al. 2005; Ferroni et al., 2013).

2.8 TEM analysis

T~~The t~~hin sections (0.08 µm) were cut with an ultramicrotome (OmU2, Reichert, Heidelberg) equipped with a glass blade, mounted on uncoated copper grids (200 mesh) and contrasted by adding uran~~yi~~l~~e~~ acetate and an aqueous solution of lead nitrate. Observations were carried out with a transmission electron microscope (TEM 400 T; Philips, Monza, Italy).

2.9 Scanning electron microscopy (SEM) and energy dispersive X-ray (EDX) microanalysis

Semi-thin sections were dehydrated and metalli~~s~~z~~z~~ed with graphite by "Q150T Turbo-Pumped Sputter Coater/Carbon Coater" (Quorum Technologies Ltd., Laughton, United Kingdom); EDX microanalysis was performed in a Zeiss SEM LEO 1525 (LEO Electron Microscopy Inc., One Zeiss Drive, Thornwood, NY).

2.10 Statistical analysis

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263 Experiments and assays were carried out with three replicates unless otherwise indicated and
264 data were averaged with standard error or standard deviation. P-value was obtained by t-test t
265 with $P < 0.01$ or $P < 0.05$ considered to be significant. To perform the statistical analysis, the
266 Analysis ToolPak, an Excel add-in program, was used.
267

3. Results

3.1 Growth rates and ~~leaf~~frond size

The average specific growth was calculated to investigate the influence of potassium dichromate ~~in~~ on the growth of *Lemna minor* (~~Tab~~Fig. 1A). After 7 days of treatment, ~~an inhibition of~~ growth in the treated samples was ~~observed~~ inhibited ~~in the treated samples~~ compared to the control. ~~This d~~Decrease in the growth rate was evident starting from the lowest concentration of potassium dichromate (0.50 mg/L) and ~~becomes~~ became more pronounced ~~rising from the lowest to~~at the highest concentrations. The effects on growth rate ~~are the~~were ~~n~~-dose-dependent and correlated to the increase ~~of~~ in the concentration of the heavy metal. After 7 days of treatment the ~~leaf~~frond size in all treated samples was lower than in the control (Fig. ~~1~~1B).~~.~~ ~~In samples treated with concentration of potassium dichromate between 0.5 to 3.22 mg/L (from C1 to C4), leaf size was very similar while I~~in samples treated with 6 mg/L (C5) ~~the~~ decrease ~~of~~ in the ~~leaf~~frond size was more accentuated.

3.2 Cyto-histological observations

The cyto-histological structure of the control and treated ~~leaves~~fronds was investigated by ~~observation~~ observing ~~of~~ semi-thin and thin sections -under light and electron microscope, respectively. In the control and treated samples, ~~leaves~~fronds ~~showed the~~had an anatomical structure typical of aquatic plants; there were two single layered epidermises ~~were observed~~ with an aerenchyma rich ~~of~~ in intercellular spaces (Fig. ~~2A~~2A). In the control ~~leaves~~fronds, parenchymatic cells were rich- in chloroplasts, which had a lenticular shape and were arranged close to the cell wall (Fig. ~~2B~~2B, C); in these chloroplasts thylakoid membranes were perfectly ~~conformed~~ arranged and organized in grana, little starch grains were also present, as shown by semi-thin sections stained with Schiff's reagent (Fig. ~~2C~~2C) and TEM pictures (Fig. ~~2D~~2D). In treated ~~leaves~~fronds chloroplast maintained their conformation in C1, C2 and C3 samples, however after treatment with the highest concentration of potassium dichromate (C5, 6 mg/L), chloroplasts were very similar to amiloplast with a reduced presence of stroma, a thylakoid system constituted by few grana (Fig, ~~2E~~3E, F, G) and big starch grains.

3.4 Evaluation of starch content

The presence of starch was ~~detected~~ determined by staining with the iodine-potassium iodide solution. In the control ~~leaves~~fronds, starch was poor and ~~constituted~~ consisted ~~by~~ of little starch grains inside plastid perfectly ~~conformed~~ organized (Fig. ~~2H~~2H). In treated ~~leaves~~fronds, starting from lower concentrations of potassium dichromate, starch grains were more abundant than in the control samples; the presence of starch increase from ~~treated~~ treatment C1 (Fig, ~~2I~~2I) to ~~treated~~ C4 (Fig. ~~2J~~2J) and C5 (Fig. ~~2K~~2K). In ~~treated~~ C5 starch grains completely occupied plastids that had lost their original shape and appeared similar to amyloplasts (Fig. ~~2L~~2L). The

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amount of starch in the control and treated fronds, expressed by relative units (pixel μm^{-2}), is reported in Table 1; the quantitative analyses confirmed the qualitative observations, that the presence of starch increased from control to treated samples, with statistically significant differences in samples C3, C4 and C5.

3.5 Physiological analysis

Data from pigment analysis of control and treated plants ~~were~~ are reported in Fig. ~~3~~3A. The content of chlorophyll *a* was variable but not significantly different in the control or treated plants~~,~~. ~~the~~ The same was true ~~consideration is valid~~ for the carotenoid content. The content of chlorophyll *b* decreased in ~~treated C4 and~~ C5 and was significantly lower than in the control; in C1, C2, ~~and~~ C3 and C4~~treated samples~~, however, the chlorophyll *b* content did not significantly differ from the control.

As shown by the graphs in ~~by the graphs (~~Fig. ~~4~~3B~~)~~, the effect of potassium dichromate ~~in~~ on the maximum efficiency of PSII ~~(photosystem II)~~ was dose-dependent, and clearly more pronounced at the higher concentrations of chromium. In the C1 samples, which were treated with the ~~lower~~ lowest concentration of potassium dichromate, there were no significant effects in terms ~~were observed in terms~~ of F_V/F_M ratio. For those samples the values of F_V/F_M (0.737) were not significantly different from those of the control (0.775; $p = 0.2189$). However, starting from C2 samples, there were increasing differences ~~were observed,~~with C4 and C5 being the most affected ones with F_V/F_M being below 0.700 (Fig. ~~4~~3B). The PSII photochemistry was also estimated as a F_V/F_0 ratio since it shows a wider dynamic range of variation with respect to F_V/F_M, and thus is more sensitive to highlight the effect of stress (Lichtenthaler et al., 2005). Considering the F_V/F_0 ratio, ~~more~~ the most significant differences from the control were in samples~~and~~ C2 to C5 ~~treated sample were observed~~ (Fig. ~~4~~3B). For both values of variable fluorescence ratios, the most marked differences compared to the control were in C3 and even more so in C4 and C5 samples~~, the most marked differences compared to the control were observed~~.

3.6 Microanalysis

The microanalysis was carried out in sections obtained from different portions of ~~leaves~~fronds of treated and control plants. In the control and C1 samples no traces of Cr~~Cr~~ were observed (Fig. ~~5A~~4A). In ~~the~~ samples C2, C3, C4 (Fig. ~~5B~~4B) and C5 there were only small traces of Cr~~Cr~~ ~~were observed~~ in increasing amounts; the highest values were observed in ~~the~~ sample C5 (Fig. ~~5C~~4C), which was treated with the highest concentrations of potassium dichromate.

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4. Discussion

Cr is the seventh most abundant metal in the earth's crust and in the hexavalent form is one of the major sources of environmental pollution. The reason of its toxicity in ~~the~~ plant systems~~,~~ appears to be due to its rapid permeability through biological membranes and subsequent interaction with intercellular proteins and nucleic acids.

Our data contribute to understanding the mechanism related to Cr tolerance on the test organism ~~test~~ *Lemna minor*, which is usually used as a biomarker to biomonitor municipal, agricultural and industrial discharge.

The effects of Cr were observed in *L. minor* ~~since~~ from the lowest concentrations, as demonstrated by the decrease of growth rate in all treated samples, but the highest effects were observed at the highest concentration of the heavy metal. The ~~growth~~ reduction in growth was paired to a decrease ~~of~~ in ~~leaf~~frond size, which was more accentuated at the ~~concentration of~~ 6 mg/L, while between 0.5 and 3.22 mg/L (from C1 to C4 samples) ~~leaf~~frond size was more similar to ~~the size~~ that of the control, although always lower ~~than it~~. ~~The d~~Decrease in the number of ~~leaf~~fronds ~~number was~~has also been observed ~~also~~ in other plants treated with Cr such as wheat (Sharma and Sharma , 1993) and bean (Barcelo et al., 1985), ~~while the~~and reduction ~~of~~ in ~~leaf~~frond size has been ~~was already known~~reported in spinach (Singh, 2001). ~~The r~~Reduction of the growth rate and ~~leaf~~frond size may be related at the molecular level to an inhibition of cell division or elongation resulting from oxidative stress (Karuppanapandian and Kim, 2013; Palit et al., 1994). ~~.~~ ~~The r~~Reduction of the growth rate ~~was also observed~~has also been reported in *Lemna minor* plants after treatment with Co^{2+} (Sree at al., 2015). During the ~~the~~ cobalt~~o~~ treatment no differences in the chlorophyll content and PSII activity were observed after 4 days but only after ~~seven~~ 7 days, suggesting that ~~the~~ duckweed growth is initially inhibited to a greater extent than photosynthesis (Sree at al., 2015). However, ~~The~~ the treatment of *Lemna* with Cr^{VI} ~~Cr (VI)~~ affected ~~however~~ the chlorophyll fluorescence parameters also after a short ~~-~~term treatment (48 h ~~H long~~) (Oláh et al., 2010); this treatment did not affected photosyhthetic pigments as strongly ~~as~~ (Sree at al., 2015). Concentrations of Chls and carotenoids decreased in the presence of Cr^{VI}, ~~Cr (VI)~~ but not statistically significant differences were observed in relation to various Cr^{VI} ~~Cr (VI)~~ concentrations. Our data, after seven days of treatment, ~~collected after seven days of treatment~~ confirmed the ~~little~~ small variation in the pigment contents ~~determined~~ caused by the Cr^{VI} treatment ~~of Cr (VI)~~. There was ~~A~~ a reduction in the chlorophyll *b* content ~~was observed in the treated~~of samples C4 and C5 ~~samples,~~ after seven days, while there was no variation ~~was observed about~~in chlorophyll *a* and carotenoid concentrations.

The decrease in chlorophyll *b* could be due to destabilization and degradation of the peripheral PSII antenna complex (Shanker at al., 2005). D ~~The d~~amages of PSII reaction centers ~~were~~ was

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also demonstrated by ~~the the~~ measur~~ement~~ing ~~of~~ the maximum efficiency of PSII. In the ~~the~~ treated plants the ~~observed~~ decrease ~~of~~ in F_V/F_M and F_V/F_0 indicates ~~was representative of a~~ photo-inhibitory damage to the reaction center of PSII. This decline was due ~~Responsible for~~to ~~this decline was~~ the increase ~~of~~ in the minimum fluorescence (F_0) as shown by the comparison of F_V/F_M and F_V/F_0, the latter being much more affected. The increase of F_0 ~~was~~ is considered an expression of irreversible damage to PSII resulting in an increased fraction of energy lost by fluorescence emission, so that leaves suffered from excess ~~of~~ light (Bussotti et al., 2011). It has been reported ~~earlier~~ that hexavalent chromium alters the distribution of excitation energy *via* regulated and nonregulated non-photochemical dissipation (Ali *et al.*, ~~-~~2008; ~~-~~ Perreault *et al.*, 2009). The reduction in photosynthetic activity and chlorophyll content ~~was~~ also ~~paired~~ corresponded to modifications in the plastid structure. Van Assche and Clijster (1983) suggested that the overall effect of Cr ions on photosynthesis and excitation energy transfer could also be due to Cr^{VI} ~~Cr(VI)~~ ~~-~~induced abnormalities in the chloroplast ultrastructure such as ~~-~~a poorly developed lamellar system with widely spaced thylakoid and fewer grana. Starting from the C4 sample the chloroplasts were very similar to amy~~i~~loplast with a reduced presence of stroma, a thylakoid system constituted by few grana and ~~big~~ large starch grains. In the C5 sample the starch grains occupied the plastid almost completely ~~the plastid~~. Our studies confirm~~med~~ the hypothesis of Bassi et al. (1990), who suggested that the presence of chromium ~~damaged~~ damages the plasma membrane and the proplastids fail~~ed~~ to develop in the normal chloroplasts ~~determining~~ resulting in the ~~observed~~ ultrastructural alterations observed.

The accumulation of starch in the plastids of the treated samples could be due to one of the following mechanisms, besides others: (1) inhibition of export of photosynthate from source to sink organs, leading to an accumulation of low-molecular-weight carbohydrates which might serve as substrates for starch biosynthesis; (2) inhibition of plant growth to a larger extent than inhibition of photosynthesis, resulting in a surplus of carbohydrates that may be stored as starch (Sree *et al.*, 2015). Often, starch accumulation under heavy metal stress is considered to be a consequence of the inhibition of carbohydrate transport from leaves, which act as source for non-photosynthetic sinks (Herren and Feller, 1997). Due to the fact that duckweed~~s~~ ~~have~~ has a low level of tissue differentiation, this model (mechanism 1) does not hold true for duckweed~~s~~ (Appenroth *et al.*, 2010), and hence we suggest a correlation between the inhibition of plant growth and the starch storage. ~~-~~Sree et al. (2015) concluded that after exposure with Co^{2+} duckweed growth is initially (four days ~~in experimental setup~~) inhibited to a greater extent than photosynthesis resulting in surplus carbohydrates and starch accumulation; thereafter, photosynthesis declines in the presence of Co^{2+} leading to restricted availability of carbohydrates, while at the same time the initially stored starch is ~~remobilised~~remobilized.

5. Conclusions

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Our data demonstrated that after seven days of treatment the effects of Cr^{VI} ~~Cr (VI)~~ on the functioning of photosynthetic apparatus involve the oxidative damage~~s~~ to photosynthetic pigment rather than ~~the~~ inhibition of synthesis as suggested after a short treatment (~~-~~48 h) by Oláh *et al.* (2010). Cr^{VI} ~~Cr(VI)~~ presumably could not fundamentally interfere with the Chl synthesis, so the measured rise in ~~Fo~~ F_0 values might evolve from inhibited energy transfer from antenna to reaction ~~centre~~center.

Our data also suggested ~~also~~ a correlation between ~~the~~ starch storage and ~~the~~ reduced growth but it would be interesting to measure ~~test~~ the starch content ~~in~~ after a short period of treatment, as has been carried out with Co^{2+} (Sree *et al.*, 2015).

Finally, the microanalysis results suggest~~ed~~ that Cr was accumulated only in small quantities in the ~~leaves~~fronds of *Lemna minor*. Probably, as observed by Nematshahi *et al.* (2012) in *Allium cepa*, Cr is first accumulated in roots, ~~that are~~which was not done ~~is not observed in this work~~in the present study, and successively transported to the aerial part of the plant, where it can have a direct impact on the cellular metabolism of shoots.

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5. Acknowledgements

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Figure Legends

Fig. 1 Growth rates and frond size. A) Average specific growth rate of *Lemna minor* from moment time 0 to 7 (days) at different concentrations of potassium dichromate; B) Frond size in control and treated (C1, C2, C3, C4, C5) samples. Data ± standard error were reported in the graphs. Asterisks indicate significant differences from the control (*, $p \leq 0.05$; **, $p \leq 0.01$)

~~Fig. 1 Leaf size in control and treated (C1, C2, C3, C4, C5) samples. In graphs data ± standard error were reported.~~

Fig.~~2~~ 2 Cyto-histological observations. A-B) Transversal semi-thin sections of control ~~leaves~~fronds stained with toluidine blue;~~,~~ the two epidermis and the parenchymatic tissues, with ~~big~~ large intercellular spaces, are evident; C) Transversal semi-thin section stained with Schiff's reagent, starch grains inside chloroplast appeared purple colored; D) TEM picture of parenchymatic cells of control ~~leaves~~fronds;~~,~~ in chloroplast (black arrows) thylakoid membranes ~~were~~are organized in grana and ~~little~~ small starch grains ~~were~~ are present; E) transversal semi-thin section of ~~leaf~~frond of a C5 sample, chloroplasts (black arrows) were very similar to ~~amiloplast~~amyloplast; F-G) TEM pictures of parencymatic cells of C5 samples in which few grana and big starch grains ~~were observed~~can be seen; H-L) Transversal semi-thin sections of ~~leaves~~fronds stained with iodine-potassium iodide solution; few starch grains (black arrows) ~~were observed~~can be seen in control ~~leaves~~fronds (H) while there is an increase ~~the presence of~~in starch ~~increase~~ from ~~treated~~ C1 (I) to ~~treated~~ C4 (J) and C5 (K,L). e = epidermis; is = intercellular space; s = starch grains.

Fig. ~~3~~ 3 Physiological analysis. A) Effect of treatment with potassium dichromate [0.5 mg/L (C1); 0.93 mg/L (C2); 1.73 mg/L (C3); 3.22 mg/L (C4); 6 mg/L (C5)] on the content of chlorophyll *a*, chlorophyll *b* and carotenoids. ~~In graphs d~~Data ± standard error ~~were~~ are reported in the graph; B) Measurement of maximum efficiency of PSII both in terms of Fv/Fm (Fm-F0/Fm) and Fv/F0 (Fm-F0/F0) ratio at different concentrations of potassium dichromate [0.5 mg/L (C1); 0.93 mg/L (C2); 1.73 mg/L (C3); 3.22 mg/L (C4); 6 mg/L (C5)]. Fm = maximum fluorescence level of PSII; F0 = background fluorescence level. In graphs data ± standard deviations were reported. Presence of asterisks indicates significant differences from the control (*, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$).~~.~~

~~Fig. 4 Measurement of maximum efficiency of PSII both in terms of Fv/Fm (Fm-F0/Fm) and Fv/F0 (Fm-F0/F0) ratio at different concentrations of potassium dichromate [0.5 mg/L (C1); 0.93 mg/L (C2); 1.73 mg/L (C3); 3.22 mg/L (C4); 6 mg/L (C5)]. Fm = maximum fluorescence level of PSII; F0 = background fluorescence level. In graphs data ± standard deviations were~~

~~reported. Presence of asterisks indicates significant differences from the control (*, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$).~~

Fig. ~~5~~ 4 Pictures of ~~leaves~~fronds at Scanning electron microscopy (SEM) and energy dispersive X-ray (EDX) microanalysis to detect chromium storage after treatment with potassium dichromate. In C1 samples no traces of chromium were observed (Fig. ~~5A~~4A), in the samples C4 (Fig. ~~5B~~4B) and C5 (Fig. ~~5C~~4C) only small traces of chromium were observed in increasing amounts.

Tab. 1 Average specific growth rate of *Lemna minor* from moment time 0 to 7 (days) at different concentrations of Potassium dichromate. Symbol "LN" refers to natural logarithm.

CONC. (mg/L)	Replicas	N° fronds T_0	N° fronds T_7	LN	LN	μ	average μ

Errors given are standard errors of the mean.

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				(Fronds T_0)	(Fronds T_7)		
0,00	CTRL A	12	102	2,48	4,62	0,31	
	CTRL B	12	100	2,48	4,61	0,30	0,30 ± 0,001
	CTRL C	12	101	2,48	4,62	0,30	
0,50	C1A	12	82	2,48	4,41	0,27	
	C1B	12	75	2,48	4,32	0,26	0,26 ± 0,012
	C1C	12	69	2,48	4,23	0,25	
0,93	C2A	12	67	2,48	4,20	0,25	
	C2B	12	57	2,48	4,04	0,22	0,22 ± 0,032
	C2C	12	43	2,48	3,76	0,18	
1,73	C3A	12	46	2,48	3,83	0,19	
	C3B	12	60	2,48	4,09	0,23	0,20 ± 0,029
	C3C	12	40	2,48	3,69	0,17	
3,22	C4A	12	42	2,48	3,74	0,18	
	C4B	12	44	2,48	3,78	0,19	0,19 ± 0,006
	C4C	12	46	2,48	3,83	0,19	
6,00	C5A	12	43	2,48	3,76	0,18	
	C5B	12	42	2,48	3,74	0,18	0,18 ± 0,005
	C5C	12	45	2,48	3,81	0,19	

Tab. 1 Starch amount in the adventitious roots (pixel μm^{-2} ± standard error). Presence of asterisk indicates significant differences from the control ($p \leq 0.01$).

Sample	pixel μm^{-2}
Control	1100.18 ± 0.04
C1	2230.52 ± 0.25
C2	1970.73 ± 0.13
C3	1340.45 ± 0.03 *

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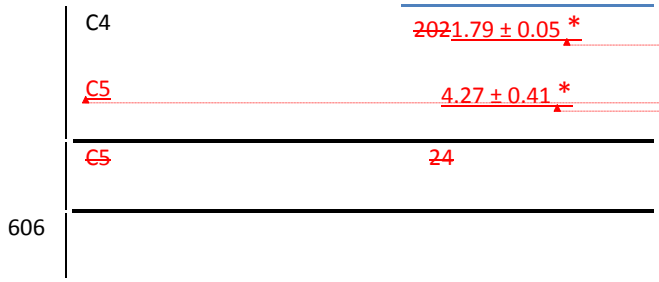

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***Highlights (for review)**

- Chromium represents an important environmental pollutant
- We investigated the effects of Cr(VI) treatments on *Lemna minor* plants
- Cr(VI) did not interfere with the Chl synthesis but with the energy transfer from antenna to reaction centre.
- A correlation between the starch storage and the reduced growth was also observed.
- Cr was accumulated only in small quantities in the leaves of *Lemna minor*

Cyto-histological and morpho-physiological responses of common duckweed (Lemna minor L.) to chromium

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Abstract

Along with cadmium, lead, mercury and other heavy metals, chromium is an important environmental pollutant, mainly concentrated in areas of intense anthropogenic pressure. The effect of potassium dichromate on *Lemna minor* populations was tested using the growth inhibition test. Cyto-histological and physiological analyses were also conducted to aid in understanding the strategies used by plants during exposure to chromium. Treatment with potassium dichromate caused a reduction in growth rate and frond size in all treated plants and especially at the highest concentrations. At these concentrations the photosynthetic pathway was also altered as shown by the decrease of maximum quantum yield of photosystem II and the chlorophyll *b* content and by the chloroplast ultrastructural modifications. Starch storage was also investigated by microscopic observations. It was the highest at the high concentrations of the pollutant. The data suggested a correlation between starch storage and reduced growth; there was greater inhibition of plant growth than inhibition of photosynthesis, resulting in a surplus of carbohydrates that may be stored as starch

The investigation helps to understand the mechanism related to heavy metal tolerance of *Lemna minor* and supplies information about the behavior of this species widely used as a biomarker.

Key words*:* Chromium; growth inhibition test; *Lemna minor*; photosystem II; plastid; starch.

Abbreviations

Car	Carotenoids
Chl	Chlorophyll
Cr	Chromium
Cr^{III}	Chromium (III)
Cr^{VI}	Chromium (VI)
EDX	Energy dispersive X-ray analysis
FM	Maximum fluorescence in the dark-adapted state
F0	Minimum fluorescence in the dark-adapted state
F_V	Variable fluorescence
PAM	Pulse amplitude modulated fluorimetry
PSII	Photosystem II
SEM	Scanning electron microscopy
TEM	Transmission electron microscopy

1. Introduction

Non-essential heavy metals, such as cadmium (Cd), chromium (Cr), lead (Pb) and mercury (Hg) are very important environmental inorganic pollutants, concentrated in areas characterized by the presence of waste products of many industrial processes.

Cr exists in the environment in two stable forms: chromium (III) and chromium (VI) originating from natural processes and human activities.

The phytotoxicity of both Cr^{III} and Cr^{VI} has been studied in many higher and lower plants. Cr^{VI} is more phytotoxic than Cr^{III} (Han et al., 2004) and retards growth, reduces the number of palisade and spongy parenchyma cells of leaves, and increases the number of vacuoles and electron dense material along the walls of xylem and phloem (Han et al., 2004). Cr phytotoxicity can also inhibit seed germination, degrade pigment status, alter nutrient balance, modify antioxidant enzymes activity and induce oxidative stress in plants (Poschenrieder et al., 1991, Barcelo and Poschenrieder, 1997, Panda and Choudhury, 2005). Apart from these effects, Cr can also alter the chloroplast and membrane ultrastructure in plants (Bassi et al., 1990, Panda and Choudhury, 2005). In greater detail, Cr inhibits photosynthesis and the PSII is known to be the main target, also in relation to structural changes within the PSII complex (Fasulo et al., 1983; Bishnoi et al., 1993; Davies et al., 2002; Shanker et al., 2005; Ait Ali et al. 2006; Rocchetta et al., 2006; Olah et al., 2010). Heavy metals must be extracted from polluted areas but they cannot be degraded in the environment like other organic xenobiotics (Augustynowicz et al., 2010). Using living plants to remove metal ions from a polluted area with organic and inorganic compounds is commonly called phytoremediation.

One of the most important aquatic family in phytoremediation research is Lemnaceae. Members of Lemnaceae, especially *Lemna minor*, are now test organisms extensively used for assessing the potential impact of environmental chemicals in ecotoxicology and plant physiology. The International Organization for Standardization (ISO) and the Organization for Economic Co-operation and Development (OECD) have developed standard growth inhibition tests using duckweed, namely *L. minor* (clone St; clone no. 9441) and *L. gibba* (clone G3; clone no. 9260), respectively (ISO, 2004; OECD, 2004). Being important elements in primary production and in the food chain, sensitivity of such aquatic macrophytes to various toxic chemicals may impact the functioning of the whole aquatic ecosystem.

Unpredictable industrial accidents can result in high loads of toxic chemicals entering the environment within short time intervals as happened to River Tisza in Hungary in 2000 when heavy-metal and cyanide contamination entered the river causing an ecological catastrophe (Lakatos *et al*. 2003). Hence, it is essential to predict the possible effects of toxic substances on vital processes and species composition of aquatic biota. Duckweed is a freefloating plant with wide distribution in different types of aquatic ecosystems. In spite of their small size, they

exhibit great potential for vegetative reproduction and thereby rapid biomass growth, in fact they are known to be the fastest growing angiosperms (Ziegler *et al*., 2015).

Olah *et al*., (2010) suggested that various duckweed species respond with different sensitivity to the same environmental concentrations of Cr^{VI} in the growth medium, and presumably to other environmental stresses too. This may have an influence on their competitive relations when heavy metal pollution occurs in an aquatic ecosystem.

In *L. minor* plants exposed to chromate (Appenroth *et al*., 2003) and nickel (Xyländer *et al*., 1993; Appenroth *et al*., 2010), one of the evident effects was starch accumulation in plastids. High biomass production, especially when rich in starch, is of immense biotechnological importance (Sree and Appenroth, 2014; Zhao *et al*., 2015; Ziegler *et al*., 2015). In the present paper the extent to which Cr^{VI} altered starch accumulation in *L. minor* was investigated and we attempted to determine the underlying mechanism(s).

We investigated the effects of Cr^{VI} treatments on *Lemna minor* plants taking into account the photosynthetic and cyto-histological parameters, which in the past had been studied independently and, for the first time to our knowledge, microanalysis of the frond sections was carried out .

2. Materials and Methods

2.1 Plant material

The organisms were originally supplied by the "Friedrich-Shiller University of Jena - Botanic Institute" and the stock cultures were subcultured in the Test Facility BioTecnologie B.T..

The test was performed according to OECD Guideline for the testing of chemicals n° 221 ("Lemna sp, Growth Inhibition Test", adopted on 23 March 2006).

Duckweed colonies with total of 12 fronds were taken from the stock culture and placed in crystallizer glass dishes with 100 mL nutrient solution (SIS growth medium, pH 6.5±0.2), containing different concentrations of potassium dichromate [0.50 mg/L (C1); 0.93 mg/L (C2); 1.73 mg/L (C3); 3.22 mg/L (C4); 6.00 mg/L (C5)]; an untreated group was prepared using 100 mL of SIS growth medium.

Expressed as concentrations of chromium, the test concentrations were the following: 3.4 μM (C1); 6.3 μM (C2); 11.7 μM (C3); 21.9 μM mg/L (C4); 40.7 μM (C5).

Cultures were grown in an incubator chamber at 24±2°C and continuous illumination in the range 6500 - 10000 Lux.

The toxicity of potassium dichromate was assessed after seven days of exposure, under static conditions.

Three replicates (test vessels) were carried out for each untreated and treated group.

The experiments were repeated three times and the results of one representative experiment are reported in this paper.

2.2 Calculation of growth rates

The average specific growth rate (μ) for a specific period (from time *i* to time *j*) was calculated as the slope of the logarithmic growth curve from the equation:

$$\mu = \frac{\ln N_{tj} - \ln N_{ti}}{t_j - t_i}$$

where:

- μ : average specific growth rate from time *i* to time *j*
- N_{ti} : number of fronds observed in the test or control vessel at time *i*
- N_{tj} : number of fronds observed in the test or control vessel at time *j*
- *ti* : time at the start of the period
- *tj* : time at the end of the period

158 The "*i*" corresponds to the start of experiment while the time "*j*" corresponds to seven days of
159 treatment with potassium dichromate. Growth rates used for the calculation of inhibition are
160 usually given as the average of 3 independent replicates (*n*=3).
161
162 *2.3 Determination of frond size*
163 Images of the fronds were taken using a stereo-microscope and quantified by the Leica IM 1000
164 software.
165
166 *2.4 Cyto-histological observations*
167 To obtain semi-thin and ultra-thin sections, portions of fronds were fixed in 3% (w/v)
168 glutaraldehyde in 0.075 M phosphate buffer, pH 7.2, for 5h. The samples were then washed four
169 times for 15 min in 0.075 M phosphate buffer, pH 7.2, post-fixed in 1% (w/v) OsO_4, dehydrated
170 in increasing concentrations of ethanol and, finally, embedded in resin (Epon, 2-
171 dodecenylsuccinic anhydride, and methylnadic anhydride mixture) (Reale et al. 2014).
172 The semi-thin sections (1–2 μm) were cut with an ultramicrotome (OmU2, Reichert,
173 Heidelberg) equipped with a glass blade, stained with 0.5% (w/v) toluidine blue in 0.2 %
174 $NaHCO_3$ buffer and observed under a light microscope (DMLB, Leica, Wetzlar, Germany).
175 Semi-thin sections were also stained by Periodic Acid Schiff's reaction (O'Brien and McCully,
176 1981). Slides were observed under a light microscope; the presence of starch was indicated by a
177 magenta colour, while proteins appeared blue.
178
179 *2.5 Evaluation of starch content*
180 To evaluate the starch content, semi-thin sections of fronds were treated with sodium metoxide
181 and methanol to remove the resin. After deresination, sections were rehydrated and treated with
182 an iodine-potassium iodide solution (Johansen 1940) and observed under a light microscope.
183 Starch grains appeared dark blue. To quantify starch content, the image of each section was
184 analysed by the image processing program "ImageJ" (Abramoff *et al*., 2004). The amount of
185 starch is reported as relative units (pixel μm^{-2}), calculated as the number of pixels, measured by
186 the software, with respect to the surface observed (μm^2).
187
188 *2.6 Photosynthetic pigment analysis*
189 Samples were cut into small pieces and extracted with 80% acetone. Extracts were maintained
190 at 4 °C until analysis and all manipulations were performed in dim green safe light to avoid
191 photo-degradation.
192 Absorption spectra (400–750 nm range) of extracts were recorded at room temperature (25 °C)
193 by the spectrophotometer. For Chls and carotenoids determinations, the extracts were measured

at 661 nm (Chl a), 644 nm (Chl b), and 470 nm (carotenoids) and pigment concentrations were evaluated as mg/g of fresh substance according to the equations proposed by Wellburn (1994).

2.7 Maximum quantum yield of photosystem II

A portable pulse amplitude modulated (PAM) fluorometer (ADC OS1-FL, ADC BioScientific Ltd., Hoddesdon, UK) was used for *in vivo* measurements of chlorophyll fluorescence. Fluorescence measurements were performed on fronds positioned onto a wet filter paper placed on ~~the floor of~~ the clip floor of the instrument. The background fluorescence level F_0 was measured on the fronds adapted to darkness for at least 20 min. The maximum fluorescence level of PSII (F_M) was obtained by exposing the sample to a saturating pulse of white light (0.8 s). The PSII maximum photochemistry was measured using the variable fluorescence ratios $F_V/F_M = (F_M - F_0)/F_M$ and $F_V/F_0 = (F_M - F_0)/F_0$ (Lichtenthaler et al. 2005; Ferroni et al., 2013).

2.8 TEM analysis

Thin sections (0.08 µm) were cut with an ultramicrotome (OmU2, Reichert, Heidelberg) equipped with a glass blade, mounted on uncoated copper grids (200 mesh) and contrasted by adding uranyl acetate and an aqueous solution of lead nitrate. Observations were carried out with a transmission electron microscope (TEM 400 T; Philips, Monza, Italy).

2.9 Scanning electron microscopy (SEM) and energy dispersive X-ray (EDX) microanalysis

Semi-thin sections were dehydrated and metallised with graphite by "Q150T Turbo-Pumped Sputter Coater/Carbon Coater" (Quorum Technologies Ltd., Laughton, United Kingdom); EDX microanalysis was performed in a Zeiss SEM LEO 1525 (LEO Electron Microscopy Inc., One Zeiss Drive, Thornwood, NY).

2.10 Statistical analysis

Experiments and assays were carried out with three replicates unless otherwise indicated and data were averaged with standard error or standard deviation. P-value was obtained by t-test with $P < 0.01$ or $P < 0.05$ considered to be significant. To perform the statistical analysis, the Analysis ToolPak, an Excel add-in program, was used.

3. Results

3.1 Growth rates and frond size

The average specific growth was calculated to investigate the influence of potassium dichromate on the growth of *Lemna minor* (Fig. 1A). After 7 days of treatment, growth in the treated samples was inhibited compared to the control. Decrease in the growth rate was evident starting from the lowest concentration of potassium dichromate (0.50 mg/L) and became more pronounced at the highest concentrations. The effects on growth rate were dose-dependent and correlated to the increase in the concentration of the heavy metal. After 7 days of treatment the frond size in all treated samples was lower than in the control (Fig. 1B). In samples treated with 6 mg/L (C5) decrease in the frond size was more accentuated.

3.2 Cyto-histological observations

The cyto-histological structure of the control and treated fronds was investigated by observing semi-thin and thin sections under light and electron microscope, respectively. In the control and treated samples, fronds had an anatomical structure typical of aquatic plants; there were two single layered epidermises with an aerenchyma rich in intercellular spaces (Fig. 2A). In the control fronds, parenchymatic cells were rich in chloroplasts, which had a lenticular shape and were arranged close to the cell wall (Fig. 2B, C); in these chloroplasts thylakoid membranes were perfectly arranged and organized in grana, little starch grains were also present, as shown by semi-thin sections stained with Schiff's reagent (Fig. 2C) and TEM pictures (Fig. 2D). In treated fronds chloroplast maintained their conformation in C1, C2 and C3 samples, however after treatment with the highest concentration of potassium dichromate (C5, 6 mg/L), chloroplasts were very similar to amiloplast with a reduced presence of stroma, a thylakoid system constituted by few grana (Fig, 3E, F, G) and big starch grains.

3.4 Evaluation of starch content

The presence of starch was determined by staining with the iodine-potassium iodide solution. In the control fronds, starch was poor and consisted of little starch grains inside plastid perfectly organized (Fig. 2H). In treated fronds, starting from lower concentrations of potassium dichromate, starch grains were more abundant than in the control samples; the presence of starch increase from treatment C1 (Fig, 2I) to C4 (Fig. 2J) and C5 (Fig. 2K). In C5 starch grains completely occupied plastids that had lost their original shape and appeared similar to amyloplasts (Fig. 2L). The amount of starch in the control and treated fronds, expressed by relative units (pixel μm^{-2}), is reported in Table 1; the quantitative analyses confirmed the qualitative observations, that the presence of starch increased from control to treated samples, with statistically significant differences in samples C3, C4 and C5.

3.5 Physiological analysis

Data from pigment analysis of control and treated plants are reported in Fig. 3A. The content of chlorophyll *a* was variable but not significantly different in the control or treated plants. The same was true for the carotenoid content. The content of chlorophyll *b* decreased in C5 and was significantly lower than in the control; in C1, C2, C3 and C4, however, the chlorophyll *b* content did not significantly differ from the control.

As shown by the graphs in Fig. 3B, the effect of potassium dichromate on the maximum efficiency of PSII was dose-dependent, and clearly more pronounced at the higher concentrations of chromium. In the C1 samples, which were treated with the lowest concentration of potassium dichromate, there were no significant effects in terms of F_V/F_M ratio. For those samples the values of F_V/F_M (0.737) were not significantly different from those of the control (0.775; $p = 0.2189$). However, starting from C2 samples, there were increasing differences with C4 and C5 being the most affected ones with F_V/F_M being below 0.700 (Fig. 3B). The PSII photochemistry was also estimated as a F_V/F_0 ratio since it shows a wider dynamic range of variation with respect to F_V/F_M, and thus is more sensitive to highlight the effect of stress (Lichtenthaler et al., 2005). Considering the F_V/F_0 ratio, the most significant differences from the control were in samples C2 to C5 (Fig. 3B). For both values of variable fluorescence ratios, the most marked differences compared to the control were in C3 and even more so in C4 and C5 samples.

3.6 Microanalysis

The microanalysis was carried out in sections obtained from different portions of fronds of treated and control plants. In the control and C1 samples no traces of Cr were observed (Fig. 4A). In samples C2, C3, C4 (Fig. 4B) and C5 there were only small traces of Cr in increasing amounts; the highest values were observed in sample C5 (Fig. 4C), which was treated with the highest concentrations of potassium dichromate.

4. Discussion

Cr is the seventh most abundant metal in the earth's crust and in the hexavalent form is one of the major sources of environmental pollution. The reason of its toxicity in plant systems appears to be due to its rapid permeability through biological membranes and subsequent interaction with intercellular proteins and nucleic acids.

Our data contribute to understanding the mechanism related to Cr tolerance on the test organism *Lemna minor*, which is usually used as a biomarker to biomonitor municipal, agricultural and industrial discharge.

The effects of Cr were observed in *L. minor* from the lowest concentrations, as demonstrated by the decrease of growth rate in all treated samples, but the highest effects were observed at the highest concentration of the heavy metal. The reduction in growth was paired to a decrease in frond size, which was more accentuated at the 6 mg/L, while between 0.5 and 3.22 mg/L (from C1 to C4 samples) frond size was more similar to that of the control, although always lower. Decrease in the number of fronds has also been observed in other plants treated with Cr such as wheat (Sharma and Sharma , 1993) and bean (Barcelo et al., 1985), and reduction in frond size has been reported in spinach (Singh, 2001). Reduction of the growth rate and frond size may be related at the molecular level to an inhibition of cell division or elongation resulting from oxidative stress (Karuppanapandian and Kim, 2013; Palit et al., 1994). Reduction of the growth rate has also been reported in *Lemna minor* plants after treatment with Co^{2+} (Sree at al., 2015). During the cobalt treatment no differences in the chlorophyll content and PSII activity were observed after 4 days but only after 7 days, suggesting that duckweed growth is initially inhibited to a greater extent than photosynthesis (Sree at al., 2015). However, the treatment of *Lemna* with Cr^{VI} affected the chlorophyll fluorescence parameters also after a short-term treatment (48 h) (Oláh et al., 2010); this treatment did not affected photosyhthetic pigments as strongly (Sree at al., 2015). Concentrations of Chls and carotenoids decreased in the presence of Cr^{VI}, but not statistically significant differences were observed in relation to various Cr^{VI} concentrations. Our data, after seven days of treatment, confirmed the small variation in the pigment contents caused by the Cr^{VI} treatment. There was a reduction in the chlorophyll *b* content of samples C4 and C5 after seven days, while there was no variation in chlorophyll *a* and carotenoid concentrations.

The decrease in chlorophyll *b* could be due to destabilization and degradation of the peripheral PSII antenna complex (Shanker at al., 2005). Damages of PSII reaction centers was also demonstrated by measuring the maximum efficiency of PSII. In the treated plants the decrease in F_V/F_M and F_V/F_0 indicates photo-inhibitory damage to the reaction center of PSII. This decline was due to the increase in the minimum fluorescence (F_0) as shown by the comparison of F_V/F_M and F_V/F_0, the latter being much more affected. The increase of F_0 is considered an expression

of irreversible damage to PSII resulting in an increased fraction of energy lost by fluorescence emission, so that leaves suffered from excess light (Bussotti et al., 2011). It has been reported that hexavalent chromium alters the distribution of excitation energy *via* regulated and nonregulated non-photochemical dissipation (Ali *et al*., 2008; Perreault *et al*., 2009). The reduction in photosynthetic activity and chlorophyll content also corresponded to modifications in the plastid structure. Van Assche and Clijster (1983) suggested that the overall effect of Cr ions on photosynthesis and excitation energy transfer could also be due to Cr^{VI}-induced abnormalities in the chloroplast ultrastructure such as a poorly developed lamellar system with widely spaced thylakoid and fewer grana. Starting from the C4 sample the chloroplasts were very similar to amyloplast with a reduced presence of stroma, a thylakoid system constituted by few grana and large starch grains. In the C5 sample the starch grains occupied the plastid almost completely. Our studies confirm the hypothesis of Bassi et al. (1990), who suggested that the presence of chromium damages the plasma membrane and the proplastids fail to develop in the normal chloroplasts resulting in the ultrastructural alterations observed.

The accumulation of starch in the plastids of the treated samples could be due to one of the following mechanisms, besides others: (1) inhibition of export of photosynthate from source to sink organs, leading to an accumulation of low-molecular-weight carbohydrates which might serve as substrates for starch biosynthesis; (2) inhibition of plant growth to a larger extent than inhibition of photosynthesis, resulting in a surplus of carbohydrates that may be stored as starch (Sree *et al*., 2015). Often, starch accumulation under heavy metal stress is considered to be a consequence of the inhibition of carbohydrate transport from leaves, which act as source for non-photosynthetic sinks (Herren and Feller, 1997). Due to the fact that duckweed has a low level of tissue differentiation, this model (mechanism 1) does not hold true for duckweed (Appenroth *et al*., 2010), and hence we suggest a correlation between the inhibition of plant growth and the starch storage. Sree et al. (2015) concluded that after exposure with Co^{2+} duckweed growth is initially (four days) inhibited to a greater extent than photosynthesis resulting in surplus carbohydrates and starch accumulation; thereafter, photosynthesis declines in the presence of Co^{2+} leading to restricted availability of carbohydrates, while at the same time the initially stored starch is remobilized.

5. Conclusions

Our data demonstrated that after seven days of treatment the effects of Cr^{VI} on the functioning of photosynthetic apparatus involve the oxidative damage to photosynthetic pigment rather than inhibition of synthesis as suggested after a short treatment (48 h) by Oláh *et al.* (2010). Cr^{VI} presumably could not fundamentally interfere with the Chl synthesis, so the measured rise in F_0 values might evolve from inhibited energy transfer from antenna to reaction center.

Our data also suggested a correlation between starch storage and reduced growth but it would be interesting to measure the starch content after a short period of treatment, as has been carried out with Co^{2+} (Sree *et al*., 2015).

Finally, the microanalysis results suggest that Cr was accumulated only in small quantities in the fronds of *Lemna minor*. Probably, as observed by Nematshahi *et al*. (2012) in *Allium cepa*, Cr is first accumulated in roots, which was not done in the present study, and successively transported to the aerial part of the plant, where it can have a direct impact on the cellular metabolism of shoots.

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Figure Legends

Fig. 1 Growth rates and frond size. A) Average specific growth rate of *Lemna minor* from moment time 0 to 7 (days) at different concentrations of potassium dichromate; B) Frond size in control and treated (C1, C2, C3, C4, C5) samples. Data ± standard error were reported in the graphs. Asterisks indicate significant differences from the control (*, $p \leq 0.05$; **, $p \leq 0.01$)

Fig.2 Cyto-histological observations. A-B) Transversal semi-thin sections of control fronds stained with toluidine blue; the two epidermis and the parenchymatic tissues, with large intercellular spaces, are evident; C) Transversal semi-thin section stained with Schiff's reagent, starch grains inside chloroplast appeared purple colored; D) TEM picture of parenchymatic cells of control fronds; in chloroplast (black arrows) thylakoid membranes are organized in grana and small starch grains are present; E) transversal semi-thin section of frond of a C5 sample, chloroplasts (black arrows) were very similar to amyloplast; F-G) TEM pictures of parencymatic cells of C5 samples in which few grana and big starch grains can be seen; H-L) Transversal semi-thin sections of fronds stained with iodine-potassium iodide solution; few starch grains (black arrows) can be seen in control fronds (H) while there is an increase in starch from C1 (I) to C4 (J) and C5 (K,L). e = epidermis; is = intercellular space; s = starch grains.

Fig. 3 Physiological analysis. A) Effect of treatment with potassium dichromate [0.5 mg/L (C1); 0.93 mg/L (C2); 1.73 mg/L (C3); 3.22 mg/L (C4); 6 mg/L (C5)] on the content of chlorophyll *a*, chlorophyll *b* and carotenoids. Data ± standard error are reported in the graph; B) Measurement of maximum efficiency of PSII both in terms of Fv/Fm (Fm-F0/Fm) and Fv/F0 (Fm-F0/F0) ratio at different concentrations of potassium dichromate [0.5 mg/L (C1); 0.93 mg/L (C2); 1.73 mg/L (C3); 3.22 mg/L (C4); 6 mg/L (C5)]. Fm = maximum fluorescence level of PSII; F0 = background fluorescence level. In graphs data ± standard deviations were reported. Presence of asterisks indicates significant differences from the control (*, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$).

Fig. 4 Pictures of fronds at Scanning electron microscopy (SEM) and energy dispersive X-ray (EDX) microanalysis to detect chromium storage after treatment with potassium dichromate. In C1 samples no traces of chromium were observed (Fig. 4A), in the samples C4 (Fig. 4B) and C5 (Fig. 4C) only small traces of chromium were observed in increasing amounts.

515

516 Tab. 1 Starch amount in the adventitious roots (pixel μm^{-2} ± standard error). Presence of
517 asterisk indicates significant differences from the control ($p \leq 0.01$).

Sample	**pixel μm^{-2}**
Control	0.18 ± 0.04
C1	0.52 ± 0.25
C2	0.73 ± 0.13
C3	0.45 ± 0.03 *
C4	1.79 ± 0.05 *
C5	4.27 ± 0.41 *

Figure 1

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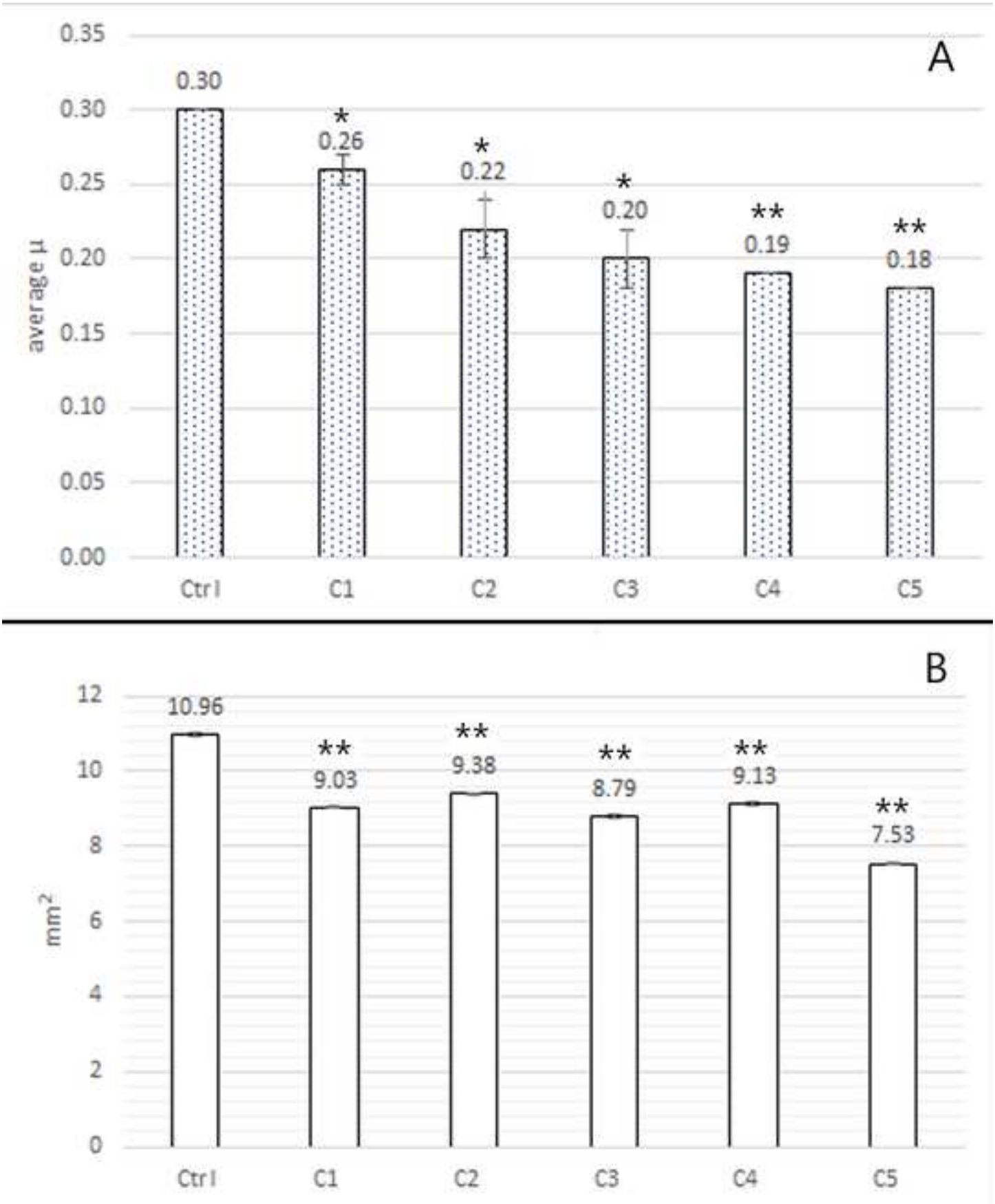

Figure 2

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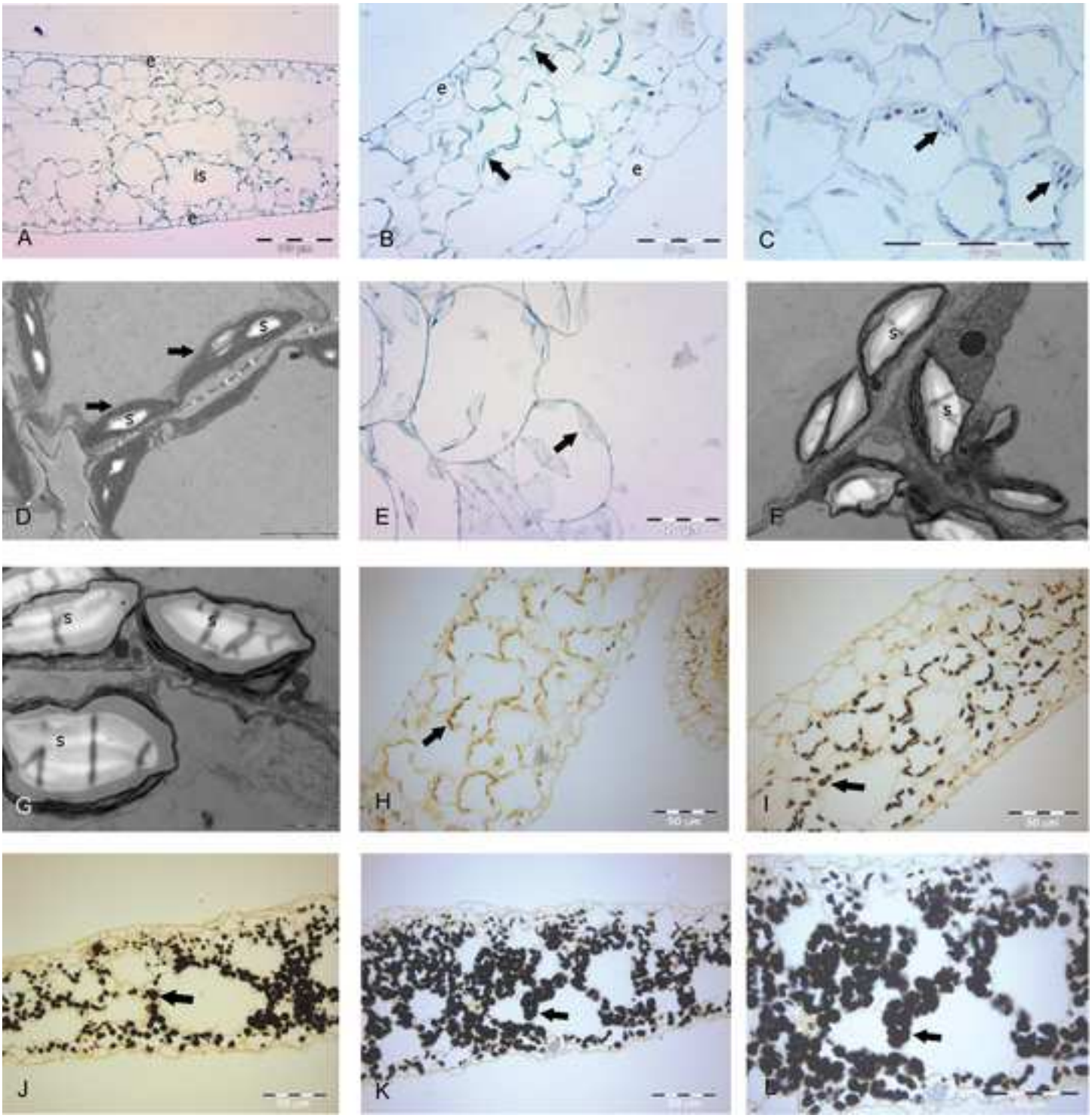

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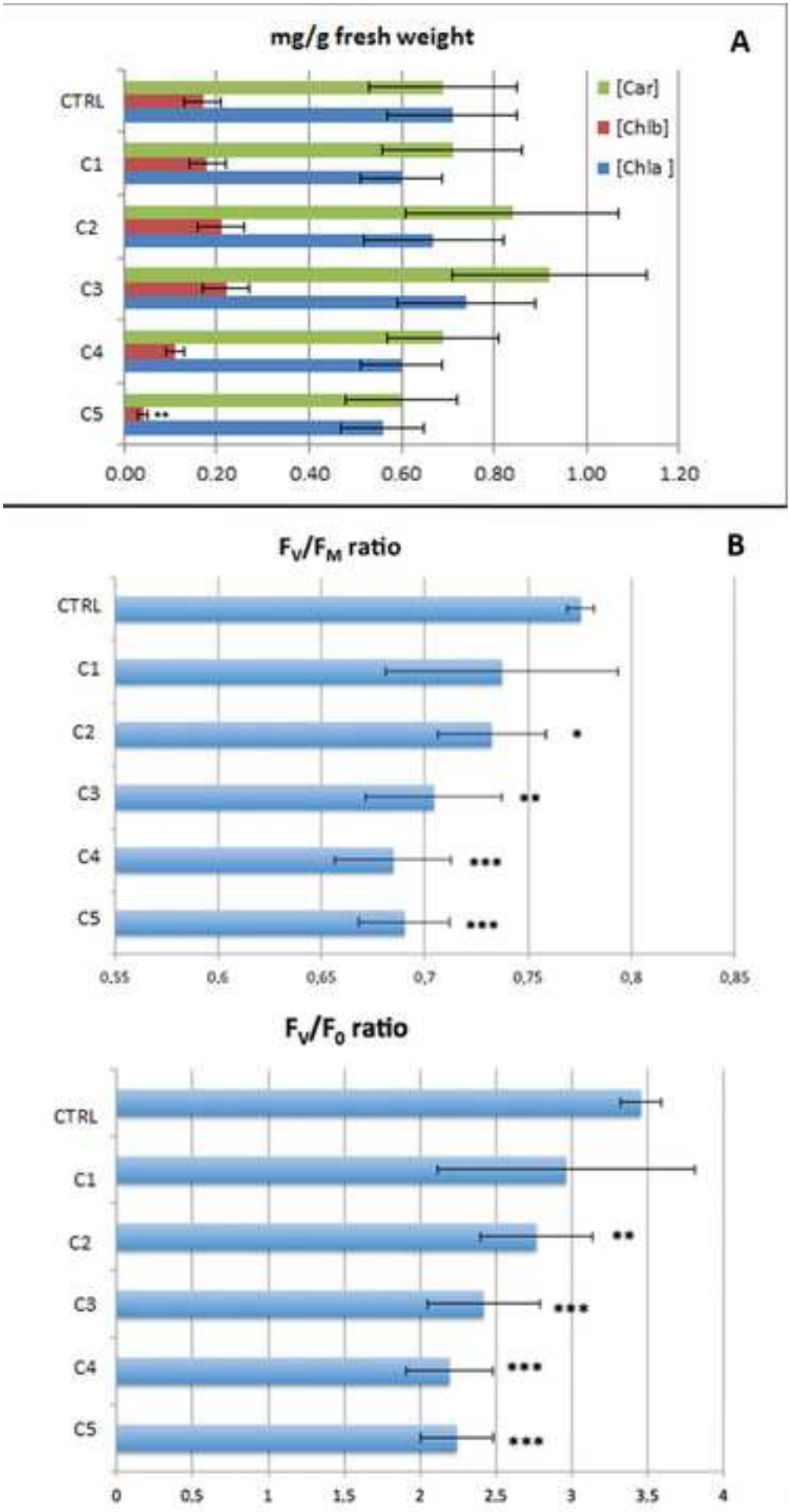

Figure 4

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