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Article

Bioaccumulation, Growth and Photosynthetic Response of a New Found in Bulgaria Invasive Species *Lemna minuta* and *L. valdiviana* to Heavy Metal Pollution

*Bioacumulação, Crescimento e Resposta Fotossintética de Duas Novas Espécies Invasivas Encontradas na Bulgária, **Lemna minuta** e **L. valdiviana**, à Poluição de Metal Pesado*

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ABSTRACT - Heavy metals can meet in the surrounding environment as natural ingredients or from agricultural, industrial and chemical industries. The study was conducted in order to trace the potential of the aquatic plant *L. minuta* and *L. valdiviana* for the bioaccumulation of Cu, Cd, and Pb from contaminated water at low levels of these elements. Each of the duckweed species was treated separately with $CuSO_4.5H_2O$, $CdSO_4$, Pb SO_4 (Valerus, Bulgaria) at 0.5 and 1 mg L^{-1} concentrations of for 96 hours. After conducting the experiments, relative growth rate (RGR), bioconcentration factor (BCF), tolerant index (T*i*) and photosynthetic pigments of two *Lemna* species were studied. The solution with higher metal concentration more inhibits the growth of macrophytes. The BCF of the metals on the two species were in decreasing order: Cu > Pb > Cd. Our study showed that *L. minuta* and *L. valdiviana* at a concentration of 0.5 mg L^{-1} copper have better affected on the photosynthetic apparatus compared to the control. Better bioaccumulation ability was established in *L. minuta* compared to *L. valdiviana*.

Keywords: new weed, *Lemna* species, environment pollution.

*RESUMO - Metais pesados podem ser encontrados no meio ambiente como ingredientes naturais ou de práticas agrícolas, industriais e químicas. Este estudo foi conduzido com o objetivo de traçar o potencial das plantas aquáticas **L. minuta** e **L. valdiviana** para a bioacumulação de Cu, Cd e Pb a partir de água contaminada com baixos níveis desses elementos. Cada uma das espécies de lentilha foi tratada separadamente com* $CuSO_4.5H_2O$, $CdSO_4$ *e* $PbSO_4$ *(Valerus, Bulgária) em concentrações de 0,5 e 1 mg* L^{-1} *durante 96 horas. Após a realização dos experimentos, estudou-se a taxa de crescimento relativo (RGR), o fator de bioconcentração (BCF), o índice de tolerância (Ti) e os pigmentos fotossintéticos de duas espécies de **Lemna**. A solução com maior concentração de metal inibe o crescimento de macrófitas. O BCF dos metais, nas duas espécies, estava em ordem decrescente: Cu > Pb > Cd. Este trabalho mostrou que **L. minuta** e **L. valdiviana**, na concentração de 0,5 mg* L^{-1} *de cobre, são mais afetadas no aparato fotossintético, em comparação ao controle. Melhor capacidade de bioacumulação foi estabelecida em **L. minuta** em relação a **L. valdiviana**.*

Palavras-chave: novas plantas daninhas, espécies de *Lemna*, poluição ambiental.

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INTRODUCTION

Heavy metals can meet in the surrounding environment as natural ingredients or from agricultural, industrial and chemical industries. They are biologically non-degradable and have to be removed from the water. Copper (Cu), cadmium (Cd) and lead (Pb) have a deleterious effect on living organisms above certain values (Khellaf and Zerdaoui, 2009). Copper is involved in the metabolic processes of plants and is important microelements, which above certain concentrations becomes toxic (Teisseire and Vernet, 2005). Copper is participates in photosynthesis and breathing processes of the plant cell (Kanoun-Boulé et al., 2009). Cadmium is a common element in the environment and negatively affects on some enzymatic reactions (Pietrini et al., 2005). Cd and Cu cause morphological (necrosis, colony disintegration, root break-up) and physiological (photosynthesis, pigment synthesis and enzyme activity) alterations of aquatic plants (Khellaf and Zerdaoui, 2010; Xing et al., 2010; Doǒanlar, 2013). In high doses, lead accelerates the process of formation of free radicals and leads to the activation of the glucose-6-phosphate dehydrogenase and catalase in the cells of the organisms. The European Water Policy Directive reported that cadmium, nickel and lead are toxic to the aquatic environment and take action to limit them to groundwater (Directive 2000/60/EC).

Aquatic plants such as duckweeds are quickly multiply and adjusts to changing conditions in eutrophic ditches and ponds. Because of small fronds and rapid reproduction Lemna sp. are good test objects for toxic effects (Verma and Suthar, 2015). In a laboratory condition, these aquatic plants are cultivated easily and have a high sensitivity to pollution (Goswami and Majumder, 2015). Due this reason, the duckweeds are good phytoremediators for different contaminated water bodies (Modlitbová et al., 2018). *Lemna* sp. also is used in the purification of wastewater (Velichkova et al., 2017). Accumulation of *Lemna* metals and metalloids allows them to be absorbed into cellular biomass (Zhao et al., 2017).

Lemna valdiviana Phil. and *L. minuta* Kunth originate from the Americas but began increasingly to be found in Europe and Asia (Iberite et al., 2011). In the flora of Bulgaria were found a new invasive species *L. minuta* and *L. valdiviana* (Kirjakov and Velichkova, 2016). The study was conducted in order to trace the potential of the aquatic plant *L. minuta* and *L. valdiviana* for the bioacumulation of Cu, Cd, and Pb from contaminated water at low levels of these elements.

MATERIALS AND METHODS

Plant material and metal treatment

L. minuta was collected from a natural pond Tvardica Dam Lake (42°24'29"N 27°28'19"E), *L. valdiviana* from Nikolaevo fishpond (42°37'1"N 25°49'1"E) and transported in plastic bottles. The plants were cultivated laboratory in open aquarium glass in sterilised medium (1.25 mM $Ca(NO3)_2$ x $4H_2O$, 3.46 mM KNO_3, 0.66 mM KH_2PO_4, 0.072 mM K_2HPO_4, 0.41 mM $MgSO_4$ x $7H_2O$, 1.94 µM H_3BO_3, 0.63 µM $ZnSO_4$ x $7H_2O$, 0.18 µM, Na_2MoO_4 x $2H_2O$, 0.91 µM $MnCl_2$ x $4H_2O$, 2.81 µM $FeCl_3$, 4.03 µM Na_2EDTA x $2H_2O$; pH 5.5. The plant cultures were maintained at room temperature (23±2 °C) with air condition and a 16 h photoperiod.

After two weeks of cultivation, each aquatic plant species (2 g) was put in to a glass tub with a total of 1 L of spiked metal solution. Each of the duckweed species was treated separately with $CuSO_4.5H_2O$, $CdSO_4$, $PbSO_4$ (Valerus, Bulgaria) at 0.5 and 1 mg L^{-1} concentrations for 96 hours period. The *Lemna* fronds were daily check for frond disconnection, chlorosis and necrosis. After duration of four days of cultivation to different metal concentrations, the species were harvested, washed and deposited for analysis. The experiments were performed in triplicate.

Analytical methods

To determine the effect of the impact of metals, RGR (relative growth rate), BCF (bioconcentration factor), T*i* (tolerant index) and photosynthetic pigments of two *Lemna* species were studied.

L. minuta and *L. valdiviana* relative growth rates were determined as follows (Hunt, 1987):

$$RGR = \ln W_2 - \ln W_1 / T_2 - T_1$$

where R is the relative growth rate (g g^{-1} day^{-1}), W_1 and W_2 - the initial and final dry weights, T_2 - T_1 – period of the experiment.

The bioconcentration factor (BCF) was determined (Rahmani and Stenberg, 1999):

BCF = Metal concentration in plant (mg kg^{-1})/Metal concentration in solution (mg L^{-1}).

Tolerance index (T*i*) was determined (Lux et al., 2004):

T*i* = dry weight of plants grown in metal solution/dry weight of plants grown in control solution.

Cu, Cd and Pb determination

Determinations of heavy metals concentration in plant by atomic absorption spectrometer (AAS) “A Analyst 800” - Perkin Elmer were analyzed. For analysis in a microwave oven, Perkin Elmer Multiwave 3000 by wet combustion the samples of aquatic plants were prepared. The extracts were extended up to 25 mL with distilled water. The metal concentrations in the acid solutions were amended of AAS in accordance with ISO 11047. The concentrations of the investigated element of aquatic plants were expressed as mg kg^{-1} dry weight.

Photosynthetic pigments

The samples were homogenised with 80% (v/v) cold acetone, centrifuged at 3500 *g* for 10 min. The absorbance of the pigment extract (665, 652 nm for chlorophyll content (a+b) and 470 for carotenoids content) were measured by using spectrophotometer DR 2800 (Hach Lange) (Vidakovic-Cifrek et al., 2015). According to Lichtenthaler (1987) the chlorophyll (a+b) and carotenoid content were determined.

Data analyses

Data analyses were conducted by using descriptive statistics, one-way Analysis of Variance (ANOVA).

RESULTS AND DISCUSSION

Cadmium, copper and lead have an inhibitory effect on the growth of the studied species at all selected test concentrations. The relative growth results obtained for the of the two species show that copper has the strongest impact. The higher concentration of the metal in the solution more inhibits the growth of macrophytes. The highest RGR reduction of *L. minuta* was found with Cu (1 mg L^{-1}) treatment which is 76.4% lower compared to control (Figure 1). Similar results were also observed at *L. valdiviana* where Cu (1 mg L^{-1}) treatment lead to RGR reduction 81% lower compared to control (Figure 2). With increasing Pb and Cd concentrations (1 mg L^{-1}) the growth rates of *L. minuta* and *L. valdiviana* declined respectively with 55.4% and 66.3% for *L. minuta* and with 65% and 75.1% for *L. valdiviana*.

To evaluate the ability of *L. minuta* and *L. valdiviana* to concentrate Cd, Pb and Cu in their tissues were calculated the bioconcentration factor (BCF). These results were given in Table 1 and BCF of *L. minuta* and *L. valdiviana* were increased in all studied metal concentrations. In aquatic plants exposed to 0.5 mg L^{-1} metal treatment was measured the highest BCF values. Removal ability was found to decrease with the increase in initial concentration. According to Zayed et al. (1998) the plant with BCF over 1000 is a good accumulator. We found values of BCF upper to 1000 in two studied macrophytes. From BCF value, our aquatic plants were found to be a hyperaccumulator of copper at concentration of 0.5 mg L^{-1}, as *L. minuta* was with 4.5% higher compare to *L. valdiviana*. The BCF of the metals on the two species were in decreasing order: Cu > Pb > Cd.

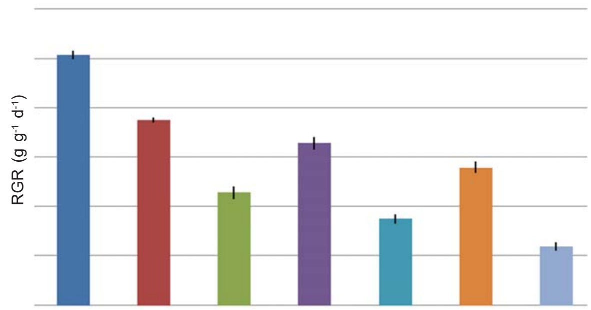

Figure 1 - Relative growth rate of *L. minuta* in the presence of 0 (Control), 0.5, 1 mg L^{-1} $CuSO_4.5H_2O$, $CdSO_4$, Pb SO_4 for periods of four days.

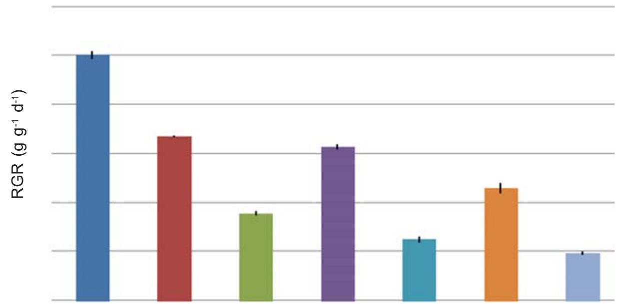

Figure 2 - Relative growth rate of *L. valdiviana* in the presence of 0 (Control), 0.5, 1 mg L^{-1} $CuSO_4.5H_2O$, $CdSO_4$, Pb SO_4 for periods of four days.

Table 1 - Values of bioconcentration factor (BCF), tolerance index (Ti) and cadmium, copper, lead measured ingestion for periods of four days in fronds of *L. minuta* and *L. valdiviana*

Species	Metal concentration	BCF	Ti
L. minuta	0.5 mg L^{-1} Cd	188.6±1.35	0.92±0.03
	0.5 mg L^{-1} Cu	1967.3±1.02	0.84±0.01
	0.5 mg L^{-1} Pb	518±0.4	0.88±0.03
	1 mg L^{-1} Cd	45.7±2.04	0.91±0.02
	1 mg L^{-1} Cu	944.6±5.03	0.82±0.02
	1 mg L^{-1} Pb	99.5±0.5	0.86±0.02
L. valdiviana	0.5 mg L^{-1} Cd	147.8±2.35	0.92±0.01
	0.5 mg L^{-1} Cu	1879.6±1.1	0.83±0.01
	0.5 mg L^{-1} Pb	438.7±0.62	0.87±0.03
	1 mg L^{-1} Cd	42.7±3.03	0.91±0.02
	1 mg L^{-1} Cu	832.9±7.5	0.81±0.02
	1 mg L^{-1} Pb	85.2±0.8	0.85±0.02

Mean ± standard deviation.

An important indicator of the ability of plants to grow in the presence of a certain concentration of metal is the tolerance index (Ti). According to Lux et al. (2004) plants are considered tolerant if they have Ti higher than 0.6. The two studied plants have values of Ti higher than 0.6 which show a good tolerance to Cu, Cd and Pb in plants exposed to 0.5 and 1 mg L^{-1}. The highest tolerance index was reported for plants treated with cadmium at both concentrations (Table 1).

Copper, although a key element in metabolic and physiological processes, is one of the most toxic (Xia and Tian, 2009). Our study showed that *L. minuta* and *L. valdiviana* at a concentration of 0.5 mg L^{-1} copper have better affected on the photosynthetic apparatus compared to the control. This appeared from the data in Table 2, where the amount of chlorophyll a+b was higher in *L. minuta* with 6.82% and in *L. valdiviana* - 2.86% compared to the control. This trend was even more noticeable with regard to the amount of carotenoids - 7.69% (*L. minuta*) and 9.1% (*L. valdiviana*) compared to the control. The pigmentation decrease in both species at a higher concentration of 1 mg L^{-1} of copper was observed. *L. minuta* and *L. valdiviana*, respectively, they decreased by 6.1% and 8.82% chlorophylls and carotinoids by 8.33% and 6.66% relative to the control. Our study has shown that *L. minuta* and *L. valdiviana* are sensitive to copper for concentrations 1 mg L^{-1}. The copper brings the photosystem alteration by reducing electron transport at above 0.5 mg L^{-1} concentration (Dewez et al., 2005). Therefore appear chlorosis of *Lemna* fronds.

Cadmium and lead inhibited photosynthetic pigments in duckweed fronds at all concentrations selected for this study. The toxic effect of Cd on *Lemna* causes changes in cytoplasm

Table 2 **-** The content of photosynthetic pigments (chlorophyll and carotenoids) in *L. minuta* and *L. valdiviana* after four days of metal treatment

Species	Metal concentration	Chlorophyll a+b (mg.g^{-1})	Carotenoids (mg.g^{-1})
L. minuta	Controle	0.82±0.04**	0.36±0.02**
	0.5 mg L^{-1} Cd	0.77±0.02	0.31±0.01*
	0.5 mg L^{-1} Cu	0.88±0.01	0.39±0.02
	0.5 mg L^{-1} Pb	0.72±0.02*	0.30±0.01**
	1 mg L^{-1} Cd	0.70±0.01*	0.28±0.01**
	1 mg L^{-1} Cu	0.77±0.01	0.33±0.01*
	1 mg L^{-1} Pb	0.69±0.02**	0.26±0.01***
L. valdiviana	Controle	0.68±0.03**	0.30±0.01**
	0.5 mg L^{-1} Cd	0.66±0.02	0.28±0.02
	0.5 mg L^{-1} Cu	0.70±0.02	0.33±0.01*
	0.5 mg L^{-1} Pb	0.54±0.02**	0.26±0.01**
	1 mg L^{-1} Cd	0.58±0.03**	0.25±0.01**
	1 mg L^{-1} Cu	0.62±0.01*	0.28±0.02
	1 mg L^{-1} Pb	0.49±0.03***	0.23±0.01***

Mean ± standard deviation, $p<0.05$*, $p<0.01$**, $p<0.001$***, $p>0.05$ – ns.

and mitochondria resulting in difficulty in respiration process (Prasad et al., 2001; Khellaf and Zerdaoui, 2009). After four days, in plants exposed of Cd 0.5 mg L^{-1} the Chl a+b content was lower in *L. minuta* with 6.1% and in *L. valdiviana* - 2.94% compared to the control levels. Regarding of carotenoids with the same treatment was also lower - 13.9% (*L. minuta*) and 6.7% (*L. valdiviana*) compared to the control. Increasing of the cadmium (1 mg L^{-1}) concentration the amount of photosynthetic pigments was further reduced. In *L. minuta* and *L. valdiviana*, respectively, they reduced with 14.63% and 14.71% for chlorophylls and with 22.22% and 16.67% for carotinoids compared to the control.

Treatment with 0.5 mg L^{-1} Pb concentration in fronds can reduce the number of photosynthetic pigments. At higher concentrations, it can limit growth and photosynthetic activity (Vavilin et al., 1995; Frankart et al., 2002). Pb ions can displace magnesium from the chlorophyll molecule and cause peroxidation (Sandmann and Boger, 1980; Mal et al., 2002). Treatment with Pb 0.5 mg L^{-1} lead a decrease of Chl a+b in *L. minuta* with 12.2% and in *L. valdiviana* - 20.6% compared to the control levels in this investigation. Carotenoid content decreased with 27.8% (*L. minuta*) and 23.33% (*L. valdiviana*) compared to the control.

The concentration of elements in water decreasing with time in the experimental duckweed treatments (Parra et al., 2012). Different species of *Lemna* bioabsorption different amounts of dissolved metals in water. Although the elements have the same initial concentration in the water, they accumulate in different degrees from the two tested species in this study. According many authors the relative growth of *Lemna* sp. decreases with increasing metal concentrations which was also confirmed in our study (Khellaf and Zerdaoui, 2009; Leblebici and Aksoy, 2011; Kaur et al., 2012; Bianconi et al., 2013). In our study cadmium, copper and lead inhibited the growth of *L. minuta* and *L. valdiviana* at 0.5 and 1 mg L^{-1} concentrations, as at the higher concentration the growth is reduced almost twice. This is particularly noticeable for copper, which has the strongest impact on the relative growth of the two studied species.

Bioaccumulation of some metal could depend on their chemical speciation, organic chelators, temperature, oxygen level, light intensity (Greger, 1999). Plants are considered good accumulators if they BCF reach 1000. Bioconcentration factors of Cu (0.5 mg.L^{-1}) were 1967.3±1.02 for *L. minuta* and respectively 1879.6±1.1 ($P \leq 0.01$) for *L. valdiviana* in this research. Higher BCF values were measured in plants exposed on lower metal concentration (Bianconi et al., 2013). For Cd (1 mg L^{-1}) a bioconcentration factor of 45.7±2.0 4 ($P \leq 0.05$) *L. minuta* and 42.7±3.03 ($P \leq 0.05$) for *L. valdiviana* was observed. In the end of an experiment, this metal has the lowest toxicity to the growth of the two investigated species.

The tolerance index (Ti) is indicator of the ability of plants to grow in the presence of a certain concentration of metal (Bianconi et al., 2013). In our study the best Ti 0.92±0.03 ($P \leq 0.05$) for Cd pollution in two *Lemna* species was reported. This result is in line with higher growth of *L. minuta* and *L. valdiviana* in cadmium concentration compared to lead and copper.

According to Xia and Tian (2009) copper is considered to be one of the most toxic metals for plants, although it is required as a basic element for metabolic and physiological processes. Copper is a potent inhibitor of the electron transport activity of Photo System II associated with the water separation system (Dewez et al., 2005). This reduces energy storage through photosynthesis, which leads to a decrease in biomass growth. Cu is an essential trace element, necessary for chlorophyll biosynthesis and is present in many metalloenzymes involved in oxidation reduction reactions (Ouzounidou, 1994). In this study, low copper concentrations led to increased levels of chlorophyll (0.88±0.01) and carotenoids (0.39±0.02) of *L. minuta* compared to control (0.82±0.04 and 0.36±0.02), but the differences were not statistically proven. The same effect was also observed in *L. valdiviana* photosynthetic pigments.

The quantity of chlorophyll and carotenoid in two *Lemna* sp. had negative correlation with Cd and Pb treatment (Table 2). The value of chlorophyll a+b content in *L. minuta* with 1 mg L^{-1} Pb (0.69±0.02) and Cd (0.70±0.01) were close and statistically proven compared to control. The concentration of chlorophyll a+b in *L. valdiviana* was lowest with Pb treatment. In *L. valdiviana* fronds treated with Pb concentration of 1 mg L^{-1} reduces the amount of photosynthetic pigments, despite that lead is a non-essential metal ion. Therefore high doses of lead ions can impact negatively on the photosynthesis process and development of plants (Vavilin et al., 1995; Frankart et al., 2002). The photosynthetic apparatus can be destroyed due to the drastic reduction of chlorophyll molecules (John et al., 2008). Of the three metals tested, lead was found to be the strongest inhibitor of photosynthetic pigments to the two studied *Lemna* species.

The six tested metal concentration had an impact on duckweed growth and photosynthetic pigments. The bioconcentration factor and tolerance index are greater at a lower concentration of metals. Of the three tested metals with the best BCF was copper. The copper content of 0.5 mg L^{-1} led to an increase in photosynthetic pigments. Better bioaccumulation ability was established in *L. minuta* compared to *L. valdiviana*.

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