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Sensitivity of *Hydrocharis morsus-ranae* L. to selected metals and its suitability for phytoremediation of waters contaminated with metals. A mesocosm study

Małgorzata Gałczyńska, Jacek Wróbel, Katarzyna Bednarz

West Pomeranian University of Technology in Szczecin, Faculty of Environmental Management and Agriculture, al. Piastów 17, 70-310 Szczecin, Poland

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Abstract: Anthropogenic pollution leads to increased concentrations of metals in the freshwater and macrophyte. Aquatic plants substantially contribute to the structure, function as well as and service provision of aquatic ecosystems. Our microcosm experiments were to test the possibility of the physiological response of *Hydrocharis morsus-ranae* to metal (Cd, Pb, Cu, Zn, Mn, Fe at three level of concentration) contaminated waters. Biomass was analysed at the beginning and at the end of the experiment. At the same time contents of photosynthetic pigments in leaves were estimated spectrophotometrically. We found that this macrophyte had the ability to grow in contaminated waters, but the effects of high concentration of isolated metals in water will indicate changes consisting in the disappearance of a significant part of biological populations were which manifested in alteration of the content of photosynthetic pigments as well as this plant's growth. We show that generally stress of Zn and Cu influenced the drop of dry biomass which was connected with a positive correlation between the amount of dry biomass and the content of chlorophyll *a* and carotenoids, or only carotenoids, respectively. The highest stress of Pb and Fe (third concentrations of these metals) also influenced the drop of biomass. We concluded that none of Cd concentrations were toxic to this plant, but the effect of Mn stress was not unequivocal. Moreover, plant growth was stimulated by low Fe concentrations (first concentration) demonstrating the hormesis effect. When plants were exposed to this metal, there was no evidence of damage to the photosynthetic processes.

Keywords: biomass, *Hydrocharis morsus-ranae*, mesocosm study, metals, photosynthetic pigments, phytoremediation

INTRODUCTION

Chemical properties of surface waters as well as the presence of bioindicators are the key elements in water monitoring as defined in the European Water Framework Directive (WFD) [Directive 2000/60/EC]. In the European Union, the contamination of surface water with metals has significantly decreased over 30 years, but in many countries, this problem still occurs due to local or regional pollution. The reaction of macrophytes to the elevated levels of metals in water affects their development and can be used as one of the three modes of monitoring surface water according to WFD, that is investigative monitoring to determine the environmental objectives. High dynamics of change in climate conditions associated with higher air temperature or low precipitation causes a local alteration in the concentration of many metals and affects the development of many macrophytes [Datta *et al.* 2022; Martinez-Haro *et al.* 2015].

In aquatic systems, free-floating species absorb metal ions in roots and also directly in the leaves owing to the deposition of particles on the foliar surfaces [Lahive *et al.* 2011; Newete *et al.* 2016]. Metals affect numerous physiological and biochemical processes occurring in plants. Trace amounts of a few metals – Cu, Mn, Zn, and Fe, are crucial to plant metabolism. However, excessive levels or bioavailable forms of metals may potentially cause toxic effects in plants [Kamal *et al.* 2004]. Some metals like Cd and Pb have no established biological functions and are considered as non-essential metals and potentially highly toxic [Nagajyoti *et al.* 2010].

Heavy metals were found to have an invariable effect on photosynthetic functions, both directly as well as indirectly [Malec *et al.* 2011; Shiryaev *et al.* 2021]. The direct effect on photosynthesis due to heavy metals is manifested in the damage to the photosystems reaction centres (PS I and PS II), the water splitting complex, LHCII (light-harvesting complex II) antenna complex, as well as other elements of the photosynthetic transport chain. Moreover, heavy metals may cause a decrease in photosynthetic efficiency owing to inhibited chlorophyll biosynthesis [Myśliwa-Kurdziel, Strzałka 2002], pigment degradation [Harguinteguy *et al.* 2013], metal-induced alterations in the structure of chloroplasts, the resulting imbalance of water uptake, and, among others, stomatal closure. When exposed to different heavy metals, plants manifest decreased photosynthetic efficiency which in turn leads to lower biomass production, inhibition of plant growth and, potentially, plant death. Studies on several different plant species revealed decreased chlorophyll levels owing to metals: Pb [Dogan *et al.* 2009; Qiao *et al.* 2014; Veselý *et al.* 2012], Cd [John *et al.* 2008; Malec *et al.* 2010; Mohammed 2016], Cu [Zeynep 2013], Zn [Yilmaz *et al.* 2012], Fe [Xing *et al.* 2009], Mn [Doganlar *et al.* 2012].

European frogbit (*Hydrocharis morsus-ranae*) is a common free-floating or rooted plant [Efremov *et al.* 2021] of Eurasia and North America. This plant grows mainly in slow-moving waters in bays, ponds, open marshes and ditches, as well as along protected lakes and rivers edges. It favours eutrophic and mesotrophic waters [Catling *et al.* 2003; Engin *et al.* 2015; Gałczyńska, Bednarz 2012; Hansen *et al.* 2022; Zhu *et al.* 2018]. Numerous systems of assessing the trophic state in running waters make use of frogbit, namely Mean Trophic Rank (MTR) in the United Kingdom, Macrophyte Index for Rivers (MIR) in Poland, and Macrophyte Biological Index for Rivers – IBMR (France). These systems employ plant species which are considered as biological indicators of water trophy status [Szczerbińska, Gałczyńska 2015].

The studies conducted so far in natural aquatic ecosystems [Gałczyńska, Bednarz 2012; Polechońska, Dambiec 2014; Polechońska *et al.* 2017; Polechońska, Samecka-Cymerman 2015; 2016; Skwierawski, Skwierawska 2013] show the possibility of occurrence of *H. morsus-ranae* in waters contaminated with metals, including heavy metals. The results confirming the accumulation of substantial amounts of metals in the plant's tissues as well as high efficiency of such accumulation in defined environmental conditions, support including *H. morsus-ranae* to a group of bioindicators of waters contaminated with metals [Gałczyńska 2012; Polechońska, Dambiec 2014; Polechońska *et al.* 2017; Polechońska, Samecka-Cymerman 2015]. Chlorophyll content is one of the parameters which may indicate the viability of a bioindicator when conducting studies. The analysis of this photosynthetic pigment is fairly common, and its content is determined for various concentrations of metals [Maleva *et al.* 2004]. Concentrations of metals in water, not tolerated by a hydrophyte, have a generally negative effect on its development. However, it is possible that chlorophyll content rises with an increase in the concentration of a given contaminant – as has been observed with *Lemna gibba* [Doganlar *et al.* 2012]. Therefore, it is valid to identify the vital parameters of the bioindicator, i.e. *H. morsus-ranae*, depending on the varied contamination of water with metals considered indispensable as well as redundant for the plant's development.

Due to high phytoremediation potential of *H. morsus-ranae* and quick decomposition time of the plant combined with the release of the elements accumulated in the leaves to water [Gałczyńska 2012; Polechońska, Samecka-Cymerman 2015; 2016], the possibility of using the plant for phytoremediation of waters contaminated with metals are limited mainly by the length of application time.

Given the usability of *H. morsus-ranae* both in monitoring as well as phytoremediation of waters, it was assumed that the changes in the content of assimilation pigments in the plant's leaves and biomass change constituting the response to the environmental stress:

- provide information on the plant sensitivity to water contamination with metals (above value for bad quality water) and the possibility of colonising water reservoirs;
- may help in deciding on acceleration of the date of the plants' removal from a contaminated reservoir, in case *morsus-ranae* is used for phytoremediation of waters contaminated with the metals under analysis.

MATERIALS AND METHODS

PLANT MATERIAL AND GROWING CONDITIONS IN NATURAL WATER ECOSYSTEM

For the purpose of conducting the hydroponic experiment with the use of European frogbit (*Hydrocharis morsus-ranae* L.), plant material that was unpolluted with metals was selected. The study material was obtained from the catchment of Lake Świdwie, located in agricultural land in the northwest of Poland. Plants of *H. morsus-ranae* were collected from Struga Żurawia Canal (N 53°33'13.81' and E 14°21'10.95'), which empties into Lake Świdwie. The water samples were also taken to measure the concentration of nitrogen nitrate, ammonium cation, and orthophosphate. For the determination of N and P in the waters, the colorimetric methods were used in accordance with the Polish standards [PN–82/C-04576/08; PN–89/C-04537/02; PN-ISO 7150-1].

It was found that *H. morsus-ranae* was grown in waters of low nitrogen compound concentration (0.148 mg·dm^{-3} N-NO_3^- and 0.093 mg·dm^{-3} N-NH_4^+) and elevated concentration of phosphorus compounds (0.140 mg·dm^{-3} P-PO_4^{3-}). Additionally, concentrations of selected elements, including potassium, were identified in water as well as in *H. morsus-ranae*. The measurements were taken after mineralisation of the two types of samples with the use of atomic absorption spectrometry using Solaar S AA spectrometer. All of the elements were measured against the recognised standards (AAS standard solution by Sigma Chemical). Aqueous samples were mineralised with concentrated nitric acid and plant samples were hot mineralised with a mixture (3:1) of concentrated acids: nitric and perchloric [Dhir *et al.* 2009]. In the aqueous environment of *H. morsus-ranae* growth, there were no ions of cadmium, lead, copper, zinc, or manganese (Tab. 1).

MICROCOSM EXPERIMENT – GROWING CONDITIONS, KINDS OF MEASUREMENTS

A microcosm experiment with *H. morsus-ranae* was performed in a greenhouse of the West Pomeranian University of Technology in Szczecin in semi-controlled conditions with a natural photo-

Table 1. Metal concentration in water and content in *Hydrocharis morsus-ranae* from Struga Żurawia Canal

Medium	Concentration[1)]/Content[2)]						
	Cd	Pb	Cu	Zn	Mn	Fe	K
Water	BDL	BDL	BDL	BDL	BDL	0.54	3.18
Plant	BDL	0.92	12.94	33.65	$3.04 \cdot 10^3$	$0.79 \cdot 10^3$	$10.2 \cdot 10^3$

[1)] In water mg·dm^{-3}. [2)] In plant mg·kg^{-1} d.w.
Explanations: BDL = below detection limit. Detection limits of element in water samples represent ranges: 0.004–12.5 mg·dm^{-3} Cd; 0.01–25.0 mg·dm^{-3} Pb; 0.004–12.5 mg·dm^{-3} Cu; 0.02–25 mg·dm^{-3} Zn; 0.03–50 mg·dm^{-3} Mn; 0.03–50 mg·dm^{-3} Fe; 0.3–150 mg·dm^{-3} K and in plant samples: 0.02–2.00 mg Cd·kg^{-1} d.w.; 0.05–15.0 mg Pb·kg^{-1} d.w.; 0.5–160 mg Cu·kg^{-1} d.w.; 4.5–700 mg Zn·kg^{-1} d.w.; 2.5–1600 mg Mn·kg^{-1} d.w.; 5–3200 mg Fe·kg^{-1} d.w.; 0.9–50 g K·kg^{-1} d.w.
Source: own study.

period of the first week of June, mean temperature (night-day) of 20–25°C. Prior to the experiment, the plants were thoroughly cleaned and rinsed with distilled water. Two rosettes of *H. morsus-ranae* (each rosette with a runner with a developing new plant) of comparable mass (fresh weight of two rosettes: 4.1 ±0.2 g) were selected for the experiment. The selected plants were placed for the period of one day in 20 containers with a solution of biogenic compounds: 0.156 mg·dm^{-3} N-NO_3^-, 0.119 mg·dm^{-3} N-NH_4^+, 0.183 mg·dm^{-3} P-PO_4^{3-}, 0.675 mg·dm^{-3} K^+ (acclimation period). The concentrations of ionic forms of N and P in growing medium were similar to those found in the natural environment of growth. In conformity with the Regulation of the Minister of Environment [Rozporządzenie … 2004], both concentrations correspond with very good water quality standards (class I: 5 mg·dm^{-3} NO_3^- and 0.5 mg·dm^{-3} NH_4^+) with regards to the analysed forms of nitrogen, whereas in terms of orthophosphates, the concentrations correspond with satisfactory water quality standard (class III: 0.7 mg·dm^{-3} PO_4^{3-}).

After one day, to 18 containers with *H. morsus-ranae* grown on culture medium the following were separately added: solutions of cadmium (Cd), lead (Pb), copper (Cu), zinc (Zn), manganese (Mn), and iron (Fe) in the form of $3CdSO_4 \cdot 8H_2O$, Pb $(CH_3COO)_2 \cdot 3H_2O$, $CuCl_2 \cdot 2H_2O$, $ZnSO_4 \cdot 7H_2O$, $MnSO_4 \cdot H_2O$, $FeCl_3$ (Tab. 2). Three concentrations of the previously prepared solutions of metal salts (C1, C2 = 2C1, C3 = 2C2 = 4·C1) were selected in such a way so as to reflect poor water quality standard (class V – bad quality water: biological values of water quality indicators show, as a result of anthropogenic impacts, changes consisting in the disappearance of a significant part of biological populations) as defined in the Regulation of the Minister of Environment [Rozporządzenie … 2004]. The aforementioned regulation, contrary to the currently applicable Regulation of the Minister of Environment [Rozporządzenie … 2016], accounts for only four of the analysed metals, i.e. Cd, Pb, Cu, and Zn, in the analysis of the chemical status of water. On the second day of the experiment, in the first control, i.e. the container with two rosettes of *H. morsus-ranae* grown on culture media, the content of the photosynthetic pigment was determined in leaves: chlorophyll *a* (Chl *a*), chlorophyll *b* (Chl *b*), chlorophyll *a* + *b* (Chl *a* + *b*) and carotenoids (Car). After seven days, the fresh and the dry weight were weighed. The conditions provided to the culture containers corresponded with a natural photoperiod of

Table 2. Metal concentration in water at the beginning of the mesocosm experiment

Concentration	Measurement unit	Concentration					
		Cd	Pb	Cu	Zn	Mn	Fe
C1	mg·dm^{-3}	0.012	0.76	0.15	3.02	1.52	3.06
	µM	0.1	3.7	2.4	46	28	55
C2	mg·dm^{-3}	0.025	1.52	0.30	6.04	3.04	6.12
	µM	0.2	7.4	4.7	82	55	110
C3	mg·dm^{-3}	0.050	3.04	0.60	12.08	6.08	12.24
	µM	0.4	14.7	9.4	185	111	220

Explanations: C_1, C_2, C_3 = class V according to the Regulation of the Minister of Environment [Rozporządzenie … 2004]: >2 mg·dm^{-3} Zn; >0.005 mg·dm^{-3} Cd; >1.0 mg·dm^{-3} Mn; >0.1 mg·dm^{-3} Cu; >0.05 mg·dm^{-3} Pb; >2.0 mg·dm^{-3} Fe).
Source: own study.

16 h light/8 h dark cycle, the intensity of quantum radiation was from 0.72 to 576 µmol·m^{-2}·s^{-1} (FF-01 Sonopan), and the temperature ranged from 20 to 25°C.

MEASUREMENTS OF DRY AND FRESH WEIGHT OF *Hydrocharis morsus-ranae* AND PHOTOSYNTHETIC PIGMENTS

Biomass was analysed at the beginning and at the end of the experiment. The measurement of fresh weight was carried out in each case in the same way, i.e. the *H. morsus-ranae* rosettes were rinsed with distilled water and then dried on filter paper. Then the rosettes were weighed. The biomass of *H. morsus-ranae* was dried in an air circulation oven for 48 h at 65°C and weighed.

The contents of Chl *a*, Chl *b*, and Chl *a* + *b* were determined in the fresh weight of *H. morsus-ranae* leaves (usually second, third and fourth) from two (at the beginning) or three rosettes (at the end of the experiment) according to a method presented by Arnon *et al.* [1956] and further modified by Lichtenthaler and Wellburn [1983]. The content of Car was assessed following Hager and Mayer-Berthenrath [1966]. In order to estimate the content of photosynthetic pigments, a known mass of leaf (about 0.05 g fresh weight) was homogenised in 10 cm^3 of 80% acetone. Subsequently, the homogenate was centrifuged at 2500 rev·min^{-1} at 4°C for 8 min. Measurements of the absorbance of the acetone extracts were conducted at 440, 645, and 663 nm with a spectrophotometer UV-VIS (UV-1800 *Shimadzu, Japan*). For all parameters, the analysis was triplicate. Chl *a*:Chl *b*, Chl *a* + *b*: Car were also calculated.

STATISTICAL ANALYSIS

The experimental design was completely randomised. All results were elaborated statistically using Statistica 13.3 software. The normality of distribution of continuous variables was verified using the Shapiro–Wilk test. The homogeneity of the variance for the two-factor analysis of variance was assessed by the Brown–Forsythe test. Continuous variables with a normal distribution (biomass, Chl *a*, Chl *b* and Chl *a* + *b*, Car) were expressed as mean values ±standard deviation (*SD*) and mean values ±standard

error (*SE*). Nine leaves samples ($n = 9$) were used to determine the photosynthetic pigments content and three rosettes ($n = 3$) were used to determine the plant dry weight. The statistical significance of differences between the two groups (control and selected levels of metal concentration) was tested using Student's *t*-test. The results were developed using a two-way analysis of variance (1st factor: the metal – 6 levels, 2nd factor: the level of metal concentration – 3 levels). For the purpose of determining the significance of differences between the mean measurement values Tukey's confidence interval was used. The associations between variables were assessed using the univariate Pearson coefficients; $P < 0.05$ value was considered as statistically significant.

RESULTS

GENERAL EFFECT OF METALS TOXICITY ON THE GROWTH OF *Hydrocharis morsus-ranae* AND THE CONTENT OF PHOTOSYNTHETIC PIGMENTS

The experiment shows that the general addition of different metals to culture medium had a significant effect both on dry weight of *H. morsus-ranae* (Fig. 1: ANOVA – 1st factor – the metal, $F = 4.58$ and $P < 0.001$) and on the content of photosynthetic pigments in leaves (Fig. 2, Chl *a* + *b*: ANOVA – 1st factor – the metal, $F = 3.20$ and $P < 0.001$; Chl *a*: $F = 3.65$ and $P < 0.005$; Chl *b*: $F = 3.35$ and $P < 0.008$; Car: $F = 4.89$ and $P < 0.001$). Moreover, the concentration of the metals also proved to have a significant effect on the dry weight of *H. morsus-ranae* (Fig. 3: ANOVA – 2nd factor – the level of metal concentration, $F = 5.33$ and $P < 0.003$) and on the content of photosynthetic pigments in leaves (Fig. 4, Chl *a* + *b*: ANOVA – 2nd factor – the level of metal concentration, $F = 13.44$ and $P < 0.001$; Chl *a*: $F = 10.57$ and $P < 0.001$; Chl *b*: $F = 11.27$ and $P < 0.001$; Car: $F = 9.18$ and $P < 0.001$).

Significantly the lowest dry weight of *H. morsus-ranae* was found generally in plants cultivated on culture media contaminated with Zn and Cu. In turn, as compared with the control, no significant differences were found in the dry weight of plants cultivated on media contaminated with the remaining metals (Mn, Pb, Fe, Cd) – Figure 1.

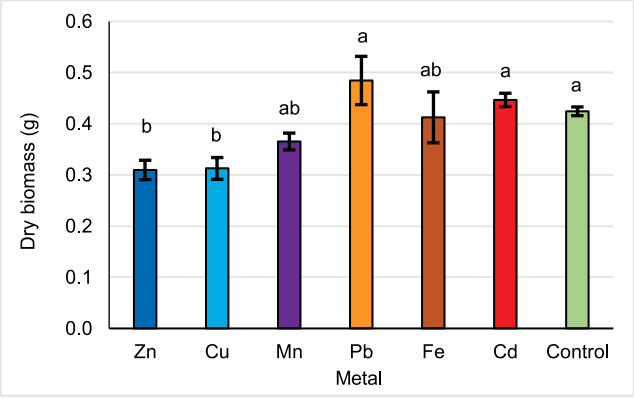

Fig. 1. Effect of metal supply on dry weight of *Hydrocharis morsus-ranae*; data represent the mean (bars) ± *SE* ($n = 9$); *SE* = standard error; different letters indicate significant differences verified by two-way ANOVA (1st factor – the metal) and Tukey's test at $P < 0.05$; source: own study

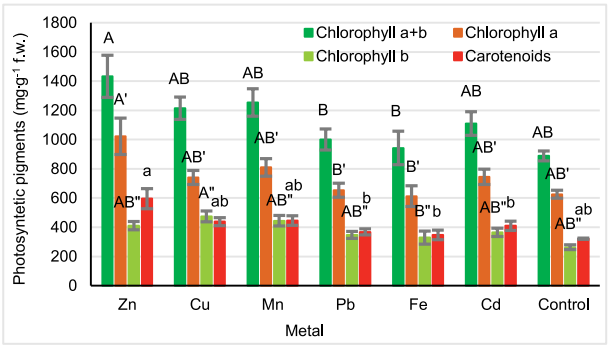

Fig. 2. Effect of metal supply on pigments content in leaves of *Hydrocharis morsus-ranae*; data represent the mean (bars) ±*SE* ($n = 27$); *SE* = standard error; different letters indicate significant differences verified by two-way ANOVA (1st factor – the metal) and Tukey's test at $P < 0.05$; source: own study

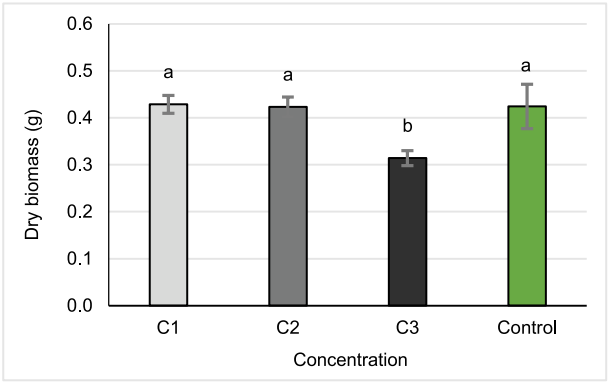

Fig. 3. Effect of metal concentrations on dry weight of *Hydrocharis morsus-ranae*; solutions of metal salts (C1, C2 = 2C1, C3 = 2C2 = 4C1); data represent the mean (bars) ± *SE* ($n = 27$); *SE* = standard error; different letters indicate significant differences verified by two-way ANOVA (2nd factor – the level of metal concentration) and Tukey's test at $P < 0.05$; source: own study

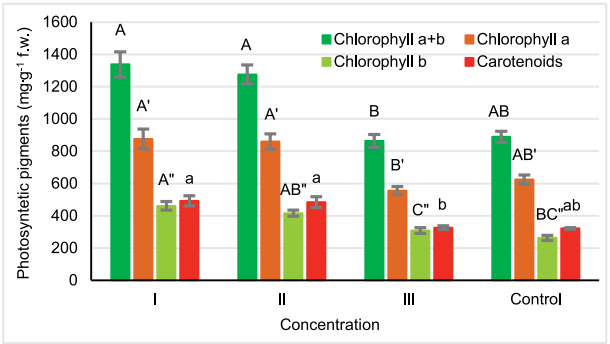

Fig. 4. Effect of metal concentrations on pigments content in leaves of *Hydrocharis morsus-ranae*; solutions of metal salts (C1, C2 = 2C1, C3 = 2C2 = 4C1); data represent the mean (bars) ± *SE* ($n = 54$); *SE* = standard error; different letters indicate significant differences verified by two-way ANOVA (2nd factor – the level of metal concentration) and Tukey's test at $P < 0.0$; source: own study

A decrease in dry weight of *H. morsus-ranae*, as compared with control, was recorded at a third concentration of the analysed metals (Fig. 2).

Among all the experimental combinations, the highest content of total Chl *a* + *b* was found for plants grown on culture media with the addition of Zn. The lowest content of total chlorophyll was found for plants grown on media with the addition

of Pb and Fe. The differences were statistically significant (Fig. 3). The same relationship was identified in terms of Chl *a* content. As for Chl *b*, the content was markedly lower in the control and in the plants grown on culture media with the addition of Fe as compared with the plants grown on media with Cu, which exhibited the highest content. The highest concentration of Car was determined in plants grown on culture media with the addition of Zn. The control and the plants grown with the addition of Pb, Fe, and Cd (Fig. 3) showed a significantly lower concentration.

The control and the plants grown at third concentrations of particular metals were characterised by the comparable content of the analysed pigments in plants, yet the content was significantly higher at the first and second concentrations than at third concentrations (Fig. 4).

EFFECT OF DIFFERENT DOSES OF METALS ON THE GROWTH OF *Hydrocharis morsus-ranae* AND THE PHOTOSYNTHETIC PIGMENTS CONTENT

The biomass and the amounts of Chl *a*, Chl *b*, Chl *a* + *b* and Car in the leaves of *H. morsus-ranae* were determined at the completion of the experiment. The obtained results (Tab. 3) varied depending on the metal and its concentration.

It was observed that upon stress caused by Zn, namely the increase of Zn concentration in culture medium, there was a decrease of *H. morsus-ranae* biomass in comparison with the control conditions – to 86, 79, and 57% respectively. At the same time, it was found that the first and the second concentration of Zn showed a stimulating effect on the content of the analysed photosynthetic pigments. The significantly highest content of Chl *a* was recorded at the first concentration of the metal in the culture medium (Tab. 3). A similar reaction of *H. morsus-ranae* was found following the addition of Cu to culture medium. A significant decrease in biomass was found for second and third concentrations of Cu – to 69 and 62% of the control respectively. In turn, the addition of Cu in the first and second concentrations had a significant effect on the increase of photosynthetic pigments content. Regardless of the concentration, the presence of Mn in culture medium resulted in an increased content of all photosynthetic pigments. However, as compared with the control, a significantly greater content was found for the first concentration, and for Chl *b* also for the second and third concentrations. A significant decrease in plant biomass, i.e. to 74% of the control, was identified only for the second Mn concentration. In turn, Pb-contaminated culture medium, at first and second concentrations, showed a marked increase in photosynthetic pigments content in comparison with the control. As for the second

Table 3. Content of photosynthetic pigments and dry weight of *Hydrocharis morsus-ranae* depending on the six metal contamination and their three concentrations

Metal	Solution	Metal concentration ($mg \cdot dm^{-3}$)	Photosynthetic pigments ($\mu g \cdot g^{-1}$ f.w.)				Dry weight of one rosette (g)
			Chl *a*	Chl *b*	Chl *a* + *b*	Car	
Control	O_s	0.00	586 ±35	270 ±24	857 ±27	343 ±21	0.41
	O	0.00	626 ±47	263 ±27	889 ±59	321 ±7	0.42 ±0.01
Zn	C1	3.02	**1319 ±213**	**472 ±53**	**1791 ±262**	**696 ±138**	*0.36 ±0.03*
	C2	6.04	**1187 ±143**	409 ±97	**1596 ±171**	**735 ±135**	*0.33 ±0.02*
	C3	12.08	562 ±40	351 ±74	912 ±63	357 ±38	*0.24 ±0.02*
Cu	C1	0.15	**782 ±84**	**550 ±111**	**1332 ±35**	**484 ±44**	0.39 ±0.03
	C2	0.30	**845 ±85**	**501 ±87**	**1346 ±164**	**477 ±67**	*0.29 ±0.03*
	C3	0.60	593 ±134	373 ±77	967 ±210	354 ±73	*0.26 ±0.02*
Mn	C1	1.52	**924 ±128**	**536 ±109**	**1460 ±238**	**524 ±67**	0.39 ±0.03
	C2	3.04	856 ±205	**450 ±63**	1307 ±267	463 ±101	*0.31 ±0.02*
	C3	6.08	648 ±97	**348 ±44**	997 ±141	351±51	0.40±0.02
Pb	C1	0.76	**715 ±2**	**382 ±35**	**1097 ±37**	**400±11**	0.43±0.03
	C2	1.52	759 ±99	**402 ±42**	**1161 ±139**	**412±41**	**0.67±0.04**
	C3	3.04	487 ±94	258 ±32	745±125	293±19	*0.36±0.02*
Fe	C1	3.06	770 ±246	453 ±155	1223 ±400	417±121	**0.55±0.04**
	C2	6.12	644 ±163	317 ±63	961 ±223	351±74	0.46±0.03
	C3	12.24	*425 ±51*	219 ±53	*644 ±105*	276 ± 67	*0.23 ± 0.03*
Cd	C1	0.012	747 ±230	373 ±131	1121 ±360	434 ±132	0.45 ±0.04
	C2	0.025	**868±51**	**417 ±21**	**1285 ±68**	**473 ±25**	**0.48 ±0.03**
	C3	0.050	620 ±15	303 ±22	923 ±7	324 ±11	0.41 ±0.02

Explanations: data represent mean ±*SD* (n = 9; *SD* = standard deviation) of photosynthetic pigments content. Values signed in bold means significant higher value than value of control, value signed in italic letter means significant lower value than value of control (n = 9 leaves samples were used to determine the photosynthetic pigments content or n = 3 rosettes were used to determine the plant dry weight), according to Student's t-test at $P < 0.05$. O_s = control at the start of experiment, O = control at the end of experiment, Chl *a* = chlorophyll *a*, Chl *b* = chlorophyll *b*, Chl *a* + *b* = chlorophyll *a* + *b*, Car = carotenoids; solutions of metal salts (C1, C2 = 2C1, C3 = 2C2 = 4C1).
Source: own study.

concentration of Pb, the highest pigments concentration and the largest plant biomass were found – approximately 60% higher than control. The third concentration of Pb resulted in a clear decrease of the photosynthetic pigment content (by 16% Chl *a* + *b* and approx. 10% Car), and 16% decrease in plant biomass. The addition of Fe at the first concentration to culture medium resulted in an increase in biomass by 31% in comparison to control. Additionally, there was an inconsiderable increase in photosynthetic pigments (Tab. 3). With the third concentration of Fe, there was a significant decrease in biomass to 55% coinciding with a decrease in pigment content, particularly Chl *a*. The presence of Cd in culture media at the first concentration, and particularly at the second concentration, caused a marked increase in the content of photosynthetic pigment as well as biomass. The third concentration of Cd in culture media showed no effect neither on the content of photosynthetic pigments nor biomass.

CORRELATION BETWEEN DRY BIOMASS OF *Hydrocharis morsus-ranae* AND CONTENTS OF PHOTOSYNTHETIC PIGMENTS IN THE LEAVES OF THE PLANT

Significant linear correlations were found between dry biomass of *H. morsus-ranae* and the contents of photosynthetic pigments in the leaves of the plant as the effect of metal supply. The content of Chl *a* + *b*, Chl *a* and Car in *H. morsus-ranae* was increased by increasing the biomass of plants in Zn contaminated waters.

After the addition of Cu to culture medium, the increase in dry biomass was accompanied only by an increase of Car content. In turn, with respect to Pb, there were positive correlations between the biomass of the plant and Chl *a* + *b*, Chl *b* and Car. As for Cd, there was a distinctive correlation between biomass and the analysed photosynthetic pigments (Tab. 4).

Table 4. Effect of metal supply on correlation between dry biomass of *Hydrocharis morsus-ranae* and contents of photosynthetic pigments in the leaves of plant

Metal	Pearson correlation coefficient for			
	Chl *a* + *b*	Chl *a*	Chl *b*	Car
Control	NS	NS	NS	NS
Zn	0.88	0.89	NS	0.76
Cu	NS	NS	NS	0.72
Mn	NS	NS	NS	NS
Pb	0.67	NS	0.68	0.69
Fe	NS	NS	NS	NS
Cd	0.86	0.90	0.77	0.86

Explanations: Chl *a* = chlorophyll *a*, Chl *b* = chlorophyll *b*, Chl *a* + *b* = chlorophyll *a* + *b*, Car = carotenoids; NS = not statistically significant Pearson correlation coefficient at $P < 0.05$.
Source: own study.

The experiment did not show any effect of the level of metal concentration (C_{Me}) on the correlation between the biomass of *H. morsus-ranae* and ratios of photosynthetic pigments in the leaves of a plant.

DISCUSSION

SENSITIVITY OF *Hydrocharis morsus-ranae* TO ZINC

Despite the lack of redox activity, Zn is a trace metal crucial for numerous vital physiological events occurring in plants [Sagardoy *et al.* 2009]. It is a constituent of six classes of enzymes and of special proteins known as Zn fingers [Gupta *et al.* 2012]. This element plays a role in the regulation of nitrogen metabolism and in the formation of carbohydrates, chlorophyll, and root development [Kleckerova *et al.* 2011]. Rout and Das [2003] concluded that the high levels of Zn may result in limited growth as well as cause symptoms of toxicity (inhibiting the root elongation, shoot stunting, curling and rolling of young leaves, leaf tips death and chlorosis).

As has been reported by Lahive *et al.* [2011], an excess of Zn is accumulated by *Lemna minor* mostly in its roots, with a reduction of Zn amount stored in the fronds providing protection to vital metabolic processes. They observed some root detachment (not quantified) at exposure to the highest Zn concentration (30 mg·dm^{-3}). The toxic response in the form of detachment of roots is commonly reported, yet rarely quantified [Megateli *et al.* 2009]. It is speculated that root detachment may constitute a protective mechanisms, particularly for *L. minor*, since the total loading of the plant with Zn would be markedly reduced (by 35%) had the roots been released. The inhibition of growth was about 37% at 10 mg·dm^{-3} and about 50% at 30 mg·dm^{-3} of Zn.

In this study, it was observed that *H. morsus-ranae* did not tolerate all concentrations of Zn used in these experiments (Tab. 3). The dry biomass decreased to 86, 79 and 57%, respectively. A similar trend of the decrease in biomass of *Pistia stratiotes* alongside the increase of concentration of zinc in the growth medium was observed by Odjegba and Fasidi [2004] after seven days. It was connected with a decrease of root elongation and the creation of new roots and a reduction in the rate of leaf expansion. Zinc stress in *H. morsus-ranae* induced an increase in all analysed photosynthetic pigments at 3.02 mg·dm^{-3} and in Chl *a*, Chl *a*+*b*, Car in the leaves at 6.04 mg·dm^{-3} (Tab. 3), mainly due to the favourable effect of metals supply on chlorophyll synthesis. However, with the increased metal exposure in the growth medium, the chlorophyll content slowly decreased to the level in the control condition. A temporal decline in the content of chlorophyll was determined given that heavy metals are capable of substituting the central Mg ion or inhibiting the synthesis of chlorophyll through inhibition of the chlorophyll-synthesising enzyme activity [Manios *et al.* 2003]. Yilmaz *et al.* [2012] described the similar results for *Lemna gibba, L. minor* and *Spirodela polyrrhiza*, but at lower Zn concentration (0, 0.01, 0.05, 0.1, 0.5 and 1.5 mg·dm^{-3}). In the present study, a positive linear correlation was identified between dry biomass of this plant and contents of Chl *a*, Chl *a* + *b* and Car. As has been reported by Ye *et al.* [1997] the seedlings of *Typha latifolia* were found to be chlorotic at ~80 μM Zn. This was contrary to the present research because the results suggested that no leaf chlorosis occurred.

SENSITIVITY OF *Hydrocharis morsus-ranae* TO COPPER

Coppper is a redox element and an essential micronutrient engaged in various vital physiological functions of plants such as the biosynthesis of Chl, Car, quinones, plastocyanin, and

superoxide dismutase [Yruela 2005]. Nonetheless, when Cu concentration in plant tissue exceeds the optimal levels, it becomes toxic to many aquatic plants [Khellaf, Zerdaoui 2010; Prasad *et al.* 2001]. Coppper accumulates primarily in root tissue and there is a little upward movement to the aerial plant parts and leaves [Hammad 2011; Khellaf, Zerdaoui 2010]. Consequently, the initial determination of Cu toxicity hinders root elongation as well as growth [Khellaf, Zerdaoui 2010]. Chlorosis, necrosis as well as leaf discolouration are listed, among others, as the consequent symptoms [Yruela 1999].

In this study, it was demonstrated that when present in the nutrient solution at 0.15 mg·dm^{-3} Cu was tolerated by *H. morsus-ranae* (Tab. 3), but the Cu concentrations of 0.3 and 0.6 mg·dm^{-3} considerably decreased the biomass to 69 and 61%, respectively. These results are comparable to those previously reported for *Lemna gibba* [Manios *et al.* 2003]. With the use of similar tests, it has been demonstrated that inhibition of *Elodea canadensis* growth could also be observed in solutions of Cu salts, within concentration range 0.016–0.16 mmol·dm^{-3}, at 25 days long incubation period of the tested samples [Mal *et al.* 2002]. Other experiments also confirmed the toxic influence of Cu on this macrophyte [Malec *et al.* 2009]. Prasad *et al.* [2001] reported that the decrease of pigment concentrations in leaves of *Lemna trisulca* was found only at 25 and 50 μM of Cu content in the medium. Interestingly, there was a markedly slower decrease in Chl *b* concentration in comparison with Chl *a* and Car. At a lower dose of Cu (1, 2, 5, 10 μM) photosynthetic pigments content (Chl *a*, Chl *b*, Car) did not change as a function of the Cu dose. In this study, Cu at concentrations of 2.4 and 4.7 μM promoted pigment biosynthesis (Tab. 3). In this situation, contrary to the results by Prasad *et al.* [2001], Chl *b* concentration decreased significantly faster than Car. This fact could be explained by different sunlight conditions in these experiments.

Zeynep [2013] reported significant increases in the content of pigment for 0.05 and 0.1 mg·dm^{-3} Cu in *Lemna gibba*. The increased pigment content can be due to the Cu-induced stimulation of pigment synthesis at low concentrations such as in this study (Tab. 3). However, high levels of Cu (0.5 and 1.5 mg·dm^{-3}) resulted in the inhibition of synthesis in *L. gibba*.

SENSITIVITY OF *Hydrocharis morsus-ranae* TO MANGANESE

Manganese is an essential metal with a crucial role in various metabolic and growth-related processes for plants, such as photosynthesis, respiration, and enzyme biosynthesis. Additionally, this redox-active metal is a necessary cofactor in multiple plant enzymes (e.g. superoxide dismutase). This chemical element participates in carbohydrate and nitrogen metabolism, as well as the synthesis of fatty acid, acyl lipids, carotenoid, and hormonal activation. It is found to have significant importance to the functionality of photosystem II (PSII), particularly during the course of water molecules splitting into oxygen, as well as to the protection of PSII against photodamage [Millaleo *et al.* 2010]. In aquatic plants, Mn is found to saturate metal-binding sites thus providing protection against the effects of more toxic heavy metals on the plants [Moore 1991]. Reduced biomass and photosynthesis as well as biochemical disorders such as oxidative stress are the manifestations of Mn phytotoxicity. The effects frequently observed in plants and algae in the conditions of excess Mn are reduced photosynthesis, reduced chlorophyll *a* and *b* contents and their biosynthesis, and reduced carotenoids [Millaleo *et al.* 2010].

Doganlar *et al.* [2012] investigated the effects of Mn stress (0.25, 1, 4, and 16 mg·dm^{-3}) in *Lemna gibba* and reported that Mn exposure at low levels (0.25, 1, 4 mg·dm^{-3}) resulted in increased pigment content. This is in line with the results of the present study, all pigment levels in the leaves of *H. morsus-ranae* increased under Mn stress at 1.52 mg·dm^{-3} (first concentration). The increased pigment content at a high concentration of Mn may be attributed to a precursor role of Mn in the synthesis of chlorophyll [Millaleo *et al.* 2010]. It was suggested that the said increase in the photosynthetic pigment content is to be considered as the plant's ability to tolerate or overcoming such low Mn concentrations. An increase in Mn concentration (3.04 and 6.08 mg·dm^{-3}) showed a stimulatory effect on the conversion of Chl *a* to Chl *b* (Tab. 4). Contrary to Doganlar *et al.* [2012], a decrease in pigment content at the third concentration of Mn (6.08 mg·dm^{-3}) was not observed. The study by Lizieri *et al.* [2012] analysed morphophysiological responses of *Azolla caroliniana*, *Salvinia minima*, and *Spirodela polyrhiza* to a supra-optimal manganese supply. It was indicated that these free-floating plants exposed to higher Mn concentrations (0.4 mM) showed a decreased plant growth and a reduction in pigment content. In this study, it was pointed out that photosynthetic pigment content did not influence dry biomass (Tab. 4).

SENSITIVITY OF *Hydrocharis morsus-ranae* TO LEAD

Lead belongs to nonredox active metals, does not have any biological functions and is toxic to aquatic plants [Samardakiewicz, Woźny 2005; Veselý *et al.* 2012]. The absorption of Pb by plants occurs mainly through the root system and, though only in minor amounts, through the leaves. In the plants, Pb is primarily accumulated in roots, but some of it is translocated to the aerial parts of a plant [Kumar *et al.* 2012; Sengar *et al.* 2008]. Inhibition of cell division in *Lemna minor* roots due to Pb has been reported by Samardakiewicz and Woźny [2005].

Principally, the negative effects of Pb on vegetative growth of a plant stem from the following factors: distorted chloroplast ultrastructure, obstruction in the electron transport, inhibition of Calvin cycle enzymes, impairment of essential elements uptakes, such as Mg and Fe, as well as induced deficiency of CO_2 owing to stomatal closure [Pourrut *et al.* 2011]. Veselý *et al.* [2012] observed that the total chlorophyll content in the leaves of *Pistia stratiotes* decreased with an increase of Pb concentration and also with a higher chelates content. According to Dogan *et al.* [2009], Pb accumulation and its toxic effect on Chl *a*, Chl *b*, Car and protein contents in *Elodea canadensis* were found to be dependent on the concentration of Pb. It was also found that toxicity increased with the increasing Pb concentration. This is in accordance with Qiao *et al.* [2014] who reported a decreased Chl *a* content in the leaves of *Nymphoides peltatum* only of 100 μM Pb application. However, they also confirmed that Pb stress caused an increase in Chl *a*:Chl *b* ratio to a different extent. Generally, our results did not show that the application of Pb produced a decrease in the production of photosynthetic

pigments of *H. morsus-ranae* (Fig. 3), yet the value of Chl *a*:Chl *b* ratio (calculated on data from Tab. 3) decreases (1.88) when compared to the control condition (2.38). Qiao *et al.* [2014] showed a significant increase in Chl *a*, Chl *b* and Chl *a* + *b* levels at high concentrations of Pb (i.e. 12.5 and 25 μM). This is in line with Singh *et al.* [2010] who reported an increase in total chlorophyll content up to 10 μM Pb concentration till day 4, followed by a decrease at 100 μM Pb concentration. These types of response have also been demonstrated in *H. morsus-ranae* (Tab. 4) of 0.76 and 1.52 mg·dm^{-3} application (only without Chl *a*). In this study Chl *a* seemed to be more sensitive than Chl *b*. Such substitution was observed for chlorophyll in *Nymphoides peltatum* [Qiao *et al.* 2014]. Here, it was shown that an increase in metabolic activity led to a significant increase in dry biomass of *H. morsus-ranae* (Tab. 3) at the second concentration of Pb (Tab. 2). Such an effect suggested that metabolic activity partakes in compensating for Pb toxicity, but the impact on biomass is not unequivocal (Tab. 3), despite the correlation between the content of the photosynthetic pigment (Chl *b*, Chl *a* + *b* and Car), and the dry biomass (Tab. 4).

SENSITIVITY OF *Hydrocharis morsus-ranae* TO IRON

Iron is a trace metal essential for plant growth. In various biochemical processes, Fe is readily reduced and oxidised. It also constitutes an important cofactor of many enzymes involved in respiration, photosynthesis, and nitrogen assimilation, as well as in various cellular processes such as synthesis of DNA and hormone production, chloroplast development and chlorophyll biosynthesis. Fe is a constituent of hemeprotein and iron sulphur protein [Arif *et al.* 2016]. In plants, Fe toxicity is manifested by bronzing characteristics (tissue necrosis in leaf), observed in plants cultivated at 40 mM Fe solutions [Rout, Sahoo 2015]. The second classic symptom of Fe toxicity is a stunted root system [Xing, Liu 2011].

Xing *et al.* [2009] studied physiological responses of *Spirodela polyrrhiza* to various doses of Fe (1, 10, and 100 mg·dm^{-3} Fe^{3+}, added as $FeCl_3 \cdot 6H_2O$). After 24 h short-term exposure, 10 and 100 mg·dm^{-3} Fe^{3+} caused plant necrosis or even death, the disintegration of colonies and roots abscission. In this study, the pigment levels in the leaves of *H. morsus-ranae* did not change under Fe stress at 3.06 and 6.12 mg·dm^{-3}. At this first concentration, the biomass of the plants was found to be higher than that in the corresponding control, which may be attributed to the hormesis effect. As is presented by Poschenrieder *et al.* [2013] hormesis is the stimulated phase in the growth response which is induced by low concentrations of toxic metal ions without any evidence of underlying mechanisms.

The third concentration of Fe (12.24 mg·dm^{-3}) decreased the content of Chl *a* and Chl *a* + *b* (Tab. 3). Notably, the extent of the increase was higher for Chl *b* than Chl *a*, resulting in a reduced Chl *a*:Chl *b* ratio to 1.94 (2.38 in control condition, ratios calculated on data from Tab. 3), indicating that the increase of chlorophyll content in response to this concentration of Fe was predominantly attributed to the improved production of Chl *b*. In this situation, leaf necrosis and necrotic patches with browning of the root system were observed, which contributed to the decrease in the plant's dry matter (Tab. 3). This element has had a stimulatory effect on the growth of the plant only at a high concentration of Fe (Tab. 3). This dose of iron has secured all the needs for the growth and development of *H. morsus-ranae*.

SENSITIVITY OF *Hydrocharis morsus-ranae* TO CADMIUM

Cadmium is a non-redox active metal without any known biological function. It is toxic to aquatic plants [John *et al.* 2008]. It is believed that the uptake of Cd by plants predominantly occurs via roots through specific and non-specific transporters of essential nutrients since Cd-specific transporter has not yet been identified. In aquatic plants, Cd causes phytotoxicity through decreasing plant biomass and photosynthetic features such as chlorophyll pigments, net CO_2 assimilation rate, sub-stomatal CO_2 concentration as well as stomatal conductance [Zahoor *et al.* 2018].

In the present research, physiological responses of *H. morsus-ranae* to applications of Cd (up to 0.4 μM) were investigated. Cadmium stress induced an increase in Chl *a*, Chl *b*, Chl *a* + *b*, Car, and biomass (Tab. 3) only at 0.2 μM Cd. This is in accordance with Mohammed [2016], who observed that as compared to the control, *Lemna minor* and *L. gibba* increased in Chl *a*, *b* and total carotenoids up to 10 and 15 μg Cd·dm^{-3} after exposure duration. However, at 20, and 30 Cd μg·dm^{-3}, a significant decrease ($P < 0.005$) in Chl *a*, Chl *b* and total carotenoids content were found after seven days of exposure as compared to the control. Biomass of duckweed species (*L. minor* and *L. gibba*) increased at lower doses of Cd in contrary to the present research because a positive linear correlation was found between the biomass of *H. morsus-ranae* and the content of the photosynthetic pigment in leaves (Tab. 4). Contrary to the findings by Malec *et al.* [2010], the calculated Chl *a* + *b*:Car ratio increased as well as a function of the Cd application (ratios calculated on data from Table 3: first concentration of Cd – ratio 2.58, second concentration – ratio 2.72 and third concentration – ratio 2.85).

CONCLUSIONS

Stress caused by the analysed trace metals had various effects on the physiological response of *Hydrocharis morsus-ranae* i.e.: the content of photosynthetic pigments as well as the amount of biomass. It would be important to identify the adaptive mechanisms for this species that may be involved in balancing low and high doses of trace metals. In the present study, at first and second concentrations of Zn and Cu, an increase of pigment content was observed. On the other hand, an inhibitory effect of metal stress on the growth of *H. morsus-ranae* was noted. However, non-essential metals such as Pb and Cd at second concentrations influenced the increase of pigments content and amount of dry biomass. At third concentrations, it was shown that the content of photosynthetic pigments did not change, but dry biomass decreased under Zn, Cu and Pb stress. The stress of essential metals such as Mn and Fe demonstrated varied growth and physiological response. Due to the observed decrease in biomass at the highest concentrations of Zn, Cu, Pb and Fe observed in the analysis, the application time of *H. morsus-ranae* for remediation of waters contaminated with metals is to be limited to one week.

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