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Growth and photosynthetic responses of *Lemna minor* L. exposed to cadmium in combination with zinc or copper

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Metals have a variety of negative outcomes on plants, essential components of any ecosystem. The effects of $CdCl_2$ (5 μmol L^{-1}), $ZnCl_2$ (25 or 50 μmol L^{-1}), and $CuCl_2$ (2.5 or 5 μmol L^{-1}) and combinations of $CdCl_2$ with either $ZnCl_2$ or $CuCl_2$ on the growth, photosynthetic pigments, and photosystem II (PSII) efficiency of duckweed (*Lemna minor* L.) were investigated. All of the treatments caused growth inhibition and remarkable metal accumulation in plant tissue after 4 and 7 days. In the combined treatments, the accumulation of each metal applied was lesser in comparison to treatments with single metals. After 4 days, all of the treatments generally diminished chlorophyll *a* content and decreased the maximum quantum yield (F_v/F_m) and effective quantum yield ($\Delta F/F'_m$) of PSII. However, after 7 days of exposure to a combination of Cd and Zn, pigment content and PSII activity recovered to control levels. A higher concentration of Cu (5 μmol L^{-1}) as well as Cd in combination with Cu had a prolonged inhibitory effect on photosynthetic features. Our results suggest that growth inhibition was due to the toxic effect of absolute metal quantity in plant tissue. Zn counteracted Cd uptake, as seen from the recovery of pigment content and PSII efficiency in plants exposed for 7 days to the Cd and Zn combination. Cu-induced oxidative stress led to a prolonged inhibitory effect in plants treated both with a higher concentration of Cu (5 μmol L^{-1}) and simultaneously with Cd and Cu. Our findings could contribute to general knowledge on anthropogenic and environmental contaminants that endanger plant communities and significantly disrupt the sensitive balance of an ecosystem by influencing photosynthetic mechanisms.

KEY WORDS: *chlorophyll fluorescence; duckweed; metal uptake; photosynthetic pigments; PSII efficiency*

Excess amounts of heavy metals are present in the environment due to natural conditions or industry, mining, and agricultural and other human activities. They influence not only plant growth and reproduction but also human health (1). The impact of heavy metals on plant growth and metabolism has been studied extensively; however, the effect of plant exposure to a single heavy metal has received much more attention than the simultaneous effects of multiple metals, although contaminated terrestrial and aquatic environments usually contain a mixture of substances.

Cadmium (Cd) is a non-essential toxic heavy metal. Its salts are dangerous due to their high water solubility and relatively high soil mobility. It is easily taken up by plants and introduced into the food chain, which is why it can have adverse effect not only on plants, but also on animal and human health (2-4). Apart from growth inhibition, leaf chlorosis, and a disturbed uptake and distribution of water and essential elements, Cd has also been reported to bind to functional groups (sulfhydryl, carboxyl, imidazole, etc.) and replace essential elements (e.g. Zn) in active sites resulting in the inhibition of enzyme activity (5-7). Cd also inhibits photosynthesis by impairing chlorophyll (Chl) biosynthesis, the activity of photosystem II (PSII), photosynthetic electron transport, the Calvin cycle, and chloroplast organization (8-11). Furthermore, Cd can cause oxidative stress by interference with the components of antioxidant defence system resulting in a disturbed cellular equilibrium between the generation and the neutralization of reactive oxygen species (ROS) (11-13).

Zinc (Zn) is an essential plant nutrient involved in the catalytic function of many enzymes, structural stability of cell membranes and proteins, DNA-binding proteins (Zn-fingers) as well as protection of biomembranes against oxidative damage (14, 15). It carries out biochemical functions as a divalent cation that is redox-stable under physiological conditions in biological medium, and cannot be either further reduced or oxidised (13, 15). For optimal growth of most plant species, Zn ion activity in nutrient media has to fall within the range of $1x10^{-5}$-$1x10^{-4}$ μmol L^{-1} (16). Zn toxicity symptoms include reduced and stunted growth, chlorosis induced by Fe-deficiency followed by

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decreased Chl biosynthesis, chloroplast degradation as well as interference with phosphorous, magnesium, and manganese uptake (15).

Copper (Cu) is an also essential nutrient, involved in a wide range of biochemical and physiological processes in plant cells. It participates in electron-transfer reactions of photosynthesis in the form of plastocyanin, acts as a cofactor of Cu-Zn superoxide dismutase, polyphenoloxidase, and other enzymes and participates in the structural stability of chromosomes (17). Redox cycling between its two ion forms (Cu^{2+} and Cu^{+}) can lead to ROS production. Activity of Cu ions in nutrient media for optimal growth of most plant species ranges from 10^{-8} µmol L^{-1} to 10^{-10} µmol L^{-1} (16). At high levels, Cu is strongly phytotoxic and can cause growth inhibition, chlorosis, necrosis, and leaf depigmentation. Further modes of Cu toxicity at the molecular level include binding onto sulfhydryl groups of proteins, impairing essential element uptake and cell transport processes (18-21).

It has long been established that the toxic effect of Cd can be modified by essential elements such as Zn, Ca, Fe, Cu, and Mn (4, 5, 22). The effects of Cd, Zn and Cu, separately and in combination, were studied in the hydroponically grown bean *Phaseolus vulgaris* (23), duckweeds *L. minor* (20), *L. gibba* (24, 25) and *L. trisulca* (26), rice *Oryza sativa* (27), and cucumber *Cucumis sativus* (28). An extensive study of the combined effect of Cd and Zn on metal accumulation (29), oxidative stress (30), and photosynthesis (8) in freshwater macrophyte *Ceratophyllum demersum* showed an alleviating effect of Zn on Cd-induced toxicity.

In our previous studies (31, 32) we investigated the effect of Cd on the induction of oxidative stress and DNA damage in duckweed (*L. minor* L.), a rapidly growing aquatic monocotyledon commonly used in ecotoxicological studies (33). To further elucidate the effects of Cd (5 µmol L^{-1}) alone and in combination with Zn (25 and 50 µmol L^{-1}) or Cu (2.5 and 5 µmol L^{-1}) on photosynthetic features, the present work evaluated growth, chlorophyll and carotenoid content as well as photosynthetic efficiency by chlorophyll fluorescence. As an added value, principal component analysis (PCA) was applied to discriminate the responses to individual metals and mixtures as well as their time dependence.

MATERIALS AND METHODS

Plant material and growth conditions

Duckweeds (*L. minor* L.) were collected from the Botanical Garden of the Faculty of Science, University of Zagreb, and sterilised according to Krajnčič and Devidé (34). The stock cultures have been maintained in chamber conditions (24±2 °C) on Pirson–Seidel nutrient medium (35) since 1996, by transferring the plants to a fresh medium every two weeks. Illumination (16:8 hours light:dark cycle) was provided by cool white fluorescent light with an intensity of 40 µmol m^{-2} s^{-1} at plant level.

Cd, Zn, and Cu treatment

The chemicals used for the nutrient media were purchased from Sigma-Aldrich (St. Louis, MO, USA) or Kemika (Zagreb, Croatia), $CdCl_2$ from Fluka (Buchs, Switzerland), $ZnCl_2$ and $CuCl_2$ from Kemika (Zagreb, Croatia).

The experiment was carried out under static conditions after pre-cultivation (2×7-days) of plants on modified Steinberg medium prepared according to ISO 20079 protocol (36): 3.46 mmol L^{-1} KNO_3, 1.25 mmol L^{-1} $Ca(NO_3)_2$ x4 H_2O, 0.66 mmol L^{-1} KH_2PO_4, 0.072 mmol L^{-1} K_2HPO_4, 0.41 mmol L^{-1} $MgSO_4$x7 H_2O, 0.91 µmol L^{-1} $MnCl_2$x4 H_2O, 1.94 µmol L^{-1} H_3BO_3, 0.63 µmol L^{-1} $ZnSO_4$x7 H_2O, 0.18 µmol L^{-1} Na_2MoO_4x2 H_2O, 4.03 µmol L^{-1} Na_2EDTAx2 H_2O, 2.81 µmol L^{-1} $FeCl_3$x6 H_2O; pH value was adjusted to 5.5 by using KOH.

For the growth experiment, individual colonies comprising 2–3 fronds were inoculated to 100 mL Erlenmeyer flasks containing 60 mL of Steinberg medium. For metal analysis, determination of pigment content as well as chlorophyll fluorescence measurement, 5-10 colonies were inoculated into 300 mL Erlenmeyer flasks containing 150 mL of medium.

For single metal treatments, Steinberg nutrient medium was supplemented with 5 µmol L^{-1} $CdCl_2$, 25 µmol L^{-1} or 50 µmol L^{-1} $ZnCl_2$ and 2.5 µmol L^{-1} or 5 µmol L^{-1} $CuCl_2$. The same concentrations were applied for the combined treatments, i.e. $CdCl_2$+$ZnCl_2$ and $CdCl_2$+$CuCl_2$. Control plants were grown on Steinberg medium without the addition of tested salts.

Treatments lasted for seven days in the same growth conditions as for stock cultures. Every treatment group as well as control was prepared in six replicates.

Cd, Zn, and Cu determination

Plants were harvested on the 4th and 7th day of the experiment, carefully washed with distilled water, oven-dried at 80 °C for 24 h, and subjected to microwave digestion in two steps. The first step was digestion of 20-30 mg of dry tissue in 10 mL of 16 mol L^{-1} HNO_3 (Kemika, Zagreb, Croatia) at 70 °C for 5 min, then 5 min at 130 °C and 4 min at 150 °C. The second step was carried out after the addition of 1 mL H_2O_2 (30 % v/v, Kemika, Zagreb, Croatia) at 85 °C for 5 min and additionally for 4 min at 130 °C. After cooling, the samples were diluted with 1 % (v/v) HNO_3 up to the total volume of 50 mL. Metal concentrations were determined using inductively coupled plasma-optical emission spectroscopy (ICP-OES, IRIS INTREPID II XSP – Thermo Fisher Scientific, Waltham, MA, USA) according to the ISO 11885 standard (37). The obtained results were processed using TEVA software.

Metal concentrations were calculated according to the calibration curve obtained with a set of standards of known concentrations (Merck, Darmstadt, Germany). A concentration range of 1 to 50 μg L^{-1} for Cd, and a concentration range of 50 to 5000 μg L^{-1} for Zn and Cu were used. The detection limits were 0.5 μg L^{-1} for Cd and 10 μg L^{-1} for Zn and Cu, respectively. The limit of quantification (LOQ) was <1 μg L^{-1} for Cd and <20 μg L^{-1}, for Zn and Cu. Metal contents in plant material were expressed as μg g^{-1} dry weight.

Bioconcentration factor (BCF) for Cd, Zn or Cu was calculated by dividing metal concentration in plant tissue (μg g^{-1} dry weight) measured at harvest with the initial concentration of the metal in nutrient solution (μg mL^{-1}) (38).

Growth assessment

Plant growth was estimated by counting fronds on the 4th and 7th day of the experiment and expressed as relative plant growth (RG) according to the equation (39):

$$RG = \frac{N_t - N_0}{N_0}$$

where N_t is the number of fronds at day t (day 4 or 7) and N_0 is the number of fronds at the beginning of the experiment (day 0).

Photosynthetic pigments

Fresh samples were homogenised with 80 % (v/v) cold acetone, centrifuged at 5000 *g* for 10 min and the absorbance of the supernatant measured at 663, 646, and 470 nm. The photosynthetic pigments, Chl *a*, Chl *b*, and carotenoids (Cars) were calculated according to Lichtenthaler (40) and expressed as mg g^{-1} fresh weight.

In vivo *measurement of chlorophyll fluorescence*

In vivo chlorophyll fluorescence was measured from the upper face of duckweed fronds by pulse-modulated chlorophyll fluorometer (Qubit Systems Inc., Kingston, Canada). Prior to the measurements, plants were dark-adapted for 30 min. After minimal fluorescence (F_0) determination, a 0.7 s pulse of saturating light (>5000 μmol quanta m^{-2} s^{-1}) was applied to induce maximum fluorescence (F_m). Steady-state fluorescence (F) and maximum fluorescence (F'_m) of the sample under illuminated conditions (150 μmol m^{-2} s^{-1} actinic light) were also measured. From the obtained parameters (F_0, F_m, F'_m, and F), maximum quantum yield of PSII photochemistry (F_v/F_m) and effective quantum yield of PSII ($\Delta F/F'_m$) were calculated according to Maxwell and Johnson (41).

Data analysis

Statistical analysis was performed using STATISTICA 12.0 (StatSoft Inc., Tulsa, OK, USA) software package. Data were compared by analysis of variance (ANOVA) followed by Newman-Keul's test to determine the significant difference between treatments (P<0.05). Results were obtained from two individual experiments. For metal content in plant tissue, values were means of at least four replicates while for all other measurements each data point is the mean value of at least six replicates.

Data obtained for day 4 and day 7 of the experiment were used for principal component analysis (PCA) in order to evaluate the most important responses of *L. minor* exposed to metals, discriminate the responses to individual metals and mixtures as well as to determine their time-dependence. Data sets used for PCA were comprised of 10 variables including growth, Cd, Zn, and Cu accumulation, Chl *a*,Chl *b*, total chlorophylls and carotenoid content, maximum quantum yield (F_v/F_m) and effective quantum yield ($\Delta F/F'_m$). Also, malondialdehyde (MDA) content, reported previously (31, 32), was added as a variable representing oxidative stress parameter. PCA was applied to the standardised data set and the factor loadings were classified as "strong", "moderate", and "weak" corresponding to absolute loading values of >0.75, 0.75-0.50, and 0.50-0.30, respectively (42).

RESULTS

Toxicity symptoms

The applied concentrations of the tested salts caused visible morphological changes. Plants treated with $CdCl_2$ started to show signs of chlorosis and rejected roots on the 3rd day of the exposure. Fronds were smaller in comparison to control plants and induction of frond abscission was noted. Zn caused an appearance of light green daughter plants, which remained connected to mother plants thus creating larger colonies. Plants exposed to a combination of $CdCl_2$ and $ZnCl_2$ were smaller and also showed delayed frond abscission. In comparison to treatment with $CdCl_2$ only, chlorosis was not so pronounced. Plants exposed to 5 μmol L^{-1} of $CuCl_2$ showed the signs of chlorosis and were smaller than control plants and those treated with 2.5 μmol L^{-1} $CuCl_2$. A mixture of $CdCl_2$ and $CuCl_2$ caused inhibition of separation of daughter plants resulting in formation of colonies with overlapping plants. In plants treated with $CdCl_2$ combined with $CuCl_2$ (5 μmol L^{-1}), considerable chlorosis was noticed.

Metal content in plant tissue and BCF

Duckweed showed a remarkable accumulation of all of the three metals tested (Table 1). Zn, as an essential element, was detected in plants from all treatment groups, including controls. After four days of exposure to 25 μmol L^{-1} $ZnCl_2$, the Zn content in plants was approximately eight times above control level, and in plants exposed to 50 μmol L^{-1} nine times. After seven days of exposure, Zn content

Table 1 *Cadmium, zinc and copper content and bioconcentration factor (BCF) values in duckweed (*Lemna minor *L.) after four and seven days of exposure to single Cd, Zn, and Cu salts and their combinations*

	Treatments	Content (μg g^{-1} dry weight)		
		Cd	Zn	Cu
Day 4	Control	<LOQ	285.25±21.6^{d} (BCF 6923.5)	<LOQ
	5 μmol L^{-1} Cd	834.32±33.66^{a} (BCF 1484.5)	164.73±13.47^{d} (BCF 3998.3)	<LOQ
	25 μmol L^{-1} Zn	<LOQ	2219.66±68.52^{b} (BCF 1324.6)	<LOQ
	50 μmol L^{-1} Zn	<LOQ	2571.88±107.76^{a} (BCF 777.2)	<LOQ
	25 μmol L^{-1} Zn+5 μmol L^{-1} Cd	396.98±9.72^{c} (BCF 706.4)	1508.88±12.24^{c} (BCF 900.4)	<LOQ
	50 μmol L^{-1} Zn+5 μmol L^{-1} Cd	342.82±28.30^{c} (BCF 610.0)	2293.99±137.42^{b} (BCF 693.2)	<LOQ
	2.5 μmol L^{-1} Cu	<LOQ	117.71±5.17^{d} (BCF 2857.0)	244.71±25.53^{c} (BCF 1540.9)
	5 μmol L^{-1} Cu	<LOQ	115.25±6.62^{d} (BCF 2797.3)	363.81±14.71^{a} (BCF 1144.9)
	2.5 μmol L^{-1} Cu+5 μmol L^{-1} Cd	536.1±65.86^{b} (BCF 953.9)	126.98±16.52^{d} (BCF 3082.0)	232.22±17.71^{c} (BCF 1462.3)
	5 μmol L^{-1} Cu+5 μmol L^{-1} Cd	431.54±11.65^{c} (BCF 767.9)	76.47±2.25^{d} (BCF 1856.0)	310.9±9.76^{b} (BCF 978.4)
Day 7	Control	<LOQ	265.58±23.82^{D} (BCF 6446.1)	<LOQ
	5 μmol L^{-1} Cd	1055.64±37.94^{A} (BCF 1878.4)	174.01±12.38^{D} (BCF 4223.5)	<LOQ
	25 μmol L^{-1} Zn	<LOQ	3715.60±136.74^{B} (BCF 2217.3)	<LOQ
	50 μmol L^{-1} Zn	<LOQ	4517.66±270.54^{A} (BCF 1365.1)	<LOQ
	25 μmol L^{-1} Zn+5 μmol L^{-1} Cd	720.54±51.14^{B} (BCF 1282.1)	2608.82±94.14^{C} (BCF 1556.8)	<LOQ
	50 μmol L^{-1} Zn+5 μmol L^{-1} Cd	539.54±44.47^{C} (BCF 960.0)	3595.83±132.63^{B} (BCF 1086.6)	<LOQ
	2.5 μmol L^{-1} Cu	<LOQ	111.48±7.79^{D} (BCF 2705.8)	342.91±36.36^{C} (BCF 2159.4)
	5 μmol L^{-1} Cu	<LOQ	111.17±2.66^{D} (BCF 2698.3)	480.98±27.82^{A} (BCF 1513.7)
	2.5 μmol L^{-1} Cu+5 μmol L^{-1} Cd	808.84±52.44^{B} (BCF 1439.2)	96.96±5.20^{D} (BCF 2353.4)	264.51±12.11^{D} (BCF 1665.7)
	5 μmol L^{-1} Cu+5 μmol L^{-1} Cd	698.81±60.3^{B} (BCF 1243.4)	94.44±14.65^{D} (BCF 2292.3)	405.87±18.43^{B} (BCF 1277.3)

The values are expressed as mean±SE of at least four replicates from two individual experiments.
Values obtained for each treatment period (day 4 and day 7) are compared separately and marked with lowercase letters for day 4 and uppercase letter for day 7. Values followed by the same letter are not significantly different (Newman-Keuls test).
Instrument's limit of quantification (LOQ) was <1 μg L^{-1} for Cd and <20 μg L^{-1} for Cu
Metal content in plants was reported also in our previous studies of Zn and Cd (31) as well as Cu and Cd (32) effects in L. minor.
BCF for corresponding metal is shown in the second row in parentheses and expressed in μg g^{-1} dry weight

increased up to approximately 14 times and 17 times above control level, respectively.

Cu was expected to be present, but in much lower quantities; however, it was detectable only in plants cultivated on media supplemented with excess amounts of $CuCl_2$. Its content in plants was also dependent on Cu concentration in culture medium and duration of treatments, being the highest after seven days of treatment with 5 µmol L^{-1} $CuCl_2$.

Cd was detected only in plants cultivated on media supplemented with $CdCl_2$. Plants exposed to 5 µmol L^{-1} $CdCl_2$ for seven days contained a 1.25 times higher amount of Cd than plants exposed to the same Cd concentration for four days. Comparison of Cd and Cu accumulation in plants during single metals exposures revealed a much higher accumulation of Cd.

In plants treated with the mixtures of $CdCl_2$ and $ZnCl_2$ or $CuCl_2$ the concentration of both essential metals was decreased in comparison to plants treated with only $ZnCl_2$ or $CuCl_2$. The only exception was the treatment with $CdCl_2$ and 2.5 µmol L^{-1} $CuCl_2$, where addition of $CdCl_2$ did not decrease the Cu content in plant tissue. The analysis of Cd content in plants exposed to the mixture of $CdCl_2$ and $ZnCl_2$, as well as to the mixture of $CdCl_2$ and $CuCl_2$, showed also a lower content of Cd in plant tissue after combined treatments. After the 4-day treatment period, plants treated with $CdCl_2$ and 25 µmol L^{-1} $ZnCl_2$ contained only 47 % and plants treated with $CdCl_2$ and 50 µmol L^{-1} $ZnCl_2$ only 41 % of total amount of Cd accumulated in plants treated with $CdCl_2$ only. After seven days, Cd content was also significantly lower and contained 68.2 and 51.1 %, respectively, of Cd content in plants treated with $CdCl_2$ only. In cultures exposed to $CdCl_2$ in combination with 2.5 or 5 µmol L^{-1} $CuCl_2$, content of Cd was 64 and 51.7 %, respectively, of Cd-content in plants treated with $CdCl_2$ alone. After the 7-day treatment, Cd content was also lowered to 75 % in plants treated with 2.5 µmol L^{-1} $CuCl_2$, and to 65 % in plants treated with 5 µmol L^{-1} $CuCl_2$, in comparison to plants treated seven days with $CdCl_2$ alone. The accumulation of all three metals was dependent on time, which means that accumulation of metals after seven days was greater than after four days.

BCF values after the separate addition of either of the three tested metals decreased with the increase of metal concentrations in the nutrient medium (Table 1). Furthermore, the BCF for the corresponding metal was even lower in plants simultaneously exposed to excess amounts of two metals. BCF values for Cd and Cu increased with time, while BCF for Zn was the highest at the end of the experiment only in plants treated with $ZnCl_2$.

Effect on growth

In comparison to control plants, all treatments significantly reduced duckweed growth rate on day 4 and day 7 of the experiment (Figure 1). Analysis of the relative growth of plants treated with $CdCl_2$ alone and plants exposed to the combinations of salts showed no significant effects on Cd-toxicity.

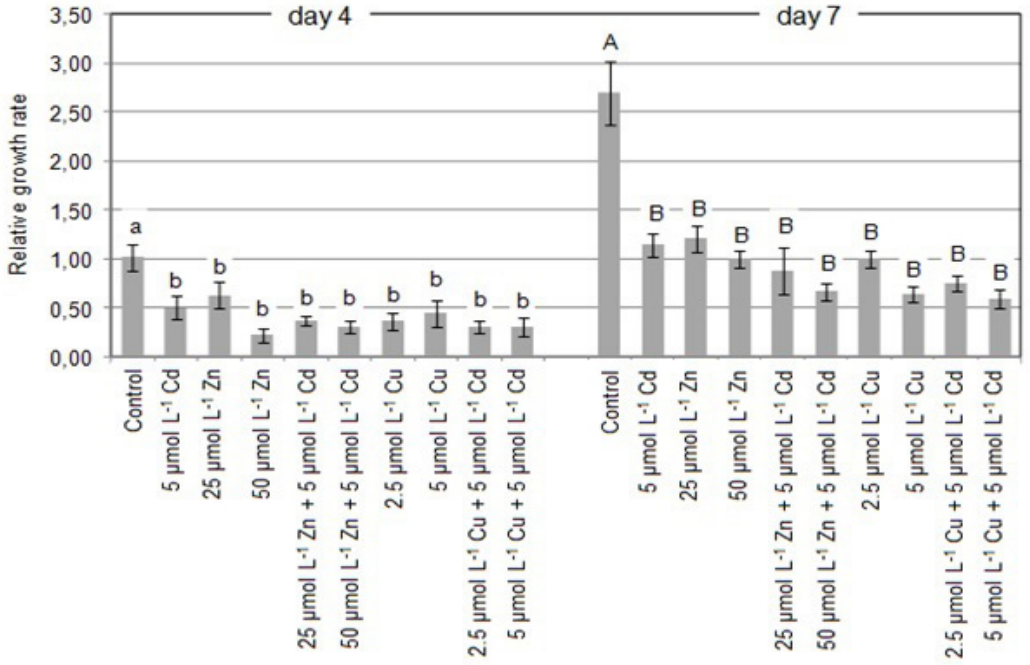

Figure 1 *Relative growth of duckweed (*Lemna minor *L.) after four and seven days of exposure to single Cd, Zn and Cu salts and their combinations*
Values (expressed as means±SE of six replicates) for each treatment period are compared separately and marked with lowercase letter for day 4 and uppercase letter for day 7
Values followed by the same letter are not significantly different (Newman-Keuls test)

Effect on photosynthetic pigments content

On day 4, all treatments, except 50 μmol L^{-1} $ZnCl_2$, caused a decrease in Chl *a* content and total Chls (Table 2). Chl *b* was affected by treatments with 5 μmol L^{-1} $CdCl_2$ and combination of $CdCl_2$ with 2.5 μmol L^{-1} $CuCl_2$, while total Cars was affected by 5 μmol L^{-1} $CuCl_2$. Treatment with 50 μmol L^{-1} $ZnCl_2$ caused an increase of Chl *a*, Chl *b*, and Cars in comparison to control.

After seven days, in plants exposed to combinations of $CdCl_2$ and $ZnCl_2$ (25 or 50 μmol L^{-1}) and in those treated with 2.5 μmol L^{-1} $CuCl_2$, content of Chl *a*, Chl *b*, total Chls and Cars did not change in comparison to control levels. Higher concentrations of $CuCl_2$ and both combinations of $CdCl_2$ with $CuCl_2$ lowered the pigment content.

Effect on chlorophyll fluorescence

In plants exposed to the tested metals for four days, all of the applied treatments caused a decrease of maximum quantum yield (F_v/F_m) of PSII (Figure 2a). The most prominent decrease was noticed in plants treated with both of $CdCl_2$ and $CuCl_2$ combinations and in plants treated with 5 μmol L^{-1} $CuCl_2$.

The effective quantum yield of PSII ($\Delta F/F'_m$) after four days was also significantly decreased by the applied treatments (Figure 2b). The most prominent effect was noticed in plants exposed to $CdCl_2$ in combination with higher concentration of $ZnCl_2$, as well as to $CdCl_2$ in combination with higher concentrations of $CuCl_2$.

After seven days, in most of the treated plants, values of both fluorescence parameters were at control levels. The exceptions were treatments with $CdCl_2$, both concentrations of $CuCl_2$ and combination of $CdCl_2$ with 5 μmol L^{-1} $CuCl_2$. These treatments caused lower values of F_v/F_m, while exposure to 5 μmol L^{-1} $CuCl_2$ showed a decrease of $\Delta F/F'_m$.

PCA analysis

PCA applied to the combined data sets obtained for day 4 and day 7 of the experiment yielded three significant PCs capturing almost 80 % of the total data variance. The first two PCA components (PC1 and PC2) explained 69 % of the total variance (Figure 3a). PC1 (45 %) was largely determined by pigment content and fluorescence parameters with strong negative loadings, as well as MDA and Cu content with moderate positive loading. PC2 (24 %) had a moderate positive loading on MDA and Cu content and moderate negative loading on growth and Zn accumulation. The scores plot (Figure 3b) revealed a grouping of plants exposed to $CuCl_2$, $CdCl_2$ and combinations of these salts in one cluster based on the induction of oxidative stress (increased MDA content), decreased photosynthetic features, and/or Cu content. Most plants exposed to $ZnCl_2$ for four days and combinations of $ZnCl_2$ and $CdCl_2$ as well as control plants grouped together based on responses of all photosynthetic parameters. Plants exposed seven days to both concentrations of $ZnCl_2$, $CdCl_2$ as well as combination of $CdCl_2$ and 2.5 μmol L^{-1} $CuCl_2$ grouped together corresponding mostly in terms of decreased pigments content. PC3 representing 10 % of the total variance correlated strongly with Cd accumulation in the negative part and moderately with plant growth in the positive part but did not provide any additional information and was not included into further analyses.

DISCUSSION

All of the three tested metal salts influenced duckweed growth and development, as indicated by morphological changes and reduced growth rates based on frond number. This was to be expected, since 5 μmol L^{-1}, and an even lower concentration of Cd, have already been confirmed to affect plant metabolism (20, 23, 43, 44). It is also well-known that Zn and Cu added in amounts above optimal level have adverse effects on plant growth (20, 45). In our experiment, contrary to the studies on the water plant *C. demersum* (29) and rice (27), Zn or Cu in combination with Cd did not alleviate the inhibitory effect of Cd on growth.

When added in excess amount, the tested metals tended to accumulate with time but also, when in combination, influenced their mutual uptake. Lower Zn and Cd uptake in plants treated with a mixture of metals indicates their strong competition for transport proteins because they are both taken up into plant cells by Fe, Ca, and Zn channels/transporters of low specificity (4, 46). There are data about the participation of transporters for other divalent cations (e.g. Fe and Zn) in Cu uptake (47) so competition between Cu and Cd is also possible, as shown after simultaneous treatment with Cd and Cu (5 μmol L^{-1} Cu). Therefore, the effects of Cd on growth and photosynthesis in our experiment could be due to the interference of Cd with the uptake of other nutrients (Ca, Mg, Fe, Mn, S, and P) and not only to the presence of Cd alone (4, 48).

The calculated BCF values for Cd, Zn and Cu were lower in plants exposed to combined treatments in comparison to single metal treatments, thus confirming the competition of metals for uptake mechanisms. Surprisingly, the reduced uptake of metals in combined treatments did not alleviate the effect on growth, which suggests that growth inhibition was due to the toxic effect of absolute metal quantity. Contrary, Aravind and Prasad (14) reported a suppression in Cd uptake due to increased Zn accumulation leading to an alleviated effect of Zn on Cd-induced toxicity in *C. demersum*. However, comparing the metal content in *L. minor* and. *C. demersum*, we can see that Cd as well as Zn content were much higher in *L. minor*. In our study, treatment with 50 μmol L^{-1} $ZnCl_2$ affected *L. minor*, while in *C. demersum* this concentration was not toxic (14). Therefore, it could be concluded that these two water plant species differ in metal uptake, accumulation, and/or response to metals.

Table 2 *The content of photosynthetic pigments (expressed as mg g^{-1} fresh weight) in duckweed (*Lemna minor *L.) after four and seven days of metal treatments*

Treatments	Content (mg g^{-1} fresh weight)							
	Chlorophyll a		Chlorophyll b		Total chlorophylls		Total carotenoids	
	4th day	7th day	4th day	7th day	4th day	7th day	4th day	7th day
Control	0.569±0.039^{a}	0.453±0.032^{A}	0.178±0.014^{b}	0.160±0.008^{A}	0.776±0.056^{a}	0.662±0.036^{A}	0.144±0.009^{b}	0.133±0.005^{A}
5 µmol L^{-1} Cd	0.343±0.029^{b}	0.266±0.017^{CD}	0.122±0.009^{c}	0.090±0.006^{C}	0.458±0.035^{b}	0.369±0.024^{C}	0.127±0.004^{bc}	0.108±0.005^{B}
25 µmol L^{-1} Zn	0.424±0.020^{b}	0.171±0.018^{E}	0.178±0.007^{b}	0.063±0.005^{D}	0.566±0.018^{b}	0.244±0.024^{D}	0.121±0.005^{bc}	0.085±0.003^{C}
50 µmol L^{-1} Zn	0.639±0.035^{a}	0.251±0.018^{CD}	0.226±0.015^{a}	0.095±0.005^{C}	0.900±0.052^{a}	0.360±0.023^{C}	0.169±0.008^{a}	0.104±0.003^{B}
25 µmol L^{-1} Zn + 5 µmol L^{-1} Cd	0.408±0.034^{b}	0.467±0.017^{A}	0.143±0.012^{bc}	0.169±0.006^{A}	0.573±0.049^{b}	0.663±0.024^{A}	0.120±0.009^{bc}	0.136±0.003^{A}
50 µmol L^{-1} Zn + 5 µmol L^{-1} Cd	0.419±0.027^{b}	0.428±0.029^{A}	0.147±0.009^{bc}	0.160±0.011^{A}	0.596±0.038^{b}	0.612±0.040^{A}	0.123±0.004^{bc}	0.132±0.006^{A}
2.5 µmol L^{-1} Cu	0.413±0.010^{b}	0.442±0.013^{A}	0.138±0.005^{bc}	0.155±0.006^{A}	0.572±0.016^{b}	0.620±0.020^{A}	0.118±0.005^{bc}	0.127±0.005^{A}
5 µmol L^{-1} Cu	0.383±0.032^{b}	0.341±0.012^{B}	0.133±0.009^{bc}	0.122±0.003^{B}	0.513±0.042^{b}	0.498±0.021^{B}	0.113±0.003^{c}	0.112±0.003^{B}
2.5 µmol L^{-1} Cu + 5 µmol L^{-1} Cd	0.401±0.025^{b}	0.187±0.009^{E}	0.120±0.010^{c}	0.067±0.003^{D}	0.493±0.046^{b}	0.264±0.013^{D}	0.119±0.014^{bc}	0.083±0.003^{C}
5 µmol L^{-1} Cu + 5 µmol L^{-1} Cd	0.413±0.018^{b}	0.305±0.013^{BC}	0.136±0.005^{bc}	0.105±0.004^{C}	0.589±0.026^{b}	0.426±0.017^{C}	0.129±0.004^{bc}	0.100±0.002^{B}

Values (expressed as means±SE of six replicates) for each treatment period (day 4 and day 7) are compared separately and marked with lowercase letter for day 4 and uppercase letter for day 7
Values followed by the same letter are not significantly different (Newman-Keuls test)

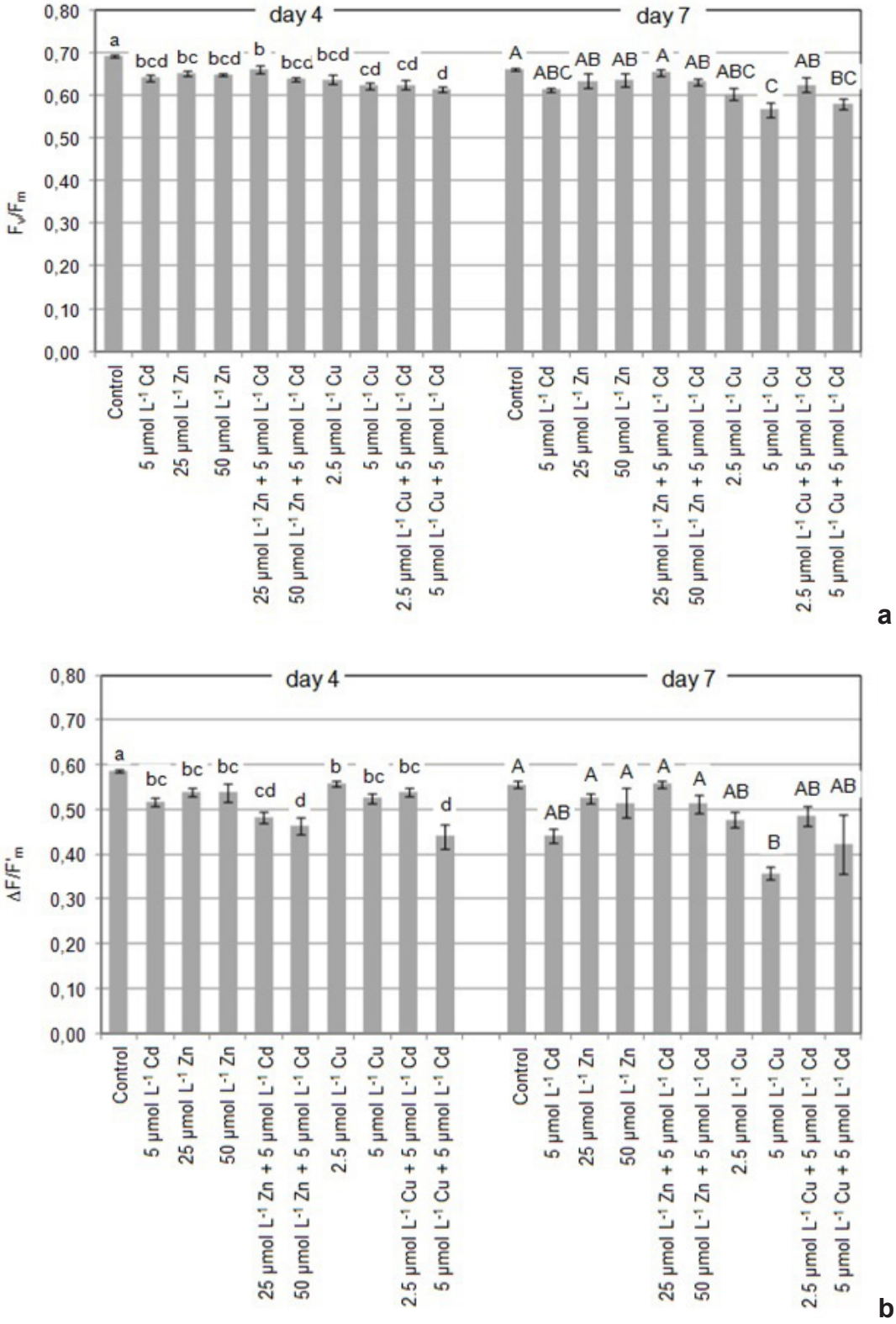

Figure 2 *a) Maximum quantum yield – F_v/F_m and b) effective quantum yield – $\Delta F/F'_m$ of PSII in* Lemna minor *L. after four and seven days of exposure to single Cd, Zn, and Cu salts and their combinations*
Values (expressed as means±SE of six replicates) for each treatment period are compared separately and marked with lowercase letter for day 4 and uppercase letter for day 7
Values followed by the same letter are not significantly different (Newman-Keuls Test)

Reduced photosynthetic pigments content and related chlorosis due to Cd and excess amounts of Zn and Cu as well as some other metals (Pb, Ni, and Hg) has been well documented in different plant species (44, 49). Therefore, chlorosis accompanied with reduced pigments content observed in Cd- and Cu-treated plants was not surprising. Cd inhibits Chl biosynthesis through δ-aminolevulinic acid dehydratase and protochlorophyllide reductase due to interaction with –SH groups which leads to a diminished production of δ-aminolevulinic acid - the first common precursor for all tetrapyrroles (29). The observed effect of $CdCl_2$ on pigments could partly be caused by Cd-induced oxidative stress (31). Contrary to redox active metals, Cd is involved in the production of ROS indirectly - by interactions with antioxidative defence system, substitution of essential elements at enzyme active sites, and disruption of electron transport chain (29, 50).

An unexpected response in pigments content was found after treatment with $ZnCl_2$, where the lower concentration (25 μmol L^{-1}) decreased Chl *a* and total Chls content on day 4 and day 7 while the higher concentration (50 μmol L^{-1}) increased Chl *b* and Cars on day 4 but decreased their content on day 7. Ralph and Burchett (51) suggested that a greater impact of the lower metal concentration could be correlated to the Zn accumulation capacity of the plant. In their work on *Halophila ovalis*, low levels of Zn in cells were tolerated. However, the concentration of Zn exceeding the tolerance level triggered a mechanism that limits Zn uptake into the cell, therefore reducing the damage of the photosynthetic pigments. However, there are reports that Zn could maintain Chl synthesis through protecting –SH group of the oxidation-prone δ-aminolevulinic acid dehydratase and protochlorophyllide reductase (49). Moreover, δ-aminolevulinic acid dehydratase requires Mg^{2+} or Zn^{2+}, so Zn possibly plays a role in activating this enzyme, facilitating the formation of the Chl molecule (8). This role of Zn could also explain the higher pigment content and alleviated symptoms of chlorosis on day 7 of our experiment in plants exposed to the combination of $CdCl_2$ and $ZnCl_2$ in comparison to $CdCl_2$-treated plants. Our results are in agreement with experiments on *C. demersum* (8, 29, 30), which proved that Zn-addition to Cd-treated plants restored Chls and Cars levels and protected chloroplasts from Cd toxicity. In rice, the addition of Zn to the nutrient solution also alleviated photosynthesis inhibition and Chl content reduction caused by Cd (27). Considering the effect of $CuCl_2$, the lower concentration (2.5 μmol L^{-1}) decreased photosynthetic pigment content after four days but after prolonged treatment pigment content reached control levels, suggesting a tolerance to the lower concentration of Cu. Higher Cu concentration (5 μmol L^{-1}) affected the pigment content during the whole experiment.

Contrary to the biologically inert Zn and Cd, Cu is highly reactive due to the existence of two interconvertible oxidation states (52). It can catalyse the formation of free radicals through the Haber-Weiss reaction, and consequently cause oxidative stress in plant cells leading to the breakdown of membrane lipids, degradation of proteins, and other macromolecules, including pigments. Therefore, it was not surprising that $CuCl_2$ enhanced $CdCl_2$-induced chlorosis and reduction of pigments content in combined treatments. Moreover, Cvjetko et al. (32) confirmed that Cu applied simultaneously with Cd increased Cd-induced oxidative stress remarkably.

Changes in duckweed photosynthetic functions under stress caused by the heavy metals were determined by chlorophyll fluorescence analysis. After four days of exposure, the maximum quantum yield of PSII (F_v/F_m) and

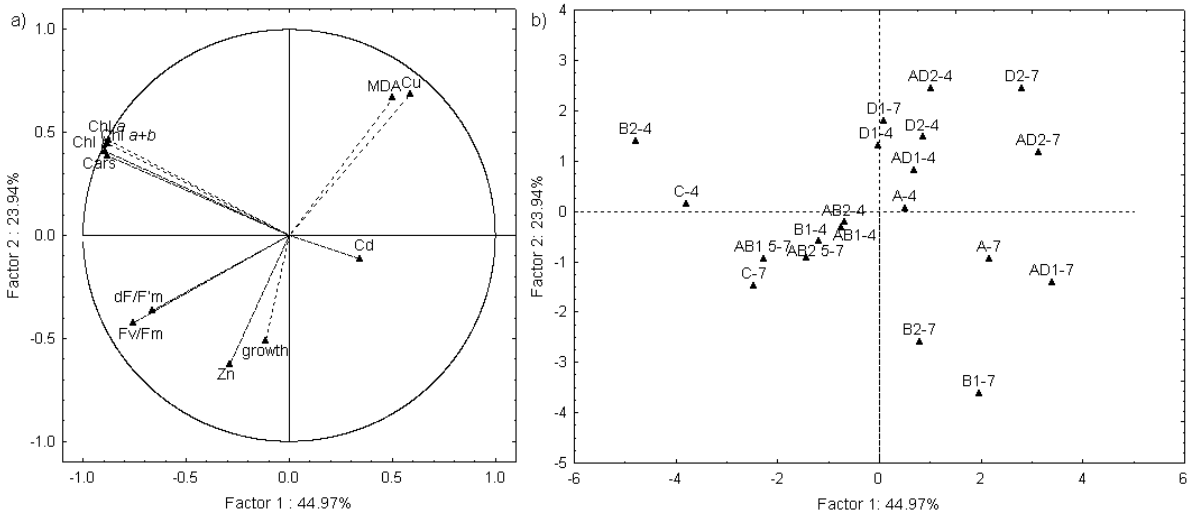

Figure 3 *Principal component analysis - a) loadings and b) scores. Growth, accumulation of Cd, Zn and Cu content of Chl a, Chl b, total chlorophylls (Chl a+b) and carotenoids (Cars), maximum quantum yield (F_v/F_m) of PSII, effective quantum yield ($\Delta F/F'_m$) of PSII and malondialdehyde (MDA) content [reported previously, (31, 32)], represent variables*
Treatments: C-control, A-Cd, B1-25 μmol L^{-1} Zn, B2-50 μmol L^{-1} Zn, D1-2.5 μmol L^{-1} Cu, D2-5 μmol L^{-1} Cu
Numbers 4 and 7 represent days of treatment

the effective quantum yield of PSII ($\Delta F/F'_m$) were diminished by all of the applied metal treatments. Lower value for F_v/F_m and $\Delta F/F'_m$ can indicate a certain proportion of inactive PSII reaction centres. Reduced pigment content could also decrease these parameters, which is consistent with the observed lower Chls content in plants exposed to heavy metals for four days. Diminished PSII efficiency can also indicate the inhibition of electron transport at other sites in the electron transport chain downstream of PSII and can be explained by the toxic effects of the tested metals, as both Cd and Cu are well-known for such an effect (21, 49). Also, PSII is highly sensitive to Cd and Cu because they can bind to various parts of the acceptor and donor side of PSII (9, 17, 21, 49).

Excess Zn can also disturb photosynthesis. It inhibits O_2 evolution and interacts with the donor side of PSII and may also affect its acceptor side (53). Cd as well as Zn and Cu can affect enzymes of the Calvin cycle through inactivation of sulfhydryl groups (50) leading to down-regulation of electron transport chain due to excessive amounts of ATP and NADPH.

Recovery of PSII activity after 7-day treatment with $ZnCl_2$ and its combination with $CdCl_2$ could be explained by subcellular compartmentalisation and extrusion of Cd and Zn, mediated by the activation of specific transport processes (4, 54). Moreover, other physiological and biochemical processes such as increased antioxidant activity and synthesis of phytochelatins (4) could also contribute to Cd and Zn tolerance. In plants exposed for 7 days to the higher concentration of $CuCl_2$ (5 μmol L^{-1}) both fluorescence parameters (F_v/F_m and $\Delta F/F'_m$) were decreased. The more prominent inhibitory effect of Cu in comparison to Zn on PSII efficiency is probably due to its ability to cause oxidative stress (18). The results of our study are consistent with those by Frankart et al. (55) who found a sensitivity of *L. minor* photosynthetic efficiency to Cu at 3.15 and 6.3 μmol L^{-1}.

The PCA analysis performed in this study contributed to knowledge on the differences in duckweed responses after exposure to single metals and their mixtures The results obtained imply that, unlike Zn, Cu could not alleviate Cd-induced toxicity. Therefore, our findings support previous observations by Cvjetko et al. (32) on Cu ability to induce oxidative stress and affect the photosynthetic apparatus in duckweed. Reduction of Cd-induced stress by Zn is probably mediated through lowering Cd uptake, which is in agreement with the results obtained for *C. demersum* (29) and tobacco (56). Interestingly, PCA did not show substantial subgrouping of the plant responses based on the treatment duration, except for the plants exposed to single Zn treatment. Furthermore, longer Zn exposure correlated with a decrease in pigment content. Such results suggest that longer exposure to Zn results in the accumulation of Zn, the excessive amounts of which exert adverse effect on plants.

The results of our study reveal significant growth-modulatory effects of Cd, Zn, and Cu in duckweed, accompanied by adverse effects on photosynthetic efficiency and pigment content, which calls for further research. Nevertheless, the findings presented here contribute to general knowledge on the specific toxicity mechanisms of metals, which both as anthropogenic and environmental contaminants may endanger plant communities and significantly disrupt the sensitive balance of an ecosystem.

Acknowledgements

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Rast i fotosinteza u vodene leće (*Lemna minor* L.) izložene kadmiju u kombinaciji s cinkom ili bakrom

Izloženost metalima može izazvati različite štetne učinke u biljaka. Vodene leće izložili smo solima teških metala $CdCl_2$ (5 µmol L^{-1}), $ZnCl_2$ (25 µmol L^{-1} ili 50 µmol L^{-1}) i $CuCl_2$ (2,5 µmol L^{-1} ili 5 µmol L^{-1}) te kombinaciji $CdCl_2$ sa svakom od navedenih koncentracija $ZnCl_2$ i $CuCl_2$. Rast biljaka, količina fotosintetskih pigmenata i učinkovitost fotosistema II (PSII) mjereni su nakon četiri i sedam dana tretmana. Utvrđeno je da su svi tretmani uzrokovali značajnu inhibiciju rasta te akumulaciju metala u biljci. U biljaka koje su bile izložene kombinacijama teških metala količina pojedinog metala u tkivu bila je niža u odnosu na količinu istog metala u biljaka izloženih samo tom metalu. Nakon četiri dana tretmana sva su tri metala, neovisno o tome jesu li bila primijenjena zasebno ili u kombinacijama, uzrokovala smanjenje količine klorofila *a* i pad vrijednosti maksimalnog (F_v/F_m) i efektivnog ($\Delta F/F'_m$) prinosa PSII. Međutim, u biljaka koje su bile istovremeno izlagane kadmiju i cinku, vrijednosti količine pigmenata i učinkovitost PSII vratile su se nakon sedam dana na kontrolnu razinu, a bakar u koncentraciji 5 µmol L^{-1} te kombinacija kadmija i bakra i dalje su imali inhibitorni učinak. Budući da smanjeno primanje pojedinog metala uočeno u biljaka izloženih kombiniranim tretmanima nije ublažilo inhibitorni učinak na rast, možemo zaključiti da je inhibicija rasta uzrokovana apsolutnom količinom metala primljenog u tkivo. Povećanje količine fotosintetskih pigmenata i učinkovitosti PSII nakon sedam dana tretmana kadmijem i cinkom upućuje na oporavak biljaka, što se može objasniti ublažavajućim djelovanjem cinka na učinak kadmija uslijed smanjenog primanja kadmija u biljku. Suprotno tome, dugotrajni inhibitorni učinak istovremenog tretmana biljaka kadmijem i bakrom te samim bakrom u koncentraciji 5 µmol L^{-1} može se objasniti oksidacijskim stresom uzrokovanim bakrom. Rezultati ovoga istraživanja pridonose saznanjima o štetnim učincima antropogenih i okolišnih onečišćivača, koji narušavanjem mehanizama fotosinteze ugrožavaju ne samo biljke i njihove zajednice nego i osjetljivu ravnotežu ekosustava.

KLJUČNE RIJEČI: *fluorescencija klorofila; fotosintetski pigmenti; primanje metala; učinkovitost PSII; vodena leća*