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Effects of metal quantity and quality to the removal of zinc and copper by two common green microalgae (Chlorophyceae) species

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SUMMARY

Appearance of metals as pollutants in the environment is an increasing global problem. Microalgae as subjects of biological remediation methods may provide a cost-effective and environmentally friendly alternative to the removal of metals during wastewater treatment. Despite the high number of data in the topic, there is still little information on how the type and the concentration of the metal affect the process of removal. In this study, correlations among the algal species, quality and quantity of metals and characteristics of metal removal mechanism were investigated at lower metal concentrations (0.2–5.0 mg L^{-1}) during zinc and copper removal of the green algae *Desmodesmus communis* and *Monoraphidium pusillum*. Analyses of the results proved that there is a statistically significant interaction ($P < 0.05$) between algal species and quality and concentration of the metals, that is, they have a significant effect on the mode and extent of removal. Both metals were mainly extracellularly bound, but at concentrations of 0.2–1.4 mg L^{-1}, intracellular proportion could exceed the extracellular adsorption. Although there were differences between the two algae, generally copper appeared in a higher intracellular proportion than zinc in the whole studied concentration range. Overall, the quality and initial concentration of the metal is decisive for the way of removal, the knowledge of which is useful for planning post treatment retention times or post treatment processes of the used biomass during wastewater treatment.

Key words: bioaccumulation, biosorption, copper, *Desmodesmus communis*, *Monoraphidium pusillum*, zinc.

INTRODUCTION

During the past decades heavy metal concentrations increased dramatically in surface and groundwater due to human activities, mainly because of industrial, agricultural and sewage disposal sectors. Accumulation of trace elements can occur in living organisms and these elements can be biomagnified at higher trophic levels across the food chain, so they are especially dangerous to final consumers (apex predators) and also to human health (Kotrba *et al*. 2011; Nyeste *et al*. 2019). Some of the heavy metals are essential micronutrients to organisms for normal growth and take part in many biochemical and physiological processes in cells, although they can inhibit the growth and can cause toxic symptoms at higher concentrations (Monteiro *et al*. 2012).

Nowadays, biological methods for removal of heavy metals got to the focus of interest, as physico-chemical methods (membrane filtration, electrodialysis, photocatalysis) may be ineffective, inefficient and very expensive at relatively low (<100 mg L^{-1}) metal ion concentration (Mehta & Gaur 2005; Lodeiro *et al*. 2006; Naja & Volesky 2009; Barakat 2011). Most of the literature agree, that constructed wetlands and algal ponds are efficient and cost effective compared to physico-chemical methods, when metal contaminants are present at the mentioned low concentration (Naja & Volesky 2009; Barakat 2011; Rajasulochana & Preethy 2016; Zeraatkar *et al*. 2016; Joseph *et al*. 2019). Macro- and microalgae may have an important role in bio-based treatments, because many of them are cosmopolitan and tolerant organisms, they may have good adaptive capacity to changing environment and are able to decontaminate and/or detoxicate various pollutants (Calahan *et al*. 2018).

Microorganisms can bind heavy metal ions by two main processes: bisorption and/or bioaccumulation. Biosorption is a metabolically independent, simple physico-chemical reaction between the surface of the cells and metal ions (Chojnacka 2010). Cell walls of green algae show very diverse chemical structure; generally they are built from multilayered cellulose fibrils consisting of exopolysaccharide, glycolipid and glycoprotein molecules, which can be potential binding sites of heavy metals (Wang & Chen 2009). In the process of bioaccumulation, the first step is also biosorption, and then metal ions are taken up into the cells by metabolism-dependent active transport systems (Monteiro *et al*. 2012). There are many complex molecular

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mechanisms in algal cells to maintain a healthy metal-balance in the cell, and to detoxicate and/or tolerate the hazardous effects of metals. In cells, heavy metals may be bound to metal binding proteins (phytochelatins, metallothioneins) and to amino- and other organic acids; they can be transformed by oxidation, reduction, methylation and dealkylation processes, and vacuolar compartmentalization also can affect their plasmatic concentrations (Blaby-Haas & Merchant 2017).

It is known that different algal species (Mehta & Gaur 2005) or different isolates of the same species (Monteiro *et al*. 2011a,b) could be characterized with different capacities and mode of removal (adsorbers or accumulators; Romera *et al*. 2006). Despite the high number of data in the topic, the question still arises, whether it is possible, that certain metals are mainly adsorbed on the cell wall while others are accumulated in the cells; and whether or not there is any effect of the metal concentration on the type of removal (biosorption or bioaccumulation). The answer could be important, since using living biomass during the remediation process, the direct toxicity of metals getting into the cells should be considered.

Here, the removal of two essential heavy metals (zinc and copper) by two green algal species (*Desmodesmus communis*, *Monoraphidium pusillum*, both are known as adsorbers) was studied. Zinc and copper were chosen, because these metals are among the most often appearing metal contaminants in surface and groundwaters and belonging to the few metal ions to which limits are given in the Water Framework Directive of the European Union (SWD 379 2012). The objective was to explore the correlations between the algal species, quality and quantity of metals and characteristics of metal removal mechanism at lower metal concentrations (0.2–5.0 mg L^{-1}). The effects of metals on growth (individual number), toxicity (EC_{50}), and the total amount of removed metal, as well as extracellular and intracellular proportions were studied. On the basis of existing literature data we assumed (i) weaker inhibitory effects (i.e. higher EC_{50} values) of zinc than copper, because of the different oxidation–reduction characteristics of the two metals (Pinto *et al*. 2003; Tripathi *et al*. 2006); (ii) higher removed amounts of zinc than copper, because of the assumed weaker toxicity; (iii) higher ratio of intracellular accumulation at lower concentration (0.2 mg L^{-1}) of both metals; and (iv) higher intracellular accumulation ratios of zinc than copper at the same low (closer to physiological) concentrations, since zinc is involved in more physiological processes in algal cells than copper (Xu *et al*. 2012).

As it is presented in detail below, the results show that members of common phytoplankton assemblages may show different, but notable sensitivity and metal removal ability. It was also proved that the quality and initial concentration of the metal is decisive for the way of removal, the knowledge of which is useful for planning post treatment retention times or post treatment processes of the used biomass during wastewater treatment.

MATERIALS AND METHODS

Experimental design and growth of the cultures

Desmodesmus communis (E.Hegewald) E.Hegewald (ACCDH-UD1004) and *Monoraphidium pusillum* (Printz) Komárková-Legnerová (ACCDH-UD0911) were maintained in the Algal Culture Collection of the Department of Hydrobiology, University of Debrecen (ACCDH-UD), in Jaworski's medium (at pH: 7–7.5; CCAP Media Recipes), in 500 mL Erlenmeyer flasks with a final culture volume of 400 mL. The cultures were bubbled with sterile air, at 24°C, under continuous illumination (40 μmol photons m^{-2} s^{-1}).

The experiments were carried out within the same conditions. Stock solution of $ZnSO_4 \times 7H_2O$ and $CuSO_4 \times 5H_2O$ were dissolved in deionized water and were added to the treated cultures to reach the adequate concentrations (0.2, 0.8, 1.0, 1.4, 2.0, 2.5 and 5.0 mg L^{-1}). Control cultures contained no added zinc or copper. Negative controls (medium + metals, without algae) were applied for all treatments to check the initial metal concentrations of the media at the start of the experiments.

Growth of the cultures was followed by counting the number of individuals (coenobia in the case of *D. communis* and single cells in the case of *M. pusillum*; European Standard EN 15204 2006). For counting, 0.2 mL samples were collected daily, individual numbers were counted in Bürker chamber using an Olympus BX50F-3 microscope at 400× magnification.

Measurement of metal removal

Samples of 5 mL were collected on zero, the third and the seventh day that is right after inoculation (within the first hour – day zero), after 72 h (third day) and after 148 h (seventh day). The samples were centrifuged (16 200 *g*, 5 min.; 24°C, Heraeus™ Fresco™ 17 Microcentrifuge), supernatants were removed. Supernatants and pellets were stored at –20°C until further processing. Aliquots of 0.5 mL supernatants were complemented with 4.5 mL 0.1 mol HNO_3 solution for the measurement of the metal content of the culturing media. Metals were measured by Microwave Plasma - Atomic Emission Spectroscopy (MP-AES) method (Agilent Technologies 4100 Spectrometer). The total amount of removed metal was calculated from the metal content loss of the supernatants (it has to be noted that this amount also contains the amount of precipitated metal).

The pellets were lyophilized (Christ Alpha 1-2 LD plus) and weighed (Ohaus Adventurer™ Pro). For measuring the extracellularly bound metal content, the lyophilized pellets of 5 mL samples were washed in 5 mL 2 mmol EDTA for 10 min to remove the cell surface bound or precipitated metals (Tripathi & Gaur 2004). After centrifugation, supernatants were measured by MP-AES. The amounts of intracellularly accumulated metals were calculated by deduction of the extracellularly bound amounts from the calculated total amounts.

Statistical analyses

All experiments were done in triplicate. One-way analysis of covariance (ANCOVA) was used to check the significances among the tendency differences of growth curves of control and treated cultures. One-way ANOVA and repeated measure ANOVA were used for the analyses of metal removal data. Three-way ANOVA was used to analyze the correlations among the algal species, quality and quantity of metals and

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characteristic of metal removal mechanisms (Hammer *et al*. 2001; Zar 2009).

RESULTS

Growth of the cultures

Zinc in 0.2 and 0.8 mg L^{-1} significantly stimulated the growth of *Desmodesmus communis* cultures ($P < 0.05$). There were no significant differences in individual number of the control and 1.0–1.4 mg L^{-1} zinc treated cultures (Fig. 1a). Individual number decreased significantly ($P < 0.05$) from 2.0 mg L^{-1} zinc (Fig. 1b).

Zinc treatments (0.2–5.0 mg L^{-1}) did not cause significant alterations in individual number of *Monoraphidium pusillum* cultures, although slight, non-significant increases were observed in 0.2–1.4 mg L^{-1} zinc treated cultures compared to control (Fig. 1c,d).

Copper in 0.2 and 0.8 mg L^{-1} concentrations did not cause significant differences in growth of *D. communis* cultures compared to control (Fig. 2a), while individual numbers were significantly lower ($P < 0.01$) in treated cultures than in control from 1.0 mg L^{-1} copper concentration (Fig. 2a,b). Above 1.0 mg L^{-1} copper concentration proliferation was almost totally inhibited (80% or higher inhibition; Fig. 2a,b).

Copper in 0.2–1.4 mg L^{-1} concentrations caused slight, but not significant growth inhibition of *M. pusillum* cultures compared to control (Fig. 2c). Individual numbers were gradually reduced with increasing copper concentration from 2.0 mg L^{-1} (Fig. 2d). Individual numbers of 2.0, 2.5 and 5.0 mg L^{-1} copper treated cultures were significantly ($P < 0.05$) lower than in control and also than in 0.2–1.4 mg L^{-1} treated cultures ($P < 0.01$; Fig. 2c,d).

Half maximal effective concentration (EC_{50}) of zinc and copper were calculated to 3- and 7-day exposure. Zinc concentration 4.42 mg L^{-1} caused 50% growth inhibition after 7-day exposure in *D. communis* cultures, whereas the applied concentrations of zinc did not cause 50% inhibition in *M. pusillum* cultures. EC_{50} values for copper were 1.13 and 1.12 mg L^{-1} after 3- and 7-day exposure in *D. communis* cultures, respectively; 3.82 mg L^{-1} copper caused 50% inhibition after 7-day exposure in *M. pusillum* cultures (Table 1).

Removal of heavy metals

Total amounts of removed zinc and copper

On dry mass basis, the highest amounts of removed zinc were observed at the beginning of the experiments and these amounts were reduced significantly ($P < 0.001$) to the seventh day in all treated *D. communis* cultures. The amount of removed zinc increased significantly ($P < 0.05$) with increasing concentration (Fig. 3a). The maximal removal of zinc per

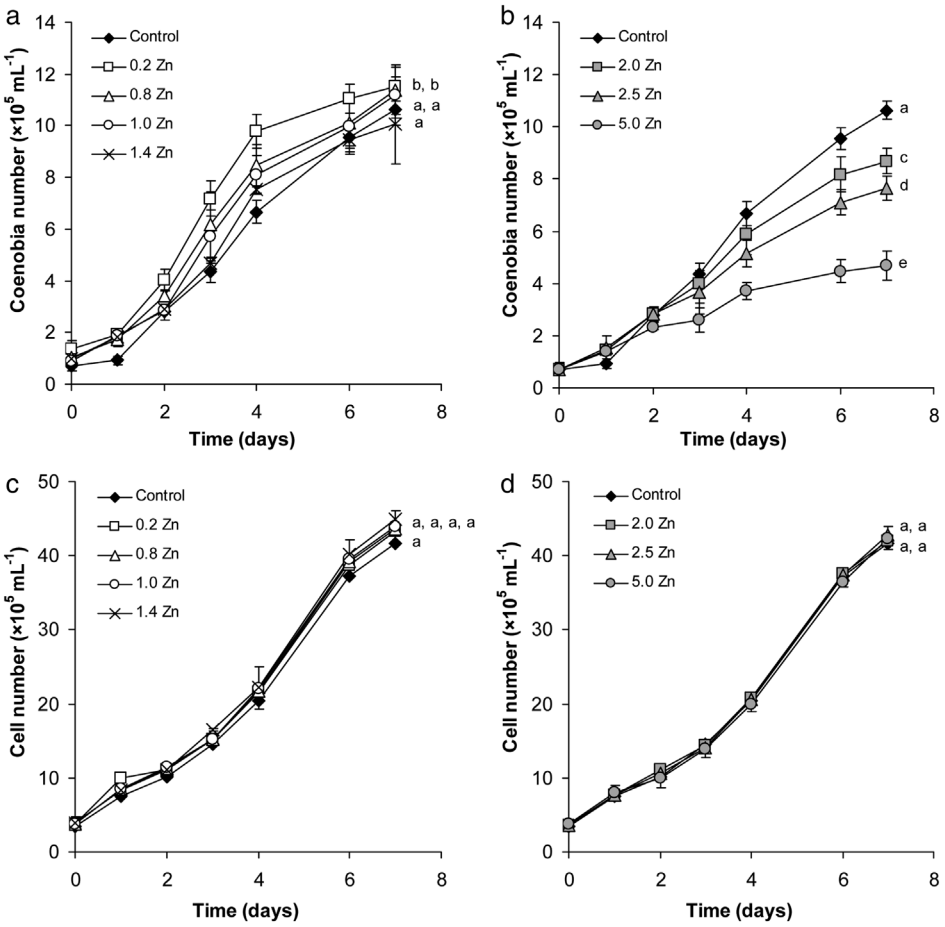

Fig. 1. Changes of individual numbers in control and in 0.2–5.0 mg L^{-1} zinc treated *D. communis* (a) and *M. pusillum* (b) cultures. Mean values ($n = 3$) and standard deviations are plotted, different lowercase letters indicate significant differences ($P < 0.05$).

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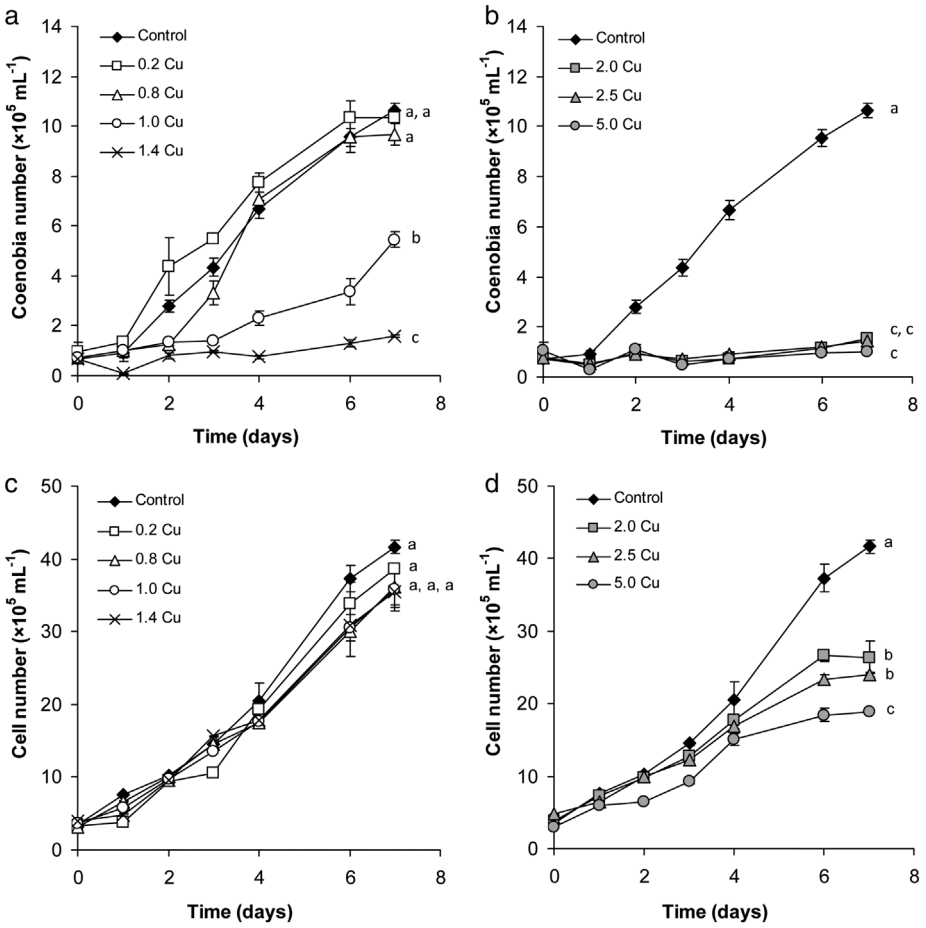

Fig. 2. Changes of individual numbers in control and in 0.2–5.0 mg L^{-1} copper treated *D. communis* (a) and *M. pusillum* (b) cultures. Mean values ($n = 3$) and standard deviations are plotted, different lowercase letters indicate significant differences ($P < 0.05$).

Table 1. EC_{50} values for zinc and copper in the case of the green algae *D. communis* and *M. pusillum*, after 3 and 7 days of exposure

Metal (mg L^{-1})	*D. communis*	*M. pusillum*
Zinc, 3 days exposure	n.c.	n.c.
Zinc, 7 days exposure	4.42	n.c.
Copper, 3 days exposure	3.82	n.c.
Copper, 7 days exposure	1.12	1.13

n.c., not calculable.

unit of dry mass was observed in 5.0 mg L^{-1} treated *D. communis* culture (~28 mg g^{-1}). Considering the zinc concentration set at the beginning of the experiments as 100%, the highest proportion of removed zinc was in the 5.0 mg L^{-1} zinc-treated culture on the seventh day (~79%; Fig. 3b). The removal percentage was the highest in 0.2 mg L^{-1} zinc treated culture on almost every sampling day and it increased by time in each treated culture (Fig. 3b). Taking initial zinc concentrations into consideration, it can be said that the amount of removed zinc increased both in time and with increasing initial zinc concentration in the case of *D. communis*.

Higher amounts of zinc calculated to a unit of dry mass occurred on day zero in 0.2–1.4 mg L^{-1} treated *M. pusillum* cultures and these amounts were gradually decreased to the end of the experiments, while in 2.0–5.0 mg L^{-1} treated cultures the most removed zinc was observed on the third day (Fig. 3c). The amount of removed zinc per unit of dry mass was the highest in the 5.0 mg L^{-1} treated *M. pusillum* culture on the third day of the experiment (~13 mg g^{-1}; Fig. 3c). The amount of removed zinc increased with the increasing concentration; however, these changes were not significant in each case. Considering the zinc concentration set at the beginning of the experiments as 100%, the proportion of removed zinc was the highest in 0.2 mg L^{-1} zinc-treated culture on day seventh day (~89%; Fig. 3d). Similarly to the observations in the case of *D. communis*, the removal percentages were higher in 0.2 mg L^{-1} zinc treated culture on every sampling day and they increased by time in each treated culture (Fig. 3d). Taking initial zinc concentrations into consideration, it can be said that the amount of removed zinc increased both in time and with increasing initial zinc concentration.

In the case of copper removal of *D. communis* cultures, the maximal amount of removed copper calculated to a unit of dry mass (~69 mg g^{-1}) occurred in 5.0 mg L^{-1} treated culture on the seventh day. There was no clear trend observable either in the case of exposition time or initial copper concentration (Fig. 4a). Considering the copper concentration set at the beginning of the experiments as 100%, the amount of removed copper was the highest in 0.2 mg L^{-1} copper-treated

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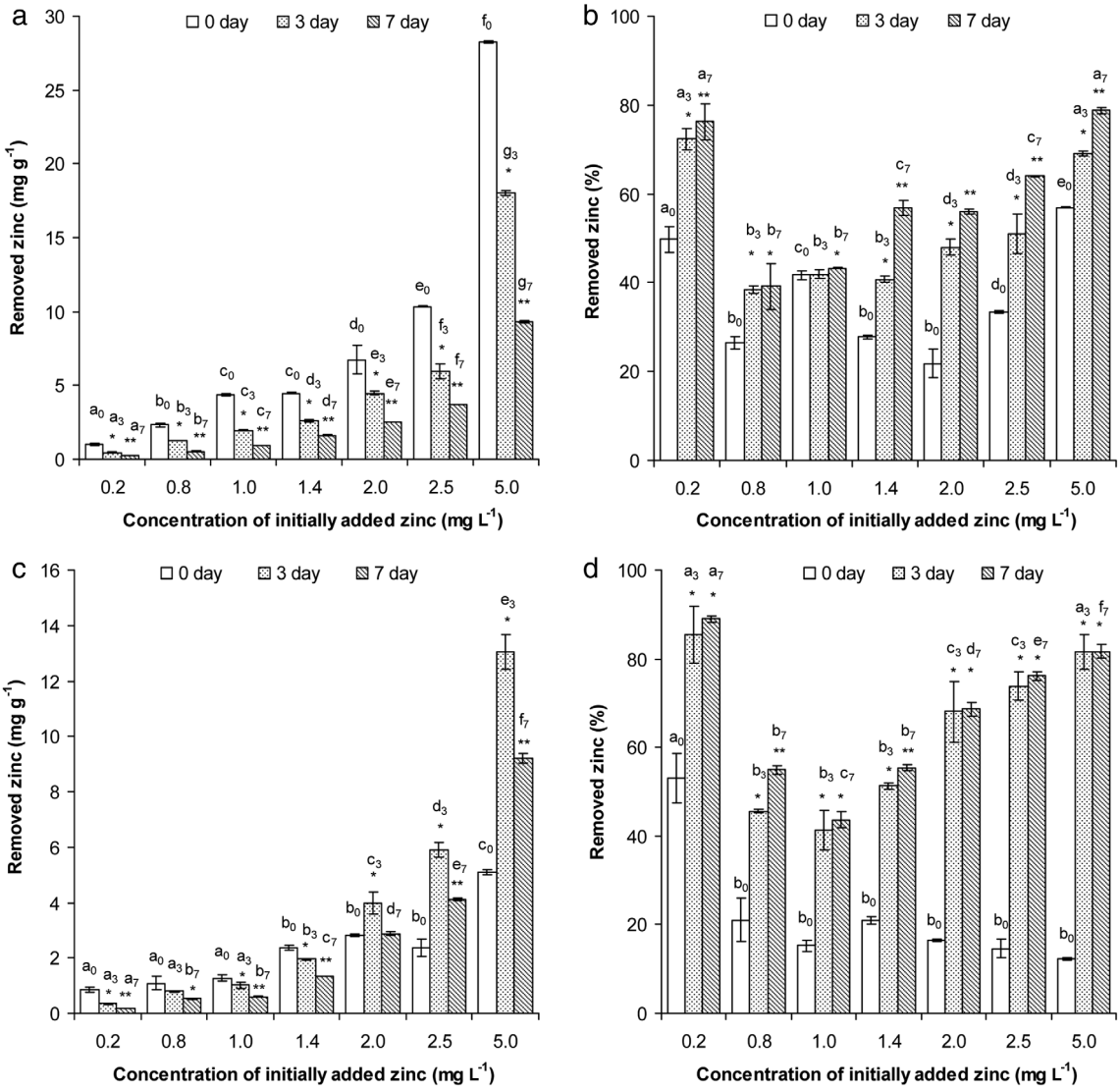

Fig. 3. Zinc removal in *D. communis* cultures calculated to a unit of dry mass (a) and given in percentage considering the zinc concentration set at the beginning of the experiments as 100% (b); and zinc removal of *M. pusillum* cultures calculated to a unit of dry mass (c) and given in percentage considering the zinc concentration set at the beginning of the experiments as 100% (d). Mean values ($n = 3$) and standard deviations are plotted. Significant differences ($P < 0.05$) among the 0 (right after inoculation - within the first hour); 3 and 7 days (72 and 148 h) of a treatment with a given concentration are marked with asterisks (*, **). Significant differences ($P < 0.05$) among the different concentrations on a given day are indicated by lowercase letters (a–g; in subscript with the given day: 0; 3; 7).

culture on the seventh day (~89%; Fig. 4b). Taking initial copper concentrations into consideration, it can be said that the amount of removed copper increased both in time and with increasing initial copper concentration.

The amount of removed copper calculated to a unit of dry mass was the highest on day zero in 5.0 mg L^{-1} treated *M. pusillum* culture (~36 mg g^{-1}) and it was gradually significantly ($P < 0.001$) decreased to the end of the experiments (Fig. 4c). The amount of removed copper increased with the increasing concentration; however, these increases were not significant in every case. Considering the copper concentration set at the beginning of the experiments as 100%, the amount of removed copper was the highest in 5.0 mg L^{-1} copper-treated culture on each day (~80–81%; Fig. 4d). There was no observed trend in the removal percentage of copper by time. The removed proportion of copper increased with the increasing copper concentration, although this increase was not always significant (Fig. 4d).

Extra- and intracellular metal proportions

Extra- and intracellular metal proportions were expressed as percentage of the total amount of removed metals. The extracellular proportion of zinc was higher than 90% at the end of the experiments in the case of all zinc treated *D. communis* cultures. The maximal extracellular proportion (~98%) was observed in 2.0 mg L^{-1} zinc treated *D. communis* culture on the seventh day (Fig. 5). The highest intracellular zinc level

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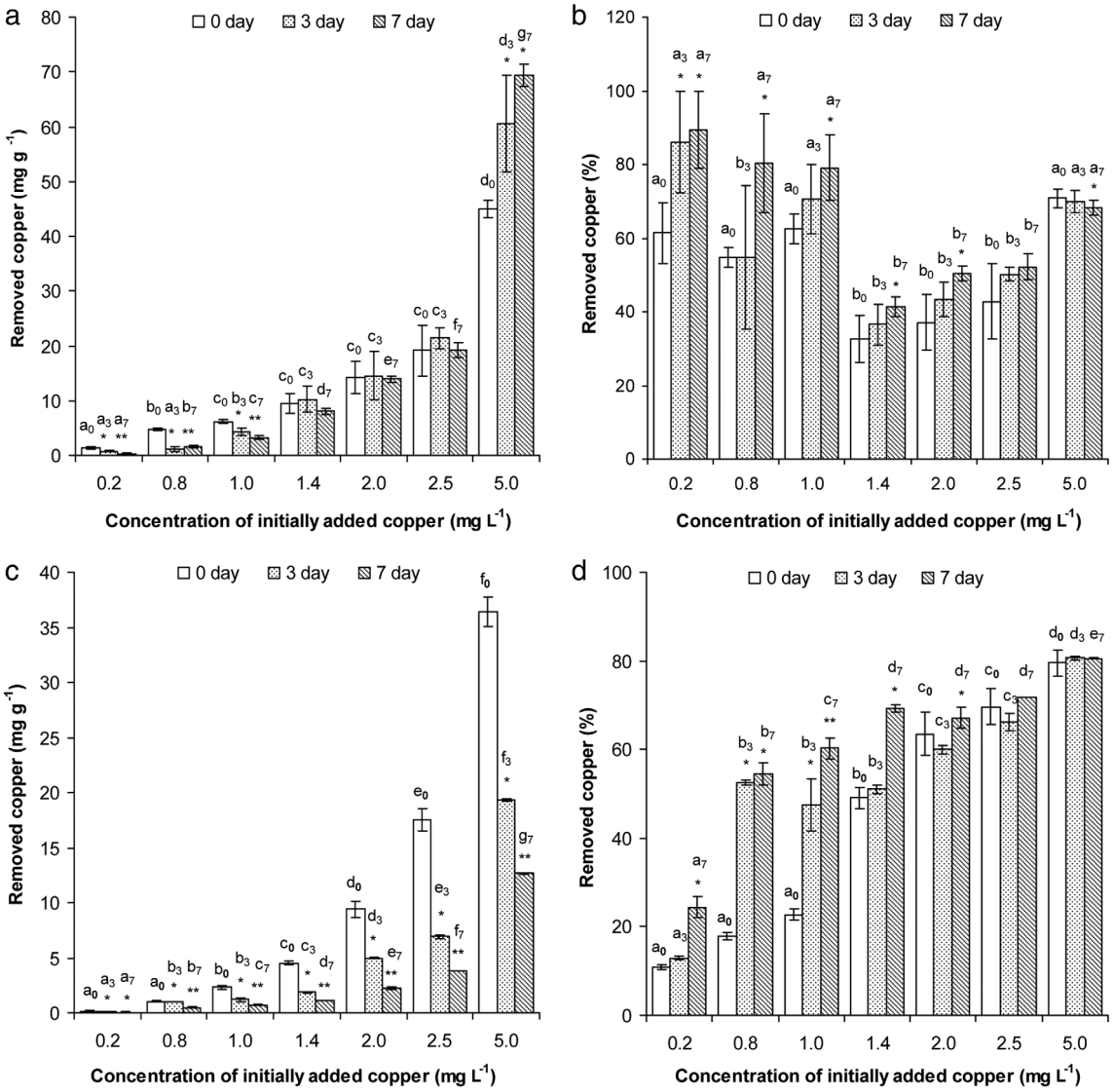

Fig. 4. Copper removal in *D. communis* cultures calculated to a unit of dry mass (a) and given in percentage considering the copper concentration set at the beginning of the experiments as 100% (b); and copper removal of *M. pusillum* cultures calculated to a unit of dry mass (c) and given in percentage considering the copper concentration set at the beginning of the experiments as 100% (d). Mean values ($n = 3$) and standard deviations are plotted. Significant differences ($P < 0.05$) among the 0 (right after inoculation - within the first hour); 3 and 7 days (72 and 148 h) of a treatment with a given concentration is marked with asterisks (*, **). Significant differences ($P < 0.05$) among the different concentrations on a given day are indicated by lowercase letters (a–g; in subscript with the given day: 0; 3; 7).

(~68%) was in 0.2 mg L^{-1} treated *D. communis* culture on the day zero. At the beginning of the experiments the proportion of intracellular zinc was higher than extracellular zinc in 0.2–1.4 mg L^{-1} treated *D. communis* cultures. The proportion of extracellular zinc increased with the exposition time and also with increasing initial concentration (Fig. 5).

Maximal extracellular proportion of zinc was the highest in the 5.0 mg L^{-1} treated *M. pusillum* culture on the seventh day, approximately 99% of removed zinc was adsorbed on the surfaces of the cells. Maximal intracellular proportion of zinc was observed in 0.2 mg L^{-1} treated *M. pusillum* culture on day zero, approximately 76% of removed zinc was accumulated into the cells (Fig. 5). The intracellular accumulation exceeded the extracellular adsorption in some cases at lower concentration; however there were no significant correlation of initial zinc concentration and exposition time on proportion of extra- and intracellular zinc content (Fig. 5).

Maximal extra- and intracellular copper removals (~74 and ~78%, respectively) were observed on the seventh and zero day in 0.8 and 1.0 mg L^{-1} treated *D. communis* culture, respectively. Intracellular accumulation exceeds the extracellular adsorption at the beginning of the experiments in 0.2 and 0.8 mg L^{-1} treated cultures, while the extra- and intracellular ratio reversed on the third and the seventh day of the experiments. The ratio of extra- and intracellular removal did not change in 1.0–5.0 mg L^{-1} treated culture during the

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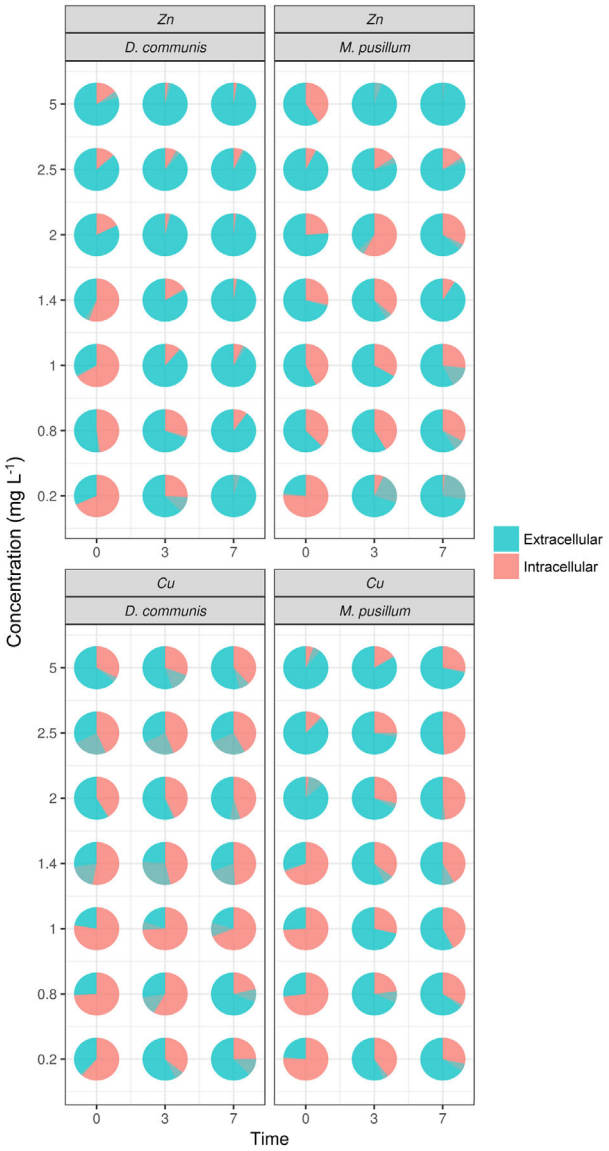

Fig. 5. Proportions of extracellularly (blue) and intracellularly (pink) removed zinc (above) and copper (below) on 0 (right after inoculation – within the first hour); third and seventh days (72nd and 148th hours) by *D. communis* and *M. pusillum* cultures treated with different initial metal concentrations. Mean values (n = 3) and standard deviations are plotted, the latter are indicated as transitional colors on pie charts. [Color figure can be viewed at wileyonlinelibrary.com]

experiments. There were no significant effects of initial copper concentration on the proportions of removed copper (Fig. 5).

Extracellular proportion of copper reached its maximum in 2.0 mg L^{-1} treated *M. pusillum* culture, it was 92% on day zero. Maximum proportion of intracellular copper was 76% on day zero in 0.2 mg L^{-1} treated culture (Fig. 5). The proportion of intracellularly accumulated copper was higher on day zero in 0.2–1.4 mg L^{-1} treated *M. pusillum* cultures, while the proportion of extracellularly bound copper was higher on the third and seventh days of the experiments. The situation was just the opposite in 2.0–5.0 mg L^{-1} treated *M. pusillum* cultures: the extracellular copper removal was higher than 90% on day zero, then it was gradually decreased to the third and the seventh day, so the proportion of intracellular copper increased at the same time (Fig. 5).

DISCUSSION

According to the individual number changes, the *M. pusillum* strain was more tolerant to the tested heavy metals than the *D. communis* strain. Weaker inhibitory effects (i.e. higher EC_{50} values) of zinc than copper were assumed, the assumption of which was proved by the results: both species were more sensitive to the presence of copper compared to zinc. Many studies have confirmed that both zinc and copper are essential micronutrients for the normal growth of living organisms (Blaby-Haas & Merchant 2017). Zinc and copper play key roles in many physiological and biochemical processes. Zinc acts as an important structural and functional component of numerous enzymes (Nguyen-Deroche *et al*. 2012), copper has a significant role in the electron transport of photosynthesis and respiration (Falkowski & Raven 2007). So these trace elements can stimulate the growth at lower concentration; however, at higher concentrations they can become toxic. It is well known from several studies that both metals inhibit the growth of algal cultures (cell division), photosynthesis and chlorophyll synthesis (Wong & Chang 1991; Cid *et al*. 1995; Omar 2002a,b; Monteiro *et al*. 2011a,b). The higher inhibitory effect of copper obviously linked to the different chemical nature of these two metals: As a metal ion with two possible oxidation states, copper is involved directly in oxidative stress by the formation of reactive oxygen species. Zinc ion with no variable oxidation state can cause weaker stress to the exposed organisms (Pinto *et al*. 2003; Tripathi & Gaur 2004; Tripathi *et al*. 2006).

Toxicity of heavy metals could differ not only among different species, but also different genotypes of the same species (Monteiro *et al*. 2011a,b). Species sensitivity or tolerance to metals can be influenced by both abiotic factors, such as pH, salinity and dissolved organic carbon (DOC) and biotic factors, such as mode of removal, size and age of organisms and detoxification processes of the cells (Levy *et al*. 2007; Borowitzka *et al*. 2016). In spite of these facts, the binding affinities for zinc to different organisms (algae, daphnids, and fishes) were found to be quite similar (Ardestani *et al*. 2014). This may suggest that the affinity of zinc for binding to biological ligands of the organisms is less species-dependent. On the other hand, the growth results presented above show that different green algal species isolated from the same 'average' environment could be characterized with significantly different tolerance.

Regarding metal removal, it can be said that zinc removal occurred more or less similarly in the two species. Similar to the literature data (Zeraatkar *et al*. 2016) removed amounts of both metals increased as the initial concentration of metal ion increased in the case of both algal species. Although the proportion of removed zinc increased over time in both strains, the amount of removed metal per unit of dry mass decreased (with some exceptions, Fig. 3). It suggests that metal removal did not increase in parallel with the increasing

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amount of biomass (and potential binding surface). Partial aggregation of individuals (as it was observed during our experiments) could be an explanation of this phenomenon. Aggregation processes could reduce the effective surface area available for sorption or by a decrease of the average distance between available adsorption sites (Ahuja *et al*. 1999; Munoz & Guieysse 2006; Gupta & Rastogi 2008; Monteiro *et al*. 2012; Kumar *et al*. 2015). Still, according to the increasing removed proportion of initial metal concentration, it can be said that using living algae, higher level of removal occur by applying longer contact times (Lamaia *et al*. 2005; Zeraatkar *et al*. 2016). *D. communis* removed more zinc than *M. pusillum* on the basis of dry mass (~28 and 13 mg g^{-1}, respectively) but *M. pusillum* removed slightly higher proportions from solvents with higher initial concentrations (2.0–5.0 mg L^{-1}; Fig. 3). These removal capacities can be considered as moderate in comparison with other micro- or macroalgae (Zeraatkar *et al*. 2016).

The main difference between the copper removals of the two strains was the remarkable change in removed proportion of copper at 1.4 mg L^{-1} or higher copper concentration in the case of *D. communis* (Fig. 4b). This phenomenon may be due to toxicity of copper to *D. communis*, removal characteristics of dead biomass could be relevant above 1.0 mg L^{-1} copper concentration. *D. communis* removed more copper than *M. pusillum* on the basis of dry mass (~69 and 36 mg g^{-1}, respectively) but *M. pusillum* removed higher proportions from solvents with higher initial concentrations (1.4–5.0 mg L^{-1}; Fig. 4). Similar to that in the case of zinc, these removal capacities can be considered as moderate in comparison with other micro- or macroalgae (Zeraatkar *et al*. 2016).

Higher removed amounts of zinc than copper were assumed, which was not proved by the results on dry mass basis: both species removed more copper than zinc per unit of dry mass. The explanation could be the excess intracellular accumulation of copper, the phenomenon of which is discussed in detail below.

Our third hypothesis was that higher proportions of both metals would be accumulated intracellularly at lower concentrations. This hypothesis was confirmed by the results: although it is difficult to draw general trends, it can be said that at lower concentrations (0.2–1.4 mg L^{-1}) higher proportion of metals were intracellular, proportion of extracellularly bound metals increased with increasing initial concentration and with aging the cultures. At the same time, it has to be noted that accumulation of copper into cells was more pronounced than in the case of zinc in both species and almost at every applied concentration. This phenomenon did not support our fourth hypothesis, according to which higher intracellular zinc proportion than copper occurred at the same low (closer to physiological) concentrations. Metal removal activity can be characterized with an initial rapid phase, which is followed by a slower uptake of metal ions into the algal cells (Monteiro *et al*. 2012; Zeraatkar *et al*. 2016). As our results show, these 'classical' characteristics occurred only in the case of *M. pusillum* copper removal between 2.0 and 5.0 mg L^{-1}. All other results suggest that the second phase, the intracellular accumulation may take place within hours, and it may exceed the extracellular adsorption at lower concentrations (0.2–1.4 mg L^{-1}). With the aging of the cultures extracellular adsorption came to the fore, as metal content supposedly distributed into daughter cells. However, higher intracellular proportion of copper than zinc is not easy to explain, since zinc is known to be involved in more physiological processes in algal cells than copper (Xu *et al*. 2012). High copper concentration may manifest as relative zinc depletion due to the highly competitive nature of copper (Blaby-Haas & Merchant 2017), so according to the hypothesis of Waldron *et al*. (2009), copper accumulation may serves to protect zinc-dependent proteins from mismetallation.

In the case of microalgae and cyanobacteria, with some exceptions, the main metal removal process is the extracellular adsorption. Some taxa can accumulate higher amounts of metals possibly due to enhanced detoxification mechanisms (synthesis of phytochelatins and metallothioneins). However, our results show, that accumulation could be relevant also above physiological concentrations, and could take place relatively early in the removal process also in the case of 'adsorbers', that is initial metal concentration and quality of metal can have a significant role on the removal mechanisms, which can also dynamically change through time. Knowledge about the tolerance and removal characteristics of common species could be important for wastewater treatment, planning post treatment retention times or post treatment processes of the used biomass. Our investigations were achieved in complex systems (culture media), which were composed of many components (they may interact with each other) increasing the difficulty of interpretation of the data and understanding the trends, at the same time it can better model the characteristics of wastewaters than using minimal media. Our study shows that different isolates from the same 'average' environment (Botanical Garden Pond of the University of Debrecen) could be characterized with different, but notable tolerance and metal removal capacity. These results suggest that even natural algal inhabitants of an artificial pond could be promising subjects of planned post-treatment strategies aiming the reduction of inorganic micropollutants as a last phase during the process of wastewater treatment.

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