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The toxic metal stress in two mosses of different growth forms under axenic and controlled conditions

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ABSTRACT:

Bryophytes are widely used in monitoring environmental changes and various types of pollution. Features such as longevity and direct intake by the whole body make them very good models for bioindication. However, there is a considerable lack of knowledge on the coping, resistance and tolerance mechanisms to toxic elements within representatives of this second largest group of terrestrial plants. We tested two different growth form mosses, acrocarpous *Atrichum undulatum* and pleurocarpous *Hypnum cupressiforme* in axenic *in vitro* fully controlled laboratory conditions, thus eliminating additional side, antagonistic or synergistic effects. These mosses were exposed to three concentrations (including control) of zinc-, copper- or cadmium-acetate for a short and long period, and their growth, development and biochemical parameters were recorded. The results showed that reactions to stress are both species and metal-specific. Also, the short and long-term exposure times and the level of toxic elements played a role in species response and showed the differences between the two moss growth forms and species. Among the investigated parameters considered in the two mosses tested, the multiplication index proved to be the most useful and sensitive in detecting the metal pollution effects. On the other hand, the concentration of chl *a* seemed to vary over a rather small range between the stressed moss species. Overall, copper was shown to be quite toxic compared to the other two metals at lower applied concentration. However, the studied mosses demonstrated decreased viability according to all of the parameters tested in the media enriched with high concentrations of cadmium when compared with copper and zinc. These results suggest that species studies related to different metals/pollutants in small scale controlled conditions are urgently needed before application in broad areas monitored by mosses.

INTRODUCTION

Metals are typically present in the environment in soluble forms and small amounts, where they contribute to the normal functioning of the biosphere with their concentrations maintained relatively constant over time through geo- and biochemical cycles. Due to anthropogenic influences, the amounts of ordinarily present metals have increased significantly to levels that may be toxic for living organisms, interfering with essential biological processes. Additionally, human activities have introduced new metal species into the environment that are harmful to different life forms even at small concentrations. This has all created the necessity for the continuous studying and monitoring of the presence, toxicity and concentrations of metals in the environment and their influence on ecosystems and living entities (Vogel-Mikuš et al. 2012; Stanković et al. 2018).

Due to their physiological and morphological characteristics, particularly the ability to absorb mineral nutrients and other substances over their entire surface, mosses have been used as one of the most efficient biomonitors and detectors of environmental changes, and of metal pollution in particular, for decades (Zechmeister et al. 2003; Aničić et al. 2009; Boquete et al. 2014; Stanković et al. 2018). Most studies of atmospheric metal pollution rely on pleurocarpous moss species, which are assumed to acquire mineral elements mostly from atmospheric sources (Gerdol et al. 2002; Zechmeister et al. 2003). These mosses are highly branched and form dense carpet-like covers loosely attached to the substratum from which they have a minimal intake of elements, making them a primary choice for use in biomonitoring studies (Izquieta-Rojano et al. 2016). Conversely, acrocarpous mosses are tufted, more attached to the substrates, and generally underrepresented in environmental studies of air pollution (Sabovljević et al. 2020). However, acrocarpous mosses are generally more drought-resistant, and can thrive even in hostile industrial and urban environments. These features make them an essential alternative for metal pollution surveys in areas where pleurocarpous species are scarce or absent (Fabure et al. 2010; Izquieta-Rojano et al. 2016).

Although mosses have been extensively used as biological monitors and bioindicators of air pollution, there is a general lack of research into the effects of metals on moss growth and their physiological parameters (Aboal et al. 2008; Shakya et al. 2008; Varela et al. 2013). Only a few studies conducted under totally controlled laboratory conditions have investigated these effects (Sassmann et al. 2015a, b; Ares et al. 2018; Sabovljević et al. 2018a). That is a critical issue to address, since the changes in moss physiology and growth caused by metal toxicity may alter the accumulation properties of the selected species (Wolterbeek 2002). Furthermore, monitoring moss behaviour or measuring the changes in their morphological and physiological traits, such as changes in chlorophyll and carotenoid content, could offer an alternative approach to environmental studies based on elemental and composition measurements and the levels of accumulated metals in mosses. These also provide information about the metal influence on the vegetation in qualitative (bioindication) and in some cases in quantitative terms (Wolterbeek 2002; Markert et al. 2003; Varela et al. 2013; Mahapatra et al. 2019).

Another critical issue concerning the data about metal pollution directly from the environment based on different moss species and the same species from different places is that they are often not comparable (Onianwa 2001; Bargagli et al. 2002). This is particularly true for those species that differ in growth forms and/or nutrient acquisition mechanisms (Fabure et al. 2010). Some studies have made a direct comparison between pleurocarpous and acrocarpous mosses in terms of metal accumulation capacities (Fabure et al. 2010; Gorelova et al. 2016; Izquieta-Rojano et al. 2016; Sabovljević et al. 2018b), but there are virtually no in vitro (controlled condition) studies which directly investigate the effects of different metals on the species characterized by these two growth forms.

Therefore, we investigated the morphogenetic responses of the two moss species [namely acrocarp *Atrichum undulatum* (Hedw.) P. Beauv. (Polytrichaceae) and pleurocarp *Hypnum cupressiforme* Hedw. (Hypnaceae)] with these distinctive growth forms. They were cultured axenically under controlled laboratory conditions and tested with zinc, copper and cadmium added separately to the solid media in different concentrations. Furthermore, we assessed the selected mosses' physiological responses by measuring the concentration of individual photosynthetic pigments and calculating the chlorophyll *a/b* ratio as an indicator of stress in the selected bryophytes caused by the addition of metal ions. A further goal of the study is to determine whether the observed responses are correlated with metal stress duration and the subsequent growth on media without metal salts achieved by transfer to metal-free media. Finally, we analyzed any possible similarities and differences in the responses of the selected mosses to particular trace metals in broad areas monitored by mosses.

MATERIALS AND METHODS

The selection of moss species. One pleurocarpous moss often used in environmental studies and one acrocarpous moss, both widely distributed, were selected for this study. The comparison of these two moss species, grown in controlled *in vitro* conditions and stressed by selected toxic elements, was obtained by analyzing their morphogenetic and physiological parameters.

Hypnum cupressiforme is one of the most common moss species used for biomonitoring atmospheric metal pollution (Onianwa 2001; Carballeira et al. 2008; Nickel & Schröder 2017; Izquieta-Rojano et al. 2018). Though it has a preference for boreal habitats (Zechmeister 1998),

this epiphytic species is more resistant to desiccation (Lee & Stewart 1971) and pollution (Izquieta-Rojano *et al.* 2018) compared to other pleurocarpous mosses and thus can be found in a broad spectrum of climates and environments (Vujičić *et al.* 2011). Still, its growth is restricted to dry areas, emphasizing the need for an alternative acrocarpous species that can be used for environmental pollution studies in these habitats (Izquieta-Rojano *et al.* 2016). *Atrichum undulatum* is a rather large, highly desiccation-tolerant acrocarpous moss, with a wide geographical distribution (Abderrahman & Smith 1982; Hu *et al.* 2016; Sabovljević *et al.* 2020). Interspecific analysis of 23 bryophytes showed that *A. undulatum* has an accumulation capacity similar to that of the pleurocarpous species routinely used and recommended for biomonitoring studies (Gorelova *et al.* 2016).

The axenic material originated from Avala Mt. and was taken from the pool of established *in vitro* cultures from the Bryophyte Biology Group Belgrade (BBGB) as stated in Sabovljević *et al.* (2006). The newly developed gametophyte shoots were propagated on half-strength solid Murashige & Skoog (MS) medium (Murashige & Skoog 1962), with the addition of 0.70% (w/v) agar and 1.5% (w/v) sucrose to achieve optimal shoot production for further experiments.

An axenic *in vitro* culture of *Hypnum cupressiforme* was taken from the Bryophyte Biology Group Belgrade (BBGB) *in vitro* bryophyte collection. The axenic culture originated from Petnica in Western Serbia and was established by Vujičić *et al.* (2011). The developed gametophyte plantlets were further subcultured in minimal KNOP medium (Reski & Abel 1985), supplemented with 0.70% (w/v) agar and 1.5% (w/v) sucrose until enough biomass was achieved.

All of the gametophores were grown axenically in controlled laboratory conditions before and during the experiments. The medium pH for both species was adjusted to 5.8 prior to sterilization by autoclaving at 121°C for 30 minutes. Before carrying out the experiments with toxic metals, the gametophores were subcultured at a six-week interval in sterile 90mm Petri dishes and kept at 18±2°C temperature, 60-70% ambient moisture, under cool-white fluorescent light with a 16-h photoperiod. In the experiments, 10mm long apical parts of the gametophores were used as experimental explants, and each was planted vertically in such a way that half (ca. 5 mm) was submerged in the medium, and half outside it.

Experimental design. To test the response to particular metals, the explants from the two moss species were grown independently in KNOP media containing zinc-acetate dihydrate $(Zn(CH_3CO_2)_2(H_2O)_2)$, copper-acetate $(Cu(CH_3COO)_2))$, or cadmium-acetate dihydrate $(Cd(CH_3CO_2)_2(H_2O)_2)$ at concentrations of 200 or 700 μM respectively. The plants grown in KNOP medium without the selected metal salts were used as the control groups.

Among the selected metals, copper and zinc are essential micronutrients for plants i.e. bryophytes. However, both can be highly toxic at elevated concentrations through different modes of action due to their different chemical properties (zinc is a redox-inert metal, while copper is redox-active). Conversely, cadmium is chemically similar to zinc, but is considered non-essential and toxic once it appears in the environment even at low concentrations, often inducing cell damage by displacing other chemically similar essential elements. The concentrations chosen here were sublethal and adopted from earlier studies (e.g. Sabovljević *et al.* 2018a, 2020) with the aim of maintaining comparability with previous results. In terms of exposure time, two types of separate experiments were performed with short and long exposure.

Type I (short exposure) experiments: explants from each moss species were cultured for five days in solid KNOP media enriched with 200 or 700 μM Zn-, Cu-, or Cd-acetate respectively. Subsequently, the plants were transferred to Petri dishes containing KNOP free of the selected metal media to grow for up to five weeks in total. Plantlets from the control groups were also subcultured after five days in a fresh KNOP medium but they were not exposed to any additional metal salt in the medium during the five week period.

Type II (long exposure) experiments: explants from each moss species were grown for five weeks continuously in solid KNOP media enriched with 200 or 700 μM Zn-, Cu-, or Cd-acetate, with no transfer to a fresh metal-free medium.

Since the study was 3 (3 different metal types: zinc, copper and cadmium) × 3 (3 different metal concentrations, namely 0, 200 and 700 μM) × 2 (2 moss species: *A. undulatum* and *H. cupressiforme*) × 2 (2 exposure durations: type I and type II) factorial design, for each combination of the factor levels (36 in total), four Petri dishes containing 12 explants were set as replicates (n=48).

Morphogenetic and physiological parameters. The morphogenetic response of both species in each treatment group was characterized by the index of multiplication (IM), calculated as the number of newly formed shoots per initial explant. The appearance of protonema and the survival of the plants were also observed. Pigment extraction was achieved using 20 mg of plant material from each treatment group in 2 ml of 95% ethanol for 10 minutes at 60°C in the dark. The absorbance levels of the ethanol extracts were measured using a UV–vis Spectrophotometer (HP/Agilent 8453 Spektrofotometer, Agilent technologies, Paolo Alto, CA) at three wavelengths: 470 nm, 648 nm, and 664 nm. The contents of individual chlorophylls *a* and *b* and carotenoids were calculated according to the equations given in Lichtenthaler (1987) using specific absorption coefficients for 95% ethanol as the pigment extraction solution. For the pigment analyses, all of the measurements were run in triplicate.

Data analyses. We used IBM SPSS Statistics (Version 26) predictive analytics software for all of the data analyses. All of the groups were tested for normal distribution using Shapiro-Wilk's test and normal quantile-quantile plots of residuals and data were checked for homogeneity of variance by Levene's test. In order to to assess the effects of the selected factors on the index of multiplication, the contents of individual photosynthetic pigments and the chlorophyll *a/b* ratio of the mosses, we used two-way ANOVAs, followed by Tukey's *post hoc* test for the main effects. Where significant interaction effects were detected, the simple main effects were tested. Different statistical significances were marked as: *** for $p < 0.001$, ** for $p < 0.01$ and * for $p < 0.05$. Pearson's correlation coefficient was used to characterize the relationship between the individual pigment contents. For this, statistical significance was accepted at the 0.01 level (two-tailed).

RESULTS

All of the plants survived the experiments, but depending on the treatment, they showed different visual symptoms, such as chlorosis in the case of moss grown on cadmium containing media, or browning of the gametophores in the case of copper exposure (Fig. 1). Neither of the two moss species showed any protonemal growth (sensu measurable protonemal diameter) in response to the different treatments.

Index of multiplication. Overall, *H. cupressiforme* had a higher index of multiplication than A. *undulatum* (Table 1) since it produced more lateral branches i.e. new shoots. The difference in IMs between the control groups of the two species was statistically significant, and remained similar when different concentrations of toxic metals were added to the media (Fig. 2A). The smallest difference between the IMs of *A. undulatum* and *H. cupressiforme* existed for the metal concentration of 700 μM (1.08), while the greatest difference was at 200 μM (1.46), since the IM pattern in *A. undulatum* remained similar in both of the toxic metal concentrations applied, and continuously decreased in *H. cupressiforme* (Fig. 2A).

No significant differences between the effects of the two time periods on the index of multiplication were observed in the tested mosses (Table 1). However, depending on the moss species, different periods of metal exposure had different effects on the IMs (Fig. 2B). *Hypnum cupressiforme* plants grown for five weeks on media containing toxic metal salts had significantly lower indices of multiplication than those grown for just five days. In *A. undulatum* the opposite trend was observed, although the difference between the effects of the two different periods of metal exposure in this moss was not statistically significant.

Hypnum cupressiforme and *A. undulatum* also responded differently to the type of metals in the growth media, with differences in the IM being statistically signif-

icant for the three trace metals (Fig. 2C). *Hypnum cupressiforme* shoots grown on the zinc-containing media had the highest index of multiplication compared to the other metals applied, while those cultured on the media with cadmium-acetate exhibited the lowest rate of multiplication. Interestingly, *A. undulatum* had the highest IM on the cadmium-enriched media, compared to copper which showed an inhibitory effect in this species as inferred by new shoot production. The most significant difference in the IMs between the two mosses were recorded when the plants were treated with copper (1.6) while the smallest difference was observed in the case of cadmium-acetate (0.53), which suggests a certain tolerance of *A. undulatum* to cadmium, and high intolerance to copper.

The increase of metal concentration from 200 μM to 700 μM had a significantly greater decreasing impact on the IM in the experiments with long-term exposure when compared to the type I treatments (Fig. 2D).

The interaction between the type of exposure time and the type of metal in the growth media also had a significant effect on the IM of the mosses (Fig. 2E). In the type I experiments, the mosses treated with copper had the highest index of multiplication which differed significantly from the IM of the cadmium treated mosses. Conversely, the moss plantlets from the type II experiments which grew on the copper-containing media had the lowest IM, and this was significantly lower than the IMs of both the zinc and cadmium treated mosses, thus suggesting that copper toxicity increases with exposure time more than the other two toxic metals applied in both of the tested species.

The effects of a single toxic metal on the IM depended on the concentration applied (Fig. 2F). At 200 μM, the indices of multiplication in both of the mosses grown in copper and cadmium enriched media were almost identical, while those from the zinc-containing media had significantly higher IM values. However, at 700 μM of metal acetate, the difference between the indices of the copper and cadmium treated mosses was highly significant. The cadmium treated mosses showed the lowest IM values.

Concentration of chlorophyll *a* (chl *a*) and chlorophyll *b* (chl *b*). There was a significant difference between the chlorophyll *a* concentrations of the two species. *Atrichum undulatum* had a much higher amount of chl *a* than *H. cupressiforme* (Table 1). However, there were no significant interactions between the moss species and any other investigated factor in the study (the metals applied, the concentration of the metals applied and metal exposure time). The chl *a* concentrations in both mosses changed proportionally in response to the varying experimental conditions.

The differences in the effects of the different metal-acetates on the chl *a* content of the mosses were significant. Furthermore, a significant interaction between the metal type and the concentration of the metals applied in the media was also noted (Fig. 3A). When 200 μM zinc- and

Table 1. Summary results of the two-way ANOVAs based on 48 replicates for each experiment, for the main and interaction effects with 4 factors: moss species, metal concentration (Zn, Cu, Cd), metal (Zn, Cu, Cd) and exposure time (long/short) as the independent variables and the IM and photosynthetic pigment contents and the chlorophyll *a/b* ratio as the dependent variables (mean values ± standard deviation). The mean values followed by different letters in the column are significantly different (Tukey's test, P ≤ 0.05). Different statistical significance for the interaction effects were marked as: ***: p < 0.001, **: p < 0.01 and *: < 0.05.

Factors	Factor levels	IM	Chl *a* (mg/g)	Chl *b* (mg/g)	Chl *a/b*	Carotenoids (mg/g)
Moss species (A)	*A. undulatum*	0.49±0.85 a	6.28±2.02 a	3.21±1.23 a	2.03±0.33 a	2.30±0.64 a
	H. cupressiforme	1.72±1.38 b	4.68±1.64 b	2.88±1.05 a	1.63±0.19 b	1.95±0.70 b
Metal conc. (µmol) (B)	0	1.72±1.36 a	6.52±1.27 b	3.55±0.90 a	1.88±0.25 a	2.43±0.48 a
	200	0.95±1.18 b	5.82±1.83 b	3.26±0.98 a	1.79±0.26 a	2.19±0.75 a
	700	0.66±1.11 c	4.09±2.02 a	2.31±1.18 b	1.82±0.46 a	1.75±0.66 b
Metal	Zn	1.32±1.38 a	6.68±1.00 a	3.88±0.67 a	1.74±0.23 a	2,54±0.47 a
	Cu	1.04±1.30 b	5.04±1.91 b	2.77±1.12 b	1.84±0.26 a	1.88±0.73 b
(C)	Cd	0.97±1.18 b	4.71±2.30 b	2.48±1.10 b	1.91±0.46 a	1.95±0.67 b
Exposure (D)	Type I	1.15±1.23 a	5.20±1.70 a	2.70±0.91 a	1.97±0.37 a	1.96±0.53 a
	Type II	1.07±1.36 a	5.75±2.24 a	3.38±1.27 b	1.70±0.24 b	2,29±0.79 b
Mean		1.11±1.30	5.48±2.00	3.04±1.15	1.83±0.33	2.12±0.69
A		***	***	NS	***	**
B		***	***	***	NS	***
C		***	***	***	NS	***
D		NS	NS	**	***	**
A × B		**	NS	NS	*	NS
A × C		***	NS	**	***	**
A × D		**	NS	*	***	**
B × C		***	***	***	NS	***
B × D		**	NS	NS	*	NS
C × D		***	NS	NS	NS	NS

cadmium-acetate were applied, no significant decrease in the chl *a* content was observed in the mosses. Conversely, copper led to a substantial reduction of chl *a*. At 700 μM a significant reduction in chl *a* was detected in the case of the cadmium treated plants, while this was not evident for those grown in Zn- and Cu-enriched media. The exposure time had no significant effect on the amount of chl *a* in the mosses. These results are in accordance with the indices of multiplication shown in Fig. 2.

In contrast to chl *a*, there was no significant difference in the amounts of chl b between *A. undulatum* and *H. cupressiforme* (Table 1). Similarly to chl *a*, the chl *b* content of the two mosses was significantly affected by the interaction effects of the type of metal and the concentration in

the media (Fig. 3B). At 200 μM, Cu-acetate led to a significant drop in chl *b* content, while 200 μM cadmium- and zinc-acetate failed to have a significant effect. When these metals were added to the media as 700 μM acetates, the chl *b* concentration decreased considerably more in the mosses cultured in media enriched with cadmium-acetate than in those containing the other two metals. There were no significant interaction effects between the moss species and the concentration of metals in the media on the chl b content.

However, significant interaction effects were observed between the moss species and the type of metal applied in the media on the amount of chl *b*. When cultured in the copper enriched media, the amount of chl *b* decreased
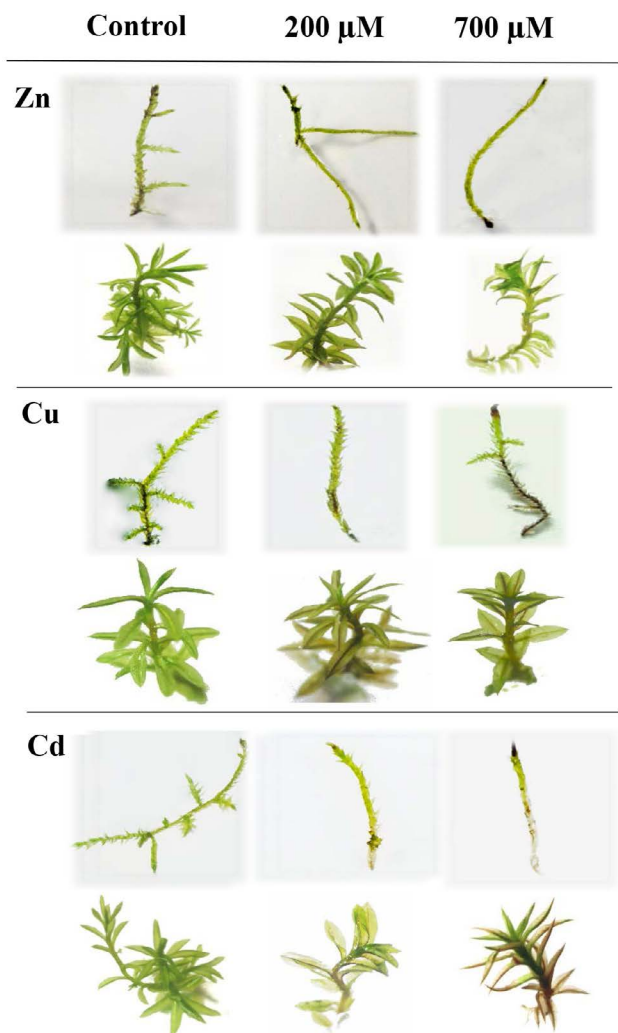
Fig. 1. Representatives of morphotypes of *Atrichum undulatum* and *Hypnum cupressiforme* developed within five weeks on basal KNOP media containing additional 0, 200 or 700 µM metal-acetetes. *Hypnum cupressiforme* is shown in the upper lines and *A. undulatum* in the lower lines for each of the metal-acetate treatments (Zn, Cu, Cd).

considerably more in *H. cupressiforme* than in *A. undulatum* (Fig. 3C). Conversely, the amounts of chl *b* in the two mosses were almost the same when they were treated with any of the other two metal-acetates.

The concentration of chl *b* in *A. undulatum* and *H. cupressiforme* reacted differently in response to the different exposure times (Fig. 3D). Long-term exposure to the metal-acetates had a more pronounced impact on the chl *b* content of *H. cupressiforme* than on that of *A. undulatum*. Conversely, these differences between the two species were not evident in the case of the short-term experiments.

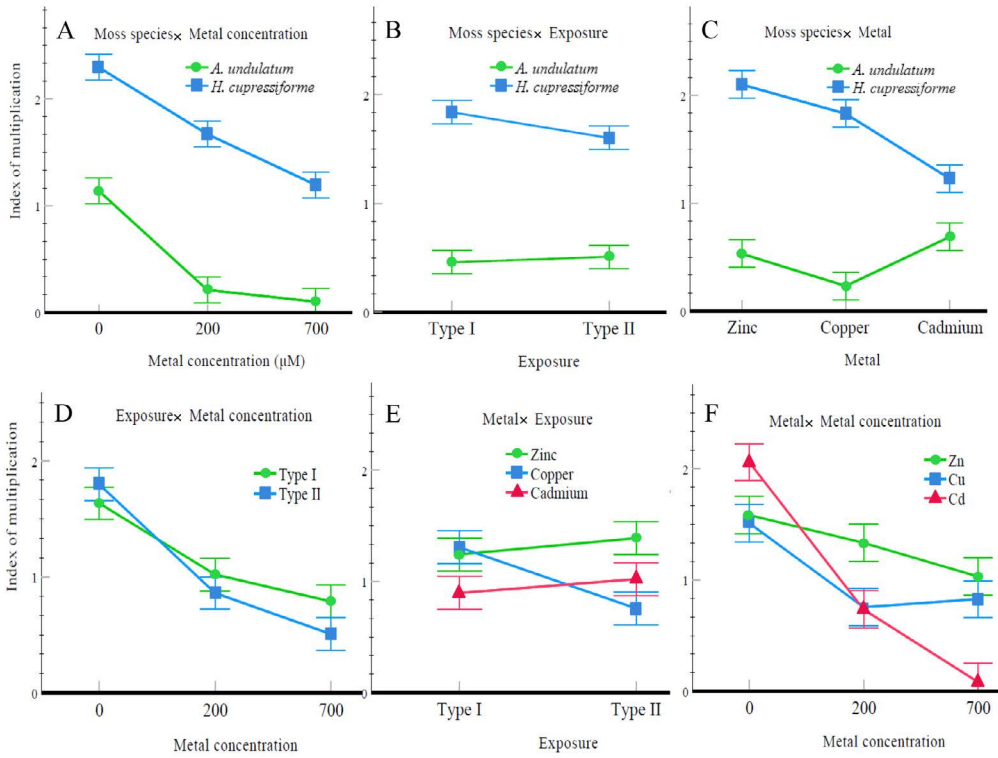**Chlorophyll *a/b* ratio (chl *a/b*).** *Atrichum undulatum* had a higher chl *a/b* ratio than *H. cupressiforme* (Table 1). The effect of metal type on the chl *a/b* ratio of the two mosses was generally not significant. However, the individual chl *a/b* ratios of *A. undulatum* and *H. cupressiforme* differed significantly depending on the type of metal acetate applied (Fig. 4A). The most significant difference between the two species was observed when the plants were treated with cadmium-acetate (0.65), while the smallest was in the case of copper-acetate (0.18).

The type of exposure time also showed a significant interaction with the moss species (Fig. 4B). In the short-term exposure experiments, the disparity between the chl *a/b* ratios of the two species was much higher (0.58) when compared to the long-term exposure experiments (0.23). In response to growing concentrations of metals in the media, the chlorophyll *a/b* ratio of the mosses did not change significantly, and there were no significant interaction effects between the moss species and the metal concentration either. However, the type of exposure time changed the chl *a/b* ratio as the mosses responded to different concentrations of metals (Fig. 4C). When the mosses were cultured in the metal-free media or that containing 200 µM metal-acetates, there were no significant differences between the chl *a/b* ratios. However, when 700 µM metal-acetates were applied, the chl *a/b* ratios differed significantly in the two different exposure times.

Carotenoids. The effects of different trace metals on the carotenoid content of the mosses depended on the concentration and presence of these metals in the media (Fig. 4D). The plants cultured on the media containing 200 µM zinc- or cadmium-acetate had almost the same quantity of carotenoids as those grown on the media without acetates. The same concentration of copper resulted in a considerable reduction of carotenoid content in the mosses. However, there was no further reduction in the carotenoid level when the mosses were grown in media enriched with 700 µM copper-acetate. At the same concentration level, a significant decrease in the value of this parameter was observed in the cadmium treated plants, and only a slight drop in the medium enriched with zinc.

Toxic metals also had different effects on the concentration of carotenoids depending on the moss species (Fig. 4E). When the mosses were cultured in the zinc-containing media, there was no significant difference in the carotenoid content in either of the two mosses. Conversely, in the case of the copper-enriched media, *A. undulatum* had much higher amounts of carotenoids than *H. cupressiforme,* with the difference being 0.66, while for the cadmium treated mosses this difference was 0.57, thus indicating the differences between the two moss species in terms of copper tolerance.

Considering the exposure times, the difference in the carotenoid contents of the two mosses was not significant when the plantlets were cultured for five days in the metal-containing media (Fig. 4F). However, long-term exposure to toxic metals had a more significant effect on the

Fig. 2. The index of multiplication (IM) of *Atrichum undulatum* and *Hypnum cupressiforme* under different treatments based on 48 replicates. Estimated mean ± 95% confidence intervals are represented.
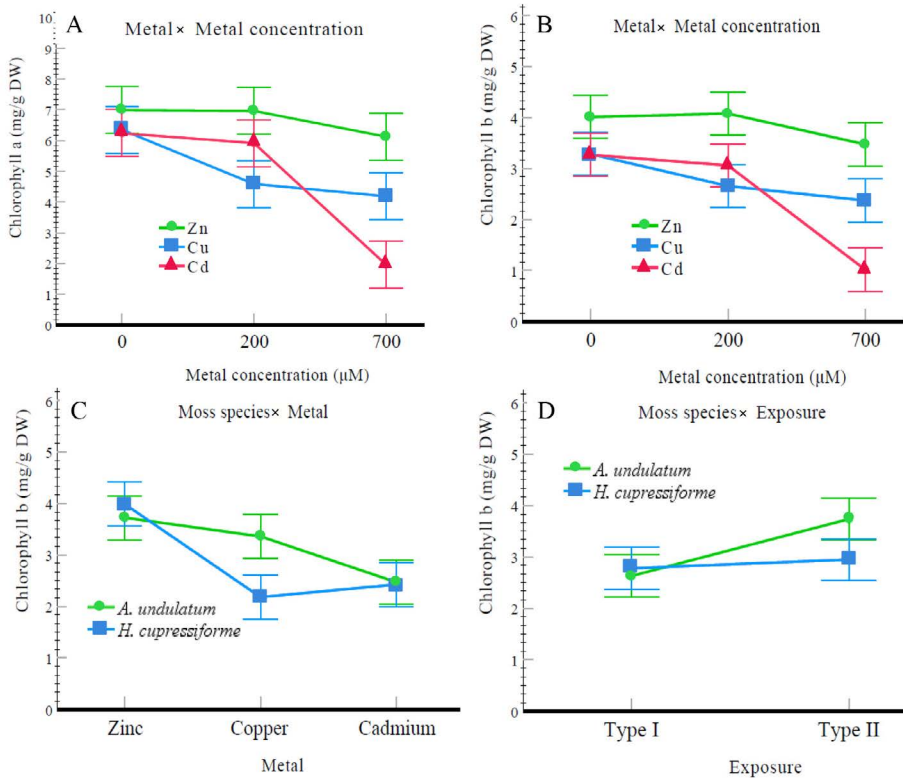
Fig. 3. The content of chlorophyll *a* (A), and chlorophyl *b* (B, C, D) of *Atrichum undulatum* and *Hypnum cupressiforme* under different stresses based on 48 replicates. Estimated mean ± 95% confidence intervals are represented.

concentration of carotenoids in *H. cupressiforme* than on that of *A. undulatum*, suggesting the increased resistance of *A. undulatum* to prolonged exposure to certain metals.

Correlation between pigments. Pearson's correlation coefficients were calculated for the studied pigments, and strong positive associations were detected between the amounts of the three pigments measured. The strongest positive correlation was observed between the contents of chl *a* and chl *b* (r = 0.922, p < 0.001), followed by that between the concentrations of chl *a* and carotenoids (r = 0.904, p < 0.001), with the correlation between the amounts of chl *b* and carotenoids being the weakest among the three, but still indicating a strong association (r = 0.894, p < 0.001).

DISCUSSION

Morphogenesis. Two growth forms (pleurocarp and acrocarp) of the perennial mosses tested in this study showed differences in coping with the three tested toxic metals in controlled conditions. In this study, all side effects including synergistic and antagonistic outcomes were excluded to prove the differences in metal toxicity survival by testing them in completely controlled conditions free of xenic cohabiting organisms and abiotic factor variation.

For the mosses grown for five-weeks on the solid media enriched with elevated concentrations of toxic metals, tolerance to metal toxicity can be considered as the ability of the mosses to survive and produce new shoots or protonema, sensu SASSMANN *et al.* (2015a). In all 36 treatment groups in our study, the survival rate of the plants was maximal (100%). However, damage was observed such as varying degrees of chlorosis in the mosses treated with 700 µM cadmium-acetate, depending on the toxic metal exposure time and moss species. The stressor effects on the growth of mosses in controlled laboratory conditions induce a shift from gametophore to protonema production (SASSMANN *et al.* 2010, 2015a; ĆOSIĆ *et al.* 2020a, b) but not necessarily (SABOVLJEVIĆ *et al.* 2018a). In this study, neither of the two tested species exhibited (visible) protonema growth after exposure to the different metal-acetates. The reason for this could be a lower effective concentration. In fact, toxic metal ions can precipitate due to the presence of phosphate anions in the nutrient media (SASSMANN *et al.* 2015a). The same authors mentioned that the toxicity of tested metals in moss species should also be regarded in view of the anions present in the growth medium. However, they did not consider the effects of the different salt anions applied in their studies, which can also act as a factor in the transition from gametophores to protonema and vice versa. In our study, it is assumed that this was not the case since we applied the toxic metal salts exclusively linked to the acetate anion, with the aim of offering an anion that is considered to have no physiological side effects and is known to be harmless

to plants i.e. mosses. The only side effects could be changes in pH, but only in cases of higher concentrations, which is outside the scope of this study.

On the other hand, acetylation of the homogalacturonan fractions could decrease the effective toxic metal ions in the media due to the lower affinity of the cell walls for cations in the tested species (KRZESŁOWSKA 2011). There is no evidence of this in the two species tested in this study. Since we applied different metals, and the patterns of the absence of visible protonema remained the same in all 36 treatments, it can be inferred that there are also other mechanisms to define the gametophore-secondary protonema development in the two species tested in the given controlled conditions.

The significant adverse effects of the metals on the shoot production in *A. undulatum* and *H. cupressiforme* in various treatments indicated that the amounts of toxic metal salts applied contained enough cations to reach the moss cells and express changes in the morpho-genetic development of the mentioned moss accessions.

However, this could also be related to the ecologically different (i.e. growth form, life strategy) and unrelated species tested by us and other authors previously. Contrary to the conclusion of SASSMANN *et al.* (2015a), based on their results and those from studies on native mosses, that the favoured growth of protonema under trace metal stress is a universal characteristic of bryophytes, we propose that this could be a species-specific feature, or that other environmental factors also affect this phenomenon. In our study, the controlled moss plantlets grown on the medium free of additional metals did not produce any visible protonemata during the experiments either, and we thus consider the absence of visible protonema in all the treatments the result of other conditions rather than the toxic metal tested.

In terms of shoot production, *A. undulatum* was more sensitive to the presence of the toxic metals than *H. cupressiforme*, exhibiting most of the reduction in the shoot production at 200 µM, with no significant further decrease at higher metal concentrations. The final effects seen in *A. undulatum* already occurred at short-term exposure. On the contrary, in *H. cupressiforme,* an increase in both the concentration and exposure time further suppressed the shoot multiplication.

The study on four moss species including *H. cupressiforme* in non-axenic *ex situ* conditions, showed that a concentration limit of metal adsorption existed in all of the investigated mosses, which is species-specific and depends on the metal type (GONZÁLEZ & POKROVSKY 2014). An *in vitro* study carried out by SASSMANN *et al.* (2015b) also found that the moss *P. patens* plantlets which grew on solid metal-containing media prevented further intake of zinc and copper after their specific intracellular concentrations had been achieved. The results from the same study showed that the amounts of zinc and copper inside the tissues have highly significant negative correlations with the growth of the moss gametophors. This is in accord-
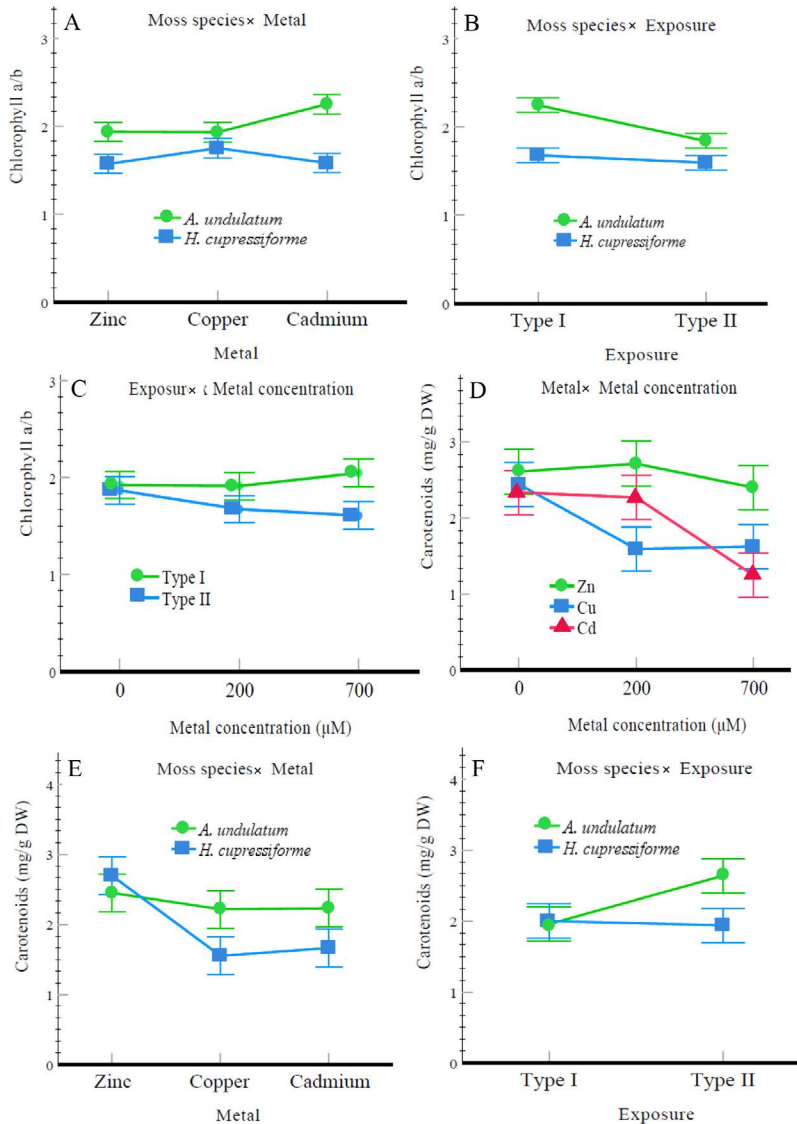
Fig. 4. The chlorophyll *a/b* ratio (A, B, C), and the carotenoid content (D, E, F) of *Atrichum undulatum* and *Hypnum cupressiforme* under different treatments based on 48 replicates. Estimated mean ± 95% confidence intervals are represented

ance with the results achieved here, making it easy to infer that in the case of *A. undulatum*, rapid saturation of the metal-binding sites in the moss tissues is achieved within the first five days of exposure. Similar effects were achieved in this species with the lower concentration of metals, as shown by the IM values. Such an effect with zinc treatment in this species was also confirmed by Sabovljević *et al.* (2018b).

However, the concentration limit inside the cells alone [sensu González & Pokrovsky (2014) and Sassmann *et al.* (2015b)] does not explain the further decrease in the shoot production of *H. cupressiforme* observed with the prolonged exposure period to the toxic metals. Most moss species are ectohydric and water and dissolved minerals are absorbed over the entire moss surface covered with a thin water solution film of the available substances from the near-environment. Subsequently transport is diffused

through external capillary spaces and the poorly differentiated stem cortex (Proctor 1982; Huttunen *et al.* 2018). The gametophytes of *H. cupressiforme*, a hypnacean pleurocarpous moss, rely mostly on these ectohydric mechanisms of water conduction, while in polytrichaceaous *A. undulatum* the water and mineral transport is additionally supported by the presence of a hydroid structure within the stem. Thus, *A. undulatum,* which has ectohydric, endohydric and cell to cell transport, is expected to conduct the water and metals dissolved in it more efficiently from the environment to the upper parts of the moss-plant than *H. cupressiforme,* where all the transport is mainly ectohydric and/or cell to cell. This is related to the faster saturation of the available cation binding sites. Sidhu & Brown (1996) showed that in *Rhytidiadelphus squarrosus* (Hedw.) Warnst. (Hylocomiaceae) when only the basal parts of the gametophytes were exposed to metal-containing media,

there was a linear relationship between the growth of the plants and their exposure to metals which is comparable with the response of *H. cupressiforme,* which in our test was standing upright. Alternatively, when the entire gametophors of *R. squarrosus* were exposed to trace metals in water solution for 20 minutes, they reached a minimal length increment by the fifth day probably due to rapid whole surface water solution saturation, without recording any further growth until the end of the experiment. In our study, the reaction of *A. undulatum* is assumed to express a similar pattern mainly due to the additional support to water solution conductivity provided by the presence of endohydry within the moss body absent in *H. cupressiforme*. It should be taken into account that the mosses tested here thrived for five weeks in constant and full hydration conditions.

The indices of multiplication of the two mosses decreased proportionally in response to zinc and copper, but *A. undulatum* showed significantly higher resistance to cadmium toxicity than *H. cupressiforme*. Cadmium is considered a non-essential element in plant physiology, and at high concentrations, it is usually more toxic than zinc or copper, which are physiologically essential micronutrients with well-governed homeostasis in plants (Küpper & Andersen 2016). Overall, the highest toxicity of cadmium at higher concentrations was also confirmed in this study. In line with our earlier conclusions on the tested species, the higher cation binding capacity of *H. cupressiforme* may have made it more susceptible to the toxic effects of cadmium. Additionally, *A. undulatum* may possess more efficient detoxifying mechanisms than *H. cupressiforme* when exposed to excess cadmium or it simply does not bind as was shown for this species and zinc (Sabovljević et al. 2018b). Parrotta et al. (2015) stated that even closely related plant species respond differently to Cd-exposure and that different coping mechanisms may exist. In mosses, trace metals are mostly bound to the anionic exchange sites on the cell walls, and this is one of the most important sites of metal accumulation and detoxification in these bryophytes (Basile et al. 2012; Parrotta et al. 2015). The specialisation towards endohydry of *A. undulatum* includes the formation of thick three-layered longitudinal walls of the hydroids, which during maturation lose living protoplast and plasmodesmata contact with the adjacent cortical cells. These walls also become encrusted with polyphenolic substances similar to lignin, favouring vertical over lateral water transport to some extent (Scheirer 1980; Ligrone et al. 2000; Glime 2017). Lavid et al. (2001) found that the accumulation of cadmium ions in two water vascular plants was predominantly in cells containing high levels of polyphenols, and that the species with constitutive levels of these substances was more tolerant to cadmium stress. Thus, it is possible that preferable binding to the longitudinal walls of the hydroids, which make the initial contact with metals, protects the other stem cells of *A. undulatum* from high cadmium concen-

trations. Conversely, transport across the external surfaces of *H. cupressiforme* would lead to a higher accumulation of this metal, affecting the formation of new shoots more than in *A. undulatum*, as shoot initiation starts in the outer layers of moss gametophyte leaf peats (Coudert et al. 2015).

The results of the *in vitro* study of Sabovljević et al. (2018b) for zinc binding sites in different bryophytes showed that the surface of *A. undulatum* contained almost no bound zinc ions. The same authors demonstrated *H. cupressiforme* to be more susceptible to zinc as inferred by the intensive labeling with a zinc-specific dye of all the outer surfaces after the mosses had been exposed to zinc, also supported by the results obtained in this study.

According to Tyler (1990), in terms of the relative toxicity of divalent toxic metals to mosses, copper was the most toxic, followed by cadmium, with zinc being the least deleterious. However, our results show that factors such as moss species, exposure time to toxic metals, and the applied concentration significantly modify the observed plant response and order of toxicity among these three metals. In accordance with our results pertaining to moss growth, Sidhu & Brown (1996) also found that the type of metal applied exhibited significant interaction effects with the concentration and mode of moss exposure to metals. Also, the synergistic and antagonistic effects should not be omitted in the search for moss-metal relations. Thus, the fully controlled conditions provide a solid basis to avoid further speculations of *in situ* and *ex situ* measurements and comparisons from distant species, populations or even genotypes in a changing environment affected by both biotic and abiotic factors.

Physiological parameters. Disruption of the photosynthetic apparatus is one of the most common effects seen in plants as a result of metal toxicity. Thus, monitoring the amounts of photosynthetic pigments could be a practical method for the assessment of metal effects in plants and their optimal function (Saxena & Saiful-Arfeen 2009; Houri et al. 2020). The chlorophyll contents of the mosses grown in media containing 200 µM Zn- or Cd-acetates did not change significantly, suggesting that the metal concentrations in the mosses were not sufficient to affect the chlorophyll levels. The similarities in the physical and chemical characteristics, the shared uptake and the transport mechanisms of zinc and cadmium could account for the similar effects on the chlorophyll contents seen in mosses treated with these two metals at lower concentrations (Das et al. 1997; Küpper & Andresen 2016). Besides, these two metals both behave as redox-inert ions, and eventually cause structural changes in/on moss cells. In the same concentration, copper significantly decreased the amounts of both chlorophylls in both of the tested species. All three toxic metals could affect the chlorophyll content through the substitution of Mg^{2+} inside the molecule of the chlorophyll, changing its stability, disrupting the association

with the axial ligands and consequently leading to the loss of the whole photosystem (Küpper *et al.* 2002; Küpper & Andresen 2016). Also, these metals can interfere with the other elements required for the biosynthesis of photosynthetic pigments (Zengin & Kirbag 2007). Küpper *et al.* (1996) showed that metal affinity to bind within the centre of the chlorophyll molecule reflects its toxicity to the photosynthetic system, with copper having the strongest tendency for Mg^{2+} substitution, followed by cadmium and finally zinc in vascular plants and green algae. Thus, the strong affinity of Cu^{2+} for Mg^{2+} substitution, even at low concentrations, could account for the differences seen among the metals and between the species in our study, when applied as 200 µM metal-acetate treatments. Furthermore, copper is a redox-active ion which in addition to the mechanisms described above can also lead to the direct production of reactive oxygen species (ROS) which affect the photosynthetic system and cause the subsequent peroxidative breakdown of pigments (Zengin & Kirbag 2007; Küpper & Andresen 2016).

In our study, the highest concentration of zinc- and copper-acetates (700 µM) led to only moderate changes in the amounts of chlorophylls compared to those observed at 200 µM. However, when applied as 700 µM acetate in the media, cadmium influenced the concentration of chlorophylls in these two mosses significantly more when compared to the other two metals. The similar direction and magnitude of the effects of zinc and copper at 700 µM suggest the existence of strong and precise homeostatic mechanisms since the chlorophyll contents were relatively stable in both of the tested species even when the concentration of the metals in the media increased. More than 1200 proteins in plants require the presence of zinc for essential functional and structural roles, including transcriptional factors, thus emphasizing the importance of the tight regulation of zinc concentration inside plant cells (Andresen *et al.* 2018). Copper is a transitional metal and essential for major physiological processes, such as photosynthesis and mitochondrial respiration, cell wall synthesis and detoxification of the reactive oxygen species (Hänsch & Mendel 2009; Andresen *et al.* 2018). In the plant photosynthetic apparatus, copper localized inside chloroplasts represents up to 30% of the total copper found in cells (Aguirre & Pilon 2006). The redox-active nature of this element allows it to interact with a wide range of molecules, making it the toxic metal with the most deleterious potential for cells. Plants have evolved strong control strategies for copper homeostasis which are tissue and ontogenesis specific in order to balance its essential functions with its toxicity (Printz *et al.* 2016). It is also evolutionarily conserved among green algae, mosses and tracheophytes (Burkhead *et al.* 2009). The homeostatic intracellular mechanisms of zinc and copper metals in plants (including mosses) may involve the exclusion of metals by predominate binding to the cell walls as the primary strategy (Lang & Wernitznig 2011; Basile *et al.* 2012; Parrotta *et al.* 2015),

the chelation inside the cytosol and vacuolar sequestration(Clemens 2001; Schmidt *et al.* 2020) or the regulated expression of specific metal transporters under high availability of trace metals in the environment (Burkhead *et al.* 2009; Küpper & Andresen 2016). However, the relative importance of individual control mechanisms in *H. cupressiforme* and *A. undulatum* is yet to be determined. Benavides *et al.* (2005) suggested it can vary greatly and depends on the concentration of metals and the metal exposure time as well as the species.

Generally in plants, low concentrations of cadmium are usually non-toxic. However, it can be imported into the cells due to the chemical similarity to other essential cations and thus easily reach toxic levels. Import mechanisms could be linked to various transporters and channels of other divalent cations (Muradoglu *et al.* 2015; Küpper & Andresen 2016; Houri *et al.* 2020). The much higher intracellular concentration of cadmium compared to essential elements could additionally be achieved by the direct cadmium-induced damage of membranes and the loss of their selective permeability, as shown in the studies carried out by Ares *et al.* (2018) on liverwort *Marchantia polymorpha* L. (Marchantiaceae) and Basile *et al.* (2012) on moss *Scorpiurum circinatum* (Brid.) Fleisch & Loeske (Hypnaceae). Consequently, excess cadmium inside chloroplasts may induce oxidative stress by non-functional binding to different molecules, intensifying the mis-transfer of electrons to oxygen or inhibiting the functioning of the components of the antioxidative system leading to the peroxidative breakdown of pigments (di Toppi & Gabrielli 1999; Küpper & Andresen 2016; Bellini *et al.* 2020). In both of the species tested in this study, cadmium significantly decreased the amounts of chlorophylls recorded when applied at 700 µM.

The different effects of copper and cadmium on the amounts of chlorophylls, depending on the applied concentration in the media, could additionally be explained by the different interaction of the two metals with the antioxidative system inside chloroplasts, particularly with the enzyme Cu/Zn superoxide dismutase 2 (Cu/ZnSOD2). This isoform is only present in chloroplasts of higher plants including mosses (Dreyer & Schippers 2019) and its expression and function are dependent on the copper availability. When plants suffer oxidative stress due to disruption of the photosynthetic apparatus, the expression of this enzyme is significantly up-regulated (Andersen *et al.* 2018). Furthermore, elevated levels of copper in the environment also favour the expression of Cu/ZnSOD2 rather than Fe-SOD in chloroplasts (Pilon *et al.* 2011; Andersen *et al.* 2018). Thus, when the concentration of copper in the chloroplast is elevated, Cu/ZnSOD2 may protect photosynthetic pigments by direct binding of free copper as well as by eliminating the toxic superoxide radicals generated at the sites of photosynthesis. Alternatively, the binding of cadmium to this enzyme, after its induction by oxidative stress, would lead to the substitution of Zn^{2+} in

Cu/Zn-SOD2 due to chemical similarity, the alternation of the enzyme structure and ultimately to its degradation, accounting for much more pronounced effects of cadmium on the chlorophyll contents applied as 700 μM. The presence of chloroplastic Cu/ZnSOD is not ubiquitous in all mosses (Pilon *et al.* 2011; Dreyer & Schippers 2019). In both of the moss species tested here, cadmium led to the significant loss of chl *b*, reaching almost the same final concentration in the two species. However, in *A. undulatum*, the content of this pigment was only slightly affected by the presence of copper, while *H. cupressiforme* suffered a significant loss, similar in magnitude to that seen under cadmium stress. These results possibly indicate the absence of Cu/Zn-SOD2 in *H. cupressiforme*, while in *A. undulatum* it can be induced by excess copper in the media.

Regarding the amounts of chl *a* and *b*, the results obtained are in accordance with other studies of the metal effects on different moss species. Chen *et al.* (2015) reported that zinc was accumulated in much lower quantities in two mosses, *Eurhynchium eustegium* (Besch.) Dix. and *Taxiphyllum taxirameum* (Mitt.) Flesich, compared to copper and cadmium, despite the increasing concentrations of metals in the environment. At the same time, they found that zinc and copper diminished the content of chlorophylls to a far lesser extent than cadmium at the same concentrations present in the substrates. Shakya *et al.* (2008) also reported a significant decrease in both chl *a* and *b* content in two mosses, *Thuidium delicatulum* (L.) Mitt. and *T. sparsifolium* (Mitt.) Jaeg. after exposure to solutions of different copper concentrations (10^{-10} to 10^{-2}M). As demonstrated by the results obtained in this study, the differences in chlorophyll content were higher among mosses from the control and treated plantlets compared to those among the treatments themselves. Shakya *et al.* (2008) also found that elevated zinc levels had no significant effect on the amounts of the two chlorophylls compared to the control. Tremper *et al.* (2004) used much lower concentrations compared to those in our experiments and found that the highest concentration of approximately 10 μM of copper in the solution led to a significant drop in the concentration of both individual chlorophylls in the moss *R. squarrosus*, while zinc had no significant effect. Saxena & Saiful-Arfeen (2009) showed that the lowest applied concentration of cadmium in their study (0.01 M) even had stimulatory effects on the amounts of chl *a* and *b* in the moss *Racomitrium crispulum*(Hook. f. et Wils.) Hook. f. et Wils., while higher concentrations decreased the content of chl *a*, as shown in the results obtained in this study.

Carotenoids are accessory pigments in photosynthesis, which in addition to this function, also have a photoprotective role since they can quench both singlet oxygen ($^1O_2$) and triplet chlorophyll (Havaux 2014). Depending on the type of metal and its concentration in the media, the content of carotenoids in the mosses in our study changed proportionally with the amounts of the two chlorophylls. The strong correlation between the three pigments in response to different factors implies the existence of a unifying underlying mechanism that affects all three photosynthetic pigments in the same way, probably through the destabilization of the photosynthetic units (photosystems). As in the study of Saxena & Saiful-Arfeen (2009), the carotenoid content decreased significantly under copper and cadmium stress, but the effect on the carotenoid contents of the two metals in the species tested in our study was dependant on their concentration. As seen in the other investigated parameters, the effect of zinc on the concentration of carotenoids was insignificant. The degradation of carotenoids affected by the high level of toxic metals could also be regarded as part of the acclimation mechanism to photooxidative stress once the defence capacity of chloroplasts has been exceeded. It has been shown that the degradation products of carotenoids lead to the induction of the $^1O_2$-responsive genes and that their expression is associated with an increase in photoresistance (Havaux 2014). The stabilization of the carotenoid content posterior to the initial decrease at 200 μM copper-acetate in the results obtained here supports this view.

The ratio of the two chlorophylls is often used as an indicator of metal stress inside plant cells and to distinguish whether the change in chlorophyll content is due to the inhibition of chlorophyll biosynthesis or chlorophyll degradation (Zengin & Munzuroglu 2005; Houri *et al.* 2020). Shakya *et al.* (2008) suggested that a reduction in the chlorophyll contents together with the chl *a/b* ratio indicates copper specific action, due to the inhibition of chlorophyll synthesis. However, in our study the chlorophyll *a/b* ratio did not differentiate between the effects of the specific metals or even between the different concentrations in the media and did not show any clear pattern. This parameter also varied significantly between the two moss species. In line with our results, other studies investigating the effects of trace metals on mosses (Tremper *et al.* 2004; Chen *et al.* 2015) also concluded that the chl *a/b* ratio was a less sensitive indicator of metal stress when compared to other parameters such as the concentration of individual pigments. However, in the present study, the ratio of the two chlorophylls proved to be a useful indicator of the cumulative negative effect of the longer exposure time and the higher concentration of metals applied in the media on photosystem II (PSII). The drop in the chl *a/b* value at 700 μM in the mosses from the type II experiments indicates the decrease in the ratio of PSII to PSI (Zengin & Munzuroglu 2005). This led to the conclusion that the prime effect of toxic metal stress in the chloroplasts was in PSII, which was more sensitive compared to PSI (also documented by Paunov *et al.* 2018). Among the investigated parameters regarding photosynthesis in our study, the concentration of chl *a* proved to be the most robust parameter changing similarly in both moss species. Regarding the carotenoids and chl *b*, however, *H. cupressiforme* was significantly more sensitive to copper, while

both species reacted similarly to the other two metals. Considering the redox-active nature of copper, the differences observed between the two mosses could be the consequence of different coping mechanisms for the oxidative stress caused by this metal. The interaction between the sensitivity of the particular plant, metal type and its form in the environment, as well as the applied dose (calculated as the product of metal concentration and duration of exposure) determines the final response of the plant to a particular toxic metal (Baker & Walker 1989), as was also confirmed by our experiments.

CONCLUSION

The development of mosses under controlled metal stress and the measure of species multiplication compared proved to be the most sensitive and useful parameter, reflecting the changes in many different underlying processes responsible for the production of the optimal morphological phenotype in the presence of excess trace metals in the substratum. Also, it showed the differences between the two moss growth forms and species. This further infers that the metal content present in various moss species used in environmental biomonitoring studies is not comparable and not easily applicable over large areas with varying conditions. Nevertheless, confirmation from the *in situ* experiments is welcomed when also taking into consideration the impacts of the positive and negative synergy in varying environmental parameters, different species genotypes (i.e. genetic plasticity between species) and the possibility of metal ion exclusion by immobilization within or outside moss cells which means that metals can be detected in high concentrations without affecting viability. However, the moss life history studied and presented here is a valuable contribution often lacking in field studies.

Both species, *A. undulatum* and *H. cupressiforme,* express the potential to be used widely in environmental monitoring, but the results obtained should be carefully explained since minor environmental changes or species misidentification could lead to incorrect conclusions. Additionally, the metal supply to mosses from the substrates and atmosphere, or both, should always be taken into consideration in the explanation of the results as well as the life history and life form of mosses along with the environmental factors (e.g. dryness, solar radiation, distribution and amount of precipitates). Among the toxic metals in the study, zinc proved to be the least deleterious to both mosses. On the other hand, copper was more toxic than the other two metals at the lower applied concentration, while at the highest concentration, cadmium generally led to a pronounced decrease in all of the studied parameters. The results obtained here suggest that the studies of species biology related to different metals in small scale controlled conditions are urgently needed prior to application in wide areas monitored by mosses.

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Stres toksičnim metalima kod dve vrste mahovina različitih formi rasta u akseničnim i kontrolisanim uslovima

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Briofite imaju široku primenu u monitoringu različitih tipova zagađenja i promena životne sredine. Direktno usvajanje supstanci iz okruženja celom površinom tela jedno je od svojstava koje je ove biljke izdvojilo kao jako dobre bioindikatore. Uprkos tome, malo je informacija vezanih za mehanizme odgovora, otpornosti i tolerancije predstavnika ove, druge po veličini, grupe kopnenih biljaka na toksične elemente iz okruženja. U ovom istraživanju, analizirane su dve mahovine koje karakteriše različita forma rasta, akrokarpna vrsta *Atrichum undulatum* i pleurokarpna *Hypnum cupressiforme.* Istraživanje je rađeno *in vitro* u akseničnim, potpuno kontrolisanim laboratorijskim uslovima, čime su eliminisani dodatni negativni, antagonistički i/ili sinergistički efekti**.** Mahovine su tretirane sa tri koncentracije (uključujući kontrolu) cink-, bakar-, ili kadmijum-acetata, kratak ili dug vremenski period, pri čemu su analizirani biohemijski i parametri njihovog rastenja i razvića. Dobijeni rezultati pokazali su da su reakcije specifične za vrstu mahovine, kao i da su one zavisne od tipa metala. Osim toga, period izloženosti mahovina metal-acetatima, kao i koncentracija metala u medijumu, dodatno su modifikovali reakciju mahovina na različite metale u medijumu. Među analiziranim parametrima kod dve testirane mahovine, indeks multiplikacije se izdvojio kao najkorisniji i najsenzitivniji za detekciju efekata zagađenja metalima. S druge strane, promene u koncentraciji hlorofila *a* bile su u relativno alom opsegu variranja između dve stresirane vrste mahovina. Pri nižoj koncentraciji u medijumu, bakar je generalno bio toksičniji u odnosu na druga dva metala. S druge strane, imajući u vidu sve analizirane parametre, najviša koncentracija kadmijuma dovela je do znatno većeg smanjenja vijabilnosti mahovina u poređenju sa istom koncentracijom cinka ili bakra. Sveukupno, naši rezultati ističu neophodnost sprovođenja malih studija u kontrolisanim uslovima, koje se bave biologijom pojedinačnih vrsta mahovina u odgovoru na različite metale ili druge polutante, pre nego što takve vrste budu korišćene u studijama za monitoring životne sredine.

Ključne reči: *Hypnum cupressiforme*, *Atrichum undulatum*, cink, bakar, kadmijum, *in vitro*