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PHYSIOLOGICAL RESPONSES IN *LEMNA MINOR FROND* TO HIGH CONCENTRATIONS OF ZINC, LEAD, COPPER AND CHROMIUM

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Abstract

Robust, rapid bioindicators of heavy metal water pollution, which are responsible for increasing environmental threats globally, are required. In the present study, we investigated the possibility of using short-term (≤ 12 hour) physiological responses of *Lemna minor* to high concentrations (up to 10 mmol L^{-1}) of zinc (Zn), lead (Pb), copper (Cu) and chromium (Cr) for this purpose. The Results showed that: (a) increase in Pb, Zn, Cu, and Cr levels increased fronds' malonaldehyde (MDA) contents, whereas increase in Pb, Cu, and Cr levels also reduced peroxidase activity (POD), although some of these effects were only observed at high concentrations; (b) high Cu and Cr levels reduced fronds' chlorophyll contents, but Zn increased chlorophyll content from 0.0016 mmol L^{-1}; (c) all four heavy metals induced frond abscission, and the percentage of frond abscission remain stable (except for Pb) after exposure for 10 h. The maximal concentrations of Zn, Cu and Cr resulted in > 50% frond abscission rates ($E_{FA}C_{50}$) within 10 h, but Pb induced much weaker responses. Hence frond abscission would not be a suitable short-term indicator of Pb pollution.

Key words: *Lemna minor*, Frond, Heavy metal, Biotoxicity.

Introduction

Environmental preservation and the threat of water pollution caused by heavy metals are issues of growing concern all over the world. Numerous studies have suggested that heavy metals can damage various components of the plant cell, *inter alia*, membranes, chlorophyll and proteins (Cervantes *et al.*, 2001; Valko *et al.*, 2005). Non-essential metals are structurally similar to essential elements, and thus can enter plant cells via non-selective ion channels and damage cellular components either directly by competing with native ions and blocking enzyme function or indirectly by producing reactive oxygen species, ROS (Shah *et al.*, 2001; Ucuncu *et al.*, 2013). Both the direct and indirect effects can significantly damage chloroplasts and other organelles. Furthermore, heavy metals can cause serious health hazards via food-chain magnification (Toppi & Gabbrielli, 1999; Wang, 2013b). Therefore, it is necessary to establish an effective way to assess and monitor heavy metal pollution.

However, developing robust tools to monitor ecosystem is challenging, because ecosystems are highly complex and a limited number of ecological indicators cannot fully represent the actual situation (Böcük *et al.*, 2013). Moreover, direct chemical analysis approachs have several drawbacks, for example, requirements for complex sample preparation procedure and expensive analytical equipment, as well as potential interference from secondary pollutants (Park *et al.*, 2013). Thus, there are clear needs for a cost-effective, rapid and accurate alternative to direct chemical analysis and diverse organisms have been used as bio-indicators to assess ecotoxicological risks. Duckweed, a collective term for 37 globally distributed species of five genera (*Spirodela, Landoltia, Lemna, Wolffia* and *Wolffiella*) (Zhao *et al.*, 2015), has several advantages as a bio-indicator. These include high ability to absorb nitrogen and phosphorus, rapid growth rate (doubling time of 1–4 d) (Blaise & Férard, 2005), high sensitivity to toxicants even at a low loads (Henke *et al.*, 2011; Böcük *et al.*, 2013; Wu & Zhang, 2013), and easy of both cultivation and harvest.

Lemna minor has a simple structure, consisting of small fronds and a single root. Reproduction occurs predominantly through vegetative propagation, as daughter fronds are released from the mother frond when they reach maturity. This asexual mode of reproduction results in a high degree of homogeneity within *L. minor*, and most clones are morphologically similar. In addition, Fenske *et al.*, (2006) found that among bacteria (*Vibrio fischeri*), human Fogh and Lund cells, protozoans (*Paramecium* spp.), nematodes (*Rhabditis oxycerca*), aquatic plants (*Lemna minor*), and fishes (*Leuciscus idus melanotus*) and *L. minor* were the most sensitive indicators. Furthermore, Park *et al.*, (2013) noted that *Lemna* spp. have gained broad acceptance as bioindicators in ecotoxicological research. Iram *et al.*, (2012) found *L. minor* can accumulate heavy metals in the water, it can be used for phytoremediation purpose. Megateli *et al.*, (2009) also suggested that aquatic plants could have applications in phytoremediation by reducing organic matter contents of polluted waters, and/or removing metallic pollutants from water.

For these reasons, *Lemna gibba* and *Lemna minor* have been extensively used in phytotoxicity testing, and several standard methods based on their use have been adopted by major international standardization agencies, e.g. U.S. Environmental Protection Agency (USEPA,1988), Organization for Economic Cooperation and Development (OECD, 2004), and International Standardization Organization (ISO20079, 2004). Moreover, *Lemna* spp. are the only aquatic vascular plants that are used to assess the toxicity of newly registered pesticides (Davy *et al.*, 2001). Hence, *L. minor* is an important bio-indicator for the assessment and monitoring of water pollution, and previous studies have demonstrated that it can be used for simple, rapid, cost-effective, sensitive, and precise assessments of

metal toxicity risks. However, most of these studies involved 7-14 days experimental period, and toxicity was assessed by growth rate inhibition, based on EC_{50} or IC_{50} values (Li & Xiong, 2004b; Kanoun *et al.,* 2009; Park *et al.,* 2013). Moreover, the maximum test concentration used in most studies has been 1 mmol L^{-1} or (in some case) 4 mmol L^{-1} (Table 1), but environment concentrations can be substantially higher than that in some polluted areas of China (Wang *et al.,* 2013a).

In this study we examined short-term (≤12 h) physiological responses of *L. minor* fronds to higher concentrations of four heavy metals (up to 10 mmol L^{-1}): zinc (Zn), lead (Pb), copper (Cu), and chromium (Cr). More specifically, we measured concentrations of malonaldehyde (MDA) and chlorophyll, as well as the activity of peroxidase (POD), in *L. minor* fronds exposed to the metals and frond abscission rates. The insights into the physiological and biochemical responses of *L. minor* to heavy metals should provide valuable complementary information that enhances its use as a bio-indicator, and potentially enables more robust and faster bioassays.

Materials and Methods

Plant material and pre-culture: Duckweed (*Lemna minor*) was collected from a lotus pond at Sun Yet-sen University, Guangzhou, Guangdong Province, China. *Lemna minor* specimens were then sterilized with 1% sodium hypochlorite to remove gastropods, algae and other undesired organisms. The duckweed was cultivated in Steinberg nutrient solution (ISO20079, 2004) with a photoperiod of 16 h (photon irradiance 85~135 uE/m^2/s^1 during light phases) and a constant day/night temperature of C25°C.

Physiological responses to different heavy metal concentrations: Analytical grade lead sulfate ($PbSO_4$), copper sulfate ($CuSO_4$), potassium dichromate ($K_2Cr_2O_7$), and zinc sulfate ($ZnSO_4$) were diluted in distilled water to obtain suitable concentrations (Table 2). Set of 10 *Lemna* colonies, each with three cultivated fronds as described above, were transferred to triplicate sets of Petri dish containing the heavy metal solutions listed in Table 2 or ultrapure water, used as a control (CK). Either 30 colonies (if intact) or 90 fronds (if colonies had disintegrated) of *Lemna* were used for assessing the physiological indices (malonaldehyde concentration, peroxidase activity and chlorophyll concentration) under each heavy metal treatment. The duckweed was cultured for 8 h in light (photon irradiance at 85~135 uE/m^2/s^1 and wavelength at 400~700 nm) at a constant temperature of 25℃ in these solutions, then the fronds' malonaldehyde (MDA) contents (Xiang & Wang, 1990), photosynthetic pigments contents (Zhang *et al.,* 2009), and the peroxidase (POD) activity (Zou, 2000), were determined.

Assays of the heavy metals on *L. minor* frond abscission: To determine the heavy metals' effects on *Lemna minor* frond abscission, the solutions of lead sulfate, copper sulfate, potassium dichromate, and zinc sulfate were applied (Table 3). Triplicate sets of 10 *Lemna* colonies were cultivated in each treatment solution, in constant light as described above, but for 12 h rather than 8 h, and the number of fronds in each petri dish was counted after 20 min, 30 min, 1 h, 2 h, 4 h, 6 h, 8 h, and 12 h exposure.

Table 1. Published metal toxicity tests with indicated duckweed species and concentrations of metals.

Species	Metals	Concentration	Unit	References
L. minor	CrO_4^{2-}	0.01-100	μM	(Henke *et al.*, 2011)
	Cu^{2+}	0.01-100		
	Zn^{2+}	0.01-100		
L. paucicostata.	Cu^{2+}	0.05-0.8	μM	(Li & Xiong, 2004a)
	Zn^{2+}	5-80		
	$Cr_2O_7^{2-}$	5-80		
L. gibba, L. minor, L. paucicostata	Cu^{2+}	62.5-5000	μg/L	(Park *et al.*, 2013)
	$Cr_2O_7^{2-}$	46.86-3000		
L. minor	Pb^{2+}	1-1.0	mM	(Mohan & Hosetti, 1997)
L. minor	Pb^{2+}	2.5	μg/L	(Fenske *et al.*, 2006)
	Zn^{2+}	23		
	$Cr_2O_7^{2-}$	1.7		
	Cu^{2+}	3.9		
L. minor	Pb^{2+}	0-4.0	mM	(Wu & Zhang, 2013)
	$Cr_2O_7^{2-}$	0-2.5		
L. minor	Cu^{2+}	0-10	μM	(Teisseire & Guy, 2000)
L. gibba, L. Minor, Spirodela polyrrhiza	Zn^{2+}	0.01-1.5	mg/L	(Uruc & Demirezen, 2012)
Lemna trisulca	Cu^{2+}	up to 50	mM	(Prasad *et al*., 2001)
L. minor	Zn^{2+}	0.15, 0.3	mM	(Kanoun *et al*., 2009; Radic *et al*., 2010)
L. minor, Spirodela polyrrhiza	Cu^{2+}	25, 50, 100	μM	(Kanoun *et al*., 2009)
Lemna minor	Pb^{2+}	0-10	mM	This study
	Zn^{2+}	0-10		
	$Cr_2O_7^{2-}$	0-10		
	Cu^{2+}	0-1; 0-0.64		

Table 2. Concentrations of heavy metals in water samples used for the physiological response experiment.

Heavy metal	Pb	Cr	Cu	Zn
Water concentration (mmol L^{-1})	0.016	0.016	0.0016	0.016
	0.08	0.08	0.008	0.08
	0.4	0.4	0.04	0.4
	2	2	0.2	2
	10	10	1	10

Table 3. Concentrations of heavy metals in the water samples used for the frond abscission experiment.

Heavy metal	Pb	Cr	Cu	Zn
Water concentration (mmol L^{-1})	n.d.	n.d.	0.00064	n.d.
	n.d.	n.d.	0.00032	n.d.
	0.1	0.1	0.0064	0.1
	0.5	0.5	0.0032	0.5
	1	1	0.064	1
	5	5	0.32	5
	10	10	0.64	10

Note: n.d.= Not determined

The percentage of frond abscission (%) was calculated as follows:

Frond abscission (%) = FNt /TFN*100 with FN_t being the single frond number caused by abscission in heavy metal treatment at a given evaluation time (t = 20 min, 30 min, 1 h, 2 h, 4 h, 6 h, 8 h or 12 h), and TFN being the total number of fronds in the 30 initial colonies. The formula has been developed based on the following assumptions: 0% and 100% frond abscission indicate no frond abscission and total colony disintegration, respectively. $E_{FA}C_{50}$ means the effective concentration which can cause 50% of frond abscission.

Statistical analysis: Data from both experiments described above were analyzed with least significant difference multiple-comparison tests based on one-way analysis of variance (ANOVA) using SPSS 17.0 (IBM, Armonk, NY). Differences were considered statistically significant if $p<0.05$.

Results

Effects of heavy metals on MDA content in *L. minor* fronds: The MDA contents in *L. minor* fronds were increased with exposure to increasing concentrations of Pb, Zn, Cu, and Cr (Fig. 1). There were no differences in MDA contents of fronds between exposed to these metals at concentration of Pb <0.4 mmol L^{-1}, Zn <2 mmol L^{-1}, Cu <0.04 mmol L^{-1}, Cr<2 mmol L^{-1}, and controls. But fronds exposed to the maximum test concentration of each metal have significantly higher MDA content ($p<0.05$) compared to controls. Exposure to 10 mmol L^{-1}, Zn, Pb, and Cr increased fronds' MDA contents 1.37, 1.44, 3.29-fold, respectively, while exposure to 1 mmol L^{-1} Cu resulted in a 3.62-fold increase, relative to the control group (CK) (Fig. 1c). Furthermore, the MDA content increased significantly when the Cu concentration exceeded 0.2 mmol L^{-1} (Fig. 1c). Thus, *L. minor* is most sensitive to Cu, followed by Cr, Pb, and Zn.

Effects of heavy metals on peroxidase (POD) activity in *L. minor* fronds: As shown in Fig. 2, POD activity was decreased with the concentrations of Pb>2 mmol L^{-1}, Zn>0.08 mmol L^{-1}, Cu>0.0016 mmol L^{-1}, Cr>0.08 mmol L^{-1}, except for the case of Zn, as POD activity did not decrease following exposure to different concentrations of Zn. However, two trends were noticed under low heavy metal concentrations. The first trend was an initial steep increase in POD activity when Zn and Cr concentrations were low (Zn<0.08 mmol L^{-1}, Cr<0.08 mmol L^{-1}), after which POD activity was decreased as Zn and Cr concentrations continued to increase (Fig. 2a, d). Under the Zn treatment the POD activity gradually approached the baseline value, as at the highest concentrations (2 and 10 mmol L^{-1}) there was no significant difference in POD activity between Zn and CK group treatments. On the other hand, when Cr concentration was 10 mmol L^{-1}, no POD activity was observed. The difference between the Zn and Cr treatments with increasing heavy metal concentration was that in the Zn treatment POD activity declined progressively and in the Cr treatment POD activity dropped rapidly. The second trend was that no significant variations in POD activity were observed at Pb <2 mmol L^{-1} and Cu <0.0016 mmol L^{-1} with CK (Fig. 2b, c). However, as the Pb and Cu concentrations increased to 10 and 1 mmol L^{-1}, respectively, there was a sharp decrease in POD activity. These results illustrate that each of these four heavy metals can unbalance the *L. minor* antioxidant system and weaken the ability of the plant cell to counteract scavenging ROS by increasing POD activity. As at a concentration of 10 mmol L^{-1} Pb and Zn treatments showed a 48% and 117% increase in POD activity, respectively, compared to the CK group.

Effects of heavy metals on chlorophyll content in *L. minor* fronds: There was no effect on chlorophyll contents, of Pb at any test concentration (Fig. 3). However, chlorophyll content was increased significantly under a low concentration of Zn (Zn=0.016 mmol L^{-1}) compared to CK group (Fig. 3b). In contrast, chlorophyll content was decreased sharply at Cu concentrations >0.04 mmol $L^{-1}$and at the maximum Cr concentration (10 mmol L^{-1}). At a Cu concentration of 1 mmol L^{-1} chlorophyll content was 29.6% that of CK and at a Cr concentration of 10 mmol L^{-1} chlorophyll content was 52.1% that of CK (Fig. 3c, d).

The *L. minor* fronds bleached at Cu and Cr concentrations of > 0.2 mmol L^{-1} and 10 mmol L^{-1}, respectively, but not under Zn and Pb treatments. Thus, Cu and Cr clearly affect the plant's chlorophyll contents stronger than Zn and Pb.

Effects of heavy metals on *L. minor* frond abscission: As shown in Figs. 4-7, all four heavy metals induced frond abscission in *L. minor*, in a quantitative and specific manners. Release of daughter fronds began after 20 min exposure to Pb, Cu, or Cr, but only after 2 h exposure to Zn. After 10 hours, abscission rates became stable under the Zn, Cu, and Cr treatments, but the patterns of response varied. Rates of frond abscission were higher at 0.1-1.0 mmol L^{-1} Zn than at 5 and 10 mmol $^{-1}$ Zn (Fig. 5), while concentrations of Cr and Cu were positively correlated with abscission rates (Figs. 6, 7).

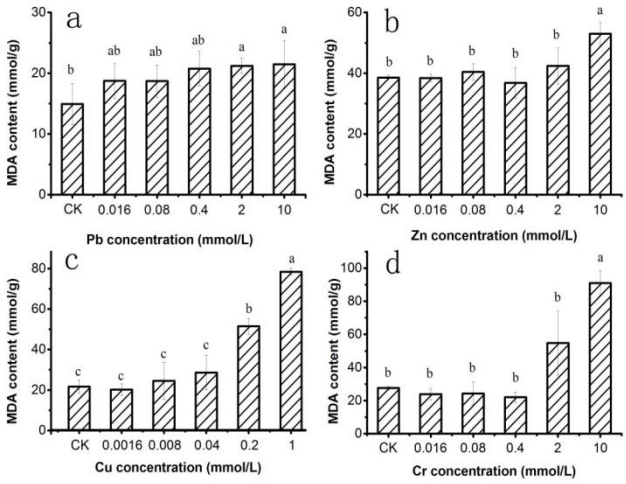

Fig. 1. MDA contents of *L. minor* fronds exposed to indicated concentrations of Pb, Zn, Cu, and Cr (and controls, CK) for 8 h.
Note: Differences in lower-case letters indicate statistically significant differences (p<0.05). The error bar is standard deviation.

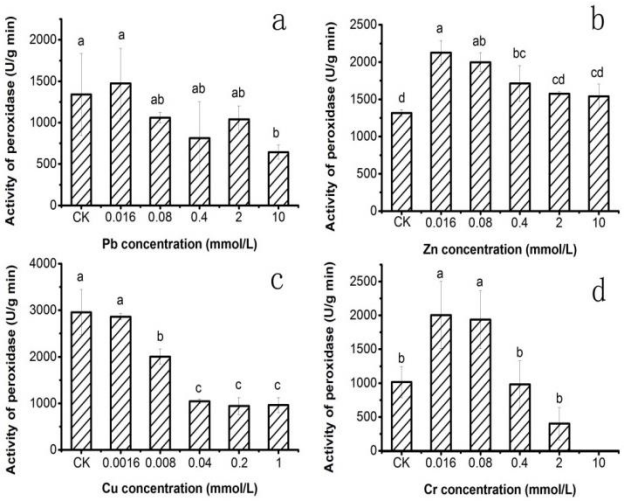

Fig. 2. Peroxidase activites in *L. minor* fronds exposed to indicated concentrations of Pb, Zn, Cu, and Cr (and controls, CK) for 8 h.
Note: Differences in lower-case letters represent statistically significant differences (p<0.05). The error bar is standard deviation.

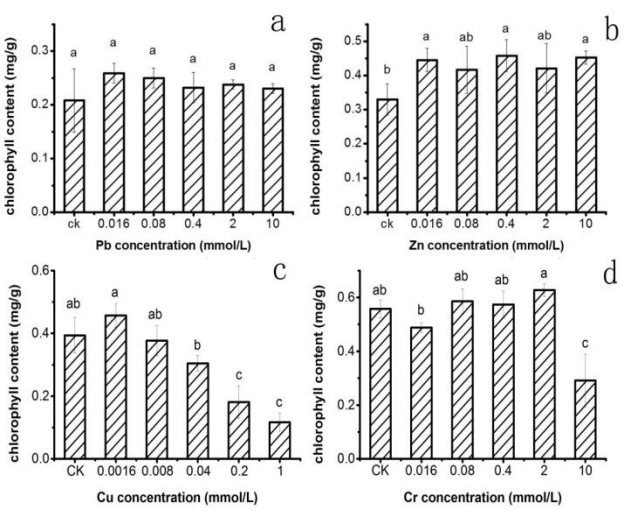

Fig. 3. Chlorophyll contents of *L. minor* fronds exposed to indicated concentrations of Pb, Zn, Cu, and Cr (and controls, CK) for 8 h.
Note: Differences in lower-case letters represent statistically significant differences (p<0.05). The error bar is standard deviation.

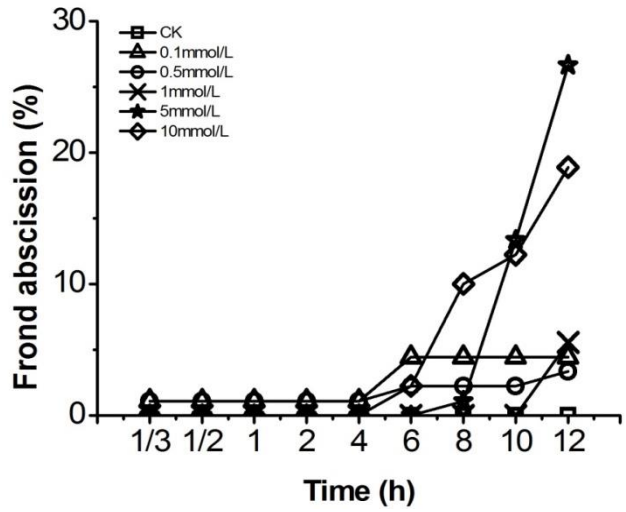

Fig. 4. The effects of various Pb concentrations on *L. minor* frond abscission.

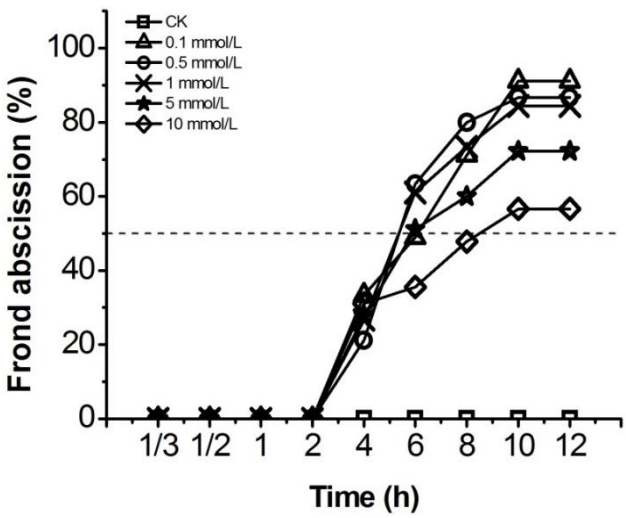

Fig. 5. The effects of various Zn concentrations on *L. minor* frond abscission.
Note: the dash line stands for $E_{FA}C_{50}$.

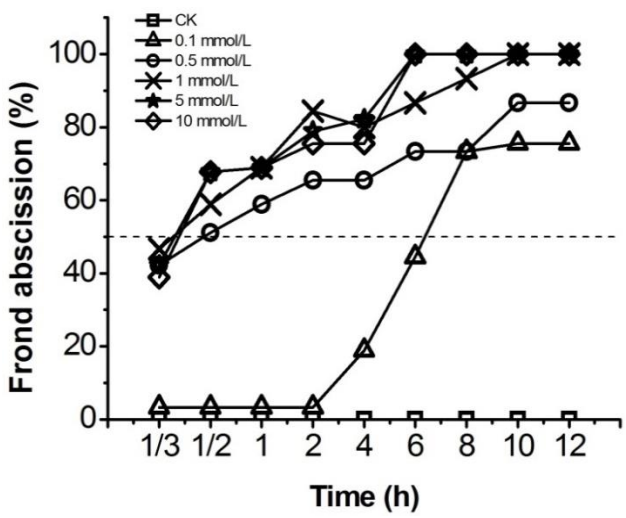

Fig. 6. The effects of various Cr concentrations on *L. minor* frond abscission.
Note: the dash line stands for $E_{FA}C_{50}$

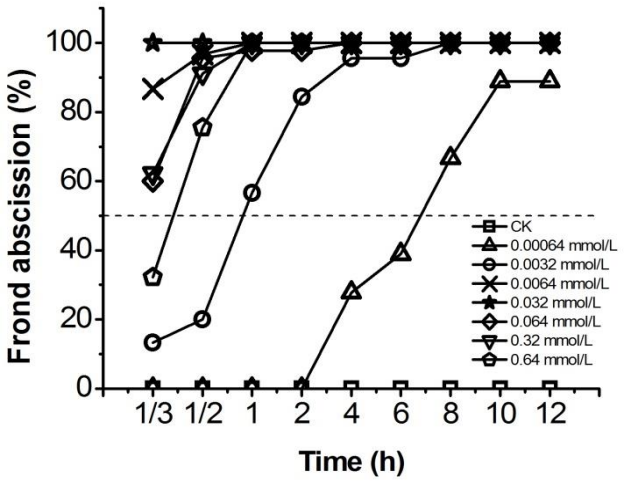

Fig. 7. The effects of various Cu concentrations on *L. minor* frond abscission.
Note: the dash line stands for $E_{FA}C_{50}$.

L. minor frond abscission was most sensitive to copper (Fig. 7). All fronds were released after just 20 min exposure to 0.032 mmol L^{-1} Cu, and even at 6.4 µmol L^{-1} Cu induced ca. 85% frond abscission after 10 h. It was next most sensitive to Cr (Fig. 6) and the frond abscission rate exceeded 50% after 30 min exposure to Cr concentrations ≥ 0.5 mmol L^{-1}. Moreover, even the lowest concentration (0.1 mmol L^{-1}) induced about 76% frond abscission and concentrations ≥1 mmol L^{-1} induced complete colony disintegration within 10 h. Zn began to induce frond abscission after 2 h, at all test concentrations abscission rates exceeded 50% after 10 h, and they ranged from 57% at 10 mmol L^{-1} to 91% at the lowest concentration (0.1 mmol L^{-1}). Frond abscission was least sensitive to Pb even after 12 h exposure to 10 mmol L^{-1} of this metal only about 18.9% of the fronds had abscised (Fig. 4).

Discussion

***L. minor* rapidly responds to heavy metal exposure:** All of the heavy metals used in the test (Pb, Zn, Cu, and Cr) induced increase in MDA contents. Pb, Cu, and Cr also induced reduction in POD activity. Some of these changes only occurred at high concentrations, and Zn didn't induce a reduction in POD activity at any concentrations. Nevertheless, these observations indicate that the metals induced increases in ROS production that (*inter alia*) increase rates of membrane peroxidation and trigger responses including increase in POD activity that are sufficient to counter the damage at low, but not high, concentrations of the metals.

Interestingly, two POD activity trends emerged under low concentrations of heavy metals. Zn and Cr had hormetic effects on POD activity, while Pb and Cu concentration were positively correlated with POD activity across the test concentration ranges. Hu & Liu (2012) confirmed that certain concentrations of Zn will stimulate the production of antioxidases in duckweed (*Spirodela polyrhiza*), thereby increasing ROS scavenging. Moreover, Wu *et al.,* (2013) found that Cr or Pb induced increases in MDA contents and activities of POD in duckweed fronds at low concentrations, but the ROS scavenging system was irreversibly destroyed when Cr and Pb concentrations exceeded 1.5 and 0.5 mmol L^{-1}, respectively.

Chlorophyll concentrations reflect plants' photosynthetic capacities. None of the tested Pb concentrations had a noticeable effect on fronds' chlorophyll contents, which reflect plants' photosynthetic capacities. However, Mohan & Hosetti (1997) found that exposure to 1.0 mM Pb reduced total chlorophyll concentration in *L. minor* frond from 2.61–1.11 mg/g after four days. Thus, Pb has chronic rather than rapid toxicity towards *L. minor*. In contrast, the fronds' chlorophyll contents were decreased at Cr and Cu concentrations of 2 and ≥ 0.04 mmol L^{-1}, respectively. Heavy metals may destroy photosynthetic pigments by impairing the electron transport chain, replacement of Mg^{2+} in the tetrapyrrole ring of chlorophyll molecules, inhibition of enzymes involved in chlorophyll biosynthesis, and/or facilitation of chloroplast membrane lipids' peroxidation by reactive oxygen species (Hou *et al.,* 2007). Chu *et al.,* (2004) reported that chlorophyll contents of cells may be increased by exposure to low concentrations of Cu, through Cu compensating for the lack of plastocyanin, while high Cu concentrations lead to imbalances in chloroplast enzymes activities and accelerated chloroplast degradation. This is partially consistent with our finding that Cu induced a non-significant increase in chlorophyll content at a very low concentration (1.6 µmol L^{-1}). Our finding that Cr significantly decreased chlorophyll content in *L. minor* fronds at a concentration of 10 mmol L^{-1} corroborates a report by Augustynowicz *et al.,* (2010) that Cr can strongly decrease plants' photosynthetic capacity.

Effects of heavy metal exposure on *L. minor* frond abscission: Frond abscission is a complex process involving numerous, interacting factors and the mechanisms involved have not yet been elucidated. However, an increase in ROS appears to be an important signal for division of new fronds in *L. minor* colonies (Huang *et al.,* 2014), although Li & Xiong (2004a) suggested that frond abscission may result from increase in ethylene production. As clearly shown in Figs. 5-7, percentage of frond abscission remained stable after 10 h of Zn, Cu, and Cr treatment, but they were still increasing under the Pb treatments. Moreover, the percentage was higher at the intermediate Zn concentrations than at the highest Zn concentrations, but they consistently increased with the increase in Cu or Cr concentration. The differences in these patterns may be due to various, complex toxicity mechanisms and/or variations in absorption kinetics (Kaszycki *et al.,* 2005). Abundant ROS in the *L. minor* fronds may also have contributed to the conflicting patterns. Henke *et al.,* (2011) obtained similar bell-shaped frond abscission response curves to the increase in Cu concentrations (with 20 min exposure), indicating the involvement of two processes: one stimulating abscission at lower concentrations and another that inhibiting it at higher concentrations.

Conclusion

Exposing *L. minor* fronds to Pb, Zn, Cu, and Cr can affect their POD activity and contents of both MDA and, chlorophyll within 8 h. However, while all of the tested heavy metals increased their MDA contents, only Pb, Cu, and Cr decreased their POD activity (no differences in POD activity were detected between fronds exposed Zn and controls).

Our results also show that the frond abscission rate frond abscission rate is not suitable for assessing heavy metal toxicity at such high concentrations: the water samples must be diluted to suitable concentrations. Moreover, the $E_{FA}C_{50}$ for this response is not the best index to assess Pb toxicity experiments with ≤ 12 h, because the abscission rate at 10 mmol L^{-1} Pb was much lower than 50%.

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References

Augustynowicz, J., M. Grosicki, E. Hanus-Fajerska, M. Lekka, A. Waloszek and H. Kołoczek. 2010. Chromium (VI) bioremediation by aquatic macrophyte *Callitriche cophocarpa* Sendtn. *Chemosphere*, 79: 1077-1083.

Blaise, C. and J.F. Férard. 2005. Overview of Contemporary Toxicity Testing. In: Blaise, C. & J.F. Férard. *Small-scale Freshwater Toxicity Investigations*. Springer, Dordrecht, pp. 1-68

Böcük, H., A. Yakar and O.C. Türker. 2013. Assessment of *Lemna gibba* L. (duckweed) as a potential ecological indicator for contaminated aquatic ecosystem by boron mine effluent. *Ecological Indicators*, 29: 538-548.

Cervantes, C., J. Campos-Garcia, S. Devars, F. Gutierrez-Corona, H. Loza-Tavera, J.C. Torres-Guzman and R. Moreno-Sanchez. 2001. Interactions of chromium with microorganisms and plants. *FEMS Microbiology Reviews*, 25: 335-347.

Chu, L., D. Liu, Y. Wang, Y. Li and H. Liu. 2004. Effect of copper pollution on seedling growth and activate oxygen metabolism of *Trifolium pratense*. *Chinese J. Appl. Ecol.*, 15(1): 119-122.

Davy, M., R. Petrie, J. Smrchek, T. Kuchnicki and D. Francois. 2001. Proposal to update non-target plant toxicity testing under NAFTA, USEPA, Washington, DC. http://www.epa.gov/scipoly/sap/

Fenske, C., G. Daeschlein, B. Gunther, A. Knauer, P. Rudolph, C. Schwahn, V. Adrian, T.V. Woedtke, H. Rossberg, W.D. Julich and A. Kramer. 2006. Comparison of different biological methods for the assessment of ecotoxicological risks. *Inter. J. Hygiene and Environm. Health*, 209: 275-284.

Henke, R., M. Eberius and K.J. Appenroth. 2011. Induction of frond abscission by metals and other toxic compounds in *Lemna minor*. *Aquatic Toxicology*, 101: 261-265.

Hou, W., X. Chen, G. Song, Q. Wang and C.C. Chi. 2007. Effects of copper and cadmium on heavy metal polluted waterbody restoration by duckweed (*Lemna minor*). *Plant Physiology and Biochemistry*, 45: 62-69.

Hu, C., X. Liu. 2012. Ectoxicity of ZnO nanoparticles on duckweed (*Lemna minor* L.) and analysis on its aggregation and dissolution. *J. Agro-Environment Sci.*, 31: 1690-1695.

Huang, R., X. Chen, L. Guo, R. Su and J. Shen. 2014. Effects of pH on growth and protective enzyme activity of duckweeds. *Acta Agriculturae Shanghai*, 30: 90-94.

Iram, S., I. Ahmad, Y. Riaz and A. Zahra. 2012. Treatment of wastewater by *Lemna minor*. *Pak. J. Bot*., 44(2): 553-557.

ISO20079. 2004. Water quality - Determination of the toxic effect of water constituents and wastewater on duckweed (*Lemna minor*) - Duckweed growth inhibition test.

Kanoun B., M., J.A. Vicente, C. Nabais, M.N. Prasad and H. Freitas. 2009. Ecophysiological tolerance of duckweeds exposed to copper. *Aquatic toxicology*, 91: 1-9.

Kaszycki, P., H. GabryŚ, K.J. Appenroth, A. Jaglarz, S. Sedziwy, T. Walczak and H. Koloczek. 2005. Exogenously applied sulphate as a tool to investigate transport and reduction of chromate in the duckweed *Spirodela polyrhiza*. *Plant, Cell & Environment*, 28: 260-268.

Li, T.Y. and Z.T. Xiong. 2004a. A novel response of wild-type duckweed (*Lemna paucicostata* Hegelm.) to heavy metals. *Environ. Toxicol*, 19: 95-102.

Li, T.Y. and Z.T. Xiong. 2004b. Cadmium-induced colony disintegration of duckweed (*Lemna paucicostata* Hegelm.) and as biomarker of phytotoxicity. *Ecotoxicol. and Environm. Safet.*, 59: 174-179.

Megateli, S., S. Semsari and M. Couderchet. 2009. Toxicity and removal of heavy metals (cadmium, copper, and zinc) by *Lemna gibba*. *Ecotoxicol. & Environ. Saf.*, 72: 1774-1780.

Mohan, B.S. and B.B. Hosetti. 1997. Potential phytotoxicity of lead and cadmium to *Lemna minor* grown in sewage stabilization ponds. *Environm. Pollut.*, 98: 233-238.

OECD. 2004. Test No. 221: *Lemna* sp. Growth Inhibition Test. *In*: *OECD Guidelines for the Testing of Chemicals, Section 2*. OECD Publishing, Paris, http://dx.doi.org/10.1787/9789264016194-en.

Park, A., Y.J. Kim, E.M. Choi, M.T. Brown and T. Han. 2013. A novel bioassay using root re-growth in Lemna. *Aquatic Toxicol.*, 140: 415-424.

Prasad, M.N.V., P. Malec, A. Waloszek, M. Bojko and K. Strzalka. 2001. Physiological responses of *Lemna trisulca* L. (duckweed) to cadmium and copper bioaccumulation. *Plant. Sci.*, 161: 881-889.

Radic, S., M. Babic, D. Skobic, V. Roje and B.P. Kozlina. 2010. Ecotoxicological effects of aluminum and zinc on growth and antioxidants in *Lemna minor* L. *Ecotoxicol. & Environm. Safety*, 73: 336-342.

Shah, K., R.G. Kumar, S. Verma and R.S. Dubey. 2001. Effect of cadmium on lipid peroxidation, superoxide anion generation and activities of antioxidant enzymes in growing rice seedlings. *Plant Sci.*, 161: 1135-1144.

Teisseire, H. and V. Guy. 2000. Copper-induced changes in antioxidant enzymes activities in fronds of duckweed (*Lemna minor*). *Plant Sci.*, 153: 65-72.

Toppi, L. and R. Gabbrielli. 1999. Response to cadmium in higher plants. *Environ. & Experim. Bot.*, 41: 105-130.

Ucuncu, E., E. Tunca, S. Fikirdesici, A.D. Ozkan and A. Altindag. 2013. Phytoremediation of Cu, Cr and Pb mixtures by *Lemna minor*. *Bul. Environ. Contamin. & Toxicol.*, 91: 600-604.

Uruc, P.K. and Y.D. Demirezen. 2012. Response of antioxidant defences to Zn stress in three duckweed species. *Ecotoxicol. & Environm. Safety*, 85: 52-58.

USEPA. 1998. Aquatic Plant Toxicity Test Using *Lemna* spp. United States Environmental Protection Agency, EPA 712-C-96-156.

Valko, M., H. Morris and M. Cronin. 2005. Metals, toxicity and oxidative stress. *Current Med. Chem.*, 12: 1161-1208.

Wang, S.L., X.R. Xu, Y.X. Sun, J.L. Liu and H.B. Li. 2013a. Heavy metal pollution in coastal areas of South China: a review. *Mar. Poll. Bul.*, 76: 7-15.

Wang, Y., Q. Qiu, G. Xin, Z. Yang, J. Zheng, Z. Ye and S. Li. 2013b. Heavy metal contamination in a vulnerable mangrove swamp in South China. *Environm. Monit. & Asses.*, 185: 5775-5787.

Wu, J. and S. Zhang. 2013. Effect of Metal Icon Cr, Co, Pb on SOD, POD, MDA in Lemna minor L. *Chin. Agricul. Sci. Bull.*, 29: 188-194.

Xiang, R. and D. Wang. 1990. Improvement of using thiobarbituric acid (TBA) to measure lipid peroxidase with spectrophotometry. *Prog. Biochem. & Biophy.*, 17: 241-242.

Zhang, Y., X. Huang and Y. Chen. 2009. *Plant Physiol. Experi.* Higher Education Press, Beijing, China.

Zhao, Y., Y. Fang, Y. Jin, J. Huang, S. Bao, T. Fu, Z. He, F. Wang, M. Wang and H. Zhao. 2015. Pilot-scale comparison of four duckweed strains from different genera for potential application in nutrient recovery from wastewater and valuable biomass production. *Plant Biology*, 171: 82-90.

Zou, Q. 2000. *Guidance of Plant Physiology and Biochemistry Experimentation.* Chinese Agricultural Press, Beijing, China.

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