DIFFERENT STRESS RESPONSES OF *LEMNA MINOR* DURING PHYTOREMEDIATION OF COPPER AND A CHLOROACETAMIDE HERBICIDE

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ABSTRACT

Common duckweed has raised interest for its application to phytoremediation due to its rapid growth and ubiquitous occurrence. In rural areas heavy metals and pesticides are frequently found in water bodies. In order to test the potential of duckweed as a species for phytoremediation, its stress response during exposure to heavy metals and the chloroacetamide herbicide were investigated in lab studies. *Lemna minor* were treated with $CuSO_4$ and pethoxamide as model pollutants. Measurements focused on plant growth, oxidative stress and basic detoxification enzymes. Duckweed was found to survive treatment with both pollutants very well, and without growth inhibition. Accumulation of reactive oxygen species were detected, as well as stress reactions of the antioxidative enzyme system. While pethoxamide was found to be conjugated with glutathione, copper was accumulated in the plants at high levels, and transient oxidative defense reactions were triggered.

We are able to confirm the significance of *L. minor* for the remediation of copper and a herbicide from water. *Lemna* might be used for phytoremediation of low level contamination with metals and organic xenobiotics, however more detailed analysis of the stress reactions following copper exposure and of the enzymatic metabolism of pethoxamide are required.

Keywords: phytoremediation, duckweed, *Lemna minor*, heavy metals, herbicide

1. Introduction

A steadily growing proportion of organic and inorganic pollutants is found in waste water treatment plant (WWTP) effluents, and the receiving surface waters are not significantly protected by state of the art tertiary wastewater treatment (Reemtsma et al 2006, Mench et al 2010). Several alternative technologies could be promising to reach the desired water quality, especially in rural regions with poorly developed or outdated sewage systems, where the high costs of state of the art WWTPs cannot be instantiated. Here, phytoremediation could be a sustainable solution (Pilon-Smits 2005). Phytoremediation exploits plants and associated organisms to decontaminate polluted media. The only prerequisites for high phytoremediation efficiency are fast biomass production, appropriate climatic conditions and tolerance towards pollutants (Lyubenova and Schröder 2010). With view to real life conditions, simultaneous phytoextraction of metals and phytodegradation of organics in a mixed pollutant situation would be desirable (McCutcheon and Schnoor 2003).

Lemna spp. is ubiquitously distributed across many continents and known for fast growth and high biomass production. Its bioaccumulation capacity as well as the high resilience to highly contaminated water bodies indicate a potential for phytoremediation (Mkandawire and Dudel 2007). Due to its small size, high growth rate and easy propagation and handling, *L. minor* is a common test species for ecotoxicological research (OECD 2006). The accumulation of heavy metals in *Lemna spp*. may depend on their concentration within the growth medium, and hereby especially on the bioavailability of the compound.

Copper (Cu) is an essential microelement and important co-factor for many enzymes, involved in photosynthetic electron transport, respiration, and oxidative stress response (Perales-Vela et al. 2007). However, it is also characterized as toxic heavy metal, due to its interactions with cellular components.

Pethoxamid (PA) is a chloroacetamide herbicide inhibiting grass and broadleaf weeds during cultivation of maize and soybean (Kato et al. 2001). Chloroacetamides disturb cell division of plants via inhibition of the synthesis of very long chain fatty acids. Their major detoxification pathway proceeds through glutathione conjugation (Böger et al. 2000).
When plants are confronted with pollutants, be they inorganic or organic, they develop stress, and under favorable conditions, defense reactions. Visible symptoms might derive from changes in chlorophylls and carotenoids, indicating that primary productivity might be influenced. Specifically accessory pigments like the xanthophylls, protecting chlorophylls from photo-oxidation, might be affected (Lichtenthaler 2014). Such changes can indicate differences in the responses following exposure toward organic and inorganic pollutants. Protection from reactive oxygen species (ROS) via non-photochemical quenching may play a role in this respect (Ruiz-Sola and Rodríguez-Concepción 2012).

Reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2) and superoxide (O_2^-), are among the earliest responses following chemical or ecological stress. The rapid enrichment of H_2O_2 and O_2^- after heavy metal exposure is well known as oxidative burst (Torres et al. 2006). ROS will further cause a damage of lipids, DNA, proteins and other biomolecules (Halliwell and Gutteridge 1986). For a healthy equilibrium of ROS, plants have developed effective enzymatic and non-enzymatic mechanisms, amongst them the Halliwell-Asada pathway (Foyer and Noctor 2011).

Organic xenobiotics may be detoxified in plants by other effective enzymatic defense mechanisms, following a three-phase-process described by Sandermann 1992. In phase I, xenobiotics are transformed to increase their reactivity. Subsequently, they are conjugated mainly to glutathione and sugars. In the third phase, compartmentation via vacuolar sequestration or formation of bound residues is catalyzed. Because of their important role in detoxification of xenobiotics, glutathione-S-transferases (GST) were analyzed in the present study. These enzymes catalyze conjugation of GSH via its addition or substitution to an electrophilic center of the xenobiotic during phase II (Dixon et al 2010).

This work investigates duckweed stress tolerance and ability to accumulate heavy metals or to take up and detoxify organic pollutants. Copper and pethoxamide are used as examples for contaminants of anthropogenic origin in WWTPs.

2. Material and methods

2.1. Plant material

Lemna minor L. was cultivated in Steinberg medium according to ISO 20079 (OECD 2006) after disinfection for 3 min with 0.5 % sodium hypochlorite. Plants were propagated in aquaria filled with 24 L of sterile Steinberg medium (pH 6.4) and cultivated for 7 days at a temperature of 24 ± 2 °C with a photoperiod of 16 to 8 h and an average light intensity of 43 µmol/(m^2s).

2.2. Treatments

Plants were treated with copper ions ($CuSO_4$ x $5H_2O$, Sigma-Aldrich, St. Louis, USA) in concentrations of 50 and 100 µg/L. Pethoxamide (2-chloro-N-(2-ethoxyethyl)-N-(2-methyl-1-phenylprop-1-enyl) acetamide) was from Dr. Ehrenstorfer GmbH (Augsburg, Germany) and applied in concentrations of 1.25 and 2.5 µg/L. Plant samples were taken after 0, 48, 96 and 168 h and frozen immediately in liqu. N2. Growth rates were determined following the standard method (OECD 2006). For each treatment, three independent biological replicates were taken and kept at -80 °C for further analysis.

2.3. Pigment analysis

For the determination of chlorophylls (Chl) and carotenoids, 0.6 mL cold 95 % ethanol was added to 0.1 g freshly ground plant material. Measurements were performed in triplicates. Absorption of

each sample was recorded at specific wavelengths of 664.1, 648.1 and 470 nm, according to Lichtenthaler and Buschman (2001).

2.4. Protein extraction

Protein extraction followed the methods developed by Schröder and coworkers (2008). The content of proteins was determined after Bradford (1976).

2.5. Enzyme assays

All enzyme assays were performed in a Spectra max 190 J spectrophotometer using 96 well plates at 25 °C. All measurements were done in triplicates and enzyme activity is expressed in µkat/mg protein. Assay conditions are summarized below.

Enzyme	Buffer	Tot. Vol./ enz. vol. [µl]	Substrates	Mol. Ext. coefficient [$mM^{-1}cm^{-1}$]	Reference
GST	TRIS/HCl 100 mM (pH 6.4 – 7.5)	190/10	CDNB, pNpa Fluorodifen	ε_{340nm} = 9.6 ε_{400nm} = 8.8 ε_{400nm} = 17.2	(Habig et al 1974)
POX	TRIS/HCl 50 mM (pH 6)	190/10	Guaiacol, H_2O_2	ε_{420nm} = 26.6	(Putter 1975)
GR	TRIS/HCl 100 mM (pH 7.5); 0.1 mM EDTA	190/10	GSSG, NADPH	ε_{340nm} = 6.2	(Kirkham and Zhang 1996)

2.6. F-AAS and etA-AAS analysis of copper concentration

The content of copper ions in both, growth media and plant material, were determined with flame atomic absorption spectrometry (F-AAS) and graphite furnace atomic absorption spectrometry (etA-AAS). Plant samples were dried at 70 °C, weighed, digested with aqua regia (DIN EN 16174) and finally boiled at 120 °C. Approx. 0.5 g of dry weight was used to analyze the content of copper ions in the *L. minor* plants. Samples of each 50 mL of growth medium were analyzed in triplicate.

3. Results and discussion

Duckweed growth was slightly retarded by both pollutants, and pethoxamide showed much stronger effects on growth than copper ions. After 168 h of exposure, no significant changes of the Chl *a* contents of all treatments compared to controls were detected. *L. minor* seems to be able to regenerate Chl *a* after longer exposure times. Plants treated with copper showed a concentration dependent decrease of Chl *b*, accompanied by strong increases of carotenoids. This underpins the protection potential of carotenoids for chlorophylls to avoid photo-oxidation and Cu influence on photosynthesis via blocking the photosynthetic electron transport (Fernandes and Henriques 1991). Plants treated with pethoxamide showed decrease of Chl *b* accompanied by an increase of carotenoids.

Detection of stress borne reactive oxygen species (ROS) confirmed that controls and *Lemna minor* treated with 50 µg/L copper did not show significant deviation of their H_2O_2 and O_2^- contents. However, 100 µg/L copper treatments induced visible increases of ROS, with an increase of H_2O_2 occurring predominantly in older fronds. The highest amount of ROS arises in veins and the youngest leaflets of the fronds. Pethoxamide concentrations were influential to the production of H_2O_2 and O_2^-. Untreated plants had the lowest ROS amounts, and with increasing pethoxamide concentration, stained areas increased. This confirms observations that higher amounts of H_2O_2 require higher peroxidase activity to reduce this ROS and equilibrate metabolism (Huber et al 2012).

The ability of duckweed to tackle oxidative stress, such as ROS, which might increase after the application of herbicides and heavy metals, has been shown previously, with similar results (Teissiere et al. 1998). In the current study it was determined via the analysis of the catalytic activity of peroxidases (POX) and glutathione reductase (GR). Both enzymes have important functions within the Halliwell-Asada-pathway (Halliwell and Gutteridge 1986). Peroxidases are well known to catalyze the oxidation of many organic and inorganic compounds within plant

secondary metabolism (Schröder et al 2008). The very high concentration dependent increase of POX-activity in plants exposed to copper after 48 h underpins the development of an oxidative burst. This burst is well known in the context of heavy metal stress and demonstrated a concentration dependent effect of copper on POX-activity (Levine et al 1994). The rapid decline after longer incubation times confirms previous observations of POX activity following an oxidative burst. Similarly, the activity of glutathione reductase and the time shift of its maxima towards 96 h underpins the functioning of the Halliwell-Asada-pathway. Within this cycle, GR reduces oxidized GSSG to GSH after the reduction of H_2O_2 via catalases and POX. A corresponding delay in GR-activity has been previously described and seems to be inherent to the antioxidant detoxification cycle of plants (Foyer 1993).

After exposure to pethoxamide, different trends of POX and GR-activities were observed. The transient response of POX and delayed GR point to defense against an oxidative burst in the presence of Cu followed by exhaustment, depicted by the presence of ROS at 168h and growth inhibition. Whereas ROS production after Cu treatment may be a primary mode of action of the metal, it is different in the case of PA where oxidative stress comes later. It is a consequence of damages from primary effects of the herbicide without relation to ROS production. At 168h ROS accumulate, necrotic spots appear, and growth inhibition starts.

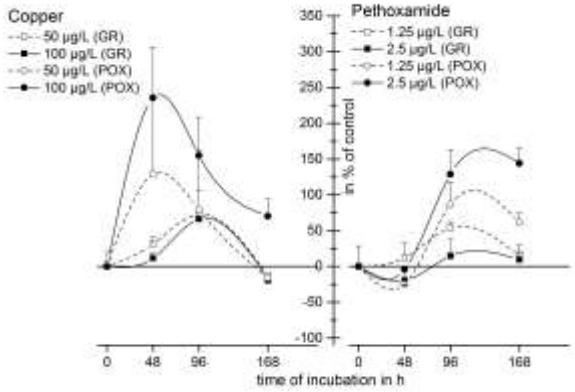

Figure 1: Activity of peroxidases (POX) and glutathione reductase (GR) in % of untreated control after different times of incubation. Data shown are means and SE of 3 biological replicates of copper and pethoxamide treated *Lemna minor* plants, respectively. Statistical significance of curve maxima was confirmed by ANOVA at P<0.05.

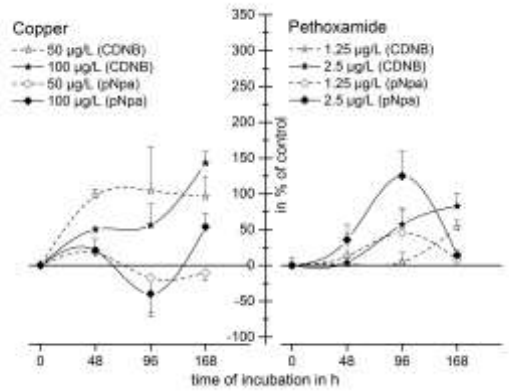

Figure 2: Specific activity of CDNB and pNpa conjugation catalyzed by GSTs in % of untreated control after different times of incubation. Data shown are means and SE of 3 biological replicates of copper and pethoxamide treated *Lemna minor* plants. Statistical significance of curve maxima was confirmed by ANOVA at P<0.05.

GST-activities coupled to different substrates showed various trends. After copper exposure, conjugation of fluorodifen was strongly concentration dependent during the first 96 h. The maxima of plants exposed to 100 µg/L copper occurred with a delay compared to the 50 µg/L treatment. After 168 h of exposure the enzyme activity decreased strongly. *Lemna* plants treated with pethoxamide showed much lower fluorodifen catalyzed GST-activities.

For further analysis of detoxification mechanisms on the enzymatic level, classical GST substrates were assayed. Plants treated with copper showed a manifest concentration dependent increase of the glutathione conjugation with CDNB after an oxidative burst (Torres et al 2006). Copper exerts a large number of effects on protein metabolism and fatty acids (Fernandes and Henriques 1991). As with pethoxamide, plants exposed to 100 µg/L copper induced a weaker increase of the CDNB-activity during the first 48 h. This disturbance of the oxidative burst may have arisen by an inhibitory effect of excess copper ions to certain GST-isoforms. After 168 h of incubation CDNB-activity in the 100 µg/L treatment even increased. pNpa conjugation of *Lemna* plants treated with Cu were transiently induced. Similarly both treatments led to a slight increase of GST-activity after 48 h and a decrease after 96 h.

Duckweed exposed to pethoxamide induced a much weaker increase of GST mediated CDNB conjugation. This activity has been found to be connected to xenobiotic detoxification in plants (Dixon et al 2010). No decrease of this activity was observed, underpinning the long-lasting effect of pethoxamide. Besides differences in CDNB-activity, GST-activity for pNpa was inversely concentration dependent after 96 h. This activity decreased and achieved finally values comparable to controls. In any case, it is shown that conjugation with GSH mediates the detoxification of herbicides such as the chloroacetamide pethoxamide. Such a connection could be made by the inhibitory assays with treated *Lemna* GSTs. A similar inhibitory effect had been shown in *Phragmites* exposed to pethoxamide (Schröder et al 2005). Further, LC-MS analysis showed that conjugation results in a pethoxamide-glutathione with a mass of 567.3 g/mol, supporting the hypothesis that conjugation with GSH is the major metabolic pathway for pethoxamide in animals, plants and soil (Kato et al 2001).

The concentration of copper accumulated was investigated to determine the ability for phytoremediation. Rapid accumulation of copper was observed during the first 96 hours. The amount of copper accumulated within plants was strongly concentration and time dependent. This saturation was not detected for *Lemna* exposed to 100 µg/L copper during the time of incubation. We hypothesize that the concentration of copper in media might be limited, but that the ability of *L.minor* for the uptake of copper is not exceeded. The accumulation of Cu inside the plants (250 - 350 µg/g dw) confirms the investigations of Mkandawire and Dudel (2007) who observed bioaccumulation between 200 – 800 µg/g dw for *Lemna spp*. Furthermore, the bioconcentration factor of 233 proves the assumption that *L. minor* is suitable for the application in phytoremediation.

The significance of *L. minor* for phytoremediation of the heavy metal copper and the herbicide pethoxamide can be confirmed. It is shown that these two xenobiotics induce different oxidative and enzymatic stress response. Copper(II)ions induced strong enzymatic and antioxidative stress responses during the first 48 hr of incubation in an oxidative burst. Pethoxamide induced different effects with increasing enzyme activity during the whole experiment (168 h), since it does not have the same mode of action as Cu, especially with regard to oxidative stress. Also, its detoxification via conjugation of glutathione was confirmed. Carotenoids seem to play a significant role for the protection of chlorophylls under such stress situations. Due to the relatively low inhibition of frond growth and the ability to handle appearing stress, it may be concluded that *L. minor* is suitable for phyto-remediation of low-level contamination in water bodies.

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