IS *LEMNA MINOR* A CANDIDATE PLANT FOR PHYTOREMEDIATION? OBSERVATIONS ON THE EFFECT OF METALS AND INDUSTRIAL POLLUTANTS ON THE SMALL DUCKWEED

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**ABSTRACT**

The removal of pollutants from wastewater with plants is an upringing technology, and several plant species have been investigated in this respect. Floating species are of some interest because of their easy propagation and potential role as adsorbers for pollutants in solution. Amongst them, *Lemna minor* has been investigated primarily because of its rapid growth and biomass production. Here we present results on the reaction of *Lemna minor* towards zinc and cadmium, two metals of widespread distribution and significant occurrence in waste water, and towards triclosan and SDS, two organic chemicals that are frequently found in WWTPs. *Lemna* plants were grown under standardized conditions and exposed to the chemicals for several days. After visual check for stress symptoms, the plant material was harvested, ground under liquid nitrogen, and the activity of major detoxification enzymes as well as the occurrence of stress markers was determined in the protein crude extracts.

*Lemna* reacts rapidly to the exposure towards heavy metals, and shows strong but transient reduction in the activity of detoxification enzymes, including glutathione transferases. After 48 hrs, a general recuperation of detoxification was found and enzymes remained significantly induced for 96 hrs.

Incubation with industrial chemicals caused chlorophyll losses and severe stress reactions, but a different set of detoxification reactions was induced. Glutathione transferases, esterases and peroxidases were selectively reduced under SDS, whereas triclosan rather induced the activity of these enzymes.

Compared to other plants used in phytoremediation, *Lemna*’s performance is poor, and the plant is sensitive to peak concentrations of pollutants. In cases where low pollution ranges prevail, it might be an additional species removing residual chemicals and metals from pretreated waters.

1. Introduction

Applications for successful phytoremediation and their limitations have been reviewed recently (Mench et al. 2010). Amongst macrophytes of choice, floating species are of some interest because of their easy propagation and potential role as accumulators of pollutants in solution. Also small species with high biomass like the small Duckweed (*Lemna minor* L.) may be of interest in this context. Duckweeds colonize lakes and rivers with reduced flow and increased levels of nutrients to an extent where the surface is completely covered. *L. minor* has the additional advantage that it tolerates a wide range of temperatures and climatic conditions.

For long, this plant genus has been regarded as an undesirable weedy species in waste water treatment processes, but since duckweed wastewater treatment systems in developing countries have been studied for dairy waste lagoons (Körner et al. 1996), domestic sewage (Oron 1994, Hammouda *et al.* 1995), the utilization of the plant has become more interesting for developing countries. Ten years ago, Iqbal (1999) has
reviewed that several full-scale treatment systems were in operation in Taiwan, China, India, Bangladesh, but also in Belgium, and the USA. Since Lemnaceae had been reported to possess a very high capacity of accumulating heavy metals and organic xenobiotics, they have been proposed to be potentially suitable for removal of these compounds from industrial wastewaters (Iqbal 1999). However, when the simultaneous removal capacity of Fe, Cu, Zn, Mn, Cr and Pb was analysed in L. minor and a related species, S. intermedia and in Pistia stratiotes, the small duckweed died, whereas other macrophytes resulted highly effective in the uptake of heavy metals (Miretzky et al. 2005).

Here we present results on the reaction of Lemna minor towards triclosan, SDS and the heavy metals zinc and cadmium, pollutants that are frequently found in WWTPs.

2. Materials and methods

Duckweed plants (Lemna minor L.) were obtained from the Botanical Garden in Munich and grown in a greenhouse (T=20°C day, 15°C night, RH 75 %). For the incubation, independent cultures were grown in glass vessels of 35 x 25 x 5 cm (l x w x d) in 2 L of modified Steinberg-Media following ISO/DIS 20079. Initially 50 plantlets were used to start each culture. Six cultures were used as controls.

Plants were incubated for one week with SDS and Triclosan, in concentrations given in table 1. Each incubation was repeated three times. After visual check for stress symptoms, and determination of growth, the plant material was harvested and ground under liquid nitrogen.

Table 1: Sample names and xenobiotic incubation schemes

<table>
<thead>
<tr>
<th>Sample</th>
<th>Concentration [mg/L]</th>
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<tbody>
<tr>
<td>SDS 0</td>
<td>control</td>
</tr>
<tr>
<td>SDS1</td>
<td>SDS</td>
</tr>
<tr>
<td>SDS2</td>
<td>SDS</td>
</tr>
<tr>
<td>Tricl 0</td>
<td>control</td>
</tr>
<tr>
<td>Tricl 1</td>
<td>Triclosan</td>
</tr>
<tr>
<td>Tricl 2</td>
<td>Triclosan</td>
</tr>
<tr>
<td>Tricl 3</td>
<td>Triclosan</td>
</tr>
</tbody>
</table>

Three independent controls without treatment were set up. The experiments were harvested after 24, 48 and 96 hrs of incubation. Zinc and Cadmium were applied as Zincsulfate Heptahydrate (ZnSO₄ 7H₂O) and Cadmium sulfatehydrate (3CdSO₄ 8H₂O), in 100 µM concentrations. Prior to incubation with xenobiotics, a control experiment was performed to check the growth of the culture. After 48 hrs the culture showed significant deviation from ideal growth patterns (hatched lines). Still the cultures had a healthy appearance and developed normally. After subculturing and incubating with the xenobiotics in the given concentrations, samples were taken after 168 hrs in the case of organic pollutants, and after 24, 48 and 96 hrs in the case of heavy metals.

Growth of subcultures was calculated using the equation:

\[ f(x) = b \cdot e^{ax} \Leftrightarrow a = \frac{1}{t} \cdot \ln \left( \frac{f(x)}{b} \right) \]
with b, the number of leaves at the start of the experiment, t, the time and a, the growth rate.

![Growth curve of untreated Lemna fronds under the given experimental conditions](image)

**Figure 1**: Growth curve of untreated Lemna fronds under the given experimental conditions

For the determination of major stress metabolites and detoxification enzymes, the plantlets were thoroughly rinsed, shortly dried on filter paper, weighed, and frozen in liquid N₂. Aliquots of 1 to 3 g FWt were transferred to a mortar and ground to a fine powder under liquid N₂. Max 3g powder were added 30ml of freshly prepared extraction buffer (0,1M Tris/HCl pH 7.8 5mM EDTA, 5mM dithioerythritol DTE, 1% Nonidet P40, 1% insoluble polyvinylpyrrilidone PVP K90). This mixture was homogenised and extracted for 30min prior to centrifugation at 20 000 rpm. The proteins in the resulting crude extract were precipitated by addition of ammonium sulfate in two steps – 40 and 80% of saturation. The protein solution was centrifuged after each step and the pellet finally was resuspended in 2.5ml of 25mM Tris/HCl buffer pH 7.8. This step was followed by desalting on Sephadex PD-10 desalting columns (Pharmacia, Freiburg, Germany).

Enzyme assays in this paper were performed either with a 6 cell Beckman 640 UV/Vis spectrophotometer, or with a Spectra max Plus 384 photometer (Molecular Devices) using 96 well plates, at 25 °C. All assays were done at least in triplicate. All enzyme activities are expressed in µkat/mg protein.

**Glutathione S-transferase (GST) assay** - Spectrophotometer assays for determination of GST activity using the model substrates: 1-chloro-2,4-dinitrobenzene (CDNB, ε₃₄₀nm=9.6mM⁻¹ cm⁻¹), 1,2-dichloro-4-nitrobenzene (DNCB, ε₃₄₅nm=8.5mM⁻¹ cm⁻¹), 4-nitro-benzyl-chloride (NBC, ε₃₁₀nm=1.8mM⁻¹ cm⁻¹), p-nitrobenzoyl chloride (NBoC, ε₃₁₀nm=1.9mM⁻¹ cm⁻¹), followed the method of Schröder (2005). In all of the above mentioned assays, the concentration of reduced glutathione (GSH) as well as of the model substrates was 1mM.

**Peroxidase** - The method was modified from the method published by Roy and Hänninen (1994). Each well contained 190 µL mixture of reaction buffer (0.05M Tris/HCl buffer, pH 6.0), 3.4 mM Guajacol (0.068 mM per reaction), 9 mM H₂O₂ (0.18 mM per reaction) and 10 µL enzyme extract (5 % per reaction). POX activity was assayed at 420 nm (ε = 26.6 mM⁻¹cm⁻¹) for 5 min.
**Protein contents** - The protein contents were evaluated according to the method described by Bradford (1976) using serum albumin as standard protein. 10% of the total volume per well is protein. The specific activities of the above mentioned enzymes were expressed in μkat/mg of protein.

3. Results & Discussion

Confrontation of duckweed plantlets with Zn and Cd in 100 µM concentrations influenced their growth and appearance during 96 hrs. Growth was slower than in controls, and fronds took more time to develop and separate. In addition, typical detoxification processes were altered, namely the activity of glutathione conjugation reactions (Table 2). These reactions, catalyzed by glutathione S-transferases (GST), are proposed to detoxify numerous organic xenobiotics and pesticides in plants, and act to mitigate pollutant stress. In exposed L. minor plants, the activity for the conjugation of the most prominent model substrate for GST, 1-Chloro-2,4-dinitrobenzene (CDNB), after an initial lag phase, increased strongly upon exposure to Zn, leading to almost doubling of the activity as compared to controls. Cd exposure caused also a lag phase, where activities were lower than in control plants, and then to a strong increase of the conjugative capacities after 48 hrs. After 96 hrs, the GST levels were again lower than in controls. Nitrobenzylchloride, a closely related substrate, was always conjugated at lower rates than in control plants, and Nitrobenz-O-ylchloride (NBoC) conjugation was completely blocked during the initial phases of the exposure. After 96 hrs exposure to Zn, control levels were reached, whereas exposure to Cd kept NBOC-GST levels at 50 % of control. Conjugation of the substrate, DCNB, was not determined in any of the HM treated samples.

Overall it has to be concluded that the detoxification of organic xenobiotics will be depressed under the influence of both, Zinc and Cadmium, and that GST mediated stress reduction is also unlikely to occur.

**Table 2:** Summary of the GST activities in Lemna minor after the exposure to 100 µM Zn or Cd. Data are means of 3 to 5 replicas ± SD.

<table>
<thead>
<tr>
<th></th>
<th>CDNB</th>
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<tbody>
<tr>
<td></td>
<td>24</td>
<td>48</td>
<td>96</td>
</tr>
<tr>
<td>Control</td>
<td>0.45 ± 0.04</td>
<td>0.84 ± 0.58</td>
<td>0.56 ± 0.14</td>
</tr>
<tr>
<td>Zn</td>
<td>0.21 ± 0.06</td>
<td>0.38 ± 0.02</td>
<td>0.88 ± 0.56</td>
</tr>
<tr>
<td>Cd</td>
<td>0.27 ± 0.02</td>
<td>1.17 ± 0.76</td>
<td>0.49 ± 0.01</td>
</tr>
<tr>
<td>NBC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.09 ± 0.04</td>
<td>0.12 ± 0.02</td>
<td>0.14 ± 0.12</td>
</tr>
<tr>
<td>Zn</td>
<td>0.01 ± 0.13</td>
<td>0.11 ± 0.09</td>
<td>0.10 ± 0.07</td>
</tr>
<tr>
<td>Cd</td>
<td>0.02 ± 0.19</td>
<td>0.14 ± 0.15</td>
<td>0.11 ± 0.07</td>
</tr>
<tr>
<td>NBoC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.07 ± 0.04</td>
<td>0.20 ± 0.08</td>
<td>0.23 ± 0.13</td>
</tr>
<tr>
<td>Zn</td>
<td>0.00 ± 0.00</td>
<td>0.01 ± 0.01</td>
<td>0.29 ± 0.06</td>
</tr>
<tr>
<td>Cd</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
<td>0.14 ± 0.09</td>
</tr>
</tbody>
</table>

When *L. minor* was exposed to organic pollutants, growth was impaired even at lowest concentration by more than 30 % and frond development was impaired even more. Moreover, a different reaction of the detoxification enzymes was recorded (Fig 2 and 3).
Glutathione S-transferase activity for CDNB and DCNB was not influenced by sodium dodecylsulfate (SDS) in concentrations of 25 mg/L, but 50 mg/L led to a 1.4fold increase of CDNB conjugation, and at the same time to a reduction of DCNB activity to 20% of controls. The determination of peroxidase (POX) activity indicated a general, but rather low (1.4fold) induction of this general stress marker enzyme (Fig. 2).

![Graph 1](image1)

**Figure 2**: Incubation of Lemna minor with Triclosan and SDS changes the activity of detoxification enzymes. Data are means of 3 to 5 replicas ± SD.

Quite different from the above results, the disinfectant, triclosan, influenced GST strongly (Figure 3). Low concentrations of 0.1 and 0.5 mg/L increased CDNB GST 1.5 fold, whereas 1 mg/L led to a 2.5fold induction. In the same extracts, DCNB conjugation remained unaffected by the lower triclosan concentrations, but increased more than 7fold at 1 mg triclosan per L. For the peroxidase activity in the same samples, no coherent pattern was observed. After a transient induction at a concentration of 0.1 mg/L, the activity ceased, to reach maximum levels of 3fold induction at 1 mg/L.

![Graph 2](image2)

**Figure 3**: Detoxification enzymes in Lemna minor under the influence of SDS and Triclosan. Data are means of 3 to 5 replicates ± SD.
Duckweed populations are frequently found on freshwater resources, and in tertiary waste water treatment plants. Under favourable conditions, they can produce remarkable amounts of biomass, and even remove unwanted substances like nitrogen and phosphorus from eutrophic water bodies (Al-Nozali et al. 2000, Porath and Pollock, 1982). Different from reports on duckweed growth in eutrophic lakes or sewage tanks from tropical regions, where the plant regularly thrives well, our experimental conditions including heavy metals and organic xenobiotics caused growth reductions even at lowest concentrations of the pollutants applied. Although defence enzymes are generally found to be well expressed in *Lemna*, they were obviously not capable of saving the plant from damage. The observed inductions might be rather seen as stress response than detoxification. This is especially true for the activity of glutathione S transferases which are thought to contribute to detoxification via conjugation of xenobiotics. Compared to other plants used in phytoremediation, *Lemna*’s performance is poor, and the plant is sensitive to peak concentrations of pollutants. In cases where low pollution ranges prevail, duckweed might be an additional species removing residual chemicals and metals from pretreated waters. Any case, duckweed for food or fertilizer production should only be grown on wastewaters with extremely low toxin concentrations. Even low concentrations in the raw wastewater may become hazardous due to the manifold bioaccumulation in duckweed and, possibly, in the food chain (Iqbal 1999).

4. References


