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Arsenate, Cadmium and Lead affect the plant detoxification cascade for organic pollutants

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ABSTRACT

Multiple sites of moderate to low pollution with heavy metals or organic xenobiotics have been identified throughout Europe, and those where no immediate pressure exists for further use, may well be remediated by plant based technologies. Hence, phytoremediation has become a green sustainable technology that has gained increasing attention during the last decade. Municipal and industrial waste water treatment plants can be optimized by the addition of lagoons with aquatic macrophytes. However, it is obvious, that quite a number of remediation projects failed due to insufficient plant performance. In this paper, we present evidence that *Typha* and *Phragmites*, two widely used plant species in phytoremediation, are capable of removal and metabolism of heavy metals and organic pollutants from water and sediments to some extent, but will fail at higher concentrations. We show also that pollution with heavy metals will interfere with both, the oxidative stress defence in plants, and with their ability to conjugate and detoxify organic xenobiotics. Despite plant species dependent differences, the general reactions seem to include oxidative stress and an induction of antioxidative enzymes. Both species respond to trace elements with oxidative stress and decay of central physiological functions. In non-hyperaccumulators like the chosen species, photosynthesis, growth, but also defense will cease at higher concentrations or prolonged exposure to heavy metals. Interestingly, defense reactions against organic xenobiotics, so far considered unrelated to heavy metal stress will also be impaired by mixed pollution, especially cadmium. Here, direct interactions with enzyme proteins are possible, but influence on transcription is frequently observed. For the practical use of plants in phytoremediation, it is discussed that only species with proven stress resistance or mixed plant consortia with differential resistances and uptake can be recommended.

Keywords: Mixed pollution; Waste water treatment; Heavy metals; Chlorobenzenes; Phytoremediation; *Typha*; *Phragmites*; Oxidative burst; Antioxidant enzymes; Glutathione; Glutathione S-transferases

1. Introduction

Decreasing water quality is one of the most challenging problems of our society, especially with view to the global warming problem and the increasing demand for irrigation in food and feed production. Confronted with an increasing number of polluted water reserves in all European countries, communities, environmentalists, and plant scientists have started to evaluate options for phytoremediation [1]. Such a phytotechnology would offer efficient tools and environmentally friendly solutions for the cleanup of contaminated sites and water,

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the improvement of food safety, carbon sequestration to reduce global warming, and the development of renewable energy sources, all of which contribute to sustainable resource management.

During recent years, different plant species have been identified as useful in the removal of organic pollutants from water, soil and sediments. Amongst them, cattail *(Typha latifolia)* and common reed *(Phragmites communis)* are species that have already been widely used for removal of nitrogen and phosphorus in constructed wetlands or lagoon type waste water treatment plants, but they have also been found to remove organic pollutants to some extent.

Because of their vigorous growth in all kinds of wetlands and their known capacity to detoxify foreign compounds [2,3], the performance of reeds in contaminated sewage sludge, crude oil contaminated soils and watercourses has been regarded an attractive alternative to technical solutions that seem to be complicated in operation and pose novel problems, i.e., the storage or removal of concentrated waste fractions [4].

Yet another focus of investigations within the field of phytoremediation was the determination of the metal uptake in plants from wetlands and mining waters with regard to the amelioration of water quality. In the meantime, and due to the activities of EU-COST actions 837 and 859, a huge amount of literature exists on uptake, sequestration and metabolomics of heavy metals [5–9], to name but a few.

Interestingly, most of the published work considers heavy metals or distinct groups of organic pollutants alone, and not in mixture. Most studies remained in important, but narrow topics, and one of the main problems for any given plant, the survival under mixed pollution situation where heavy metals might occur together with organic pollutants, as is the case in military training sites or many industrial brownfields, and which hence would be an extreme of the "real world" situation of polluted sites or water bodies, has not been investigated in any depth [10]. Still, it is obvious, that quite a number of remediation projects failed due to insufficient plant performance [7].

From in vitro studies with isolated plant enzymes, the detrimental role of heavy metals on functional proteins has become clear [11]. Some recent work addressed the physiology of mutagenized *Nicotiana tabacum* and its antioxidant system, to yield information on effects of heavy metal stress to the plant [12].

In this present paper, we demonstrate that *Typha* and *Phragmites* are capable of removal and metabolism of heavy metals and organic pollutants to some extent, but will fail at higher concentrations. We show also that pollution with heavy metals will interfere with both, the oxidative stress defence in plants, and with their ability to conjugate organic xenobiotics. Despite plant species dependent differences, the general reactions seem to include oxidative stress and an induction of antioxidative enzymes. Several processes seem to depend on direct binding of heavy metals to enzyme proteins, but effects on transcription are also observed. Induction of xenobiotic metabolism will be obtained at high heavy metal concentrations, when plant stress is elevated.

2. Material and methods

2.1. Plant material

Typha latifolia and *Phragmites communis* plants were grown in hydroponic culture in a green house at 14 h d, 10 h night and humidity of 50%. After a growing period the plants were treated with three different heavy metals at four different concentrations. The heavy metals applied are: cadmium (as cadmium sulphate), arsenic (as sodium arsenate) and lead (as lead chloride). Control plants were grown under same conditions but not treated with heavy metals. Three plant replicates for each treatment were used.

2.2. Pigment analysis

In order to extract the photosynthetic pigments from leaf samples, 10 ml cold 80% acetone were added to 0.5 g freshly ground plant material. The mixture was centrifuged 20 min by 39,250 g at 4 °C. The supernatant was collected in Falcon tubes. To the pellet were added again 10 ml 80% acetone, stirred, and centrifuged by 39,250 g at 4 °C for 20 min. The supernatant was added to the first extract. This procedure was repeated twice. One ml of the extracted pigment supernatants was withdrawn and pigments were determined spectrophotometrically in four repetitions at wavelengths: 663.2 nm, 646.8 nm and 470 nm, according to Lichtenthaler [13]. Pigment contents were expressed in µg/g fresh weight.

2.3. Protein extraction

Protein extraction followed procedures by Schröder and coworkers [14]. In short, frozen plant material was pulverized and to max 3 g powder were added 30 ml of freshly prepared extraction buffer (0.1 M Tris/HCl pH 7.8 5 mm EDTA, 5 mm dithioerythritol DTE, 1% Nonidet P40, 1% insoluble polyvinylpyrrilidone PVP K90). This mixture was homogenised and allowed to stand on ice for 30 min prior to centrifugation at 20,000 rpm. Proteins in the resulting crude extract were precipitated by addition of ammonium sulphate in two steps: 40 and 80% of saturation. The protein solution was centrifuged after each step and the pellet finally resuspended in

2.5 ml of 25 mm Tris/HCl buffer pH 7.8. This was followed by desalting on Sephadex PD-10 columns (Pharmacia).

2.4. Protein content

The protein content in the plant samples was evaluated by a Bradford-Test using Coomassie Brilliant Blue as a dye and according to [3], with serum albumin as a reference protein standard. All measurements were done in triplicate in a spectrophotometer at 595 nm.

2.5. Enzyme assays

All enzyme assays in this paper were performed in 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. Assays followed published methods but were modified accordingly (Table 1).

3. Results and discussion

Macrophytes can assimilate inorganic and organic pollutants in their tissue, create good conditions for sedimentation of suspended solids and prevent erosion by reducing the velocity of the water in a given wetland. Several ways are used to express accumulation of pollutants in plants. For heavy metals, they include the determination of the bioconcentration factor (BCF) = (total HM concentration in a plant tissue/total HM concentration in either the contaminated substrate or labile pool) or accumulation factor (AF) = (total amount in a plant tissue/total amount in the contaminated substrate) and the translocation factor (TF) or shoot:root (S:R) ratio = (total HM concentration in shoot tissue/total HM concentration in the root tissue).

These equations have been used with great success to derive plant specifications, e.g. for the identification of heavy metal hyperaccumulators or avoiders. Hyperaccumulator plants, for example, are capable of accumulating metals 100-fold higher (2% on the dry weight basis) than those typically measured in shoots of the common nonaccumulator plants. Metal avoiders would exclude heavy metals from their living tissues [9]. But most of these species are slowly growing and produce little biomass. This fact limits the use of hyperaccumulator plants for commercial phytoextraction. With respect to this definition, the plants chosen here, *Typha* and *Phragmites*, are neither heavy metal hyperaccumulators, nor heavy metal avoiders, which might make them susceptible to stresses. Table 2 summarizes uptake studies using *Typha* rhizomes and selected heavy metals. A concentration dependent uptake of the respective metals is recorded, with highest values reached for lead, and lowest for arsenic. In untreated control plants, HM concentrations were found to rank as As > Pb > Cd.

Similarly, plants in a constructed wetland will also take up organic pollutants from soils, sediments or directly from the water. The transfer of a given xenobiotic from soil or water to the plant occurs concentration dependent and diffusion driven for compounds with lipophilicity close to that of the respective plant root. Root uptake and transport of organic xenobiotics has been reviewed recently [15]. Uptake of lipophilic and amphiphilic compounds from soil has been intensively studied in the

Table 1
Conditions for the enzyme assays used indicating buffers and substrates, and wavelengths for the measurements. All measurements were done in triplicate, using blanks without protein extract, to assess the nonenzymatic reaction rates

Enzyme	Buffer	Tot. Vol./enzyme Vol. [µl]	Substrates	Photometric conditions ε [$mm^{-1}cm^{-1}$]	Reference
Catalase	KH_2PO_4, 100 mM pH 7.0	150/10	H_2O_2	$\varepsilon_{340\ nm} = 0.04$	[3,23]
Peroxidase	Tris/HCl, 50 mM pH 6.0	200/10	guajacol, H_2O_2	$\varepsilon_{420\ nm} = 26.6$	[23]
Ascorbate peroxidase	KH_2PO_4 55.56 mM pH 7.0	180/20	ascorbate, H_2O_2	$\varepsilon_{290\ nm} = 28$	[23]
Monodehydro genascorbate reductase	Hepes/KOH 1 mM pH 7.6	200/20	NADPH, ascorbate	$\varepsilon_{340\ nm} = 6.2$	[23]
Glutathione peroxidase	KPP/EDTA 50 mM pH 7.0	200/50	NADPH, H_2O_2, GSSG	$\varepsilon_{340\ nm} = 6.2$	[3]
Glutathione S-transferase	Tris/HCl 100 mM pH 6.4 – 7.5	200/50	CDNB DCNB NBC	$\varepsilon_{340\ nm} = 9.6$ $\varepsilon_{340\ nm} = 8.5$	[13]

Table 2
Uptake of arsenate, lead and cadmium into *Typha* rhizomes, all experiments were done in triplicate

Heavy metal concentration in water [µM]	Arsenic conc. in rhizomes [mg/kg Dwt]	Lead conc. in rhizomes [mg/kg Dwt]	Cadmium conc. in rhizomes [mg/kg Dwt]
Control	4.55 ± 1.03	0.851 ± 0.3	0.041 ± 0.01
10	21.80 ± 6.24	97.76 ± 46.83	24.2 ± 5.05
50	34.20 ± 12.24	319.43 ± 240	181.66 ± 86.85
250	56.5 ± 19.48	1046.00 ± 91.	271.67 ± 28.72

context of pesticide application. For organochlorines, transfer into plants depends on log P values and transpiration rates. The root concentration factor, RCF, describes the potential of given organic molecules to accumulate in plant roots, without differentiating between surface accumulation or uptake into root tissues, be it root cortex or vascular system. In any case, RCF is strongly dependent on the lipophilicity of the compound (i.e., its log $K_{O/W}$), and hence, seems to be governed by the absorptive properties of the root bark [15].

Briggs and coworkers [16] have found the following relationship between the log K_{OW} and the RCF in barley plants (adapted from [15]):

$$\text{Log (RCF} - 0.82) = 0.77 \log K_{ow} - 1.52$$

Compounds exhibiting a low K_{ow} (i.e., < 1) will not be able to penetrate the lipid containing rhizodermis, while, on the other hand, substances with log $K_{ow} > 2$ will be increasingly retained by the epidermal lipids in the root and the lipophilic mucilage surrounding the root.

The uptake of foreign compounds into the hydraulic system of a given plant might be calculated with the transpiration stream factor, TSCF. Compounds of average solubility, amphiphilic substances and weak acids are clearly in favour. Xenobiotics with a log $K_{ow} \approx 2$ are transported solely in the transpiration stream, while those with a log $K_{ow} \approx 1$ are both, phloem and xylem mobile, although only metabolites might enter the phloem. For compounds with log $K_{ow} \approx 1.0–3.5$ metabolism may occur in the leaf and stem tissue.

But more important than the mere uptake of compounds into plant tissues is the potential of plants to withstand long lasting pollution situations, e.g., when they are used for practical phytoremediation in waste water treatment plant (WWTP) lagoons that are fed with sewage of varying degree of pollution. Under such conditions, plants have to manage the xenobiotic compounds and detoxify or sequester them. Interestingly, in studies in WWTPs neither *Typha* not *Phragmites* exhibit typical stress symptoms like discolorations or pigment losses at lower concentrations of heavy metals. Table 3 indicates that *Typha* rather shows increases in pigments than yellowing under typical concentrations of arsenic and cadmium. Only at As and Cd concentrations above 50 µm, and in all incubations with lead chloride a net loss in chlorophylls is recorded. At the same time, the chla/chlb ratio changes from 1.1 in control plants to values around 1.4 to 1.7, indicating a strong adaptation of the photosynthetic apparatus to the confrontation with the HM.

On the other hand, both species, T*ypha* as well as *Phragmites*, exhibit stress on the molecular level when exposed to the toxic elements for extended periods. The reasons for this are intelligible. Disodium arsenate (Na_2HAsO_4), used in this study, can be regarded as a physiological analogue of phosphate and acts as uncoupler of oxidative phosphorylation and potent inhibitor of catalase [17]. Cadmium, due to its long biological half-life and low excretion rate, evokes a whole array of toxic effects in plant cells [18]. Acute toxicity occurs in response to oxidative stress. Cd interacts with photosynthesis, respiration and nitrogen metabolism and causes oxidative damage to lipids and enzymes [18]. Lead is one of the first discovered and widely used metals in human history and most commonly encountered in the environment [18]. Acute toxicity is revealed by its binding to enzyme prosthetic groups, and the inhibition of heme complex formation.

Table 3
Pigment contents [µg/g Fwt] in the *Typha* leaves after 72 h of incubation. Measurements and calculations were performed according to [13], and in triplicate

Treatment	Chlorophyll a	Chlorophyll b	a/b ratio
Control	7.31 ± 0.9	6.47 ± 1.03	1.1
10 µM Cd	11.10 ± 5.58	7.9 ± 3.82	1.4
50 µM Cd	21.31 ± 4.48	13.25 ± 3.27	1.6
100 µM Cd	5.70 ± 1.09	5.82 ± 0.62	1.0
10 µM As	19.66 ± 1.57	11.54 ± 6.17	1.7
50 µM As	21.07 ± 4.11	11.22 ± 1.81	1.9
100 µM As	4.62 ± 3.21	6.06 ± 0.93	0.8
10 µM Pb	21.34 ± 6.8	13.13 ± 4.73	1.6
50 µM Pb	16.29 ± 7.84	9.69 ± 4.09	1.7

We have published several reports on the oxidative stress caused by heavy metals, in different plant species. Both, arsenate and cadmium were shown to cause strong oxidative burst in cell cultures of spruce [2,15,20,21]. The metals and H_2O_2 directly influenced enzymes of the Halliwell-Asada cycle and induced antioxidative responses [3].

Recent literature has proven that H_2O_2 might also be a potent regulator of glutathione dependent reactions [22], and that the glutathione homeostasis is a driver of stress response. Of course, Cd will be sequestered by phytochelatins, derived from glutathione in all plants, and *Phragmites* exhibits strong losses of reduced GSH upon treatment with Cd after 72 h of exposure (Fig. 1). Notably, these losses start within 6 h in the rhizomes (Fig. 1b), where Cd meets with metabolically active tissues after uptake. It might be speculated that glutathione is used up in a concentration dependent manner to synthesize phytochelatins. Root GSH contents are restored in the following hours, but loss of 30% is recorded in the leaves. This may be explained by the fact that glutathione is synthesized in leaf chloroplasts and may subsequently be translocated to heterotrophic tissues, in this case the rhizome.

Correspondingly, the detoxification of organic electrophilic compounds like pesticides or aromatic pollutants is altered in the presence of heavy metals. Table 4 summarizes results of enzyme assays with glutathione S-transferases (GST) and antioxidative enzymes of the Halliwell-Asada cycle (Catalase, Peroxidase, Ascorbate peroxidase, Monodehydrogenascorbate reductase and Glutathione peroxidase) under the influence of cadmium. The latter enzymes are induced in *Phragmites* corresponding to elevated Cd concentrations, which indicates activity against oxidative stress, in order to cope with the HM. Strongest activation is observed with GR, the enzyme responsible for the reduction of GSH.

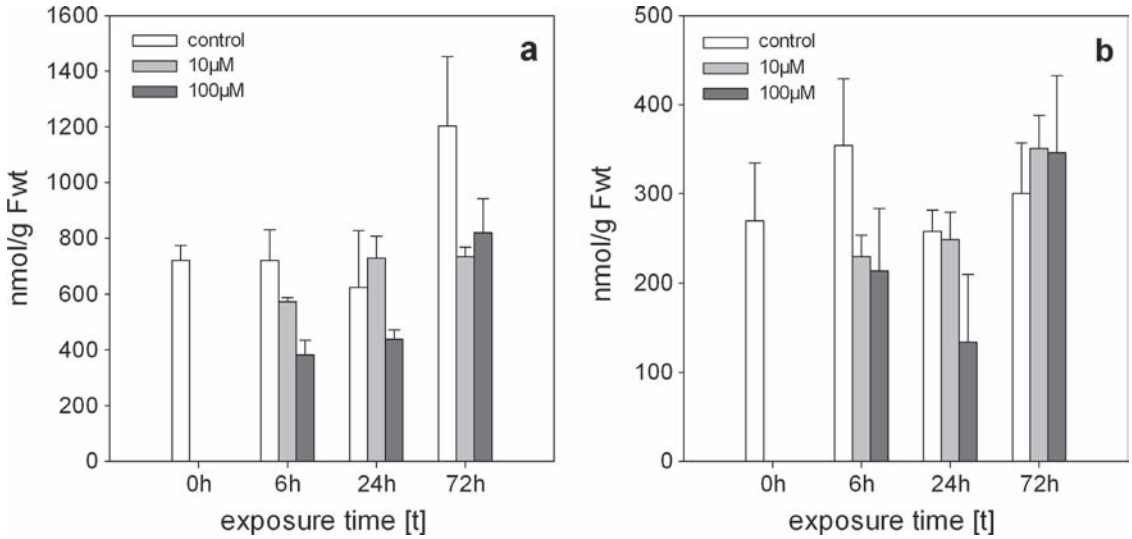

Fig. 1. The contents of Glutathione in *Phragmites* leaves. GSH contents in leaves (a) and rhizomes; and (b) have been determined after incubation with $CdSO_4$. Data were obtained by HPLC analysis of aqueous plant extracts and are means of three biological replicates ± SD.

Table 4
Activity of *Phragmites* and *Typha* detoxification enzymes under the influence of $CdSO_4$. All measurements were done in triplicate and according to the conditions summarized in Table 1

Enzyme assayed	*Phragmites* enzymes [µkat/mg]			*Typha* enzymes [µkat/mg]		
Treatment	Control	10 µM	100 µM	Control	10 µM	100 µM
GST:CDNB	6.8 ± 1.5	5.5 ± 0.9	5.2 ± 0.2	0.76 ± 0.7	0.5 ± 0.25	0.6 ± 0.2
GST:DCNB	0.01 ± 0	0.01 ± 0	0.01 ± 0	0.21 ± 0.2	0.39 ± 0.25	0.82 ± 0.4
GR	4.6 ± 1.5	5.1 ± 0.4	5.9 ± 1.3	1.6 ± 1	1.5 ± 0.25	0.95 ± 0.25
APOX	2.1 ± 1.1	2.0 ± 0.6	3.1 ± 1.4	0.32 ± 0.3	0.25 ± 0.15	0.17 ± 0.16
POX	2.6 ± 0.7	2.9 ± 0.8	3.1 ± 0.4	0.40 ± 0.3	0.56 ± 0.26	0.81 ± 0.7

This induction is consequent with view to the increased demand for GSH, as seen in Fig. 1. In contrast, the GST activity levels for the detoxification of organic xenobiotics decrease. This could mean that plants encountering multiple pollution with heavy metals and organic xenobiotics at the same time would have less potency to fight organic compounds.

Contrary to the results in *Phragmites*, in *Typha*, the antioxidant enzymes decrease under cadmium stress. Only peroxidases, a group of more unspecific defense enzymes, show increased activities towards their model substrate, guajacol. The main GST activity for the conjugation of CDNB, however, decreases by 20%, similar to reeds. This indicates the same inhibitor signalling for GST activity in both species. Remarkably and different from cattail, in reeds, a fourfold induction of a minor isoenzyme GST fraction for the conjugation of DCNB is observed.

4. Conclusions

When planning phytoremediation of a given site, sediment or water body, it is of utmost importance to consider the given pollution situation. In most water bodies and sewage multiple pollution will prevail, with trace heavy metals and organic pollutants in varying concentrations.

Plants will respond to trace elements with oxidative stress and a decay of their central physiological functions. In non-hyperaccumulators, photosynthesis, growth, but also defense will cease at higher concentrations or prolonged exposure to heavy metals. This might have specific consequences for the use of cattail, *T. latifolia*, whereas reeds, *P. communis*, seem to be less susceptible.

Interestingly, defense reactions against organic xenobiotics, so far considered unrelated to heavy metal stress, will also be impaired by mixed pollution. For the practical use of plants in phytoremediation only such species with proven stress resistance against both pollutant groups or mixed plant consortia with differential resistances and uptake can be recommended—they will be able to take up pollutants accordingly and metabolize them to products that are unavailable to the food web, and our most valuable resource, clean water.

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