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Biotransformation and antioxidant response in *Ceratophyllum demersum* experimentally exposed to 1,2- and 1,4-dichlorobenzene

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Abstract

We report the effects of 1,2- and 1,4-dichlorobenzene (1,2-DCB and 1,4-DCB) on the aquatic macrophyte *Ceratophyllum demersum*. We evaluated the response of the antioxidant system through the assay of glutathione reductase (GR), guaiacol peroxidase (POD) and glutathione peroxidase (GPx). Additionally, the effect of DCBs on the detoxication system by measuring the activity of glutathione-*S*-transferase (GST) was evaluated.

C. demersum showed elevated GST activities when exposed to 10 and 20 mg l^{-1} 1,2-DCB, and at 10 mg l^{-1} for 1,4-DCB. These results show that glutathione conjugation take place at relatively high concentrations of both isomers. Significantly increased activities of POD were also detected in *C. demersum* exposed to concentrations above 5 mg l^{-1} of the corresponding isomer.

The GR activity was enhanced in plants exposed to 1,2-DCB (5 mg l^{-1}) and 1,4-DCB (10 mg l^{-1}). GPx was also significantly increased in exposures to the corresponding isomer, each at a concentration of 10 mg l^{-1}. However, plants exposed to low doses of 1,4-DCB (1 mg l^{-1}) showed significantly decreased activities of both enzymes GR and GPx.

Consequently, it is clear that the exposure of the aquatic macrophyte *C. demersum* to DCBs is able to cause an activation of the antioxidant system, showing an isomer specific pattern, which suggests that the defence system of this plant is playing an important role in scavenging ROS, helping to protect the organism against adverse oxidative effects generated by the prooxidant action of the tested xenobiotics. Furthermore, increased GST activities give indirect evidence on the conjugation of either DCBs or the corresponding metabolites during phase II of detoxication, which supports the elimination process of toxic metabolites from cells of *C. demersum*.

Keywords: DCBs; ROS; Macrophytes; Antioxidant; Detoxification; GSH

1. Introduction

Chlorobenzenes (CBs) have been produced in large quantities and used widely for both domestic and industrial purposes (Wang et al., 1996). CB's are used as solvents, deodorants or as intermediates in the manufacture of pesticides and other chemicals (Beurskens et al., 1994). Because of their widespread use over decades, DCBs have become very common in the environment. They are found in water (Meharg et al., 2000), soil (Wang et al., 1996), sediment (Beck et al., 1996) sewage sludge (McPherson et al., 2002), and even within the aquatic biota (van Wezel and Opperhuizen, 1995). Concentrations of DCBs are variable within the environment. The amounts of dissolved DCBs are generally within the ng to μg per liter range (Oliver and Nicol, 1982; Boutonnet et al., 2004; Malcolm et al., 2004). Our own experience shows that dissolved DCBs are present in Suquía River reaching 10 μg l^{-1}, while DCBs

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associated to sediments reach 600 μg K g^{-1} (Monferrán, 2006), which is in good agreement with other reports of DCBs in sediments (Malcolm et al., 2004).

DCBs are hazardous to the health and have been ranked as priority pollutants by the USA Environmental Protection Agency (EPA), who established maximal concentration levels in drinking water for 1,2-DCB (0.6 mg l^{-1}) and 1,4-DCB (0.075 mg l^{-1}). No regulatory issues are reported for 1,3-DCB. These compounds have a high octanol–water partition coefficients of 3.4 (Wang and Lee, 1998) and consequently a biological accumulation can be expected in the aquatic ecosystem (Malcolm et al., 2004). Based on ^{14}C labelling techniques, Wang et al. (1996) reported high bioaccumulation factors (>1.0) for chlorobenzene (CB) and 1,4-DCB from soils to crop plant cells.

The fate and effects of DCB's on aquatic organisms depend on their susceptibility, which is closely related to the ability of a particular organism to perform biotransformation and excretion of these xenobiotics.

The metabolism of DCBs has been studied in mammals showing a metabolization via microsomal oxidation, proceeding either directly or through the formation of a metastable epoxid intermediate, followed by transformation to the corresponding chlorophenol, which then is excreted as mercapturic, glucuronic, or sulfate conjugate (Hawkins et al., 1980; Lake et al., 1997). The secondary P450-catalyzed oxidation of phenols to hydroquinones and benzoquinones may promote oxidative stress to macromolecules (Hissink et al., 1997; Rietjens et al., 1997). Several studies indicate that DCB metabolites covalently bind to kidney and liver tissues, producing kidney tumors in male rat and liver tumors in mice (Lake et al., 1997). Furthermore, it has been demonstrated that the metabolism of DCBs results in the formation of reactive oxygen species (ROS), which significantly contribute to their toxicity as evidenced in rat livers exposed to 1,2-DCB (Hoglen et al., 1998; Younis et al., 2000).

Little information is available on the metabolism of DCBs in plants. Metabolic pathways for biotransformation of xenobiotics in plants can be summarized as follows: A first transformation step, usually performed by cytochrome P-450 monooxigenases (phase I), followed by conjugation (e.g. through glutathione *S*-transferases and glucosyltransferases; phase II), and then internal compartmentalization, mostly in the cell wall fraction or in the vacuole (phase III) (Sandermann, 1994).

It has been demonstrated that soybean cell cultures are able to metabolize 1,4-DCB through hydroxylation and conjugation. (Wang et al., 1996), and also investigations of Topp et al. (1989) showed that some chlorobenzenes could be metabolized in soil plants. To the extent of our knowledge, there is no information available on the metabolism of chlorobenzenes in aquatic macrophytes.

The main aim of this study was to assess the response of the defence systems in *Ceratophyllum demersum*, experimentally exposed to 1,2-DCB and 1,4-DCB, by evaluating selected biotransformation and antioxidant responses. *C. demersum* was chosen as sentinel organism for the present survey as it is a free floating aquatic macrophyte of ecological importance within the aquatic ecosystem, providing shelter and habitat for young fishes, other aquatic animals and additionally has a worldwide distribution. The tested concentrations of DCB's to perform our survey were chosen within the range of previous toxicological investigations on acute effects on different diatoms, invertebrates, fish, algae and plants (Wang et al., 1996; Calamari et al., 1983; Malcolm et al., 2004).

2. Materials and methods

2.1. Plant material

C. demersum plants were purchased at Aqua Global (Dr. Jander & Co. OHG, Seefeld, Germany) and cultivated non-axenically for two weeks prior to the experiments in 100 l tanks using Provasoli's medium (ESI_{SP} 15 ml l^{-1}). Supplementary light was provided by day light lamps with an irradiance of 100 μE m^2 s^{-1} at a light:dark cycle of 14:10 h. Temperature was kept between 20 and 22 °C.

2.2. Plant exposure

Fifteen grams (fresh weight-FW) *C. demersum* were exposed separately to 1,2-DCB and 1,4-DCB at concentrations of 0.5, 1, 5, 10 and 20 mg l^{-1} in 1 l Provasoli's medium for 24 h under the light and temperature conditions described above. Culture flasks were sealed during exposures to avoid the volatilization of DCBs. All exposures were done in quintuplicate. Stock solutions were prepared by dissolving 500 mg of the corresponding DCB isomere in 10 ml methanol. Controls were assayed using an equivalent amount of pure methanol. After exposures, plants were rinsed with ultrapure water to remove the adsorbed DCB from their surface, and then immediately shock frozen using liquid nitrogen. Frozen plants were stored at −80 °C until enzyme extraction.

2.3. Enzyme extraction

The extraction of cytosolic enzymes was done according to the method described by Pflugmacher and Steinberg (1997) with minor modifications. Briefly, we used 5 g of plant material, arising from independent exposures. Frozen plant tissue was ground to fine powder with mortar and pestle under liquid nitrogen, followed by the addition of 10 ml sodium-phosphate buffer (0.1 M, pH 6.5) containing 20% glycerol, 14 mM DTE, and 1 mM EDTA. Cell debris was removed by centrifugation at 10 000*g* for 10 min. The supernatant was centrifuged at 40 000*g* for 60 min to achieve the microsomal fraction, which was re suspended in sodium phosphate buffer (20 mM, pH 7.0 supplemented with 20% glycerol and 1.4 mM DTE), and homogenized in a glass potter. Selective protein precipitation was achieved by adding solid ammonium sulphate (35% saturation) to

the supernatant. After centrifugation at 20 000*g* for 20 min, the pellet was discarded and additional ammonium sulphate (80% saturation) was added to the supernatant. Finally, after a centrifugation step at 30 000*g* for 30 min, the pellet (containing cytosolic enzymes) was resuspended in sodium-phosphate buffer (20 mM, pH 7.0), desalted by gel filtration using NAP-10 columns (GE-Healthcare), to achieve a concentrated enzyme extract (1.5 ml) which was immediately shock frozen using liquid nitrogen, and stored at −80 °C until measurement.

2.4. Measurement of enzymatic activities

Enzymatic activities were determined by spectrophotometry. The activity of soluble glutathione *S*-transferase (sGST) was determined using 1-chloro-2,4-dinitrobenzene (CDNB) as substrate, according to Habig et al. (1974). Glutathione reductase activity (GR) was assayed according to Tanaka et al. (1994). The activity of glutathione peroxidase (GPx) was determined according to Drotar et al. (1985) using H_2O_2 as substrate. The guaiacol peroxidase activity (POD) was measured using guaiacol and hydrogen peroxide (Bergmeyer, 1983). The enzymatic activities were calculated in terms of the protein content for each sample (Bradford, 1976); they are reported in nano katals per milligram of protein (nkat mg prot^{-1}), where 1 kat is the conversion of 1 mol of substrate per second. Each enzymatic assay was carried out in triplicate.

2.5. Statistics

All values are expressed as mean ± standard deviation. Normal distribution for data was analyzed by Shapiro–Willks test. One-way ANOVA was carried out to determine whether treatments were significantly different from control group ($p < 0.01$).

3. Results

3.1. Glutathione S-transferase

The dose–response curves for s-GST were determined in extracts arising from plants exposed for 24 h to 1,2 and 1,4-DCB.

We observed a significant elevation of s-GST activity in *C. demersum* exposed to 10 and 20 mg l^{-1} 1,2-DCB with no significant changes at the lowest applied doses (Fig. 1a). The exposure of *C. demersum* to 10 mg l^{-1} 1,4-DCB also caused a significant increase in s-GST; however, a significant inhibition was observed at the lowest doses (0.5 and 1 mg l^{-1} 1,4-DCB) (Fig. 2a).

3.2. Guaiacol peroxidase

The activity of guaiacol peroxidase (POD) was significantly increased in plants exposed to both isomers at 5, 10 and 20 mg l^{-1} (Figs. 1a and 2a).

3.3. Glutathione reductase

The activity of GR in *C. demersum* exposed to 5 mg l^{-1} 1,2-DCB was significantly increased (Fig. 1b). The exposure of *C. demersum* to 10 mg l^{-1} 1,4-DCB also caused a significant increase of GR in comparison to controls. Similarly to s-GST, the exposure of *C. demersum* to 1 mg l^{-1} 1,4-DCB caused a significant decrease of GR (Fig. 2b).

3.4. Glutathione peroxidase

The GPx activity was significantly increased in plants exposed to 10 mg l^{-1} of both isomers (1,2- and 1,4-DCB) (Figs. 1b and 2b). Similarly to s-GST and GR, GPx showed a significant decrease in activity at the lowest applied concentrations of 1,4-DCB (0.5 and 1 mg l^{-1}) (Fig. 2b).

4. Discussion

Organisms exposed to pollution show diverse stress responses, from structural to biochemical changes. The present study analyzes the performance of metabolic enzymes in *C. demersum* experimentally exposed to 1,2-DCB and 1,4-DCB.

From Figs. 1a and 2a, it is evident that s-GST shows a dose-dependent response pattern to DCBs, with significant activation at 10 mg l^{-1} in both cases. This suggests that either DCBs or their metabolites are being conjugated to glutathione in the phase II, mediated by GST. Our present results are coincident with those reported by Qian et al. (2004) in fish (*Carassius auratus*) exposed to DCBs by i.p. injection. Glutathione-DCB conjugates were also reported by Hissink et al. (1996) in hepatic human and rat microsomes. Additionally, Wang et al. (1996) demonstrated the metabolism of 1,4-DCB through hydroxylation and conjugation in soybean cell cultures. Thus, it seems that *C. demersum* is also able to perform biotransformation of DCBs, or their derivatives, by conjugation to GSH.

So, the present results extend the knowledge on the metabolism of DCBs in plants, in, by identifying the participation of glutathione *S*-transferases during the phase II of detoxication within the aquatic macrophyte *C. demersum*. To the extent of our knowledge, this is the first report on the probable conjugation of DCBs to GSH in *C. demersum*. Furthermore, though the toxicity of DCBs to several aquatic species has been reported, there is no mention on the toxicity of chlorobenzenes to aquatic macrophytes (Malcolm et al., 2004).

Moreover, the production of reactive oxygen species (ROS) in cells may cause an increase of peroxides levels, which can be metabolized by the action of GPx and GST (Awasthi et al., 2004). The activity of POD prevents H_2O_2 damage to cell membranes, proteins, and DNA (Di Giulio et al., 1989). GPx also catalyzes the reduction of hydrogen peroxide (H_2O_2) to water and lipid or hydroperoxides to alcohols, thus coupled to the oxidation of GSH to

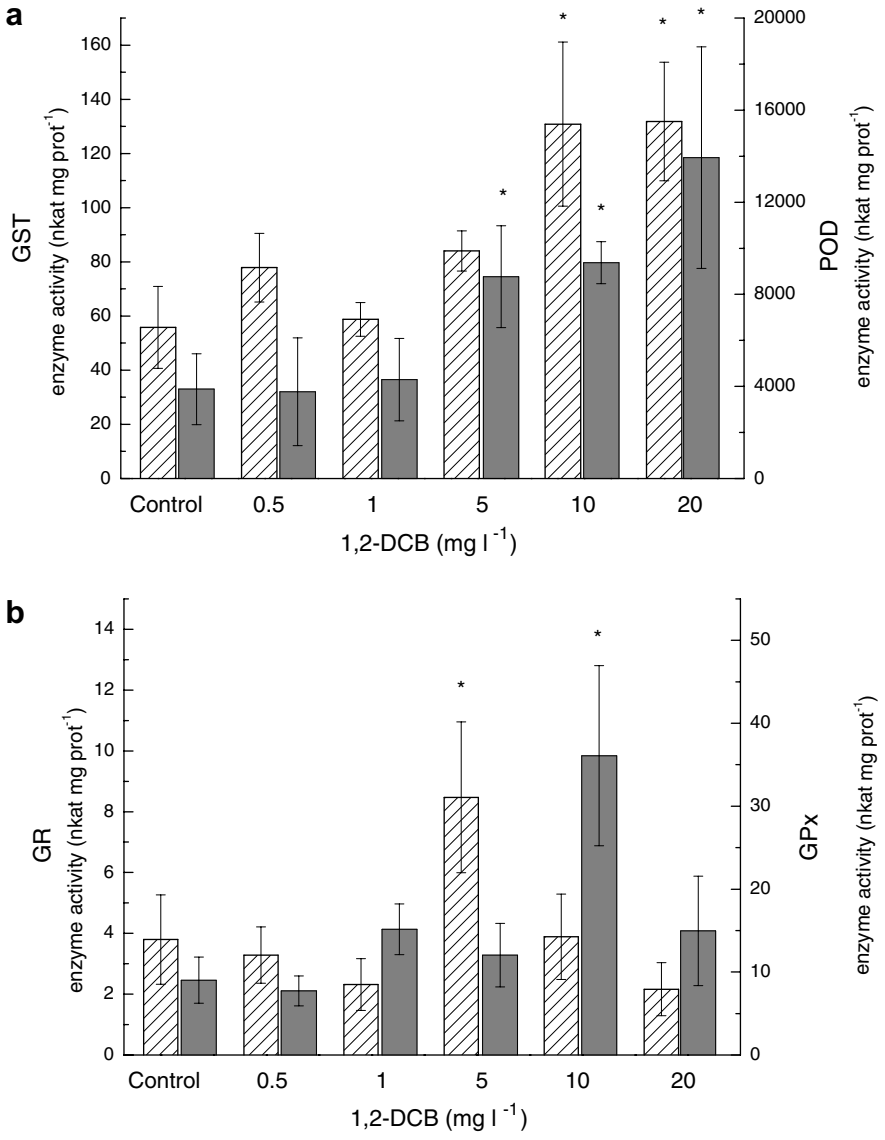

Fig. 1. Cytosolic glutathione *S*-transferase (sGST –▨–) and guaiacol peroxidase (POD –■–) activities (a); glutathione reductase (GR –▨–) and glutathione peroxidase (GPx –■–) activities (b) in *C. demersum* exposed to 1,2-DCB. Values are expressed as mean ± SD. (*) Significance levels observed are $p < 0.01$ in comparison to control group.

GSSG (Winston and Di Giulio, 1991). The key function of GR within the glutathione cycle is to maintain the GSH/GSSG ratio by recycling glutathione disulfide (GSSG) to reduced glutathione (GSH) playing a crucial role for the antioxidant system as well as for primary detoxication pathways (Mishra et al., 2006).

Several studies have demonstrated that DCBs induce the generation of reactive oxygen species (ROS) (Hissink et al., 1997; Rietjens et al., 1997; Hoglen et al., 1998; Younis et al., 2000, 2003), which may be scavenged by the antioxidant defence system. The activity of enzymes such as GPx, GR, POD, SOD and CAT prevents adverse effects of oxidative stress to cells. Oxidative stress will occur whenever the activity of the antioxidant system decrease or the production of ROS is increased (Moreno et al., 2005).

As the increased activity of antioxidant enzymes in plants indicates the formation of ROS (Pflugmacher, 2004), our survey suggests the generation of oxidative stress in *C. demersum* at the tested concentrations of both DCBs, resulting in enhanced activities for POD, GR and GPx when this macrophyte is exposed for 24 h to concentrations above 5 mg l^{-1} DCBs (Figs. 1a and b and 2a and b). A similar activity pattern has been previously reported in *C. demersum* exposed to increasing concentrations of 3-chlorobiphenyl (Menone and Pflugmacher, 2005).

Interestingly, the lowest doses of 1,4-DCB (0.5 and 1 mg l^{-1}) caused a significant drop in GST, GR and GPx activities (Figs. 2a and b), which is described in the literature as the hormesis phenomenon. Hormesis has been widely documented in pharmacological and toxicological

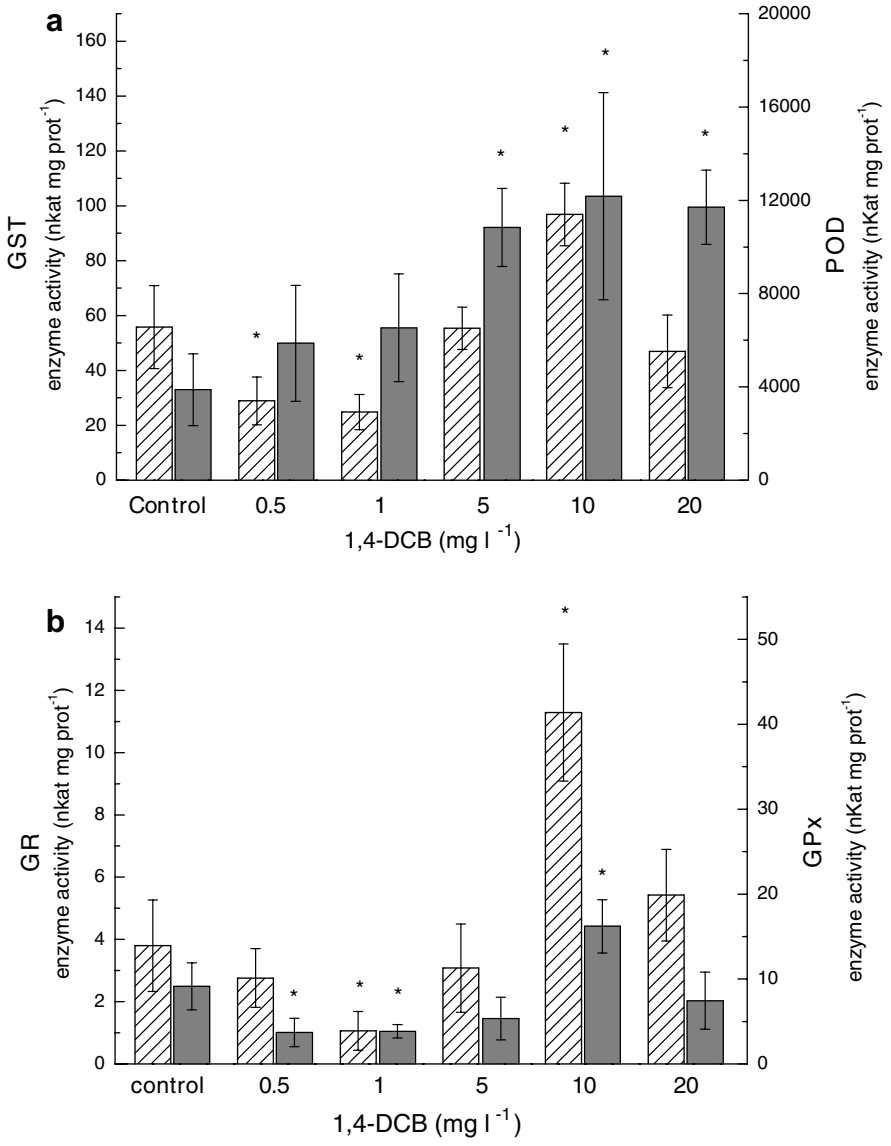

Fig. 2. Cytosolic glutathione *S*-transferase (sGST –▨–) and guaiacol peroxidase (POD –■–) activities (a); glutathione reductase (GR –▨–) and glutathione peroxidase (GPx –■–) activities (b) in *C. demersum* exposed to 1,4-DCB. Values are expressed as mean ± SD. (*) Significance levels observed are $p < 0.01$ in comparison to control group.

studies (Stebbing, 1982; Calabrese et al., 1987; Calabrese and Baldwin, 2002). The potential implications of this phenomenon for natural populations are now being considered in terms of life-history traits and ecological risk assessment (Forbes, 2000).

Hormesis has been reported in different plants exposed to heavy metals and aromatic hydrocarbons (Stebbing, 1982; Calabrese et al., 1987). Our results are consistent with other studies, reporting hormesis during the growth of four different algae species exposed to DCBs (Calabrese and Baldwin, 2002).

5. Conclusions

To the extent of our knowledge, this is the first report on the toxic effects of chlorobenzenes on aquatic macrophytes, like *C. demersum*. Our results are in line with previous reports, demonstrating the toxicity of chorobenzenes derivatives on different aquatic species (diatom, invertebrates and fish) (Malcolm et al., 2004).

Our present results also gives indirect evidences on the involvement of the GSH-mediated detoxification of DCBs in *C. demersum*, which are in good agreement with results obtained from other plants exposed to these and similar xenobiotics.

The increased activity of antioxidant enzymes in plants indicates the formation of ROS (Pflugmacher, 2004). Considering our present results, it is clear that the exposition of *C. demersum* to DCBs caused a general activation of its antioxidant system in response to oxidative stress, scavenging ROS, and protecting the plant from adverse oxidative effects of DCBs.

Exposure of *C. demersum* to relatively low concentrations of 1,4-DCB had a hormetic effect on the enzymatic activity of plants (GST, GR and GPx), although this phenomenon was not observed upon exposure to 1,2-DCB.

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