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Evaluation of photosynthetic oxygen production, pigment pattern, glutathione content and detoxication enzymes activity in the aquatic macrophyte *Ceratophyllum demersum* exposed to cypermethrin

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Summary

Cypermethrin is a widespread pyrethroid that can be very toxic for certain taxa, but there is a lack of information concerning its effects within the plant kingdom. Exposures to different concentrations as well as at different times were carried out in the aquatic macrophyte *Ceratophyllum demersum* in order to evaluate effects on photosynthetic oxygen production, chlorophyll a and b, total glutathione content and detoxication enzymes. Activity of guaiacol peroxidase (POD), glutathione reductase (GR), microsomal and cytosolic glutathione S-transferases (m- and cGST, respectively, from phase II of metabolism) were measured.

No significant effects on photosynthetic oxygen production or on chlorophyll content were found. On the other hand, there was a noticeable activation of POD at the higher concentrations tested and the time-response curve for 50 µg/L showed that its activation was significant until day 3 in comparison to the controls. At 0.5-5 µg L^{-1} of cypermethrin the activity of cGST, mGST and GR enzymes as well as the total GSH content were not significantly different from the controls. At 50 µg L^{-1} mGST was significantly activated but the activity of cGST and total GSH content were not different from the controls. GR was not activated at any concentration or time tested. Although some detoxication responses at day 3 of exposure in the physiological system of *C. demersum* when exposed to a high dose of cypermethrin seemed to happen, it appears that environmentally relevant concentrations of this insecticide do not result in any significant damage to this aquatic macrophyte.

Introduction

Cypermethrin was first sold in the late 1970s and is now used throughout the world on a wide range of crops due to its cost-effective control of insects and its low mammalian toxicity relative to other insecticides (FARMER et al., 1995). Although pyrethroid insecticides pose a serious threat to non-target animals like fish and aquatic invertebrates in downstream aquatic receiving systems (DAVIES et al., 1994) assessment of exposure and effects of these compounds on plants have hardly been explored.

Aquatic macrophytes are able to absorb pyrethroids from the water column in great extent and could have a considerable influence on the fate and behaviour of these insecticides in surface waters (HAND et al., 2001; MOORE et al., 2001). In this work the submerged aquatic macrophyte *Ceratophyllum demersum* was used as the bioassay organism because of its worldwide distribution and importance in the aquatic ecosystem. It is sensitive to other stressors such as pure humic substances (PFLUGMACHER et al., 1999), and Cd (ARAVIND and PRASAD, 2004) and it can absorb organic pollutants like cypermethrin directly from the water column (HAND et al., 2001).

Plant metabolic sequences of transformation – mostly done by cytochrome P-450 monooxygenases (phase I), conjugation through glutatione S-transferases and glucosyltransferases (phase II), and internal compartmentation reactions mostly in cell wall fractions or in the vacuole (phase III) – have been summarized by SANDERMANN (1994). Peroxidase enzymes (POD) are widely distributed in plants and they catalyse degradation of natural substrates as well as xenobiotics. They have been suggested as potential biomarkers of stress since their activity is increased in several plant species exposed to a variety of stressors and they are thought to protect cells from free radical oxidation (SIESKO et al., 1997). Another enzyme that plays an important antioxidant role in animals and plants is the glutathione reductase (GR), by catalysing the reduction of oxidised glutathione (GSSG) to GSH at the expense of NADPH (RAND, 1995), maintaining a high GSH/GSSG ratio.

Regarding enzymes of phase II of metabolism, the glutathione S-transferases (GST) are a family of proteins that detoxify a wide variety of xenobiotics in addition to their natural substrates (SANDERMANN, 1994; DIXON et al., 1998). They catalyse the conjugation of electrophilic and frequently hydrophobic toxic compounds with glutathione to form non-toxic peptide derivatives (DIXON et al., 1998). The tripeptide, L-γ-glutamyl-L-cysteinyl-glycine, or GSH, is the major low molecular-mass thiol compound in plants and animals and act as a cellular thiol „redox buffer" to maintain a given thiol/disulfide redox potential. Its role in the detoxication of many pesticides has been established (RENNENBERG, 1982).

Our general hypothesis in this study was that *C. demersum* is under physiological stress when exposed to cypermethrin and that it would be able to detoxify this pyrethroid through the activation of peroxidases and glutathione conjugation.

The aims of this study were a) to establish a dose-response curve after a short time exposure to cypermethrin, b) to establish a time-response curve after a heavy exposure to cypermethrin and c) to analyse the metabolism of cypermethrin in *C. demersum*.

Materials and methods

Plant material

Ceratophyllum demersum was collected from the Möllensee near Berlin; species identification was done according to CASPER and KRAUSCH (1980). It was cultivated axenically prior to the experiments for some months in Provasoli's medium (ESI $_{SP}$ 15ml L^{-1}) in 100 L tanks. Supplementary light was provided by daylight lamps with an irradiance of 12 µE m^{-2} s at a light/dark cycle of 14:10 hours. Temperature was maintained at 20-22 °C.

Plant exposure

Dose-response curves

To assess biochemical endpoints like photosynthesis, chlorophyll pattern and detoxication enzyme activities for objective a), samples of *C. demersum* were exposed to different concentrations (environmentally relevant and higher) of cypermethrin. For objective b), exposures to cypermethrin for 2 and 4 hours, and 1, 2, 3, 4 and 5 days were carried out. For objective c), the total glutathione content, activation of GR and GST were analysed.

Approximately 10 g freshweight (FW) of *C. demersum* was exposed to concentrations of 0.5, 5, 50, 500 or 1,000 µg L^{-1} of cypermethrin ([S,R]-α-cyano-3-phenoxybenzyl(1R,1S,*cis*,*trans*)-2,2-dimethyl-3-(2,2´- dichlorovinyl)cyclopropanecarboxylate) (Fluka, Germany) in a volume of 1.5 L each for 24 h under constant conditions concerning light and temperature. All exposures were done in quintuplicate. The concentrations tested in this study were environmentally relevant and in higher loads. The test substance was dissolved in acetonitrile (Roth, Germany) in a stock solution of 10 g L^{-1}. Aliquots of 5, 50 and 100 µl were taken to prepare 1L exposure solutions of 50, 500 and 1,000 µg L^{-1}, respectively. A solution „B" of 100 mg L^{-1} was prepared from the stock solution. Aliquots of 5 and 50 µl from solution „B" were taken to prepare 1L exposure solutions of 0.5 and 5 µg L^{-1}. In the control experiments cypermethrin was left out of the medium but pure acetonitrile was added. The acetonitrile in water solutions never exceeded 0.005 % (50 µg L^{-1}).

Time-response curves

Approximately 10 g freshweight (FW) of *C. demersum* were exposed to 50 µg L^{-1} of cypermethrin for 2 and 4 hours, and 1, 2, 3, 4 and 5 days in a volume of 1.5 L each under constant conditions concerning light and temperature. Five to ten independent exposures per treatment were done. In order to test toxicity, the concentration used was higher than the environmentally relevant ones (0.5- 5 µg L^{-1}) since these did not conferred significant damage to the plants.

Measurement of photosynthetic oxygen production

The measurement of photosynthetic oxygen production of the plants was performed using a Phosy-Mess 4000 (InnoConcept, Straussberg), 100 % light intensity (2000 lx) and a dark/light/dark cycle of 10/10/10 min under constant temperature of 20 °C. Measurement were taken with a Clark electrode (WTW EO 196-1,5). Photosynthetic oxygen production rates were calculated in µmoles O_2 min^{-1} g^{-1} FW. Measurements of photosynthetic oxygen production were performed using approximately 0.1 g FW of *C. demersum* in 5 independent replicates for the dose-response curve.

Measurement of pigments

Analysis of chlorophyll a and b content was done according to INSKEEP and BLOMM (1985) using 0.1 mg of plant material and 1 ml N,N-dimethylformamide (N,N-DMF). Samples were kept in darkness at 4° C for 3 days, until the plant material was completely discoloured. The absorption of the extracts at O.D.= 647 and 664.5 nm was measured against N,N-DMF. The following terms were used for calculation of chlorophyll content:

Chlorophyll a = [12.7 x O.D. $_{664.5}$] - [2.79 x O.D. $_{647}$]
Chlorophyll b = [20.7 x O.D. $_{647}$] - [4.62 x O.D. $_{664.5}$]

All pigment analysis was performed by 5 to 10 independent replicates.

Enzyme preparation

Preparation of microsomal and soluble enzymes was done according to PFLUGMACHER and STEINBERG (1997). 5 Gram of plant material was used in five independent preparations. Plants were ground to a fine powder with mortar and pestle under liquid nitrogen and next 10 ml of sodium-phosphate buffer (0.1 M, pH 6.5), containing 20 % glycerol, 14 mM of DTE, 1 mM of EDTA was added. Cell debris was removed by centrifuging at 10,000 G for 10 min. The supernatant was centrifuged again at 40,000 G for 60 min to obtain the membrane fraction, defined as the microsomes. The microsomes were re-suspended in sodium-phosphate buffer (20 mM, pH 7.0) containing 20 % glycerol and 1.4 mM of DTE, and homogenized in a glass potter. Solid ammonium-sulphate was added to the supernatant to a concentration of 35 % saturation. After centrifugation at 20,000 G for 20 min, the pellet was discarded and ammonium-sulphate was added to the supernatant to a saturation of 80 %. After centrifugation at 30,000 G for 30 min, the pellet (containing the soluble proteins) was resuspended in sodium-phosphate buffer (20 mM, pH 7.0) and desalted by gel filtration on NAP-10 columns.

Enzyme activity measurement

All enzyme measurements were done in triplicates of five independent samples. Protein determination in the microsomal and soluble fractions was done according to BRADFORD (1976) using bovine serum albumin as standard. Enzyme activity was calculated in nanokatals per milligram of protein from percent substrate conversion.
Measurement of the peroxidase in the soluble protein fraction using guaiacol as substrate was performed as described by DROTAR et al. (1985). Determination of microsomal and soluble glutathione S-transferase activity with the model substrate 1-chloro-2, 4-dinitrobenzene (CDNB) was done according to HABIG et al. (1974). The glutathione reductase activity was measured in the soluble protein fraction according to TANAKA et al. (1994), spectrophotometrically via the reduction in absorbance due to the reduction of NADPH in the reaction mixture at 340 nm.

Total glutathione content

Approximately 1 g of fresh pre-exposed plant material was used for the crude extract preparation. The material was grounded under liquid nitrogen and extracted with 5 volumes of sulfosalycilic acid. The slurry was centrifuged at 4 °C at 18,000 G for 12 min and the total GHS content was determined in the supernatant fraction. Five independent samples were prepared per each exposure experiment. Total glutathione was assayed by the glutathione reductase recycling assay as described by ANDERSON (1985). The following mix was prepared and pre-warmed at room temperature for 15 min: 700 µl NADPH, 100 µl 5,5'-dithiobis 2-nitrobenzoic acid (DTNB) and 165 µl water. NADPH (0.248 mg ml^{-1}) and DTNB (6 mM) were both prepared in 143 mM Na-phosphate buffer containing 6,3 mM Na_4-EDTA, pH 7.5. The sample (25 µl) was added to the reaction mix and the reaction was initiated by adding 10 µl of GSH reductase (Sigma, Germany) diluted to 266 units ml^{-1} in Na-phosphate-EDTA buffer, pH 7.5. The rate of stoichiometric formation of 5-thio 2-nitrobenzoic acid (TNB) from DTNB was followed at 412 nm, in triplicate, recording the absorbency for 2 min. The calibration curve was made using different concentrations of GSH (Sigma, Germany).

Statistics

The null hypothesis of no difference in photosynthetic oxygen production or pigment contents (and the activity of POD, mGST, cGST or GR) between samples exposed to various concentrations of cypermethrin was tested with one-way analysis of variance (ANOVA) (ZAR, 1999). A Tukey test was used to test which treatments differed from each other if a significant difference between treatments was detected.

Results

Photosynthetic oxygen production

The photosynthetic oxygen production of *C. demersum* after 24 h of exposure to cypermethrin decreased by 35 to 45 % (Fig. 1), although this decrease was not statistically significant ($F_{5,24} = 2.13$, $p = 0.096$).

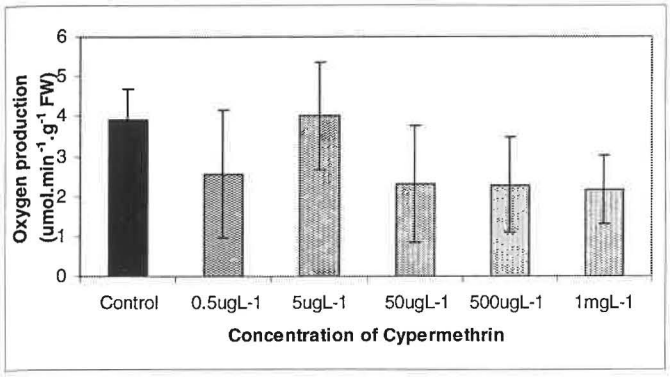
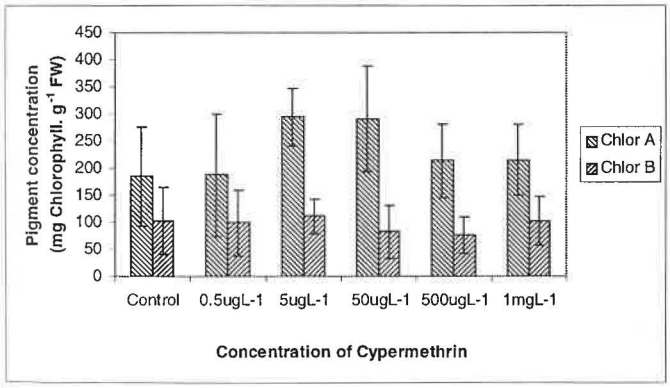


Fig. 1: Dose - response curve of the oxygen production in *Ceratophyllum demersum* exposed to cypermethrin. The data shows means and standard deviation for n = 5.

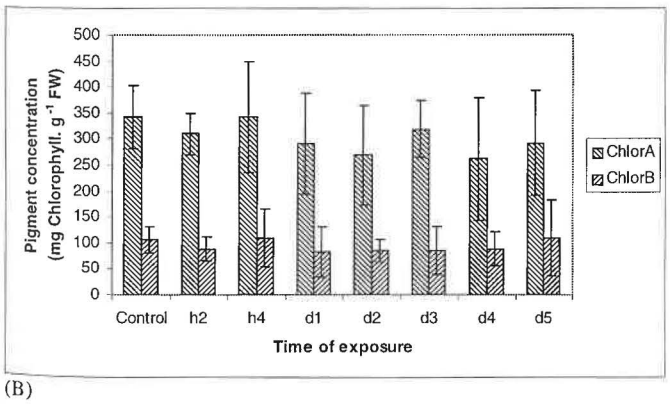
Pigment Pattern

Chlorophyll a and b levels did not change at the concentration of cypermethrin tested compared to controls (Fig. 2 a): there were no significant differences among treatments (Chlorophyll a: $F_{5,24} = 1.843$, $p = 0.142$; Chlorophyll b: $F_{5,24} = 0.540$, $p = 0.744$).

The time-response curve also showed no effect on the concentrations of these two pigments since there were no significant differences between exposed and control samples (Chlorophyll a: $F_{7,54} = 0.624$, $p = 0.733$; Chlorophyll b: $F_{7,54} = 1.204$, $p = 0.316$) (Fig. 2b). The ratio of chlorophyll a to chlorophyll b did not change at any dose or at any time of exposure.



(A)



(B)

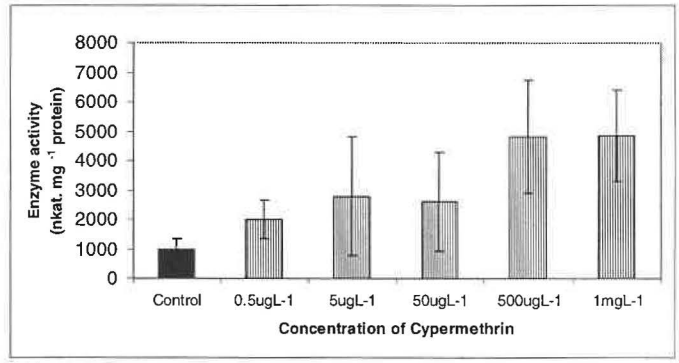
Fig. 2: Chlorophyll a and b concentration in *Ceratophyllum demersum* exposed to cypermethrin. (A) Dose - response curve, (B) time-response curve. The data shows means and standard deviation for n= 5. h2: 2 hours, h4: 4 hours, d1: day 1, d2: day 2, d3: day 3, d4: day 4 and d5: day 5.

Biotransformation enzymes

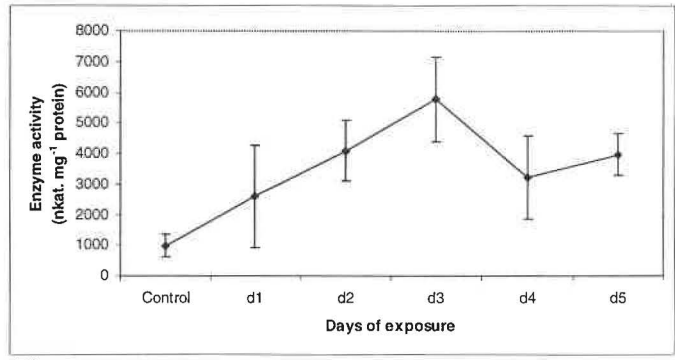
Peroxidase

The dose-response curve for guaiacol-peroxidase (POD) is shown in Fig. 3a. ANOVA test showed differences between control and exposed samples ($F_{5,23} = 5.387$, $p = 0.002$). There was a noticeable activation of this enzyme at all the concentrations tested although only significantly at 500 and 1,000 µg L^{-1} of cypermethrin (Tukey test, $p < 0.05$).

The time-response curve showed significant differences among treatments ($F_{5,22} = 5.903$, $p = 0.001$). A significant activation of POD at days 2 and 3 ($p < 0.05$) in exposed samples was observed. At days 4 and 5 activity decreased and no significant differences with the control samples were observed ($p > 0.05$) (Fig. 3b).



(A)



(B)

Fig. 3: Guaiacol-peroxidase (POD) activity in *Ceratophyllum demersum* after exposure to cypermethrin. (A) Dose - response curve, (B) time-response curve at 50 µg L^{-1} cypermethrin. The data shows means and standard deviation for n= 5. d1: day 1, d2: day 2, d3: day 3, d4: day 4 and d5: day 5.

Glutathione S-transferase

ANOVA test showed significant differences in the dose-response curve for mGST ($F_{5,19} = 6.786$, $p = 0.001$). Plants exposed to 0.5, 5, 500 and 1,000 µg L^{-1} of cypermethrin did not show significantly enhanced mGST activity ($p > 0.05$) but significant activation at 50 µg L^{-1} of cypermethrin was found ($p < 0.05$) (Fig. 4a). The time-response curve showed a significant activation of mGST at day 1 ($F_{5,20} = 13.112$, $p < 0.05$, Tukey test $p < 0.05$) but from day 2 to 5 the

activity decreased and was not significantly different from the controls ($p < 0.05$) (Fig 4b). There were significant differences in activity at days 2 to 5 compared to day 1 ($p < 0.05$).
The cGST activity was slightly enhanced in exposed samples at all concentrations tested but it was not significantly different from the control samples ($F_{5,22} = 0.422$, $p = 0.828$) (Fig. 4c). The activity of the enzyme in exposed samples was also not different from the controls at all the times tested ($F_{5,21} = 1.053$, $p = 0.413$) (Fig. 4d).

Glutathione Reductase

No significant activation of GR activity at any concentration or time of exposure tested was observed (dose-response curve, $F_{5,22} = 0.495$, $p = 0.776$; time- response curve, $F_{5,21} = 0.693$, $p = 0.634$).

Total glutathione content

The total GSH content in control plants was 8.9 (SD = 1.5) nMol g^{-1}FW; in plants exposed to 0.5 µg L^{-1} of cypermethrin it was 10.6 (SD = 1.4) nMol g^{-1}FW, and at 50 µg L^{-1} it was 10.1 (SD= 2.9) nMol g^{-1}FW, but these differences were not statistically different ($F_{2,12} = 0.846$, $p = 0.453$). The time-response curve showed values between 7.2 (SD= 1.9) and 12.7 (SD= 4.4) nMol g^{-1}FW in exposed plants (Fig. 6) but these values were not significantly different from control values ($F_{4,20} = 2.305$, $p = 0.094$) (Fig. 6).

Discussion

It has been widely recognized that aquatic organisms are less likely to be affected by pyrethroids under field conditions. The amelioration of effects results from a reduction in exposure because of the tendency of this group of insecticides to bind rapidly and extensively to suspended particulate matter, sediments and aquatic plants (MAUND et al., 2002). From this study it appears that cypermethrin does not inflict any significant damage to *C. demersum* exposed to environmentally relevant concentrations, since there were no consistent effects on any of the biochemical endpoints analysed in the plants. Only at higher concentrations some biotransformation responses occurred over the course of 5 days of exposure. At day 3 of exposure, POD reached its (statistically significant) maximum activity, while the activity of GR, m- and cGST as well as the total GST content did not show any significant change. These results could indicate a biotransformation response via peroxidases in the physiological system of *C. demersum* when exposed to a high dose of cypermethrin. The use of the GSH pool and the related enzymes do not seem to be involved in the biotransformation of this insecticide. One of the most commonly used functional endpoints in aquatic plants under stress conditions is the photosynthetic efficiency (WHITE et al., 1996; SIESKO et al., 1997; PADINHA et al., 2000). Despite a slight variation in the oxygen production, no significantly different values with the controls were observed for any of the concentrations of cypermethrin tested. The time-response curve also showed no significant changes at 2 and 4 hours, 1, 2, 3, 4 and 5 days of exposure (data not shown). From these observations it seemed that this

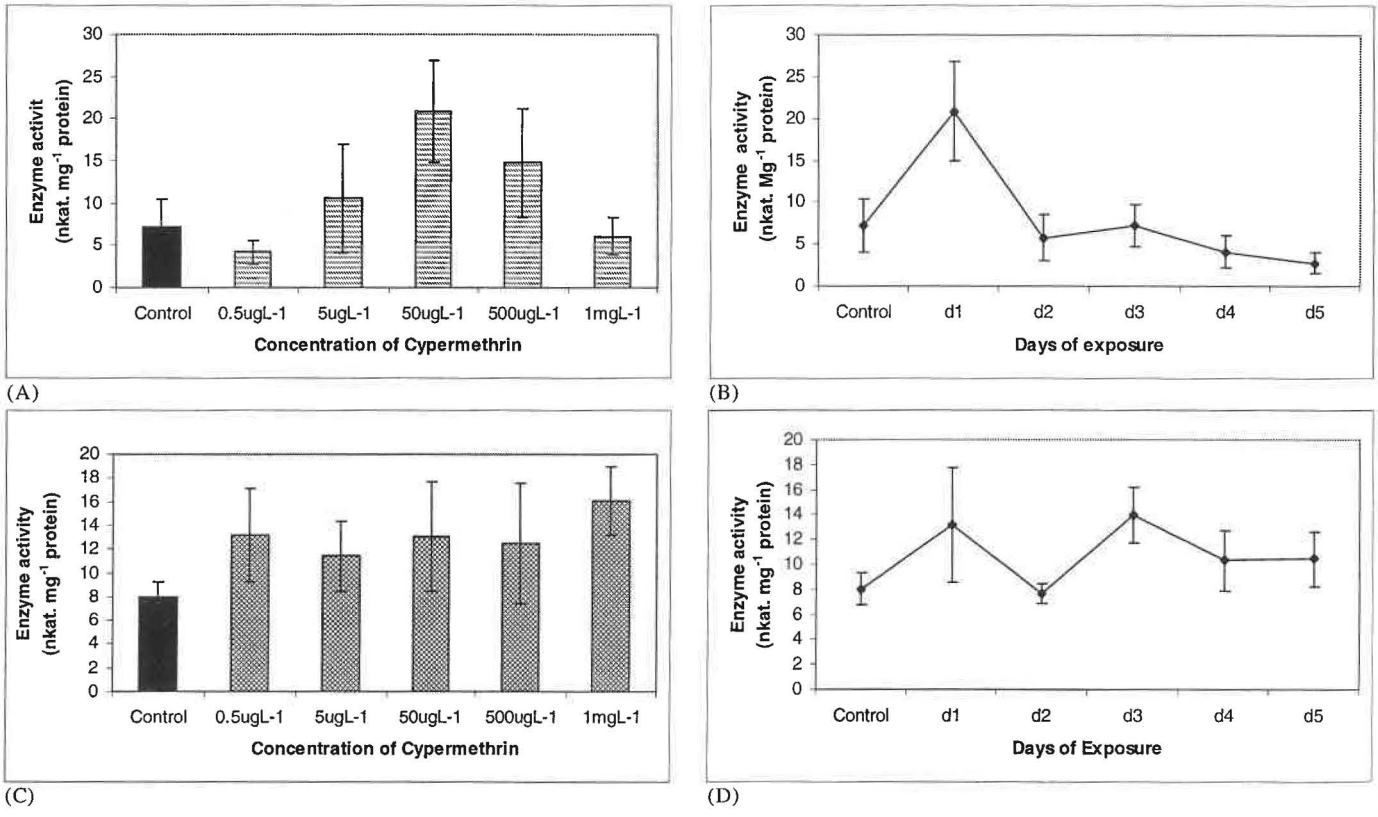


Fig. 4: CDNB- Glutathione-S-transferase activities in *Ceratophyllum demersum* after exposure to cypermethrin. (A) Dose - response curve in the microsomal fraction, (B) time-response curve in the microsomal fraction at 50 µg L^{-1} cypermethrin, (C) dose - response curve in the cytosolic fraction, (D) time-response curve in the cytosolic fraction at 50 µg L^{-1} cypermethrin. The data shows means and standard deviation for n= 5. d1: day 1, d2: day 2, d3: day 3, d4: day 4 and d5: day 5.

insecticide is not a blocking agent of the electron transport through photosystem II in *C. demersum*. Although the pigment pattern has been recognized as a sensitive indicator of a physiologic stress response to some pollutants in plants (LYTLE and LYTLE, 1998), in this study the dose- and time-response curves for chlorophyll a and chlorophyll b did not present any significant changes. Previous studies with cypermethrin have shown that there were no adverse effects on algal chlorophyll content or productivity (FARMER et al., 1995), although some authors attributed the lack of statistically significant effects to the high variability of this endpoint (HANSON et al., 2002). In this work, it is unlikely that cypermethrin is causing effects on photosynthesis and pigment pattern in *C. demersum* exposed to environmental concentrations of cypermethrin.

Activation of biotransformation enzymes after exposure to dissolved organic matter (PFLUGMACHER et al., 1999a), microcystin-LR (PFLUGMACHER et al., 1999b) and Cu (DEVI and PRASAD, 1998) have been previously reported in *C. demersum*. In this study, mainly guajacol POD was significantly activated when plants were exposed to high concentrations of cypermethrin. POD is ubiquitous in plants and it has been shown to respond to various stressful conditions (e.g. exposure to various metals, air pollutants, growth regulators and aromatic ring structures; BYL et al., 1994). It has been proposed as one possible protective mechanism against some organic pollutants, since those macrophytes that exhibited the highest POD activity also showed the highest tolerance to toxins (LYTLE and LYTLE, 1998).

POD is also included among the enzymatic antioxidants of the plants protecting cells from toxic oxygen species. Therefore, it is plausible that oxidative damage might be occurring. On the other hand, GR is also included in this type of defence as it is involved in the regeneration of the reduced form of the antioxidant glutathione. However, this study did not show any effect of exposure to cypermethrin on GR activity. The lack of significant changes in mGST, cGST and GR activities as well as in total glutathione content found in this work indicated that cypermethrin was not biotransformed in *C. demersum* through conjugation with glutathione. In animals, significant hepatic GST activities have been shown in the fish *O. mykiss* at concentrations of cypermethrin up to 0.87 $\mu g\ L^{-1}$ (DAVIES et al., 1994) and in the shore crab *Carcinus maenas* at 0.5 and 5 $\mu g\ L^{-1}$ (GOWLAND et al., 2002). In plants, reports concerning biotransformation of pyrethroids have shown that the major route of degradation of cypermethrin was the hydrolysis of the ester linkage followed by the formation of conjugates of the 3-(2,2'-dichlorovinyl)-2,2-dimethylcyclopropanecarboxylic acid with carbon hydrates (WRIGHT et al., 1980). The formation of glycoside conjugates in plants like the β-D-glucopyranose ester from cypermethrin in lettuce (WRIGHT et al., 1980) has also been detected for other pyretroids such as fenvalerate (MIKAMI et al., 1985) and seems to be the main route of biotransformation of these insecticides.

Although this work shows a lack of significant effects of exposure to cypermethrin in *C. demersum,* further studies are necessary in order to understand the mechanisms of biotransformation that confer resistance to this pyrethroid. This study indicates that cypermethrin, at typical concentrations occurring in areas receiving spray drift from agricultural usage, should pose no significant damage on *C. demersum.*

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