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The applicability of acetylcholinesterase and glutathione S-transferase in *Daphnia magna* toxicity test

Anita Jemec [a], Damjana Drobne [b], Tatjana Tišler [a], Polonca Trebše [c], Milenko Roš [a], Kristina Sepčić [b,*]

[a] *National Institute of Chemistry, Hajdrihova 19, SI-1000 Ljubljana, Slovenia*
[b] *University of Ljubljana, Biotechnical Faculty, Department of Biology, Večna pot 111, SI-1000 Ljubljana, Slovenia*
[c] *University of Nova Gorica, Laboratory for Environmental Research, P.O.B. 301, SI-5001 Nova Gorica, Slovenia*

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Abstract

The most commonly used toxicity test worldwide is the acute *Daphnia magna* test. The relevance of acetylcholinesterase (AChE) and glutathione S-transferase (GST) activity in *D. magna* exposed to chromium, cadmium, and diazinon was evaluated in connection with this standard test. We found no link between enzyme activities and immobility. Concentrations of Cr^{6+} up to 280 μg/L had no effect on AChE and GST activities, while 20% immobility was observed. At concentrations of 20–25 μg/L of Cd^{2+} AChE activity was increased by about 50%. The effect of diazinon on both enzymes was insignificant up to concentrations that caused 27% immobility. Consequently, while the use of AChE and GST activities is recommended when the mode of action of chemicals is studied, the value of these biomarkers in routine acute toxicity tests is limited because the relationship between enzyme activities and immobility of *D. magna* exposed to different chemicals is unclear.

Keywords: Acetylcholinesterase (AChE); Cadmium; Diazinon; Glutathione S-transferase (GST); Hexavalent chromium; Standard toxicity test

1. Introduction

The interest in the use of biomarkers in environmental risk assessment (ERA) has been increasing steadily (Adams, 2002). Allan et al. (2006) recently proposed the use of biomarkers on sub-individual (e.g. biochemical) level in water quality monitoring regulated by the European Union's Water Framework Directive (Directive 2000/60/EC, 2000) in order to "provide more realistic assessment of impacts and exposure of aquatic organisms to specific contaminants present in water". Important characteristics of biochemical biomarkers for the use in ERA are: sensitivity, specificity, simplicity of measurement and a clear linkage to higher level effects (Peakall and Walker, 1994; Peakall, 1999; Walker, 1999).

Water flea *Daphnia magna* is a standardised test organism and has been widely used in toxicity tests for 40 years (Crosby et al., 1966). A number of biochemical biomarkers have been studied in *D. magna* (Day and Scott, 1990; Gälli et al., 1994; Guilhermino et al., 1996a; Bond and Bradley, 1997; Sturm and Hansen, 1999; De Coen et al., 2001; Diamantino et al., 2001; Printes and Callaghan, 2003; Meems et al., 2004; Barata et al., 2005). However, several authors have concluded that the immobility after acute exposure in this organism is not always directly correlated with the degree of enzyme (e.g., acetylcholinesterase, AChE) inhibition (Day and Scott, 1990; Gälli et al., 1994; Printes and Callaghan, 2004). In order to perform enzyme analyses routinely after standard acute toxicity test (EN ISO 6341, 1996), the linkage of biochemical biomarkers to immobility needs to be better established. This study, consequently, was to evaluate the usefulness of two commonly studied biochemical biomarkers AChE and glutathione S-transferase (GST) when measured after this routine acute toxicity test.

* Corresponding author. Tel.: +386 1423 33 88; fax: +386 1257 33 90.
E-mail address: kristina.sepcic@bf.uni-lj.si (K. Sepčić).

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The following ecotoxicologically important chemicals were studied: hexavalent chromium (Cr^{6+}), divalent cadmium (Cd^{2+}), and organophosphate diazinon. Cr^{6+}(in the form of potassium dichromate) is a reference chemical in standard acute *D. magna* test and is used to check the sensitivity of *D. magna* and to confirm the validity of the test procedure (EN ISO 6341, 1996). $Cd^{2+}$is a common pollutant in a variety of aquatic environments. Diazinon is one of the most used insecticides in the last 50 years and is currently a subject to phased revocation in many countries because unacceptable risk to environment was proved (US EPA, 2004). To our knowledge, only the effects of Cr^{6+} (Diamantino et al., 2000) and Cd^{2+}(Guilhermino et al., 1996b) on AChE, and Cd^{2+} on GST (Barata et al., 2005) in *D. magna* have previously been presented. Based on previous literature data, the effects of both metals on AChE and GST activities (Stohs and Bagchi, 1995; Payne et al., 1996; Guilhermino et al., 1998) were expected, as well as the inhibition of AChE activity (Booth and O'Halloran, 2001) and induction of GST activity (Chambers, 1992) in animals exposed to diazinon.

AChE (E.C.3.1.1.7.) enables hydrolysis of the neurotransmitter acetylcholine in cholinergic nerves. Inhibition of AChE, resulting in overaccumulation of acetylcholine and prolonged electrical activity at nerve endings, comprises a key mechanism of toxicity for organophosphorus and carbamate pesticides (Day and Scott, 1990; Gälli et al., 1994; Guilhermino et al., 1996b). GST (E.C.2.5.1.18) belongs to the class of phase II detoxifying enzymes that catalyse the conjugation of glutathione with xenobiotics, among them organophosphorus pesticides (Booth and O'Halloran, 2001). Induction of GST activity is an indication of a detoxification process. GST also catalyses the conjugation of glutathione with cytotoxic aldehydes produced during lipid peroxidation (Halliwell and Gutteridge, 1999).

The aims of this study were (1) to evaluate the applicability of AChE and GST assays in connection with routine standard acute toxicity tests, (2) to compare the effects of Cr^{6+}, Cd^{2+}, and diazinon on AChE and GST activities in *D. magna*, and (3) to assess the linkage of selected enzyme activities to immobility.

2. Materials and methods

2.1. Chemicals

The following chemicals were obtained from Sigma (Germany): dibasic and monobasic potassium phosphate, 1-chloro-2,4-dinitrobenzene, L-glutathione (reduced form), 5,5′ dithiobis-2-nitrobenzoic acid, sodium hydrogen carbonate, acetylthiocholine chloride, sodium sulphate and ethylenediaminetetraacetic acid. BCA Protein Assay Reagent A, BCA Protein Assay Reagent B, cadmium chloride, and potassium dichromate were purchased from Pierce (USA). Diazinon and ethyl acetate were provided by Pestanal, Riedel-de Haën (Seelze, Germany). All chemicals were of the highest commercially available grade.

2.2. Test organism D. magna Straus 1820 (water flea)

Water fleas (*D. magna* Straus 1820) were obtained from the Institut fur Wasser, Boden und Lufthygiene, des Umweltbundesamtes (Berlin). Daphnids were held in 3-L aquariums containing 2.5 L of modified M4 media (Kühn et al., 1989) at a constant room temperature of 21 ± 1 °C. The 16:8 h light/dark regime was maintained by illuminating the animals with fluorescent bulbs (1800 lx). Daphnids were fed a diet of the algae *Scenedesmus subspicatus* Chodat 1926 corresponding to 0.13 mg carbon/daphnia per day.

2.3. Acute toxicity tests

Our laboratory is accredited according to ISO/IEC 17025 (1999) for standard acute testing with *D. magna*. All toxicity tests were carried out according to EN ISO 6341 (1996) in ISO 6341, Cor.1 (98) (E). Neonates less than 24 h old, derived from the second to fifth brood, were transferred into the test containers with increasing concentrations of chemicals. Controls containing only dilution water (defined in EN ISO 6341, 1996) without chemicals were included in all experiments. Prior to tests followed by enzyme analyses, we performed several range-finding tests in order to define 48-h EC_{10} and EC_{50} values.

For the enzyme analyses, 10 test containers containing 20 daphnids/50 mL of test solution were prepared for each concentration of chemical. Each acute toxicity test was repeated at least 3 times (Tišler, 1992). Concentrations of the chemicals tested were: 180, 210, 250, and 280 μg/L of Cr^{6+}; 20, 25, 30, 35, and 40 μg/L of Cd^{2+}; and 3, 4, 5, and 7 μg/L of diazinon. After a 48-h exposure period, the immobile daphnids (those which were not able to swim after gentle agitation of the liquid for 15 s) were counted and removed (EN ISO 6341, 1996). Enzyme analyses were performed only with mobile animals.

2.4. Stability of the test chemicals in dilution water

The test solutions with the highest concentrations of diazinon and Cd^{2+} (7 μg/L and 40 μg/L, respectively) were separately exposed to the same experimental conditions as the toxicity tests. At the beginning and end of the experiment diazinon and Cd^{2+} concentrations were determined. 10 mL of diazinon solution was extracted with three portions of ethyl acetate (25, 20 and 10 mL) with the addition of 50 mL of a 10% aqueous solution of sodium sulphate. The organic phases were evaporated, the residue was redissolved in 1 mL of ethyl acetate and analysed by gas chromatography (HP 6890) using a flame ionisation detector (Bavcon et al., 2003). Cd^{2+} was determined using a Perkin-Elmer 1100B flame atomic absorption spectrophotometer in air-acetylene flame with deuterium correction of non-specific absorption. Hexavalent chromium (potassium dichromate), a reference chemical according to the standard EN ISO 6341 (1996), was assumed to be stable in water.

2.5. Determination of enzyme activities

Mobile animals from 5 test containers were combined into one enzyme sample. Since 2 samples were prepared for each concentration of chemical in one experiment, and each experiment was repeated 3 times, altogether 6 samples per concentration were prepared. Enzyme activities in each sample

Table 1
Acute toxicity of Cr^{6+}, Cd^{2+}, and diazinon to daphnids

	Cr^{6+}	Cd^{2+}	Diazinon
48 h EC_{10} (μg/L)[a]	182.7 (145.8–219.6)	22.4 (16.5–28.4)	4.3 (3.2–5.4)
48 h EC_{50} (μg/L)[a]	360.4 (243.8–477.1)	41.1 (35.0–47.2)	6.1 (4.8–7.4)

[a] Mean effective concentration from standard toxicity tests (7, 9, and 10 tests for Cr^{6+}, Cd^{2+}, and diazinon, respectively) (95% confidence interval shown in brackets).

were measured in triplicate (AChE) or duplicate (GST). Animals contained in one sample (70 to 100) were homogenized for 3 min in 0.6 mL of homogenization buffer (50 mM phosphate buffer pH 7.0), using a glass–glass Elvehjem-Potter homogenizer.

In preliminary experiments, where each experiment was repeated twice, excess chemicals were not removed from the surfaces of animals and homogenizer prior to homogenization. In subsequent experiments, the excess chemical was removed by rinsing the animals in the homogenizer 3 times with 2 mL of the homogenization buffer combined with 5 mM ethylenediaminetetraacetic acid. In this way, possible *in vitro* interactions between the exogenously derived chemicals and the enzymes present in the homogenate that could lead to artefacts were minimized. The homogenate was centrifuged for 15 min at 15,000 ×*g* and 4 °C. Supernatants were stored at −80 °C until all

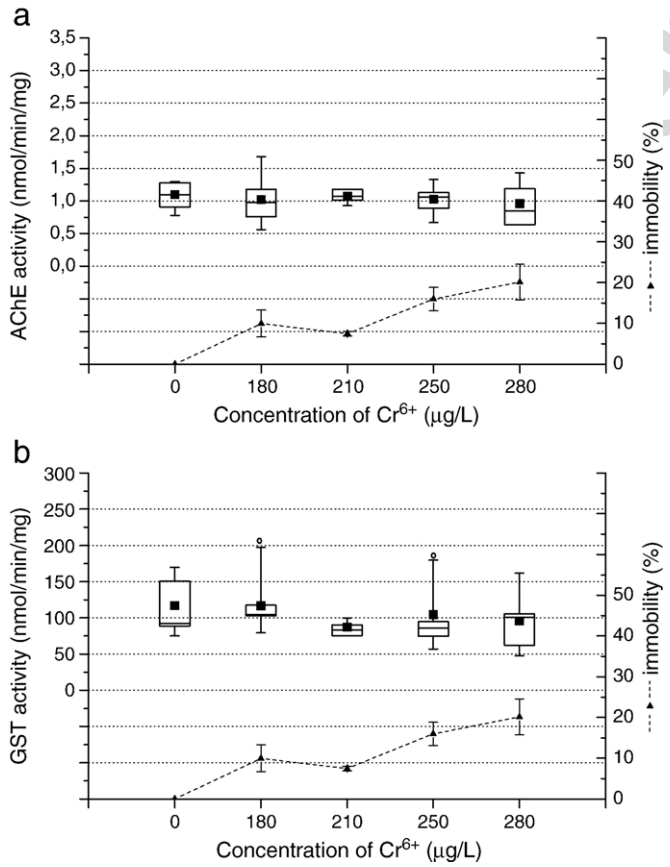

Fig. 1. AChE (a) and GST (b) activities in *D. magna* exposed to Cr^{6+}. Symbols on the box plot represent maximum and minimum values (whiskers: ⊥), mean value (■), and outliers (○). The dashed line represents the percent of immobile neonates of *D. magna* (mean for three replicates±standard error of mean).

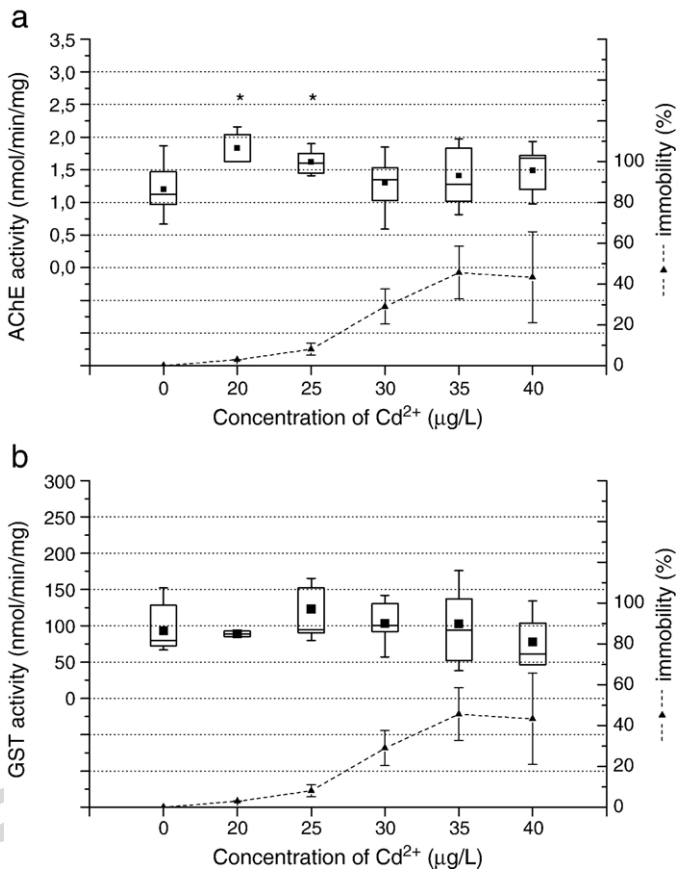

Fig. 2. AChE (a) and GST (b) activities in *D. magna* exposed to Cd^{2+}. Symbols on the box plot represent maximum and minimum value (whiskers: ⊥), mean value (■), and significant changes compared to control (*) (Kruskal–Wallis test, $P<0.05$). The dashed line represents the percent of immobile neonates of *D. magna* (mean for three replicates±standard error of mean).

toxicity tests were performed (up to 3 months). Afterwards, all enzyme activities were measured on the same day. Storage had no effect on AChE and GST activity (data not shown).

AChE activity was determined according to the method of Ellman et al. (1961), using microtiter plates (Bio-Tek® Instruments, USA; PowerWave™ XS) as described by Mancini et al. (2004). The reaction mixture was prepared in 100 mM of potassium phosphate buffer pH 7.4 containing acetylthiocholine chloride and 5,5′ dithiobis-2-nitrobenzoic acid in the final concentrations of 1 mM and 0.5 mM, respectively. 100 μL of protein supernatant were added to start the reaction, which was followed spectrophotometrically at 412 nm and 25 °C for 15 min. AChE activity was expressed in nmoles of hydrolysed acetylcholine chloride/min/mg protein (extinction coefficient $\varepsilon_{412}=13{,}600\ M^{-1}\ cm^{-1}$).

GST activity was determined using the method described by Habig et al. (1974), using microtiter plates (Bio-Tek® Instruments, USA; PowerWave™ XS). 1-chloro-2,4-dinitrobenzene was dissolved in ethanol to obtain a 50 mM solution, which was afterwards diluted with 100 mM potassium phosphate buffer pH 6.5 to the final concentration of 4 mM. This solution was used to prepare a reaction mixture containing 1 mM of 1-chloro-2,4-dinitrobenzene and 1 mM of reduced glutathione. 50 μL of protein supernatant was added to start the reaction. The final concentration of ethanol in the reaction

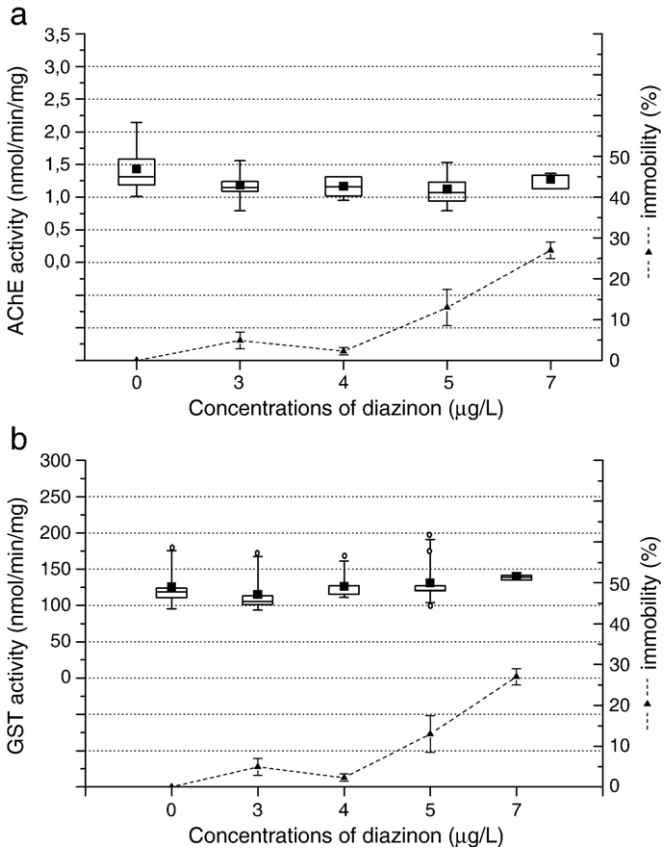

Fig. 3. AChE (a) and GST (b) activities in *D. magna* exposed to diazinon. Symbols on the box plot represent maximum and minimum value (whiskers: ⊥), mean value (■), and outliers (◦). The dashed line represents the percent of immobile neonates of *D. magna* (mean for three replicates±standard error of mean).

mixture was 2%. At this concentration, the activity of GST was not inhibited (not shown). A blank reaction without the protein supernatant was followed and the rate of the absorbance change was subtracted from the measurements containing supernatant. The reaction was followed spectrophotometrically at 340 nm and 25 °C for 3 min. GST activity was expressed in nmoles of conjugated GSH/min/mg protein (extinction coefficient ε_{340}=9600 M^{-1} cm^{-1}). The concentrations of substrates used for both enzymes were saturated and ensured the linear changes of absorbance with time and the concentration of proteins.

Protein concentration was determined using a BCA™ Protein Assay Kit, a modification of the bicinchoninic acid protein assay (Pierce, Rockford, IL, USA).

2.6. Statistical analyses

The effects of the toxicant exposure on the different parameters tested were compared by Kruskal–Wallis analysis ($P<0.05$), using Statgraphics software (Statgraphics Plus for Windows 4.0, Statistical Graphics Corporation). Homogeneity of variance was tested using Levene's test. The percentages given in the results represent the change in medians of AChE and GST activity in exposed animals compared to control. The 48-h EC_{10} and EC_{50} were calculated by Probit analysis using the USA EPA statistical program (US EPA, 1994), and the corresponding 95% confidence limits with Origin software (7.5 SR4, OriginLab Corporation, USA).

3. Results

3.1. Toxicity tests

The results of the range-finding toxicity tests performed with *D. magna* are given in Table 1. The highest toxicity was observed with diazinon, followed by Cd^{2+} and Cr^{6+}. The concentrations of diazinon and Cd^{2+} in test solution did not change during the toxicity test. No degradation products of diazinon (2-isopropyl-6-methyl-4-pyrimidinol) were detected.

3.2. AChE and GST activities in *D. magna* exposed to Cr^{6+}, Cd^{2+}, and diazinon

In animals exposed to up to 280 µg/L of Cr^{6+}, no effects on AChE and GST activities were observed (Fig. 1a and b).

The activity of AChE was significantly increased compared to control ($P<0.05$) by 58% and 43% at 20 µg/L and 25 µg/L of Cd^{2+}, respectively. The concentrations of Cd^{2+} above 25 µg/L

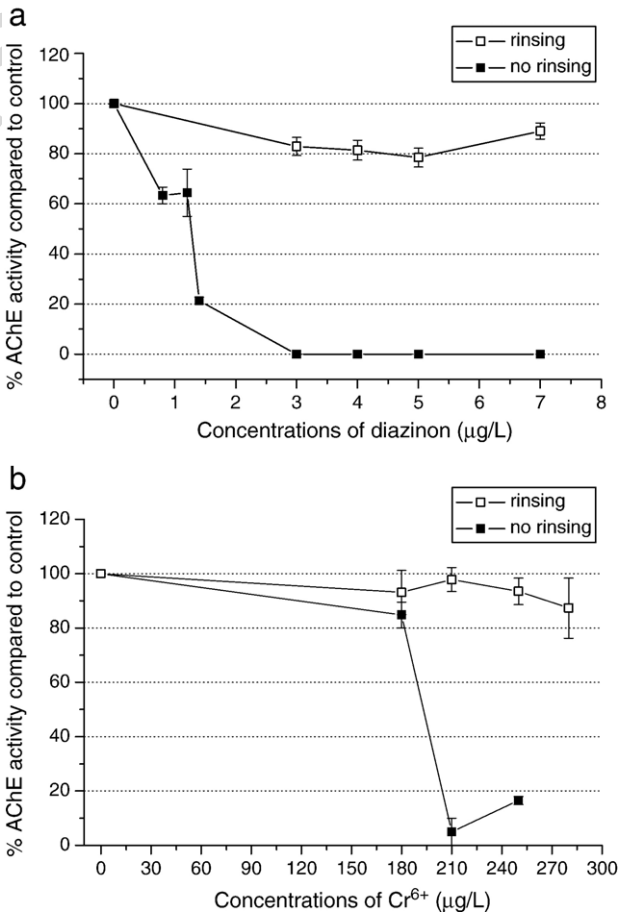

Fig. 4. The effect of the removal of test solution prior homogenization on AChE activities in *D. magna* exposed to diazinon (a) and Cr^{6+} (b). AChE activities at different concentrations of chemicals are shown as percentages compared to control (100%).

caused increase of AChE activity compared to control, but decrease in comparison to lower concentrations of Cd^{2+} (20 μg/L and 25 μg/L). On the contrary, no significant changes of GST activity were observed. (Fig. 2a and b).

No significant changes of AChE and GST activities were observed in animals exposed to up to 7 μg/L of diazinon (Fig. 3a and b).

In our preliminary experiments with no removal of the redundant chemicals, a more pronounced inhibition of AChE activity was observed at the same or lower tested concentrations of Cr^{6+} and diazinon, as used in the subsequent experiments. AChE activity was inhibited by 84% and 77% at 250 μg/L of Cr^{6+} and 1.4 μg/L of diazinon, respectively (Fig. 4a and b). In Cd^{2+} stressed animals, AChE activity was inhibited by 40% at higher concentrations (60 μg/L and 90 μg/L) than used in further experiments. GST activity was evidently inhibited only at 60 μg/L and 90 μg/L of Cd^{2+}.

3.3. The relationship between AChE and GST activities and immobility of D. magna

The degrees of immobility of *D. magna* treated with different concentrations of chemicals in standard 48-h acute toxicity tests are shown as dotted lines in Figs. 1, 2, and 3. There was no direct linkage of the two biochemical biomarkers to immobility of *D. magna* in our study. While immobility of *D. magna* was proportional to the concentration of the tested chemical in a dose-response manner, AChE and GST activities changes were independent of the tested concentration.

4. Discussion

In this study, the effects of Cr^{6+}, Cd^{2+}, and diazinon on AChE and GST activities in *D. magna* after exposure in a standard acute toxicity test were assessed and linked to immobility. The role of specific AChE and GST activity measurements as an addition to this test is examined.

This paper provides a detailed report on measurement of AChE and GST activities in *D. magna*. We find the rinsing of the excess chemicals from the surface of the animals and homogenizer an important precaution to exclude the possible *in vitro* effect of the chemicals on enzymes after sample preparation. Such effects were implied in our preliminary experiments with no rinsing, where a clear inhibition of AChE activity was noticed after exposure of daphnids to Cr^{6+}, Cd^{2+}, and diazinon. The methods for measuring AChE and GST activities in *D. magna* exposed in a standard acute toxicity test are demanding, time-consuming and hence not well suited to routine use. A large number of neonates (up to 800) of the same age and derived from the second to fifth broods are needed in these tests. We recommend the use of these two enzymes when the mode of action of chemicals and daphnids' response to them are under study and we also suggest further testing of these enzymes after longer exposure periods. Our current experiments show that this approach leads to more pronounced changes in AChE and GST activities and, as the animals are larger, fewer of them are needed (Jemec et al., unpublished).

Literature data on the effects of Cr^{6+} on GST and AChE activities are inconsistent. Based on Diamantino et al. (2000), who observed a statistically significant inhibition of AChE activity (17% compared to control) in *D. magna* exposed to 150 μg/L of Cr^{6+} for 48 h, we expected the inhibition of AChE activity in our experiments. GST activity was expected to increase due to possible oxidative stress caused by Cr^{6+} (Stohs and Bagchi, 1995). However, we observed no effects on AChE and GST activities in animals exposed up to 280 μg/L of Cr^{6+} for 48 h. Similarly, Choi et al. (2000) found no effects on GST and AChE activities in the midge (*Chironomus riparius)* after acute 24-h exposure to 1.75 mg/L of Cr^{6+}. In our current experiments, where daphnids were chronically exposed for 21 days, a significant inhibition of AChE activity above 35 μg/L of Cr^{6+} was observed (Jemec et al., unpublished).

The AChE activity in animals exposed to low concentrations of Cd^{2+} increased slightly, a result, possibly of hormesis. This phenomenon is characterized as a dose-response with stimulation at low and inhibition at high doses as result of compensatory mechanisms following a disruption in homeostasis (Calabrese and Baldwin, 2003). Cd^{2+} is known to cause different hormetic responses in a number of organisms (Calabrese and Baldwin, 2003), and in *D. magna* as well (Bodar et al., 1988). No data are currently available on the hormetic effects of Cd^{2+} on AChE, but metals (e.g. aluminium) (Calabrese and Baldwin, 2003), and some other chemicals (Day and Scott, 1990; McHenery et al., 1991; Printes and Callaghan, 2004) have been found to cause these effects on AChE activity. In contrast to our findings, no changes were found in AChE activity in *D. magna* exposed to Cd^{2+} (up to 10 μg/L) in a 24-h acute toxicity test (Guilhermino et al., 1996b), or in the marine microcrustacean *Tigriopus brevicornis* after a 96-h exposure to LC_{50} concentrations of Cd^{2+} (Forget et al., 1999).

Current literature data report different effects of Cd^{2+} on GST activity. An increase in GST activity in *D. magna* exposed to 5 μg/L of Cd^{2+} was observed (Barata et al., 2005), and inhibition of GST activity has been observed in crayfish *Procambarus clarkii* that were acutely exposed to 100 μg/L of Cd^{2+} for 96 h. Our experiments showed no effect of Cd^{2+} on GST activity in *D. magna* up to 40 μg/L.

Most studies report the decrease in AChE activity when animals are exposed to diazinon (Booth and O'Halloran, 2001), but in our study no changes of AChE activity in daphnids exposed to 7 μg/L of diazinon were noted. The absence of AChE inhibition is thought to be the result of diazinon metabolism in *D. magna*. Keizer et al. (1995) showed that diazinon toxicity is species-dependent and depends on the rate of bioactivation of diazinon by conversion to the more potent diazoxon, detoxification in the organism, and the affinity of AChE for diazinon or diazoxon. No data are currently available on the metabolism of diazinon in *D. magna*, and no studies on the effects of diazinon on AChE and GST activities in *D. magna* were previously published. We expected an increase in GST activity in daphnids exposed to diazinon, since GST is able to detoxify diazinon (Chambers, 1992), but we were not able to observe any up to 7 μg/L of diazinon.

We found no links between the change in activities of the two biochemical biomarkers and immobility of *D. magna* in our study. Consequently, these two biomarkers have limited use as early warning signals of future effects of Cr^{6+}, Cd^{2+}, and diazinon exposure at organismal level of organization. Other authors also observed different relations between the change in AChE activity and immobility. For instance, Printes and Callaghan (2004) observed no immobility of *D. magna* exposed to 100 μM of organophosphate acephate, but AChE activity was inhibited by 70% compared to control. This means that high levels of AChE inhibition do not always lead to immobility. On the contrary, Day and Scott (1990) found that some OPs did not change AChE activity in stonefly *Classenia sp.* at concentrations that resulted in significant immobility. This suggests the involvement of other mechanisms of OP toxicity. Similarly, our results show that mechanisms other than AChE and/or GST inhibition are involved in acute toxicity of Cr^{6+}, Cd^{2+}, and diazinon.

In conclusion, the *D. magna* acute toxicity test remains the most widely applied and legally adopted bioassay in many countries. Measurements of AChE and GST activities as an adjunct to this routine standard test have little value because the changes in AChE and GST activities do not always appear to be correlated with immobility. Further, the enzyme assay methodology is too demanding for routine tests. An appropriate use of these two enzyme assays is in integrated biomarker studies, and in mechanism of action studies.

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