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Biochemical biomarkers in chronically metal-stressed daphnids

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Abstract

Biochemical biomarkers are a popular measure of toxic effects on organisms due to their assumed fast response, and are usually assessed after acute exposure of the organism to the stressor. However, increasing interest in the use of biochemical biomarkers in environmental pollution monitoring calls for more laboratory long-term studies of contaminants' effects on biochemical endpoints. In this study, four biochemical biomarkers (protein content, activity of cholinesterase (ChE), catalase (CAT) and glutathione *S*-transferase (GST), were correlated with standardised reproductive and survival endpoints of water fleas (*Daphnia magna*) after chronic exposure to Cr (VI) and Cd. No effect on the reproduction and survival was noticed up to the highest tested concentration of Cr (VI) (52.5 μg/L), while the protein content, and the ChE and CAT activity decreased, and GST activity increased. Cd affected reproduction of daphnids above 0.656 μg/L, but the protein content and ChE activity were changed at 0.328 μg/L and 0.082 μg/L of Cd, respectively. Biochemical biomarkers in some cases proved to be equally or more sensitive than reproduction and mortality. We recommend more frequent use of a battery of biochemical biomarkers in combination with other higher-level biomarkers also in chronic studies and not only in the acute ones.

Keywords: Cadmium (Cd); Catalase; Cholinesterase; Chromium (Cr (VI)); Chronic toxicity test; *Daphnia magna*; Glutathione *S*-transferase

1. Introduction

The utility of biochemical approaches in environmental pollution monitoring and characterization of effect/exposure to stressor for the use in environmental risk assessment is based on the assumption that low concentrations of a toxicant will cause biochemical responses within individual organisms before these effects are observed at higher levels of biological organization (Sarkar et al., 2006). Such biochemical responses are considered to be rapidly responding endpoints (Adams, 2002), and thus most biochemical biomarkers in the laboratory studies are assessed after acute exposure to chemicals. In the water flea *Daphnia magna* Straus, a number of biochemical biomarkers have been studied after acute exposure (Day and Scott, 1990; Guilhermino et al., 1998; Sturm and Hansen, 1999; Diamantino et al., 2000; De Coen and Jassen, 2003; Printes and Callaghan, 2003; Barata et al., 2005; Jemec et al., 2007a), but they have rarely been assessed after 21 d exposure (Jemec et al., 2007b). The need for longer-term laboratory studies using biochemical biomarkers which may serve as reference points with which to develop biomarkers of chronic exposure situations, usually faced by organisms in the field, has been proposed by Handy et al. (2003).

Among the most frequently analyzed biochemical biomarkers in toxicity studies are the enzymes cholinesterase, glutathione *S*-transferase and catalase. Cholinesterase (ChE) hydrolyzes the neurotransmitters such as acetylcholine at the nerve synapse. In the absence of such hydrolysis, neurotransmitter accumulates and as a consequence, prolonged electrical activity at nerve endings occurs. Inhibition of ChE activity is usually regarded as an indicator of organophosphorus and carbamate exposure, but metals can also influence this enzyme (Ishaaya, 2001). Glutathione *S*-transferase (GST) belongs to a family of detoxification enzymes, and catalyses the conjugation of glutathione with xenobiotics including organophosphorus pesticides (Booth and O'Halloran, 2001), and cytotoxic aldehydes produced during lipid peroxidation (Halliwell and

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Gutteridge, 1999). Catalase (CAT) decomposes hydrogen peroxide formed extensively during oxidative stress (Halliwell and Gutteridge, 1999). In chemically stressed animals, increase or decrease of CAT and GST activities is possible, depending on the type of the chemical, time and dose of exposure. Protein content in *D. magna* has previously been used as a biomarker of chronic chemical exposure (Knowles and McKee, 1987, Bodar et al., 1988a), and reflects the entire physiological state of the organism (Printes and Callaghan, 2003).

In our previous work (Jemec et al., 2007a) acute (48 h) effects of Cr (VI) and Cd on the activities of ChE and GST in *D. magna* were studied. Since, except for the induction of ChE at lower concentrations of Cd, no significant changes of selected enzyme activities were detected, it was to conclude that the applicability of these biomarkers in routine acute toxicity tests is limited. In the present chronic (21 d) study the effects of Cr (VI) and Cd on biochemical, reproductive, and survival endpoints in *D. magna* were assessed. These two metals were chosen because they are common pollutants in a variety of aquatic environments, and Cr (VI) is used as a reference chemical in standard acute *D. magna* test (ISO 6341:1996). It is well established, that their toxic action is mediated through the induction of oxidative stress (Stohs and Bagchi, 1995; Halliwell and Gutteridge, 1999). Additionally, Cd has been shown to directly inhibit GST activity (Dierickx, 1982). For both metals, the inhibition of ChE activity was expected (Guilhermino et al., 1998). We compare the sensitivities of biochemical biomarkers to higher-level chronic (reproduction, survival) and acute (immobility) endpoints and discuss the use of such biomarkers in chronic toxicity studies.

2. Materials and methods

2.1. Chemicals

The following chemicals were purchased from Sigma (Germany): dibasic and monobasic potassium phosphate, hydrogen peroxide (30%), 1-chloro-2,4-dinitrobenzene, L-glutathione (reduced form), 5,5-dithiobis-2-nitrobenzoic acid, sodium hydrogen carbonate, acetylthiocholine iodide, and ethylenediaminetetraacetic acid. BCA Protein Assay Reagents A and B, cadmium chloride, and potassium dichromate were purchased from Pierce (U.S.A.). All chemicals were of the highest commercially available grade, typically 99% or higher.

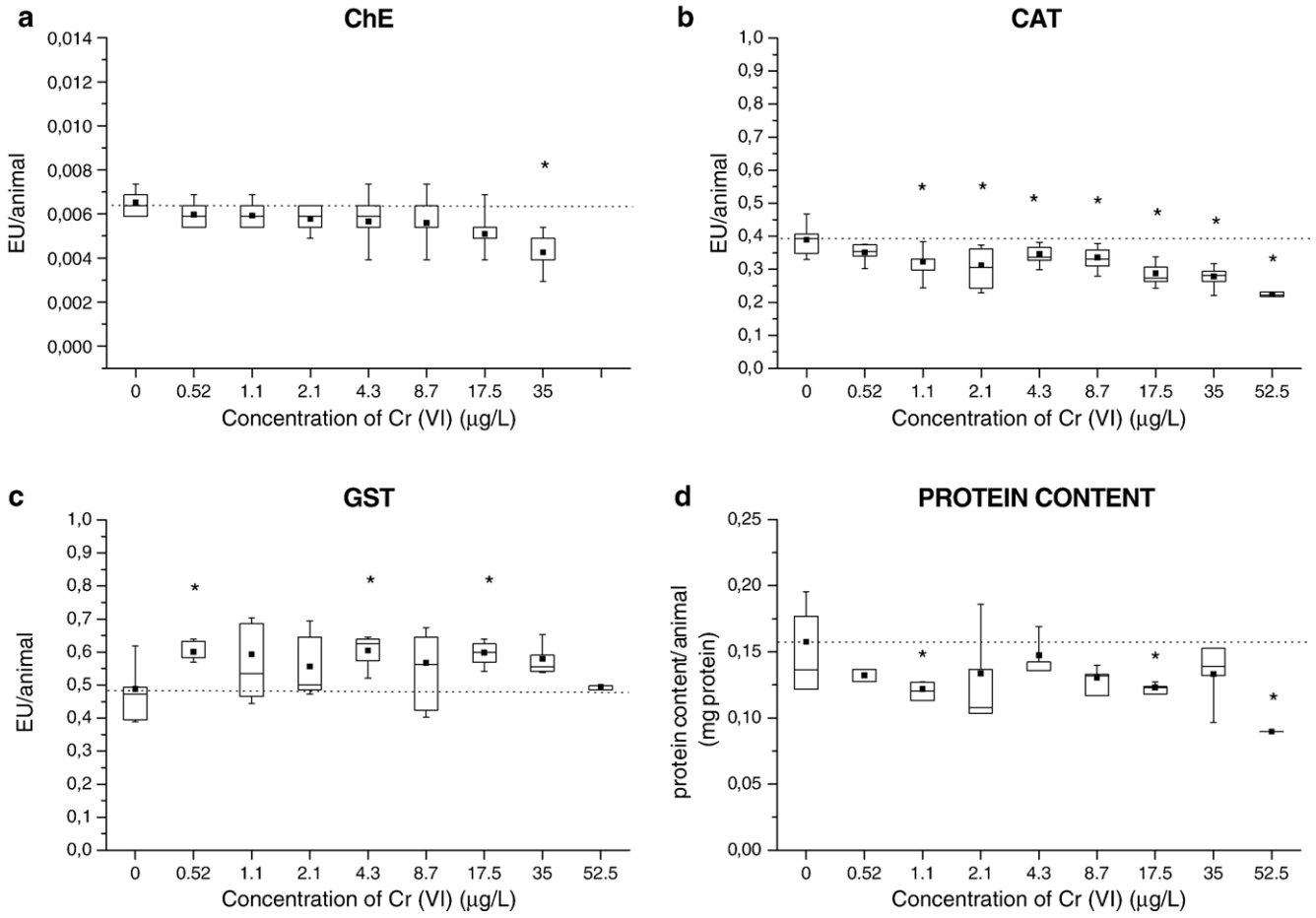

Fig. 1. ChE (a), CAT (b), GST (c) activities and protein content (d) in *D. magna* exposed to Cr (VI). Symbols on the box plot represent maximum and minimum value (whiskers: ⊥), mean value (■), and significant changes compared to control (*) (ANOVA, $P<0.05$). The dashed line represents the mean value of the control.

2.2. Test organism water flea Daphnia magna

Water fleas (*Daphnia magna* Straus 1820), obtained from the Institut für Wasser, Boden und Lufthygiene des Umweltbundesamtes (Berlin, Germany), were cultured in 2.5 L of modified M4 media (Kühn et al., 1989) at 21 ± 1 °C and 16:8 h light/dark regime (light intensity 1800 lx). They were fed a diet of the algae *Desmodesmus subspicatus* Chodat 1926 corresponding to 0.13 mg carbon/daphnia per day.

2.3. Chronic toxicity test with Daphnia magna

Our laboratory is accredited according to ISO 17025:1999 for standard acute testing with *D. magna*. Quality of the test results is regularly assured by the internal quality control (control charts) and participation in proficiency testing schemes (AQUACHECK, UK), where good performance has been demonstrated. The appropriate sensitivity of *D. magna* is regularly checked using a reference chemical potassium dichromate according to the standard (ISO 6341:1996).

Chronic toxicity to daphnids was evaluated using a semi-static exposure system according to ISO 10706:2000. Ten test containers, each with 50 mL of test solution, were prepared for each concentration and a control. Individual daphnids (less than 24 h old) were placed in the test solution. The animals were fed daily a diet of *Desmodesmus subspicatus* (0.13 mg carbon/daphnia per day) and the newly born neonates were counted and removed. The following concentrations of chemicals were tested: 0, 0.52, 1.1, 2.1, 4.3, 8.7, 17.5, 35, 52 µg/L of Cr (VI), and 0, 0.0205, 0.041, 0.082, 0.164, 0.328, 0.65, 1.31, 2.62 µg/L of Cd. Chronic tests for both metals were repeated up to three times.

The survived initial daphnids were transferred into freshly prepared test solutions every two days. The concentration of Cd in test solutions was measured at the outset and after two days using a Perkin-Elmer 1100B flame atomic absorption spectrophotometer in an air-acetylene flame. No changes in concentrations of Cd in test solutions during two days of exposure were observed. Cr (VI) (potassium dichromate), a reference chemical (ISO 6341:1996), proved to be stable in water according to previous toxicity testing in our laboratory (Tišler and Zagorc-Končan, 1997).

2.4. Evaluation of reproduction and survival endpoints

The reproduction of adult daphnids was evaluated during 21 d using the following endpoints: the number of neonates per

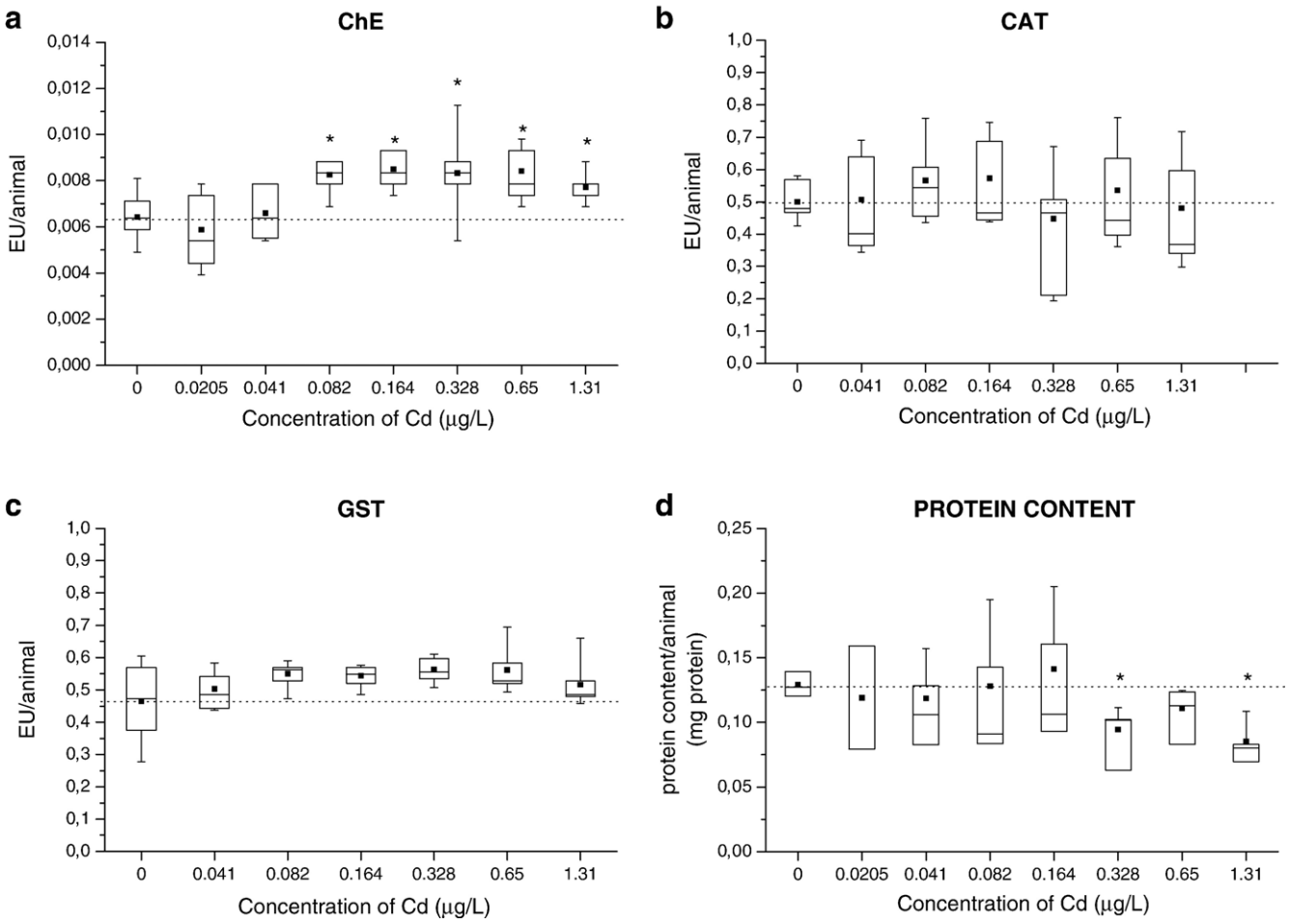

Fig. 2. ChE (a), CAT (b), GST (c), activities and protein content (d) in *D. magna* exposed to Cd. Symbols on the box plot represent maximum and minimum value (whiskers: ⊥), mean value (■), and significant changes compared to control (*) (ANOVA, $P<0.05$). The dashed line represents the mean value of control.

adult, the average brood size per adult, the number of broods per adult, and the time to the first brood. The average brood size per adult was calculated by dividing the number of neonates with the number of broods per adult. The mortality of adults at the end of the test was also monitored.

2.5. Evaluation of biochemical biomarkers

After 21 d of exposure to the chemicals, survived adult daphnids were further used to prepare the samples for enzyme analyses. Each enzyme sample was prepared by combining five adult daphnids. Altogether, six samples per concentration were prepared.

The animals were transferred to a glass–glass Elvehjem–Potter homogenizer using a glass pipette. Excess test solution introduced into the homogenizer, was removed from the surface of animals and from the homogenizer walls by rinsing three times with 2 mL of 50 mM phosphate buffer pH 7.0 combined with 5 mM ethylenediaminetetraacetic acid as previously described by Jemec et al. (2007a). The animals were then homogenized for 3 min in 0.8 mL of 50 mM phosphate buffer pH 7.0. The homogenate was centrifuged for 25 min at 15,000 *g* and 4 °C. Enzyme activities were measured on freshly prepared supernatants for each sample in triplicate.

ChE activity was determined according to Ellman et al. (1961), adapted for microtiter plates (Bio-Tek® Instruments, USA; PowerWave™ XS) as described by Jemec et al. (2007a). The reaction mixture was prepared in 100 mM of potassium phosphate buffer pH 7.4 containing 1 mM and 0.5 mM final concentrations of acetylthiocholine iodide and 5,5' dithiobis-2-

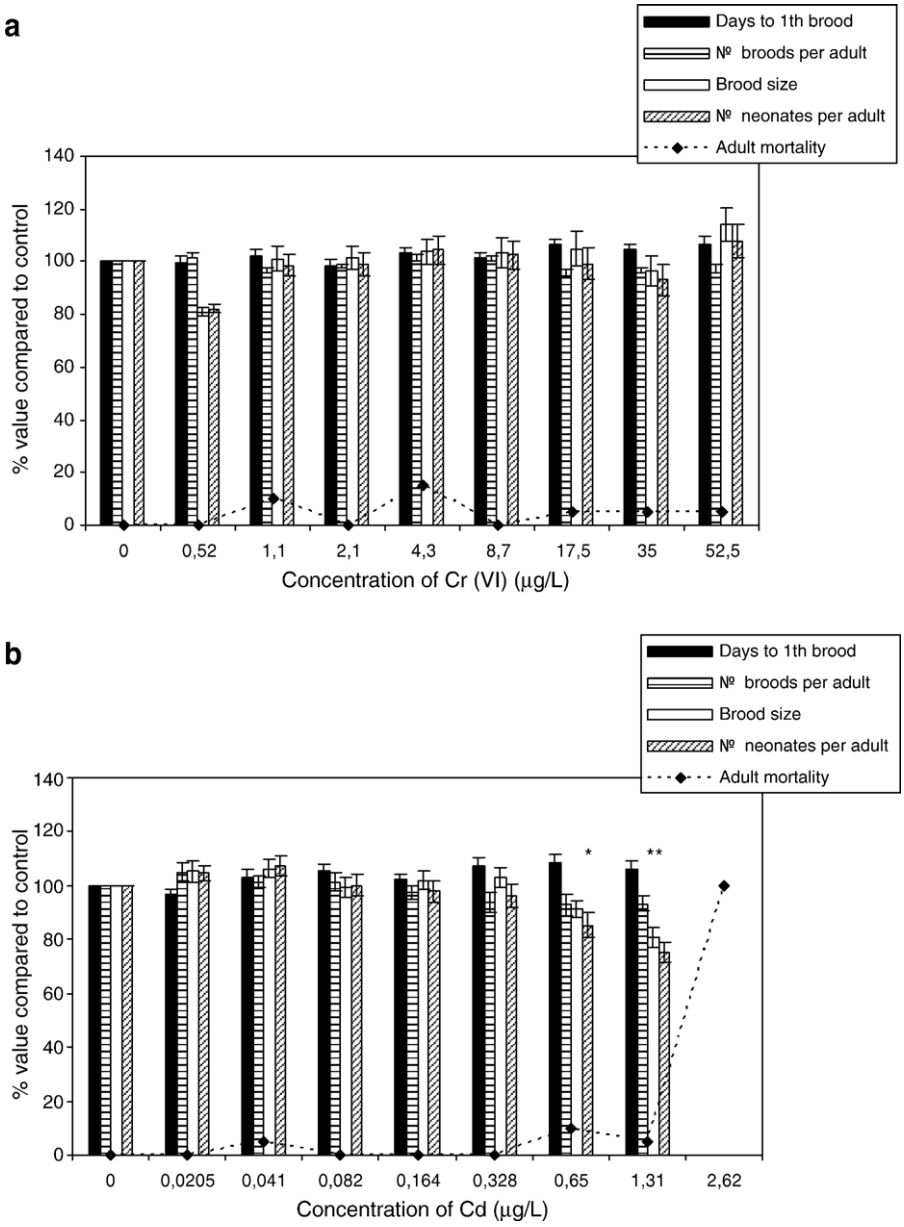

Fig. 3. The effects of Cr (VI) (a) and Cd (b) on the reproduction and mortality of *D. magna* (significant changes compared to control (*); Dunnett's test, $P<0.05$).

Table 1
The mean values±standard errors of mean for the reproduction data of the control animals

Experiments	Mortality of adults at the end of the test (%)	Days to first brood	No. broods per adult	Average brood size	No of neonates per adult
Cr (VI)	0	9.9±0.2	4.1±0.05	14.9±0.8	61.1±3.1
Cd	0	9.9±0.2	4.1±0.07	14.8±0.4	60.7±1.8

nitrobenzoic acid, respectively. The reaction was followed spectrophotometrically at 412 nm and 25 °C for 15 min after the addition of 100 µL of protein supernatant.

GST activity was measured according to the method proposed by Habig et al. (1974) and optimized for microtiter plates (Bio-Tek® Instruments, USA; PowerWave™ XS) by Jemec et al. (2007a). A 50 mM solution of 1-chloro-2,4-dinitrobenzene in ethanol was diluted with 100 mM potassium phosphate buffer pH 6.5 to a 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 were added to start the reaction, which was followed spectrophotometrically at 340 nm and 25 °C for 3 min.

CAT activity was determined according to Aebi (1984) and Jamnik and Raspor (2003). 50 µL of protein supernatant were combined with 750 µL of hydrogen peroxide solution (10.8 mM) prepared in 50 mM potassium phosphate buffer pH 7.0. The final concentration of hydrogen peroxide was 10 mM. The reaction was followed spectrophotometrically for 2 min at 25 °C and 240 nm on a Shimadzu UV-2101PC spectrophotometer (Japan).

Protein concentration of the homogenate was measured using a BCA™ Protein Assay Kit, a modification of the bicinchoninic acid protein assay (Pierce, Rockford, IL, USA). Protein content per animal was calculated by dividing the total amount of proteins in the homogenate with the number of adults included in the sample.

2.6. Interpretation of enzyme activities

Enzyme activities were expressed in enzyme units (EU) per one adult daphnia as previously proposed in case the protein content is changed due to chemical exposure (Jemec et al., 2007b). One EU was determined as the amount of ChE that hydrolyses one nmole of acetylthiocholine/min (ε_{412}=13600 M^{-1} cm^{-1}), the amount of CAT that degrades one µmole of hydrogen peroxide/min (ε_{240}=43.6 M^{-1} cm^{-1}), and the amount of GST that conjugates one nmole of reduced glutathione/min (ε_{340}=9600 M^{-1} cm^{-1}).

2.7. Data analysis

The 21 d lowest observed exposure concentration (LOEC) and lowest observed lethal concentration (LOLC) producing a statistically different response from the control response after 21 d were determined by One-way Analysis Of Variance (ANOVA; $P<0.05$), the Games–Howell post hoc test for biochemical endpoints and Dunnett's test for reproduction and survival data, using SPSS for Windows 8.0 (SPSS Inc., USA).

3. Results

3.1. Biochemical biomarkers

In Cr (VI) stressed animals, protein content decreased compared to the control at 1.1, 17.5 and 52.5 µg/L of Cr (VI). CAT activities decreased significantly at nearly all tested concentrations. GST activities increased significantly only at 0.52, 4.3, and 17.5 µg/L of Cr (VI), but the trend of the GST activity increase was observed at all tested concentrations. ChE activity decreased above 35 µg/L of Cr (VI) (Fig. 1).

Higher concentrations of Cd (above 0.328 µg/L) caused significant decrease of the amount of total protein per daphnia. No significant changes of CAT and GST activities at all tested concentrations of Cd were observed. ChE activity was increased when the Cd concentration exceeded 0.082 µg/L (Fig. 2).

The mean values (±SE) for the enzyme activities (EU/animal) of control animals obtained from the experiments with Cr (VI) were: 0.006±0.00016 (ChE), 0.488±0.024 (GST), 0.39±0.011 (CAT) and from the experiments with Cd: 0.006±0.00032 (ChE), 0.46±0.046 (GST), and 0.50±0.020 (CAT). These values are in accordance with previously published chronic study (Jemec et al., 2007b).

3.2. Reproduction and survival

Reproduction and survival of adult daphnids were assessed in standard chronic toxicity tests with *D. magna*. No effect of Cr

Table 2
LOEC for biochemical biomarkers, reproduction data and LOLC for mortality in daphnids exposed chronically (21 d) to Cr (VI) and Cd, and LOEC values for biochemical biomarkers and immobility in acutely (48 h) exposed daphnids (Jemec et al., 2007a)

LOEC/LOLC values (µg/L)	Cr (VI)		Cd	
	Acute (48 h)	Chronic (21 d)	Acute (48 h)	Chronic (21 d)
Highest concentration tested	280	52.5	40	2.62 [a]
LOEC $_{ChE}$	>280	35 (↓)	20 (↑)	0.082 (↑)
LOEC $_{GST}$	>280	n.d. [b]	>40	n.d. [b]
LOEC $_{CAT}$	–	1.1	–	n.d. [b]
LOEC $_{PROTEINS}$	>280 [c]	n.d. [b]	>40 [c]	0.328
LOEC $_{No.\ neonates\ per\ adult}$	–	>52.5	–	0.656
LOEC $_{Brood\ size}$	–	>52.5	–	1.31
LOEC $_{Days\ to\ first\ brood}$	–	>52.5	–	n.d. [b]
LOEC $_{No.\ broods\ per\ adult}$	–	>52.5	–	n.d. [b]
LOEC $_{Immobility}$	250	–	30	–
LOLC $_{Mortality}$	–	>52.5	–	n.d. [b]

–: not tested.
(↓) the activity was decreased.
(↑) the activity was increased.
[a] 100% mortality of adult daphnids was observed at this concentration. At lower tested concentration (1.31 µg/L), mortality did not exceed 20%.
[b] LOEC could not be determined.
[c] Our laboratory, unpublished.

(VI) on reproduction or survival was noted at concentrations up to 52.5 μg/L (Fig. 3a).

The effect of Cd on reproduction was observed above 0.656 μg/L. At the highest tested concentration (2.62 μg/L of Cd) 100% mortality of daphnids was observed (Fig. 3b).

The mean control values of reproduction data for both chemicals satisfied the validity criteria prescribed by the ISO standard 10706:2000. Namely, the mortality of adults at the end of the test was ≤20%, the mean number of neonates per adult was ≥60, and the time to first brood was within 11 days (Table 1).

The sensitivities of biochemical biomarkers in comparison to higher-level chronic (reproduction, survival) and acute (immobility) endpoints were evaluated by comparing 21 d LOEC values for biochemical endpoints to 21 d LOEC values for reproduction and 21 d LOLC for mortality, and 48 h LOEC for biochemical biomarkers to 48 h LOLC for immobility obtained in our previous study on the acute effects of Cr (VI) and Cd on daphnids (Jemec et al., 2007a) (Table 2). In general, biochemical biomarkers after acute exposure were less sensitive endpoints than the immobility (higher 48 h LOEC for biochemical biomarkers), but after chronic exposure in some cases (e.g. ChE, GST and protein content in Cr (VI) stressed animals, and ChE and protein content in Cd exposed daphnids) they proved to be equally or more sensitive indicators than reproduction and mortality of adult daphnids.

4. Discussion

In this study, specimens of *D. magna* were exposed chronically to Cd and Cr (VI). During the exposure, different biochemical, reproductive, and survival endpoints were assessed and compared.

In Cr (VI) stressed daphnids, no effect on survival and reproduction of *D. magna* was observed up to 52.5 μg/L. This seems to be in accordance with previously published but inconsistent values of the 21 d NOLC for survival: 75 μg/L (Diamantino et al., 2000), 6.3 μg/L (Mark and Solbe, 1998), as well as for 21 d NOEC for reproduction: 100 μg/L (Versteeg et al., 1997), 6.3–245 μg/L (Mark and Solbe, 1998), 12.5 μg/L (Diamantino et al., 2000) and 18 μg/L (Kühn et al., 1989).

At the same concentrations, the activities of CAT and GST were altered, indicating that Cr (VI) probably induced reactive oxygen species (ROS) production. Above 0.52 μg/L of Cr (VI), the decrease of CAT activity was observed. This might be due to Cr (VI) induced hydroxyl radical production, which leads to the production of H_2O_2 (Halliwell and Gutteridge, 1999). Namely, high concentrations of H_2O_2 cause rapid inactivation of CAT (Aebi, 1984). No previous studies on chronic effects of Cr (VI) on CAT and GST activities in daphnids have been published. After acute exposure, no effects on GST activities in daphnids exposed up to 280 μg/L of Cr (VI) for 48 h (Jemec et al., 2007a) and no effects on GST activities in the midge (*Chironomus riparius*) after 24-h exposure to 1.75 mg/L of Cr (VI) (Choi et al., 2000) were observed. Our study showed the decrease of ChE activity in chronically exposed daphnids above 35 μg/L of Cr (VI). The decrease of ChE activity was also observed in *D. magna* acutely exposed to 150 μg/L of Cr (VI) (Guilhermino et al., 1998).

In Cd stressed daphnids, slightly lower 21 d LOLC for survival and 21 d LOEC for reproduction were determined (2.62 μg/L and 0.656 μg/L, respectively) than previously described: 5 μg/L–8 μg/L (Knowles and McKee, 1987; Semsari et al., 2002; De Coen and Janssen, 2003), and 2.1 μg/L–5 μg/L (Knowles and McKee, 1987; Bodar et al., 1988b; De Coen and Janssen, 2003), respectively.

Activities of CAT and GST did not change when the animals were exposed up to 1.31 μg/L of Cd. This suggests that no ROS production was induced. No previous data on chronic effects of Cd on CAT and GST activities in daphnids are available. In our previous study, no acute effects were found on GST activity in *D. magna* exposed up to 40 μg/L for 48 h. However, Barata et al. (2005) observed an increase of GST activity and decrease of CAT activity in daphnids acutely (48 h) exposed to 5 and 20 μg/L of Cd, respectively. Chronic exposure to Cd resulted in elevated ChE activities above 0.082 μg/L of Cd. This is in agreement with the results previously obtained in this laboratory (Jemec et al., 2007a), in which a proposed hormetic increase of ChE activity was observed in daphnids acutely exposed to 20 μg/L and 25 μg/L of Cd. An increase of ChE activity exposed to metals (Calabrese and Baldwin, 2003, Brown et al., 2004) and some other chemicals (Day and Scott, 1990; Printes and Callaghan, 2004) has been reported previously. In contrast to our findings, no changes in ChE activity were found in *D. magna* exposed up to 10 μg/L of Cd in a 24-h acute toxicity test (Guilhermino et al., 1996).

The decrease in the protein content of daphnids was observed in both Cd and Cr (VI) exposure. This biomarker is a reflection of the entire physiological state of the organism and a measure of its energy budget, which can be affected by chemically induced allocation of energy (Koojiman, 2000; De Coen and Janssen, 2003). This and previously published work (Jemec et al., 2007b) suggest more frequent evaluation of this endpoint, especially if it is to be used further as a reference for the calculation of specific enzyme activities.

In some cases, the changes of biochemical biomarkers in daphnids chronically exposed to Cr (VI) and Cd turned out to be more pronounced than in the acute exposure investigated in our previous study (Jemec et al., 2007a). This was particularly true for Cr (VI), where all biochemical biomarkers were changed after chronic exposure, while none was changed after acute exposure. In Cd exposed animals, ChE activity and protein content were affected, while CAT and GST activities were not changed, similarly as after acute exposure. The comparison between acute and chronic effects of chemicals on biochemical biomarkers in daphnids has not been thoroughly investigated, but more pronounced effects of chemicals after long-term exposure have been reported by Printes and Callaghan (2003), who observed higher inhibition of ChE activity in daphnids exposed to parathion for seven days than after two and four days.

The sensitivity of biochemical biomarkers to chronic chemical exposure depends on the mode of action of a chemical. Namely, the 21 d LOEC values for Cr (VI) and Cd exposed animals were as follows: $LOEC_{CAT} < LOEC_{ChE} < LOEC_{GST} = LOEC_{PROTEIN}$ and $LOEC_{ChE} < LOEC_{PROTEIN} < LOEC_{GST} = LOEC_{CAT}$, respectively. Thus the same biochemical biomarker can be the most sensitive to one chemical, and the least sensitive to the other,

as for instance CAT activity. This implies the need to evaluate a suite of biomarkers to fully understand the integrated toxic effect of a contaminant to organism as proposed by Brown et al. (2004).

The European Union (EU) water quality criteria for Cr (VI) and Cd are currently in the proposal stage (Commission of the European communities, 2006). Cadmium and its compounds are identified as priority hazardous substances with the proposed maximum allowable concentration (e.g. concentrations that may be present without causing significant harm) of 0.9 μg/L (hardness of water 100–200 mg $CaCO_3$/L). Hexavalent chromium is not included in the EU priority substance list (Commission of the European communities, 2006). According to the U.S. EPA (2006), both Cd and Cr (VI) are recognized as priority toxic pollutants for freshwaters with final chronic values (geometric mean of the NOEC and LOEC) 0.25 μg/L (hardness of water 100 mg $CaCO_3$/L) and 11 μg/L, respectively.

On the basis of our results, the currently proposed EU water quality criteria for Cd are not protective enough for daphnia, while U.S. water criteria are. Namely, the 21 d LOEC for the reproduction of daphnids (0.656 μg/L) is lower as the EU proposed maximum allowable concentration of Cd (0.9 μg/L), and higher than U.S. final chronic value (0.25 μg/L). For Cr (VI) (21 d LOEC>52.5 μg/L), the U.S water quality criteria are protective enough for the reproduction of daphnids. However, in both cases tested biochemical biomarkers would be affected at currently proposed water quality criteria concentrations.

In the environment, very high concentrations of Cd and Cr (VI) were found. Namely, some of the recently reported levels of Cd were in the range from 0.488 μg/L (Dipsiz stream, Turkey) (Demirak et al., 2006) to 1800 μg/L (Rio Piscinas, Italy) (Concas et al., 2006) and for Cr (VI) from 0.41 μg/L (Dipsiz stream, Turkey) (Demirak et al., 2006) to 1580 μg/L (Rio Grande, Mexico) (Rios-Arana et al., 2003). Based on high environmental concentrations of both metals, and their 21 d LOEC values for the reproduction of daphnids, Cr (VI) and Cd pose high risk to daphnids.

At the current state of the knowledge, we do not suggest that water quality criteria are set based on the results on biochemical biomarkers unless they are linked to the effects at higher levels of biological complexity. There are more than 25 years of studies on the use of biochemical biomarkers in ecotoxicological studies, but they are still not as widely applied as anticipated. We believe that more data on a link between lower and higher-level biomarkers will promote their applicability in pollution monitoring and risk assessment. At present, we do recommend their use in studies on the mechanism of action and effects of new emerging chemicals and products of nanotechnologies before they are released to the market.

In conclusion, in contrast to our previously published acute effects of Cr (VI) and Cd on daphnids (Jemec et al., 2007a), where the responses of biochemical biomarkers were less sensitive than higher-levels endpoint (immobility), biochemical biomarkers in this chronic study were in some cases equally or more sensitive than reproduction and mortality. More frequent use of a battery of biochemical biomarkers in combination with other higher-level biomarkers is recommended for chronic studies and not only in the acute ones.

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References

Adams, S.M., 2002. Biological indicators of aquatic ecosystem stress. American Fisheries Society, Bethesda, Maryland.

Aebi, H., 1984. Catalase in vitro. Methods Enzymol. 105, 121–126.

Barata, C., Varo, I., Navarro, J.C., Arun, S., Porte, C., 2005. Antioxidant enzyme activities and lipid peroxidation in the freshwater cladoceran *Daphnia magna* exposed to redox cycling compounds. Comp. Biochem. Physiol. 140 C, 175–186.

Bodar, C.W.M., Van Der Sluis, I., Voogt, P.A., Zandee, D.I., 1988a. Effects of cadmium on consumption assimilation and biochemical parameters of *Daphnia magna*: possible implications for reproduction. Comp. Biochem. Physiol. C 90, 341–346.

Bodar, C.W.M., Van Leeuwen, C.J., Voogt, P.A., Zandee, D.I., 1988b. Effect of cadmium on the reproduction strategy of *Daphnia magna*. Aquat. Toxicol. 12, 301–310.

Booth, L.H., O' Halloran, K., 2001. A comparison of biomarker responses in the earthworm *Aporrectodea caliginosa* to the organophosphorous insecticides diazinon and chlorpyrifos. Environ. Toxicol. Chem. 20, 2494–2502.

Brown, R.J., Galloway, T.S., Lowe, D., Browne, M.A., Dissanayake, A., Jones, M.B., Depledge, M.H., 2004. Differential sensitivity of three marine invertebrates to copper assessed using multiple biomarkers. Aquat. Toxicol. 66, 267–278.

Calabrese, E.J., Baldwin, L.A., 2003. Inorganics and hormesis. Crit. Rev. Toxicol. 33, 215–304.

Choi, J., Roche, H., Caquet, T., 2000. Effects of physical (hypoxia, hyperoxia) and chemical (potassium dichromate, fenitrothion) stress on antioxidant enzyme activities in *Chironomus riparius* mg. (Diptera, Chironomidae) larvae: potential biomarkers. Environ. Toxicol. Chem. 19, 495–500.

Commission of the European communities, 2006. Proposal for a directive of the European parliament and of the council on environmental quality standards in the field of water policy and amending Directive 2000/60/EC, Brussels, 17.7.2006.

Concas, A., Ardau, C., Cristini, A., Zuddas, P., Cao, G., 2006. Mobility of heavy metals from tailings to stream waters in a mining activity contaminated site. Chemosphere 63, 244–253.

Day, K.E., Scott, I.M., 1990. Use of acetylcholinesterase activity to detect sublethal toxicity in stream invertebrates exposed to low concentrations of organophosphate insecticides. Aquat. Toxicol. 18, 101–114.

De Coen, W.D., Janssen, C.R., 2003. The missing biomarker link: relationship between effects on the cellular energy allocation biomarker of toxicant stressed *Daphnia magna* and corresponding population characteristics. Environ. Toxicol. Chem. 22, 1632–1641.

Demirak, A., Yilmaz, F., Tuna, A.L., Ozdemir, N., 2006. Heavy metals in water, sediment and tissues of *Leuciscus cephalus* from a stream in southwestern Turkey. Chemosphere 63, 1451–1458.

Diamantino, T.C., Guilhermino, L., Almeida, E., Soares, A.M.V.M., 2000. Toxicity of sodium molybdate and sodium dichromate to *Daphnia magna* Straus evaluated in acute, chronic and acetylcholinesterase inhibition tests. Ecotoxicol. Environ. Saf. 45, 253–259.

Dierickx, P.J., 1982. In vitro inhibition of the soluble glutathione *S*-transferase from rat liver by heavy metals. Enzyme 27, 25–32.

Ellman, L.G., Courtney, K.D., Andres Jr., V., Featherstone, R.M., 1961. A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem. Pharmacol. 7, 88–95.

Guilhermino, L., Lopez, M.C., Carvalho, A.P., Soares, A.M.V.M., 1996. Inhibition of acetylcholinesterase activity as effect criterion in acute tests with juvenile *Daphnia magna*. Chemosphere 32, 727–738.

Guilhermino, L., Barros, P., Silva, M.C., Soares, A.M.V.M., 1998. Should the use of inhibition of cholinesterases as a specific biomarker for organophosphate and carbamate pesticides be questioned. Biomarkers 3, 157–163.

Habig, W.H., Pabst, M.J., Jakoby, W.B., 1974. Glutathione *S*-transferases, the first enzymatic step in mercapturic acid formation. J. Biol. Chem. 249, 7130–7139.

Halliwell, B., Gutteridge, J.M.C., 1999. Free Radicals in Biology and Medicine. Oxford University Press, New York, USA.

Handy, R.D., Galloway, T.S., Depledge, M.H., 2003. A proposal for the use of biomarkers for the assessment of chronic pollution and in regulatory toxicology. Ecotoxicology 12, 331–343.

Ishaaya, I., 2001. Biochemical sites of insecticide action and resistance. Springer-Verlag Berlin Heidelberg, New York.

ISO 6341, 1996. Water quality — determination of the inhibition of the mobility of *Daphnia magna* Straus (Cladocera, Crustacea) — acute toxicity test in ISO 6341, Cor.1(98). International Organisation for Standardisation, Geneve, Switzerland.

ISO/IEC 17025, 1999. General requirements for the competence of testing and calibration laboratories. International Organisation for Standardisation, Geneve, Switzerland.

ISO 10706, 2000. Water quality — Determination of long term toxicity of substances to *Daphnia magna* Straus (Cladocera, Crustacea). International Organisation for Standardisation. Geneve, Switzerland.

Jamnik, P., Raspor, P., 2003. Stress response of yeast *Candida intermedia* to Cr(VI). J. Biochem. Mol. Toxic. 17, 316–323.

Jemec, A., Drobne, D., Tišler, T., Trebše, P., Roš, M., Sepčić, K., 2007a. The applicability of acetylcholinesterase and glutathione *S*-transferase in *Daphnia magna* toxicity test. Comp. Biochem. Physiol. C 144, 303–309.

Jemec, A., Tišler, T., Drobne, D., Sepčić, K., Fournier, D., Trebše, P., 2007b. Comparative toxicity of imidacloprid, of its commercial liquid formulation and of diazinon to a non-target arthropod, the microcrustacean *Daphnia magna*. Chemosphere 68, 1408–1418.

Knowles, C.O., McKee, M.J., 1987. Protein and nucleic acid content in *Daphnia magna* during chronic exposure to cadmium. Ecotoxicol. Environ. Saf. 13, 290–300.

Koojiman, S.A.L.M., 2000. Dynamic energy and mass budgets in biological systems. Cambridge University Press, Cambridge, United Kingdom.

Kühn, R., Pattard, M., Pernack, K.D., Winter, A., 1989. Results of the harmful effects of water pollutants to *Daphnia magna* in the 21 d reproduction test. Wat. Res. 23, 501–510.

Mark, U., Solbe, J., 1998. Analysis of the ecetoc aquatic toxicity (EAT) database V — the relevance of *Daphnia magna* as a representative test species. Chemosphere 36, 155–166.

Printes, L.B., Callaghan, A., 2003. Interclonal variability in *Daphnia* acetylcholinesterase activity: the implications for its applicability as a biomarker. Environ. Toxicol. Chem. 22, 2042–2047.

Printes, L.B., Callaghan, A., 2004. A comparative study on the relationship between acetylcholinesterase activity and acute toxicity in *Daphnia magna* exposed to anticholinesterase insecticides. Environ. Toxicol. Chem. 23, 1241–1247.

Rios-Arana, J.V., Walsh, E.J., Gardea-Torresdey, J.L., 2003. Assessment of arsenic and heavy metal concentrations in water and sediments of the Rio Grande at El Paso–Juarez metroplex region. Environ. Int. 29, 957–971.

Sarkar, A., Ray, D., Shrivastava, A.N., Sarker, S., 2006. Molecular biomarkers: their significance and application in marine pollution monitoring. Ecotoxicology 15, 333–340.

Semsari, S., Amar, H.A., Badani, Z., Benayad, B., 2002. Study of the inhibition of certain trophic levels by the presence of metal cations in water. Desalination 150, 177–188.

Stohs, S.J., Bagchi, D., 1995. Oxidative mechanisms in the toxicity of metal ions. Free. Radical. Bio. Med. 18, 321–336.

Sturm, A., Hansen, P.D., 1999. Altered cholinesterase and monooxygenase levels in *Daphnia magna* and *Chironomus riparius* exposed to environmental pollutants. Ecotoxicol. Environ. Saf. 42, 9–15.

Tišler, T., Zagorc-Končan, J., 1997. Comparative assessment of toxicity of phenol, formaldehyde, and industrial wastewater to aquatic organisms. Water Air Soil Pollut. 97, 315–322.

U.S. EPA, 2006. National recommended water quality criteria.

Versteeg, D.J., Stalmans, M., Dyer, S.D., Janssen, C., 1997. *Ceriodaphnia* and *Daphnia*: a comparison of their sensitivity to xenobiotics and utility as a test species. Chemosphere 34, 869–892.