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Biochemical Responses of Earthworm *Eisenia fetida* Exposed to Cadmium-Contaminated Soil with Long Duration

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Abstract The biochemical responses of the earthworms, *Eisenia fetida*, exposed to a series of Cd concentrations (0.00, 1.25, 2.50, 5.00 and 10.00 mg Cd^{2+} kg^{-1} soil) for up to 8 weeks were investigated, aiming to evaluate the sub-lethal effects of Cd with long exposure and to explore the potential for applying these responses as biomarkers to indicate the Cd-contaminated soil. The following biochemical parameters were determined: cytochrome P450 (CYP) contents and activities of superoxide dismutase (SOD), catalase (CAT) and glutathione-s-transferase (GST). Cadmium concentrations in all earthworms were apparently accumulated in 4 weeks, and showed minor changes in weeks 6–8 compared to the first 4 weeks. CYP presented a significant elevation in 2–4 weeks and a decline in 6–8 weeks in each treated group. The activities of SOD and CAT showed an obvious increase with exposure of 6–8 weeks while their levels were not affected in 4 weeks in each treated group. GST activity revealed significant activation starting from week 4. This study confirmed the significance of applying a suite of biomarkers rather than a selective choice to assess the impact of pollutants on organisms. It also indicated that the observed effects were more dependent upon exposure duration than dose.

Keywords Cadmium · Biochemical responses · Long exposure · CYP · Antioxidant enzymes · GST

Cadmium (Cd), a non-essential heavy metal, is commonly regarded as a pollutant in soils throughout the world due to natural and anthropogenic activities. It has been identified as a potential human carcinogen, and is known to cause adverse effects on organisms in soils (Friberg et al. 1985). Earthworms are common soil organisms that may comprise 60 %–80 % of the total soil biomass (Saint-Denis et al. 1999). Their ecological importance and ease of collection and culture serve to make them suitable organisms as bioindicators of soil contamination, and as test organisms for examining biological effects of soil contaminants. *Eisenia fetida* is the earthworm species commonly used for the standardization of acute and chronic ecotoxicity assays (ISO 1998; OECD 1984).

Assessment of the impact of contaminants in soils in Organization for Economic Cooperation and Development (OECD) countries has been focused upon acute toxicity and the chronic bioassay endpoints of reproduction and growth (Saint-Denis et al. 1999). However, bioassays that utilize molecular endpoints may also be valuable assessment tools. Biomarkers such as cytochrome P450 (CYP), glutathione-s-transferase (GST) and antioxidant enzymes may reveal the biological effects of contaminants in earthworms. Cytochrome P450 (CYP), of the detoxification process, catalyzes the oxidative conversion of lipophilic xenobiotics into entities which are more water-soluble, and thus can be readily excreted and detoxified. GST is a Phase II enzyme that facilitates conjugation of electrophilic

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substances with the tripeptide, glutathione, thus aiding in the elimination of toxic compounds and exerting a detoxification function. It has been documented that heavy metals affect the CYP content and GST activity in earthworms, and particularly in *E. fetida* (Bouraoui et al. 2008; Ribera et al. 2001; Saint-Denis et al. 1999).

Previous studies reported that an overproduction of reactive oxygen species (ROS) is related to Cd exposure (Muñoz et al. 2008; Qiu et al. 2008). Free radical byproducts are usually generated accompanied with harsh Phase I oxidation reactions. Cells and tissues protect themselves against ROS damage by means of antioxidant enzymes such as superoxide dismutase (SOD) and catalase (CAT). SOD can dismutate superoxide to hydrogen peroxide (H_2O_2), acting as the first line of defense against ROS. H_2O_2 is subsequently detoxified by several enzymes. CAT converts H_2O_2 to H_2O and molecular oxygen. The study of CAT and SOD in *E. fetida* indicates that this organism has a good ability of dealing with oxidants (Ribera et al. 2001). However, exposure duration has been <30 days in studies dealing with molecular biomarkers in worms (Saint-Denis et al. 1999; Sandrini et al. 2008). Sublethal tests with longer exposure time are thus needed for evaluating the biological effects of pollutants in soil, as well as their bioaccumulation potential.

The objectives of this work were (1) to evaluate the sublethal effects of Cd on selected biochemical responses in the earthworm, *E. fetida*, with longer exposure, and (2) to explore the potential of using these biomarker responses to indicate the contamination of soil with Cd. To achieve these objectives, earthworms were exposed to a series of Cd concentrations for up to 8 weeks. The biomarkers of Phase I (CYP) and Phase II (GST activity) metabolism, and oxidative stress (SOD and CAT activities) were investigated after 1, 2, 3, 4, 6 and 8 weeks of exposure.

Materials and Methods

Earthworms, *E. fetida*, with well-developed clitella weighing 300–400 mg were purchased from Shenyang Agricultural University, China and kept in control soil in the dark at (20 ± 2)°C prior to the start of toxicant exposure. No sexual differences were considered since earthworms are hermaphroditic. The test soil (0–20 cm) was collected from the Ecological Experimental Station of Chinese Academy of Sciences in Shenyang, China. The soil was screened through a 5-mm sieve, kept at 4°C and normal field moisture until use. This soil had the following characteristics: pH 6.2, Kjeldahl nitrogen 0.091 %, total phosphorus 0.04 %, total potassium 0.18 %, organic matter content 1.65 %, cation exchange capacity 12.3 cmol kg^{-1}, water holding capacity (WHC) 32 %. The particle distribution of this soil was as follows: sand (>50 μm) 22 %, silt (1–50 μm) 64 % and clay (<1 μm) 14 %. The Cd background level was 1.80 mg kg^{-1}.

Cadmium exposure doses (0.00, 1.25, 2.50, 5.00 and 10.00 mg Cd^{2+} kg^{-1} soil) were selected to mimic its ambient concentrations of soil in China and the Chinese environmental quality standard for soils (Ministry of Environmental Protection of the People's Republic of China 1995). The experiment was performed according to the method of International Organization for Standardization (ISO 1998) with some modification. $CdCl_2$ solutions (50 mL per 500 g soil) with different concentrations were added to the tested soil to obtain the above nominal concentrations, respectively. After mixing them adequately for 1 h, soils were adjusted to 40 % of the maximum water capacity, and 5,000 g treated-soil was transferred into each aerated container (45 cm × 30 cm × 20 cm) at each concentration (5 containers in total). After aging the spiked soil for 1 week, double soil samples were taken from each treatment to determine the total Cd concentrations. Mature earthworms (130) were placed into each container, cultivated with 12-h light and 12-h dark cycles in an incubating chamber with a constant temperature of (20 ± 2)°C. Worms were collected for the analysis of Cd and enzymes assays after 1, 2, 3, 4, 6 and 8 weeks of exposure. Worms were fed with cow dung during the whole incubation period and a few millilitres of distilled water were added daily into each treatment to maintain suitable humidity for earthworm activity. Five samples (4 worms per sample) at each treatment were collected every time, 2 of which were used for Cd measurement and the others for biochemical analysis. Each sample was measured three times. Both unhatched earthworms cocoons and new-born earthworms were removed throughout the experiment.

The worms (4 worms/sample) collected for preparing biochemical measurements or cadmium analysis were first rinsed with distilled water and placed in containers with moistened filter paper to purge their gut contents for 3 days. The filter paper was changed every day. Samples were subsequently immobilized in ice-cold 20 % (V/V) glycerol solution for 3 min, and the guts were separated. The guts were afterwards washed with cold KCl solution (0.15 mol L^{-1}), and homogenized by hand in a vitreous tissue homogenizer with 5 mL of homogenization buffer solution (250 mmol L^{-1} sucrose, 50 mmol L^{-1} Tris pH 7.5, 1 mmol L^{-1} DTT and 1 mmol L^{-1} EDTA). The homogenate was centrifuged at 15,000×*g* for 20 min at 4°C. The supernatant was collected, one part of which was used for the measurement of GST, SOD and CAT activities, and the other part was further centrifuged at 150,000×*g* for 90 min at 4°C in a hypervelocity centrifuge (Hitachi CP-80MX, Japan) to obtain microsomes for CYP content determination.

Cytochrome P450 content was determined by the method of Omura and Sato (1964) by means of sodium dithionite reduced carbon monoxide. Microsomal protein concentrations were evaluated by the method of Bradford (1976) using bovine serum albumin (BSA) as standard. CAT activity was assayed according to the method of Aebi (1984). The assay mixture contained 0.2 mL supernatant, 1.5 mL of 50 μM phosphate buffer pH 7.8, 0.3 mL of 0.1 M H_2O_2 and 1 mL deionized water. The decrease of the mixture absorbance was recorded at 240 nm for four min. GST activity was assayed using the method of Habig et al. (1974) using 1-chloro-2,4-dinitrobenzene (CDNB) as substrate. The assay was carried out by monitoring the appearance of the conjugated complex of CDNB and glutathione (GSH) at 340 nm. The mixture contained 190 μL of 0.1 M Tris buffer pH 7.0, 0.5 mL of 1 mM GSH, 1 mL of 1 mM CDNB and 10 μL enzyme extract. The reaction was initiated by the addition of GSH. SOD activity was assayed by measuring the inhibition of the photochemical reduction of nitroblue tetrazolium (NBT) (Beauchamp and Fridovich 1971). The assay mixture contained 1.5 mL of 50 mM phosphate buffer pH 7.8, 0.3 mL of 130 mM methionine, 0.3 mL of 750 μM NBT, 0.3 mL of 0.1 mM EDTA, 0.3 mL of 20 μM riboflavin, 0.05 mL of deionized water and 0.05 mL enzyme extract in a total volume of 3 mL. Riboflavin was added finally, and the tubes were shaken and then illuminated for 15 min. The absorbance was recorded at 560 nm and the absorbance of the nonirradiated reaction mixture served as the control.

Soil samples were dried naturally for 48 h, and then sieved to remove aggregates larger than 2 mm. Worms or soil samples were digested with 4 mL of 65 % HNO_3 and 1 mL of 70 % $HClO_4$ for 24 h at 250°C. Dried samples were reconstituted with 10 mL of HCl (20 %, V/V). The Cd content of worms or soil was measured by atomic absorption spectrophotometry (PerkinElmerAA-400, USA). Blanks were taken through the procedure in the same way as the sample. Calibration curves were carried out daily using certified standard solutions (GSB 07-1276-2000, China). Levels of Cd in worms or soil are given on a dry weight basis.

All the data are presented as the mean ± standard deviation (SD). Except for soil concentrations, statistical analyses for all measurements were performed by SPSS software (SPSS 16.0 for windows, SPSS Inc, Chicago, USA). Normality and variance homogeneity were first tested using the Kolmogornov–Smirnov and Levene's tests, respectively. For results with equal variance and normality, the effects of exposure time on Cd accumulation in worms were estimated by a parametric one-way analysis of variance (ANOVA) with Tukey's test to indicate the difference from one another. The non-parametric Kruskal–Wallis test for independent samples ($K > 2$) was applied for heteroscedastic or non-normally distributed results after data transformation. $p < 0.05$ was considered to be statistically significant.

Results and Discussion

Cadmium concentration in each spiked soil was measured and the results were shown in Table 1. It is necessary to mention that no overt signs of toxicity or behavioral or external physiological differences were observed for worms with Cd-exposure during the course of the study, and all sampled worms seemed healthy and active. Cd accumulated in the body with concentrations of 9.10, 10.70, 13.70 and 21.40 mg kg^{-1} dry mass in 1 week for treatment groups exposed to 1.25, 2.50, 5.00 and 10.00 mg kg^{-1}, respectively (Fig. 1). Cd accumulation in week 4 increased by 37.8 %, 46.4 %, 35.0 % and 47.0 % with respect to those in 1 week, respectively. However, it was calculated that the rate of increase of Cd accumulation thereafter was between 11.4 % and 14.0 % for all Cd-treated worms compared with week 4, indicating that a

Table 1 Spiked and measured Cd concentrations in soil

Cd concentrations spiked (mg kg^{-1})	Cd concentrations measured[a] (mg kg^{-1})
0 (control group)	1.77 ± 0.03
1.25	3.15 ± 0.02
2.50	4.22 ± 0.05
5.00	6.83 ± 0.05
10.00	12.54 ± 0.03

[a] Values are the mean ± SD of duplicate analyses

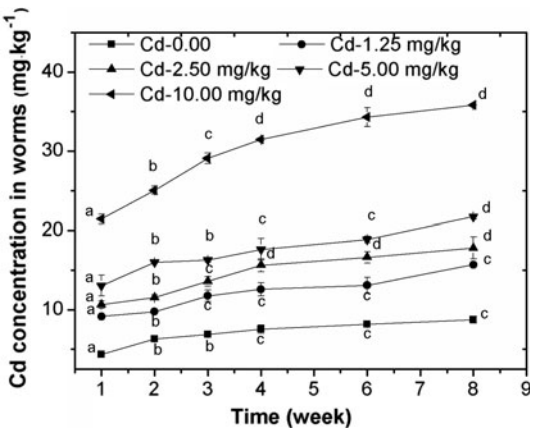
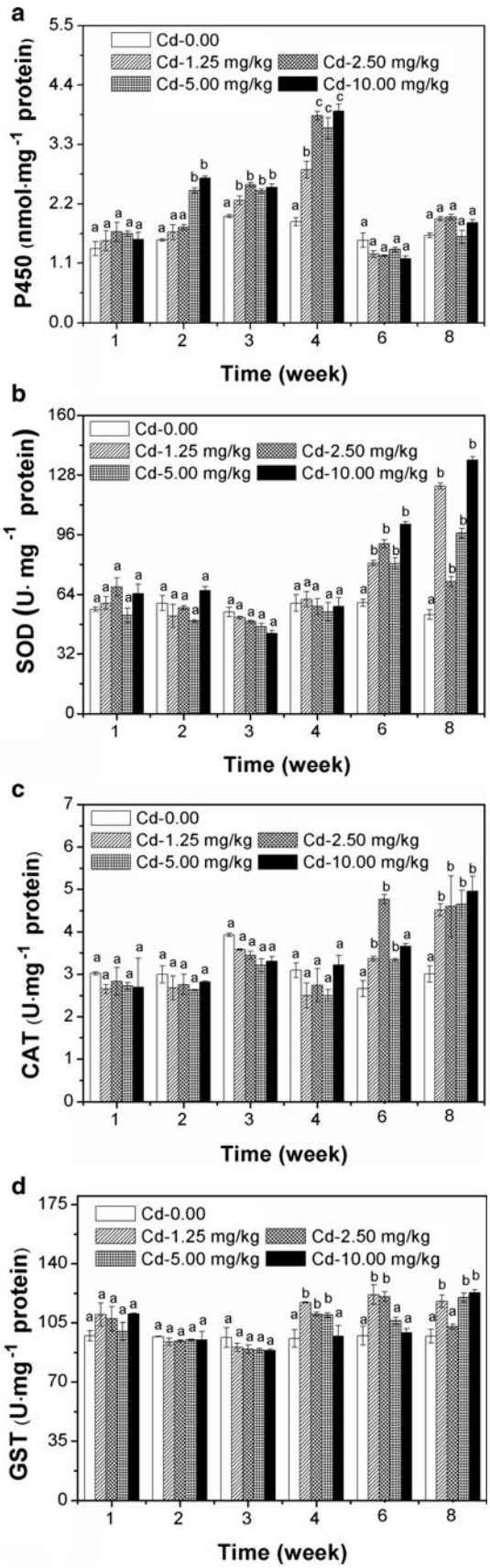


Fig. 1 Cadmium content in the gut of earthworms exposed to Cd^{2+}-contaminated soil. Data are expressed as the mean ± SD of duplicate analyses. The *same letters above the bars* represent values that do not differ significantly ($p > 0.05$)

state approaching homeostasis was obtained with regard to tissue levels of Cd.

Cytochrome P450 represents a family of important Phase I enzymes for the metabolism of many toxic substances, such as polycyclic aromatic hydrocarbons (PAHs) and heavy metals. Its induction and the subsequent increase in the related activities of enzymes are widely used as biomarkers of organic and inorganic pollution in fish and rats. In the present study, CYP in untreated worms varied from 1.40 to 1.50 nmol mg^{-1} protein during the whole experimental period, showing insignificant variation (Fig. 2). A slight and insignificant increase in CYP was observed initially in all groups during the first week. Significant increases in CYP content dependent upon Cd concentration occurred after 2–4 weeks of exposure with respect to the control group. A decline in CYP content was found at 6 weeks of exposure, recording reductions of 55.3 %, 67.8 %, 62.4 % and 69.9 % relative to the week 4 values for each treatment group between 1.25 and 10.00 mg/kg, respectively. A similar response was observed by Bouraoui et al. (2008) in a fish species for ethoxyresorufin-o-deethylase (EROD) activity after 3 weeks of exposure to Cd. Contrarily, some researchers reported that the EROD in worms exposed to Cu or Zn was reduced after 14 days of exposure (Khatun et al. 2008). CYP is a complex family of membrane-bound enzymes located in the endoplasmic reticulum. With increased time of exposure, more Cd was accumulated in worms. Cd exposure with long-term may alter the membrane structure. The permeability and integrity of the membrane is dependent upon the phospholipid bilayer, and heavy metals can bind to the lipid and modify both the charge structure on the surface and the intrinsic confirmation of the lipid molecule (Bouraoui et al. 2008). The effect of Cd on the membrane may be linked with the decrease of CYP content with exposed 6–8 weeks. A second assumption is that an accumulation of ROS, especially hydrogen peroxide (H_2O_2) resulted in the decrease of CYP. Previous work has shown that CYP genes respond to oxidation stress, and the accumulation of CYP mRNAs were thus reduced by H_2O_2 (Barker et al. 1994). Reciprocally, ROS, in particular H_2O_2, can down-regulate CYP expression acting in the signalling pathway through the stress response transcription factor, nuclear factor 1 and nuclear factor kB (Barker et al. 1994). This may also have accounted for the decline of CYP after 6 weeks. However, these results are contrary with those observed by Achazi et al. (1998) and Brown et al. (2004). These authors failed to test the CYP isoenzyme activity in worms.

The SOD-CAT system plays a vital role in fighting against oxygen damage and free radicals generated in Phase II metabolism. SOD and CAT activities in worms without Cd exposure were 56.94 ± 2.78 U mg^{-1} protein and 3.12 ± 0.42 U mg^{-1} protein (Fig. 2b, c), respectively.



◀**Fig. 2** Cytochrome P450 content **a** and activities of SOD **b**, CAT **c**, GST **d** in earthworms exposed to 0.00, 1.25, 2.50, 5.00 and 10.00 mg kg^{-1} Cd. Data are expressed as the mean ± SD of triplicate analyses. The *same letter above the bars* represent values that do not differ significantly ($p > 0.05$)

It is noteworthy that the variation tendency of SOD and CAT was very similar over the duration of the study. Interestingly, a fluctuation occurred in SOD and CAT activities in Cd-treated worms compared to the control group between 4 and 6 weeks of exposure. An apparent enhancement of activity by both enzymes was observed when exposure time was extended to 6 weeks. At the end of experiment (week 8), SOD activity was 130.0 %, 33.5 %, 82.5 % and 156.0 % higher than the corresponding control level at the Cd treatment levels of 1.25, 2.50, 5.00 and 10.00 mg/kg, respectively. CAT activity levels increased 50.4 %, 53.3 %, 54.9 % and 65.0 %, respectively, by week 8. The current results showed that the response of oxidative stress in earthworms possibly occurred after 4 weeks. A more intensive response to oxidative stress appeared with longer exposure to Cd. It may be assumed that the worms initially had the ability to combat oxidative stress. However, with increased exposure time, both Cd and ROS accumulation increased. This may have resulted in an exceedance of the worms' normal maximum antioxidative capability, leading to a need for increased antioxidative enzyme production. The elevated SOD and CAT activities at 6–8 weeks of exposure were responses to this need.

The suppressed CYP content by the apparent accumulation of ROS after 6 weeks of Cd exposure coincided with the increases in SOD and CAT activities. Though it was reported in one study with plants (Hou et al. 2007) that increased concentrations of heavy metals reduced rather than increased antioxidative enzyme activities, another study with fish showed that Cd exposure over time resulted in increased SOD and CAT activities (Messaoudi et al. 2009). Interestingly, our results are in agreement with the latter study. SOD and CAT activities increased as exposure time was increased beyond 4 weeks.

Glutathione-s-transferase as an enzyme in Phase II metabolism that also serves as an antioxidative enzyme. In the present study, GST activity did not exhibit any significant variation from controls with the different Cd exposures during the first 3 weeks (Fig. 2d). However, significant increases in GST activity occurred during weeks 4–8. This observation is in accordance with that documented by Saint-Denis et al. (1999) showing that the GST in earthworms exposed to benzo (a) pyrene remained unchanged with short exposure time. The significant activation observed starting at week 3 of exposure in GST activity is related to free radical reactions, resulting in the induction of metal-binding protein and the subsequent sequestering of the metal to result in its presence in a biologically inactive form (Radwan et al. 2010; Saint-Denis et al. 1998).

This study observed that Cd accumulation showed only minor changes in weeks 6–8 compared to the first 4 weeks. As a previous study has shown, the main detoxification pathways for Cd are sequestration within inorganic matrices or binding to organic ligands (Spurgeon and Hopkin 1999). Cd accumulation in worms exposed for 4 weeks may have been close to the maximum of the sequestration capacity, leading to the insignificant change of Cd accumulation in weeks 6–8.

In summary, current results confirmed that cadmium could be metabolized by oligochaete earthworms, just as Sandrini et al. (2008) observed in polychaete worms. One of the aims of this study was to explain the effects of longer exposure on molecular biomarkers. In previous studies with worms of 4 weeks or less of exposure (Ribera et al. 2001; Saint-Denis et al. 1999), biochemical responses were dependent on both dose and time. The present research with 8 weeks of exposure confirmed the effects of dose and exposure time, but indicated that exposure time played a more important role. CYP increased at 3 weeks, and even at 2 weeks in the higher Cd exposure, but GST activity was not affected until week 4, and the activities of SOD and CAT were not affected until week 6.

The second aim in this research was to explore the possibility of applying the biomarker responses to indicate the presence of Cd-contaminated soil. The concentrations used in our study were environmentally realistic (Lagerwerff and Specht 1970; Liu et al. 2005). A previous study has shown that CYP, GST SOD and CAT may serve as biomarkers in earthworms (Scott-Fordsmand and Weeks 2000). Our study has shown that the effect of dose and duration of Cd on the Phase I enzyme (CYP) and on the Phase II enzyme (GST), as well as the antioxidant enzymes (SOD and CAT), were very significant and appeared at different times. It is thus proposed that a suite of biochemical responses rather than a selective choice in *E. fetida* could serve as a sensitive tool for use in soil contamination surveys. Similarly, previously published study also proposed the use of a suite of biomarkers (Saint-Denis et al. 1998, 1999).

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