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Enzymatic biomarkers of earthworms *Eisenia fetida* in response to individual and combined cadmium and pyrene

Xiaoxia Yang [a,b], Yufang Song [a,*], Jianrong Kai [a,b], Xiufeng Cao [a,b]

[a] Key Laboratory of Pollution Ecology and Environmental Engineering, Institute of Applied Ecology, Shenyang 110016, People's Republic of China
[b] Graduate School, Chinese Academy of Sciences, Beijing 100049, People's Republic of China

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ABSTRACT

The responses of enzymatic biomarkers of earthworms *Eisenia fetida* to low-level exposures of cadmium (Cd) (2.50 mg kg^{-1}), pyrene (0.96 mg kg^{-1}) or their combination were investigated in this study. A set of enzymatic biomarkers, namely, cytochrome P450 (CYP) as a family of phase I enzymes, glutathione-s-transferase (GST) as one of phase II enzymes and antioxidant enzymes (superoxide dismutase (SOD) and catalase (CAT)), was selected to evaluate the responses of the earthworms in a period upto eight weeks. The earthworms exposed to the mixture of Cd and pyrene demonstrated different responses of the enzymatic biomarkers from those exposed to Cd or pyrene alone. The responses of enzymatic biomarkers to the combined exposure were time-dependent, with initial antagonistic effects on CYP content and activities of GST and SOD, but with additive effects at the end of experiment causing the reductions of CYP content and GST activity and the enhancement of activities of SOD and CAT. Our results indicated the toxicity of low-level pyrene may be prolonged by the co-presence of Cd.

1. Introduction

Soils contamination due to discharge of various organic and inorganic pollutants has been a worldwide issue for human health and agriculture. Hence, terrestrial organisms living on contaminated soils are typically exposed to a mixture of toxicants of both related and distinct classes. Combined effects of toxicants may be stronger (synergism) or weaker (antagonism) than expected from the observed effects of individual exposures of toxicants. The responses to exposure to multiple pollutants are dependent upon the components of the mixture and may vary significantly, such as additive effects for narcotic compounds (Jensen and Sverdrup, 2002), synergistic lethal effects among metals and between metals and organic compounds (Fleeger et al., 2007; Maria and Bebianno, 2011). The toxicity of mixed toxicants has been well studied in aquatic environment (Banni et al., 2009; Benedetti et al., 2007; Bouraoui et al., 2009; Dondero et al., 2011), but not in soil environment. Soil environment is so complex that different types of interactions may occur, including chemical and physico–chemical interactions affecting sorption and bioavailability, physiological interactions in the organisms affecting uptake from soil, or mechanistic processes at the target receptors. Individual toxicants may interfere with each other in these steps, leading to changes in toxicity and responses.

Heavy metals and polycyclic aromatic hydrocarbons (PAHs) usually co-occur in soils. Cadmium (Cd) as a non-essential heavy metal is well known to cause adverse effects on organisms (Friberg et al., 1985). PAHs, all made up of 2–7 benzene rings, are ubiquitous in the terrestrial environment. Of the PAHs, pyrene, a tetracyclic PAH, is considered to be non-carcinogenic by the International Agency for Research on Cancer and the US Environmental Protection Agency (Brown et al., 2004; Jensen and Sverdrup, 2003). Toxicities of this PAH are thought to result principally from non-polar narcosis. Benzo[*a*]pyrene (B[*a*]P) has been extensively studied in invertebrates due to its known mammalian carcinogenicity (Banni et al., 2009; Saint-Denis et al., 1999), however, the toxicities of pyrene in terrestrial invertebrates are not well investigated.

Earthworms may represent 60–80 percent of the total soil biomass and have favorable effects on soil structure and function (Saint-Denis et al., 1999). These traits make them one of the most suitable organisms to examine biological effects of chemicals under laboratory conditions. A set of standard test guidelines has been established (ISO, 1998; OECD, 1984) focusing upon acute toxicity and chronic bioassay endpoints of reproduction and growth of earthworms *Eisenia fetida* as the standard species.

Biomarkers that apply molecular endpoints may also be valuable tools for toxicity assessment. A range of biomarkers have been developed in recent years to evaluate organisms' stress response to individual toxicants (Banni et al., 2009; Bouraoui et al., 2009; Saint-Denis et al., 2001). Cytochrome P450 (CYP) is a huge family of phase I enzymes of detoxification process,

* Corresponding author. Fax: +86 2483970426.
E-mail address: songyufang@iae.ac.cn (Y. Song).

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catalyzing the oxidative conversion of lipophilic xenobiotics into entities which are more water-soluble and can be readily excreted and detoxified. Glutathione-s-transferase (GST) as one of phase II enzymes can facilitate conjugation of electrophilic substances or tripipetide glutathione and exert the function of detoxification. This enzyme also plays a role in cellular protection against oxidative stress. Previous studies have documented that both heavy metals (such as Cd, Copper (Cu)) and PAHs (such as B[*a*]P, pyrene) may induce the responses of CYP and GST in earthworms (Brown et al., 2004; Ribera et al., 2001; Saint-Denis et al., 1999). A by-product of the metabolism of xenobiotics by CYP is the production of free radicals. To counter these effects, earthworms possess a suite of antioxidant enzymes such as superoxide dismutase (SOD) and catalase (CAT). SOD dismutates superoxide to hydrogen peroxide (H_2O_2), acting as the first line of defense against reactive oxygen species (ROS). H_2O_2 is subsequently detoxified by CAT and other enzymes.

Many previous studies focused on the individual effects of Cd and pyrene on earthworms' survival, reproduction and even the responses of detoxification system as well. The number of earthworms did not change significantly when exposed to 1–10 mg/kg Cd in soil upto 4 months (Lapinski and Rosciszewska, 2008). Reinecke et al. (1999) concluded that earthworm *E. fetida* developed the resistance to Cd during an exposure even for 3 years to low-level Cd. It has been reported that the survival, cocoon production rate and weight change did not vary significantly for earthworms *Lubricus rubellus* exposed to soil with pyrene ranging from 0 to 40 mg/kg for 42 days (Brown et al., 2004). The CYP content and antioxidant enzymes varied subtly when earthworms *E. fetida* were exposed for 14 days to pyrene less than 0.96 mg/kg (Zhang et al., 2007). Nevertheless, significant increases of CAT and SOD activities were observed when earthworms *E. fetida* were exposed to pyrene of 50 or 100 mg /kg soil (Wu et al., 2012).

While the effects of individual exposure of Cd and pyrene on enzymatic biomarkers were documented on earthworms, the combined effects of low-level Cd and pyrene on earthworms *E. fetida* are rarely reported. Moreover, exposure duration is generally less than 30 days in most previous studies paying attention to the response of enzymatic biomarkers of earthworms. Therefore, the study on the response of earthworms *E. fetida* to environmentally relevant low-level exposure of the mixture of Cd and pyrene for a longer period is needed for better evaluating ecological risk of Cd and pyrene in laboratory and field conditions. This study aims to investigate individual and combined effects of cadmium (2.5 mg/kg) and pyrene (0.96 mg/kg) on a set of enzymatic biomarkers (CYP, SOD, CAT and GST) of earthworms *E. fetida* upto eight weeks, and to provide valuable information for ecological risk assessment of the interacted effects of Cd and pyrene at environmentally relevant levels.

2. Materials and methods

2.1. Animals and treatment

Earthworms, *E. fetida*, with well-developed clitella 300–400 mg were purchased from Shenyang Agricultural University, China and kept in control soil in the dark at $(20\pm2)^{\circ}C$ 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 Experiment Station of Chinese Academy of Science in Shenyang, China. The soil was screened through a 5 mm sieve, kept at 4 °C and normal field moisture until use. The soil had the following characteristics: pH 6.20, Kjeldahl nitrogen 0.09 percent, total phosphorus 0.04 percent, total potassium 0.18 percent, organic matter content 1.65 percent, cation exchange capacity 12.30 cmol kg^{-1}, water holding capacity 32.00 percent. The particle distribution of this soil was as follows: sand ($>50\,\mu m$) 22 percent, silt (1–50 μm) 64 percent, clay ($<1\,\mu m$) 14 percent. The background level of Cd in the soil is 1.80 mg kg^{-1}, and pyrene was not detectable in this soil.

The environmentally relevant concentrations of Cd (2.50 mg kg^{-1}) and pyrene (0.96 mg kg^{-1}) were selected in the experiment based on previous studies (Brown et al., 2004; Lapinski and Rosciszewska, 2008; Reinecke et al., 1999; Zhang et al., 2007) and the average level of Cd (2.50 mg kg^{-1}) in agricultural soil of Shenyang, China. The worms were randomly divided into four groups with four replicates: (1) control, (2) pyrene: 0.96 mg kg^{-1}, (3) Cd: 2.50 mg kg^{-1} and (4) the mixture of pyrene and Cd (pyrene: 0.96 mg kg^{-1} + Cd: 2.50 mg kg^{-1}). Pyrene and $CdCl_2$ were dissolved in acetone and water, respectively, and then transferred to the soil. The soils were placed in a well-ventilated fume hood and turned daily for 7 days in order to evaporate acetone and age the spiked soil. Following acetone evaporation, all soils were rehydrated to 40 percent of water holding capacity and left one day to equilibrate. 5000 g treated-soil for each replicate was placed in an aerated container (45 cm × 30 cm × 20 cm) and matured earthworms (130) were transferred to each container. Containers were subsequently covered with a perforated lid to limit water loss and kept for eight weeks at $(20\pm2)^{\circ}C$ in a 12:12-h light-dark regime. Worms were fed with dry and defaunated cow dung twice a month during the whole incubation period, and a few milliliters of distilled water were added daily into each container to maintain suitable humidity for earthworm activity.

Worms were collected for the biochemical analysis after one, two, three, four, six and eight weeks of exposure. Five samples (Four worms per sample) at each container were collected every time. Each sample was measured three times. Both unhatched earthworm cocoons and new-born earthworms were removed throughout the experiment.

2.2. Biochemical assays

Worms were first rinsed with distilled water, and placed on the petri dish with moistened filter paper to purge their gut contents for 3 days. Filter paper was changed every day during this period. Samples were subsequently immobilized in ice-cold 20 percent (V/V) glycerol solution for 3 min. The guts were then separated, washed afterwards with cold 0.15 M KCl solution and homogenized manually in a vitreous tissue homogenizer with 5 ml homogenization buffer (250 mM sucrose, 50 mM Tris pH 7.5, 1 mM DTT and 1 mM EDTA). Homogenates were centrifuged at 4 °C for 20 min at $15{,}000\times g$ to produce the post-mitochondrial fraction. One part of the supernatant was collected for the determination of activities of SOD, CAT and GST. The other part was further centrifuged at $150{,}000\times g$ for 90 min to obtain microsomal pellets 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. GST activity was assayed by 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. 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 absorbance of the mixture was recorded at 240 nm for 4 min.

2.3. Statistical analysis

All data were represented as the means ± standard deviation (SD). Statistical analysis for all measurements was performed by SPSS 16.0 software (SPSS Inc, Chicago, USA). Normality and variance homogeneity were first tested using Kolmogornov–Smirnov and Levene's tests, respectively. However, even though mathematical transformation was applied to dependent variables, the variations after data transformation were still heteroscedastic or non-normally distributed in this study. Therefore the non-parametric Kruskal–Wallis test for independent samples ($K>2$) was adopted to test the difference between the control and treated samples at the same exposure time. A statistical difference at $P<0.05$ was considered to be significant, and that at $P<0.01$ very significant.

3. Results

The concentrations of Cd and pyrene in the soil treated with Cd and pyrene were verified as 4.22 ± 0.05 and 0.80 ± 0.03 mg kg^{-1},