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Toxic responses of cytochrome P450 sub-enzyme activities to heavy metals exposure in soil and correlation with their bioaccumulation in *Eisenia fetida*

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ABSTRACT

The dose- and time- dependent responses of cytochrome P450 (CYP) sub-enzyme activities to heavy metals in soil, and the relationships between biomarker responses and metal bioaccumulation in *Eisenia fetida* were evaluated. Earthworms were exposed to soils spiked with increasing doses of Cd, Cu, Pb or Zn for 21 d. Results demonstrated that EROD and CYP3A4 activities responded significantly with increasing dose and exposure duration. EROD activity significantly ($P < 0.05$) correlated with CYP3A4 activity exposed to Pb and Cu. The earthworm metal burdens had significant correlation with the total metal concentrations in soil ($P < 0.01$). The bioaccumulation factor (BAF) decreased with the increasing metal concentration in soil. The order of metal bioavailability to *E. fetida* was Cd > Zn > Cu > Pb. CYP3A4 activity in Pb-exposed earthworms had a significant correlation with the accumulated metal ($P < 0.05$). Both EROD and CYP3A4 activities in Cu-exposed worms negatively correlated with BAF ($P < 0.05$). Based on Discriminant Analysis (DA), CYPs activities were sensitive biomarkers of heavy metals exposure, and we also concluded that different biomarkers with multiple durations could be conducted in the eco-toxicological diagnosis of soil pollution.

1. Introduction

Heavy metal contamination in soils is a widespread problem due to the continuously metals input into the soils through various agricultural and industrial activities (Wu et al., 2012), which may harm organisms, ecosystem structure and functioning (van Gestel et al., 2009) and eventually pose threat to human health. Various methods from chemical analyses to bio-tests (Lukkari et al., 2004; Panzarino et al., 2016) have been widely used to assess the potential toxicity of these pollutants in soils. However, chemical methods based on the analysis of the concentrations of heavy metals in the soil could not provide an intact and valuable information of deleterious effects of contaminants on the biota (Calisi et al., 2011) because chemical analysis neglects the bioavailability of metals, which could be influenced by the soil characteristics such as pH, organic matter contents, cation exchange capacity (CEC). The best integrators of these complex toxic effects are the exposed organisms themselves (Gastaldi et al., 2007). Among soil organisms, earthworms such as *Eisenia fetida* play a major role in the functioning of the soil ecosystem (van Gestel et al., 2009). They are readily available, cost-effective and easy to maintain in laboratory conditions. But more importantly, earthworms in soil expose to heavy metals through their intestine and skin via alimentary and dermal uptake routes (Homa et al., 2010), meanwhile they can accumulate heavy metals from the soil at various levels (Dallinger, 1993; Lukkari et al., 2004; Morgan and Morgan, 1999). Hence, they have been proposed as bio-indicators of soil heavy metal pollution (Carpene et al., 2006) by using endpoints described in acute and reproduction tests according to OECD guidelines (OECD, 1984), and/or by employing the biochemical index to assess the ecotoxicity of pollutants in soil.

Biomarkers respond as early-warning signals to toxicants at earlier stages and lower concentrations (Eason and O'Halloran, 2002; Fourie et al., 2007; Nam et al., 2015). By measuring biochemical and cellular responses of organisms to pollutants, biomarkers have been widely recommended to figure out the toxicity of contaminants at lower levels in the environment over the last two decades (Song et al., 2015; Spurgeon et al., 2005). In recent years, the use of biomarkers with earthworms to evaluate the toxic effects of contaminants in soil has received increased attention (Calisi et al., 2011; Li et al., 2017), so that

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biomarkers developing with the characteristics of more sensibility and reliability have become the necessary issues to be focused. As a large family of hemoproteins, CYP monooxygenase systems are composed by many iso-enzymes and play a vital role in the detoxification and biotransformation of many xenobiotic compounds (Gottardi et al., 2016; Kim et al., 2013). CYPs are mostly found to be inhibited by heavy metals. For example, exposure to Pb caused a great inhibition on EROD (CYP1A1) activity in the bivalve (*Chlamys farreri*) (Zhang et al., 2010). The transcript level of CYP1A in European flounder was repressed by Cd (Williams et al., 2006). Vieira et al. (2009) found that EROD activity in fish (*Pomatoschistus microps*) was inhibited by Cu and leaping mullet EROD activity was also significantly inhibited by Hg, Ni, Cd, Cu, Zn (Sen and Semiz, 2007). However, studies on the usage of earthworm CYPs in the monitoring of soil heavy metal pollution are relatively less. Lukkari et al. (2004) discovered that CYP1A1 in earthworms *Aporrectodea tuberculata* can be induced by Cu or Zn. While Saint-Denis et al. (2001) found that MROD (CYP1A2) in earthworm *E. fetida* exposed to Pb spiked soil was significantly lower than the control. In our previous study, we found that EROD can be induced at lower concentrations of Cd, Pb, Cu or Zn and repressed at higher concentrations of Cd in filter papers for 48 h (Cao et al., 2012). While CYP3A4 activity was significantly induced by Cd or Pb, rather than by Cu or Zn. However, if EROD and CYP3A4 activities in earthworms are to serve as early warning signals for metal contamination survey of soil, long-term heavy metal exposure in real soils need to be conducted to study the time-dependent responses, and the results derived from the filter contact experiment need to be determined to what extent to be representative of that from the soil method. In addition, since the bioavailability of heavy metals in soils can be affected by soil characteristics etc., metal concentration in earthworms may reflect the real exposure condition, thus the bioaccumulation patterns and bio-concentrations of metals in earthworms should also be determined, and the relationships between biomarker responses and metal bio-concentration can also be explored.

In this study, following the OECD standard acute toxicity test (OECD, 1984), the earthworm *E. fetida* was exposed to a real soil spiked with different doses of Cd, Cu, Pb or Zn, and biomarker responses, heavy metal bioaccumulation, and their relationships were investigated to find out 1) the response of EROD and CYP3A4 activities in earthworm *E. fetida* under long-term exposure to Cd, Cu, Pb or Zn in soil; 2) differences in biochemical responses derived from filter paper contact and real soil exposure experiment; 3) differences in uptake of Cd, Cu, Pb and Zn by earthworms; 4) the potential relationships between bioconcentrations and biochemical responses under long-term exposure of Cd, Cu, Pb or Zn; and 5) the sensitivity of EROD and CYP3A4 activities as biomarkers to monitor heavy metal contamination in soil.

2. Materials and methods

2.1. Soil, earthworms and chemicals

Soil (0–20 cm) was collected in Shenyang Ecological Station, China with characteristics of pH 6.2, K–N 0.09%, total P 0.04%, total K 0.18%, organic matter content 1.65%, cation exchange capacity 12.3 cmol kg^{-1}, water holding capacity (WHC) 32%, sand (> 50 μm) 22%, silt (1–50 μm) 64%, and clay (< 1 μm) 14%. The background values of heavy metals contents are as follows: 0.42 mg kg^{-1} for Cd, 34.6 mg kg^{-1} for Pb, 25.2 mg kg^{-1} for Cu, and 67.8 mg kg^{-1} for Zn, respectively. Soil was air-dried and sieved through a 2 mm sieve.

Cadmium chloride, copper sulfate, lead acetate, and zinc sulfate were used in this study were all of analytical grade that were purchased from Sigma-Aldrich Company (St. Louis, MO, USA) or from their commercial suppliers in Shenyang, China. NADPH, 7-Ethoxyresorufin, Resorufin was purchased from Sigma-Adrich Company (St. Louis, MO, USA).

Adult Earthworms (*E. fetida*) with well-developed clitella were purchased from a commercial breeder in shenyang, China.

2.2. Soil contact test

Earthworm soil contact test was performed according to the OECD guidelines (OECD, 1984). Cadmium chloride, copper sulfate, lead acetate, and zinc sulfate were respectively made into four different concentrations of aqueous solutions. 75 ml different concentrations of heavy metal solutions (Cd, Cu, Pb, Zn) or H_2O (control) was respectively added to 750 g (dry weight) meadow brown soil to obtain the required soil series as follows: 0, 0.5, 1, 2, 4 mg kg^{-1} for Cd, 0, 200, 300, 400, 500 mg kg^{-1} for Pb, 0, 50, 100, 200, 300 mg kg^{-1} for Cu, and 0, 200, 300, 400, 500 mg kg^{-1} for Zn. The soil was then mixed for more than 30 min to obtain homogeneously spiked soil and the water content was adjusted to 20% before being put into aerated boxes. Each treatment had 8 boxes. After aging the spiked soil for two weeks, 16 earthworms were added to per 750 g soil, and cultivated at 20 ± 1 °C under continuous light to ensure that the worms remained in the soil. Dry cow dung was added weekly on the soil surface as a food source. During the exposure periods of 3, 7, 14 and 21d, four earthworms were collected from each box. Four earthworms in each replicate were pooled together as one replicate for biochemical determination. At the same time, soil and another 16 earthworms were collected for metal determination.

2.3. Microsomes preparation and biochemical assays

Earthworms were selected and put in separate beaker with moist filter paper for 24 h to void their gut contents. After immobilization, guts were collected and washed, then homogenized and centrifuged twice. The obtained microsome pellets were resuspended to obtain microsomal solutions to determine CYP sub-enzyme activities. See more details in previous study (Cao et al., 2012).

EROD activity was determined by the fluorometric method according to Pohl and Fouts with some modifications (Pohl and Fouts, 1980). The fluorescence was measured at 586 nm in a F-2500 fluorescence spectrophotometer (Hitachi CP-80MX, Japan) using an excitation wavelength of 560 nm. CYP3A4 activity was determined as testosterone 6β-hydroxylase activity by HPLC (Waters, USA). Total microsomal protein content was determined by the Bradford method (Bradford, 1976) with a UV-2550 dual beam spectrophotometer (Shimadzu, Japan) using BSA as a reference protein. The details for the determination of EROD and CYP3A4 activities were described in our previous study (Cao et al., 2012).

2.4. Analysis of heavy metals in soil and earthworms

The collected earthworms were washed and kept on moist paper for 48 h to void their guts. Then the worms were freeze-dried and stored at −20 °C. Cd, Pb, Cu or Zn concentrations in soil and worms were obtained by $HNO_3/HClO_4$ (3:1, v/v) digestion and analyzed by a flame atomic absorption spectrometer (AAS, Perkin Elmer 400, USA) as described in the references (Ji et al., 2011; Katz and Jenniss, 1983).

2.5. Statistical analysis

All data were first tested for normality and homogeneity of variances. One-way ANOVA was used to analyze the significant difference between treatments, followed by LSD post-hoc method. Pearson correlation was calculated to evaluate the degree of relationship between EROD and CYP3A4 activities, as well as between the accumulated metal levels in earthworms and biochemical responses. Principal component analysis (PCA), DA and the above analysis were carried out with SPSS 16.0 software. Origin 8.0 was used to draw the figures.