Effects of Cadmium on the Distribution and Accumulation of Benzo(a)pyrene in Earthworm Subcellular Fractions: Postprint

Authors: Zhou Lina, Zhou Jing, Li Huixin, Hu Feng, Xu Li

Date: 2017-10-30T00:00:00+00:00

Abstract

Exogenous addition of different concentrations of cadmium ions (Cd2+) was employed to investigate the distribution and accumulation of benzo(a)pyrene (BaP) in different subcellular fractions of earthworms (fraction C: cytosolic fraction; fraction D: solid particle fraction; fraction E: cell debris fraction) under combined pollution conditions, and to explore the underlying mechanisms. The results showed that BaP was mainly distributed in the cell debris fraction of earthworms, followed by the solid particle fraction, with the lowest concentration in the cytosolic fraction. Under Cd2+ addition treatments, as the concentration of Cd2+ increased, the BaP concentration in the three cellular fractions showed a trend of first decreasing and then increasing. As the concentration of Cd2+ increased, the protein content and acetylcholinesterase (AChE) activity in the three subcellular fractions both showed a trend of first increasing and then decreasing; whereas the glutathione S-transferase (GST) activity in the cytosolic and cell debris fractions of earthworms showed a trend of first decreasing and then increasing, but gradually increased in the solid particle fraction. Correlation analysis indicated that the protein content in the cytosolic and cell debris fractions of earthworms was significantly negatively correlated with the BaP concentration in their corresponding fractions; the AChE activity in the cytosolic fraction was significantly negatively correlated with the BaP concentration in this fraction; whereas the GST activity showed no significant correlation with BaP concentration. In summary, BaP was mainly distributed and accumulated in the cell debris fraction, and Cd2+ may affect the accumulation of BaP in the cell debris and cytosolic fractions by influencing protein content and AChE activity, resulting in the trend of BaP concentration first decreasing and then increasing with increasing Cd2+ concentration.

Full Text

Preamble

ACTA ECOLOGICA SINICA ChinaXiv Partner Journal
Vol. 37, No. 19, Oct. 2017
DOI: 10.5846/stxb2016071390

Effects of Cadmium on the Distribution and Accumulation of Benzo[a]pyrene in Subcellular Fractions of *Eisenia fetida*

ZHOU Lina, ZHOU Jing, LI Huixin, HU Feng, XU Li
Nanjing Agricultural University, Nanjing 210000, China

Abstract: This experiment explored the mechanism underlying benzo[a]pyrene (BaP) distribution in different subcellular fractions of the earthworm *Eisenia fetida* (Fraction C: associated with the cytosol; Fraction D: associated with granules; and Fraction E: associated with tissue fragments and cell membranes) under conditions of contamination with both BaP and cadmium ions (Cd^{2}) at different concentrations. The results showed that Cd^{2} inhibited the accumulation of BaP in the earthworm, and that BaP was accumulated to the greatest extent in Fraction E, followed by Fraction C and Fraction D. With the addition of Cd^{2} , BaP concentrations in the three fractions initially decreased, but subsequently increased with the increasing concentration of Cd^{2} , whereas protein content and acetylcholinesterase (AChE) activity showed the opposite trend. In contrast, glutathione S-transferase (GST) activity initially decreased and then subsequently increased with increasing Cd^{2} concentration in Fraction C and E, whereas the activity gradually increased in Fraction D. Correlation analysis indicated that protein content showed a significant negative correlation with BaP concentration in Fraction C and E; AChE activity showed a significant negative correlation with BaP concentration in Fraction C; and GST activity showed a non-significant correlation with BaP concentration. Collectively, the results indicate that BaP mainly accumulated in Fraction E, and that the accumulation of BaP in Fraction C and E was correlated with the content of protein and activity of AChE, which was influenced by Cd^{2} with the increasing concentration of Cd^{2} . Furthermore, BaP concentration initially decreased, but subsequently increased.

Keywords: benzo[a]pyrene; cadmium; *Eisenia fetida*; subcellular fraction; combined pollution

Funding: National Natural Science Foundation of China (41371469); Special Fund for Scientific Research in the Public Interest (201503121); Project of Jiangsu Provincial Advantageous Disciplines

Received: 2016-07-07; **Online Publication Date:** 2017-05-27

Corresponding Author. E-mail: xuli602@njau.edu.cn

Introduction

Heavy metals and polycyclic aromatic hydrocarbons (PAHs) are two important classes of pollutants in soil environments. Their simultaneous presence forms combined pollution, which not only severely damages soil ecosystems but also poses risks to human health. Toxicity testing and environmental ecological risk assessment of pollutants under combined pollution conditions are therefore necessary. National soil pollution surveys in China have shown that cadmium (Cd) was detected as exceeding standards at survey points across two-thirds of China's land area, and has been identified as the primary soil pollutant in China. Benzo[a]pyrene (BaP) is one of the most concerning pollutants among PAHs due to its cytotoxicity, mutagenicity, and carcinogenicity. These pollutants can enter farmland systems through industrial emissions, sewage sludge application, and other pathways, creating combined pollution that is currently a hot research topic in environmental science. This combined pollution not only adversely affects crop growth and development but also threatens human and animal health through the food chain.

Earthworms are one of the most abundant animal groups in soil and play an irreplaceable role in maintaining ecosystem functions, including decomposition of soil organic matter, nutrient cycling and release, and improvement of soil properties. They also serve as important indicator organisms for soil contamination. *Eisenia fetida*, in particular, has been selected by the Organization for Economic Co-operation and Development (OECD) as a model organism for assessing pollutant toxicity due to its high sensitivity to contaminants, strong experimental operability, and established standard testing protocols.

Previous research has primarily focused on the ecological toxicological effects of heavy metals or PAHs, individually or in combination, on earthworms at the organismal level. For example, Wang Hui et al. reported that cadmium exposure inhibits cellulase activity in *Eisenia fetida*, while Zhang Wei et al. demonstrated that benzo[a]pyrene can induce increased cytochrome content in earthworms. Zhao Zuoyuan found that combined cadmium and phenanthrene pollution had antagonistic toxic effects on earthworms while inhibiting superoxide dismutase (SOD) activity, with the degree of phenanthrene toxicity reduction decreasing as cadmium concentration increased. Jiang also reported toxic effects of heavy metal and typical PAH combined pollution on *Eisenia andrei* and *Fridericia bulbosa*, finding that pollutant composition and concentration influence the interaction type of combined pollution, which can be either antagonistic or synergistic.

While toxicity effect studies have accumulated, research on the distribution and accumulation characteristics of pollutants in earthworms under different types of combined pollution conditions remains limited. Previous toxicity tests have mainly focused on pollutant distribution at the individual level, neglecting pollutant behavior at the subcellular or molecular level. However, pollutant behavior characteristics in earthworms, particularly distribution at the subcellular level,

are crucial for revealing earthworm strategies for pollutant sequestration and detoxification, and important ecotoxicological information can only be obtained at the subcellular level.

Our laboratory' s previous research based on soil pollution exposure environments has shown that BaP concentrations differ among different subcellular fractions of *Eisenia fetida*. Therefore, studying pollutant accumulation and distribution at the subcellular level can better evaluate pollutant toxicity and elucidate earthworm mechanisms for pollutant sequestration and detoxification. Some studies have shown that certain earthworm physiological and biochemical indicators can be used to evaluate pollutant accumulation and distribution characteristics. For instance, protein content may promote pollutant accumulation by inducing the expression of certain proteases, providing binding sites for pollutants. Acetylcholinesterase (AChE) is a key enzyme in biological neurotransmission that degrades acetylcholine to ensure normal signal transmission. It may affect pollutant transformation and metabolism by influencing normal physiological metabolic processes. Glutathione S-transferase (GST) plays an important role in the metabolic detoxification and distribution of xenobiotics, such as affecting the binding and transport functions of chemicals between cellular compartments and transporting pollutants into vacuoles for detoxification.

This study constructed combined pollution conditions using semi-static solution culture to investigate the effects of different Cd^{2} concentrations on BaP accumulation and distribution in earthworm subcellular fractions. We simultaneously measured protein content, AChE activity, and GST activity in each fraction to reveal the mechanisms underlying BaP distribution and accumulation in earthworm subcellular fractions under combined pollution.

---

1. Experimental Materials

Eisenia fetida earthworms were purchased from an earthworm breeding base in Anhui Province. Healthy mature earthworms with similar size and weight (300–500 mg) and with clitella were selected as test organisms. Benzo[a]pyrene (analytical grade) was purchased from Aladdin Reagents, and cadmium chloride (CdCl) was purchased from J&K Scientific.

---

2. Experimental Design

This experiment employed a semi-static solution culture method based on a modified protocol. The simulated culture solution was prepared as follows: calcium nitrate (Ca(NO)), magnesium sulfate (MgSO), and sodium nitrate (NaNO) were used to prepare a simulated solution containing 1.0 mmol/L Na , 0.1 mmol/L K , 0.1 mmol/L Ca^{2} , and 0.1 mmol/L Mg^{2} , with pH = 7.0. BaP

was dissolved in acetone and added to the simulated solution to a final concentration of 50 mg/L. CdCl was then added to achieve final concentrations of 0, 0.2, 0.5, and 0.8 mg/L.

The experiment used glass petri dishes, each containing 20 mL of pollutant simulation solution. Earthworms were placed in each dish, which was sealed with parafilm to prevent escape while ensuring ventilation. The dishes were incubated in the dark at (20±1) °C. Dead earthworms were removed daily and the culture solution was changed. Sampling was conducted on day 7. Earthworms were killed with liquid nitrogen and stored at -70 °C. Each treatment had three replicates.

---

3. Subcellular Fractionation

Earthworms were thawed and placed in a homogenization bottle containing Tris-HCl buffer (0.01 mol/L, pH = 7.0). Homogenization was performed at 1500 r/min for 5 min using a tissue homogenizer (DY89-2, Ningbo Biotechnology Company). The homogenate was then centrifuged in a high-speed refrigerated centrifuge at 10,000 r/min for 10 min at 4 °C to obtain the cytosolic fraction (supernatant). The precipitate was washed and centrifuged again at 10,000 g for 20 min at 4 °C. The resulting precipitate was fully digested by adding NaOH (1 mol/L) to obtain the cell debris fraction. The three fractions were frozen, lyophilized in a freeze dryer (ALPHA 1-2 LD plus), and stored at -70 °C for further analysis.

---

4. Benzo[a]pyrene Extraction and Analysis

BaP extraction followed the method of Contreras-Ramos et al. with slight modifications. After pretreatment, solid fractions were weighed and ground into powder in a mortar with anhydrous sodium sulfate (Na SO) at ten times their mass. The powder was transferred to brown glass centrifuge tubes, and dichloromethane was added as the extraction solvent. The tubes were placed in an ultrasonic cleaner with ice water for ultrasonic extraction, followed by vortexing on a vortex mixer for 60 s. This process was repeated, and the supernatants were combined. A 6 mL aliquot was taken and centrifuged at 3500 r/min. The extract was then passed through a silica gel column, eluted twice with dichloromethane and n-hexane solution (1:1). The eluate was collected in a rotary evaporation flask and evaporated in a water bath for 15 min. The residue was dissolved and brought to volume with 11 mL methanol, filtered through a 0.22 m organic membrane, and analyzed by HPLC.

BaP determination was performed using reverse-phase high-performance liquid chromatography (LC-20AT, Shimadzu) equipped with a UV-Vis detector (SPD-20A) and a Shim-pack VP-ODS column (250 × 4.6 mm, 5 m). The chromato-

graphic conditions were as follows: mobile phase was chromatographic grade methanol, flow rate was 1.0 mL/min, column temperature was 30 °C, injection volume was 20 L, and detection wavelength was 254 nm. Both mobile phases were filtered through 0.45 m membranes and degassed by ultrasonication before each measurement. Peak areas were recorded and processed by HW-2000 chromatography workstation (Nanjing Qianpu Software Co., Ltd.), and quantification was performed by external standard method based on retention time.

---

5. Biochemical Determinations

Protein Content: Protein content in different earthworm subcellular fractions was determined by the Coomassie brilliant blue colorimetric method. Absorbance was measured at 595 nm to calculate protein content.

Acetylcholinesterase Activity: AChE activity was determined by the Sym-Trinitrobenzene colorimetric method. Enzyme activity units were expressed as the amount of substrate hydrolyzed per milligram of tissue protein per minute, calculated from absorbance at 412 nm.

Glutathione S-Transferase Activity: GST activity was determined by spectrophotometry through catalyzing the binding of glutathione (GSH) to 1-chloro-2,4-dinitrobenzene (CDNB). Enzyme activity units were expressed as the amount of CDNB conjugated per milligram of tissue protein per minute, calculated from absorbance at 412 nm after subtracting non-enzymatic reactions. All assay kits were purchased from Nanjing Jiancheng Bioengineering Institute.

---

6. Data Analysis

Experimental results were statistically analyzed using SPSS 20.0 software. One-way ANOVA followed by Duncan' s method was used to analyze significant differences among samples treated with different Cd^2 concentrations. Pearson correlation analysis was performed using OriginPro 9.0 to examine correlations between biochemical measurements and BaP concentrations. Significance was set at $P < 0.05$.

Results

1. Effects of Cadmium on Benzo[a]pyrene Distribution and Accumulation in Earthworms

Compared with the control, BaP accumulation in earthworms was significantly reduced under all Cd^2 treatments. BaP concentrations in all three subcellular

fractions showed a trend of initially decreasing and then increasing with increasing Cd^2 concentration, reaching minimum values at 0.5 mg/L Cd^2 treatment. Comparison among subcellular fractions showed that under the same treatment, BaP mainly accumulated in the cell debris fraction, followed by the granule fraction, with the lowest accumulation in the cytosolic fraction. The addition of Cd^2 inhibited BaP accumulation in cell debris and granules, with minimum values observed at 0.5 mg/L Cd^2 treatment. However, 0.8 mg/L Cd^2 treatment promoted BaP accumulation in the cytosolic fraction [Figure 1: see original paper].

[Figure 1: see original paper] Effects of different concentrations of Cd^2 on the distribution and accumulation of benzo[a]pyrene in the earthworm. (Note: The figure represents individual total and different subcellular fraction distributions.)

2. Effects of Cadmium on Protein Content in Earthworms

Total protein content in earthworms showed a trend of initially increasing and then decreasing with increasing Cd^2 concentration, with a significant increase to maximum values at 0.5 mg/L Cd^2 treatment. Among subcellular fractions, protein content was highest in the cell debris fraction, followed by the cytosolic fraction, and lowest in the granule fraction under the same treatment. Under Cd^2 treatments, protein content in all fractions showed an initial increase followed by a decrease, with significant increases at 0.5 mg/L Cd^2 treatment and subsequent decreases at 0.8 mg/L Cd^2 treatment [Figure 2: see original paper].

[Figure 2: see original paper] Effects of different concentrations of Cd^2 on protein concentration in the earthworm.

3. Effects of Cadmium on Acetylcholinesterase Activity in Earthworms

Total AChE activity in earthworms showed an initial increase followed by a decreasing trend, reaching maximum values at 0.5 mg/L Cd^2 treatment and significantly decreasing at 0.8 mg/L Cd^2 treatment. Among subcellular fractions, AChE activity was highest in the cell debris fraction, followed by the cytosolic and granule fractions under the same treatment. Under Cd^2 treatments, AChE activity in the cytosolic fraction gradually decreased with increasing Cd^2 concentration, while activity in the cell debris and granule fractions initially increased and then decreased, reaching maximum values at 0.5 mg/L Cd^2 treatment and decreasing at 0.8 mg/L Cd^2 treatment [Figure 3: see original paper].

[Figure 3: see original paper] Effects of different concentrations of Cd^2 on acetylcholinesterase activity in the earthworm.

4. Effects of Cadmium on Glutathione S-Transferase Activity in Earthworms

Total GST activity in earthworms showed an initial decrease followed by an increase with increasing Cd^2 concentration, reaching minimum values at 0.5

mg/L Cd^2 treatment and significantly increasing to maximum values at 0.8 mg/L Cd^2 treatment. GST activities in the cytosolic and cell debris fractions were generally similar and higher than in the granule fraction. Under Cd^2 treatments, GST activity in the cytosolic and cell debris fractions initially decreased and then increased, reaching minimum values at 0.5 mg/L Cd^2 treatment and significantly increasing at 0.8 mg/L Cd^2 treatment. In contrast, GST activity in the granule fraction gradually increased with increasing Cd^2 concentration, with significant increases observed at 0.8 mg/L Cd^2 treatment [Figure 4: see original paper].

[Figure 4: see original paper] Effects of different concentrations of Cd^2 on glutathione S-transferase activity in the earthworm.

5. Correlation Analysis

Correlation analysis revealed that protein concentration in the cytosolic fraction and AChE activity showed significant negative correlations with BaP accumulation in the cytosolic fraction. Protein concentration in the cell debris fraction also showed a significant negative correlation with BaP concentration in that fraction .

Correlation analysis between enzyme activity and BaP concentration in different subcellular fractions.

Fraction	Protein	AChE	GST
Fraction C (cytosol)	-0.702*	-0.635*	0.231
Fraction D (granules)	-0.456	-0.001	-0.102
Fraction E (debris)	-0.591*	0.048	0.494

Significant correlation at $P^* < 0.05$. AChE: acetylcholinesterase; GST: glutathione S-transferase.

Discussion

The results demonstrate that Cd^2 addition inhibited total BaP accumulation in earthworms, with BaP primarily accumulating in the cell debris fraction, followed by the granule fraction, and with the lowest accumulation in the cytosolic fraction. This pattern is consistent with the findings of Duan Xiaochen. The preferential accumulation in cell debris may be attributed to the presence of cell membranes and other materials in this fraction that have strong lipophilic properties and readily bind hydrophobic chemicals like BaP.

Protein concentrations in all three subcellular fractions initially increased and then decreased with increasing Cd^2 concentration. Correlation analysis showed

significant negative correlations between protein concentration and BaP concentration in the cytosolic and cell debris fractions. This may be because Cd^{2} promotes the synthesis of proteins related to metal detoxification, sequestration, and homeostasis. For example, cadmium exposure can promote metallothionein gene expression. Metallothionein has high affinity for heavy metals, which may lead to increased cadmium accumulation in earthworms. However, the binding of BaP to proteins may be competitively inhibited by Cd^{2} , thereby reducing BaP accumulation. At high Cd^{2} concentrations, protein expression may be suppressed, decreasing cadmium accumulation in earthworms and weakening the competitive inhibition, which could explain the observed patterns.

AChE activity in the three subcellular fractions showed an initial increase followed by a decrease with increasing Cd^{2} concentration. This hormetic effect —stimulation at low concentrations and inhibition at high concentrations—has been reported in similar studies on silkworms and wheat aphids under cadmium stress. The significant negative correlation between BaP concentration and AChE activity in the cytosolic fraction suggests that low Cd^{2} concentrations may promote earthworm metabolic detoxification of BaP, leading to reduced BaP concentrations, while high Cd^{2} concentrations may inhibit these processes, resulting in increased BaP accumulation.

GST plays an important role in detoxifying xenobiotics. Glutathione is key in molecular antioxidant mechanisms, binding to xenobiotics via GST to form hydrophilic glutathione derivatives that are excreted, thereby reducing chemical reactivity. Although no significant correlation was observed between GST activity and BaP concentration in this study, GST activity showed an initial decrease followed by an increase with increasing Cd^{2} concentration. This pattern is consistent with research showing that GST responses to pollutant exposure can involve initial inhibition followed by recovery, depending on exposure time and concentration.

Conclusion

This study revealed that Cd^{2} significantly inhibited total BaP accumulation in earthworms, with BaP primarily accumulating in the cell debris fraction, followed by the granule fraction, and with the lowest accumulation in the cytosolic fraction. The addition of Cd^{2} inhibited BaP accumulation in cell debris and granule fractions, with minimum accumulation observed at 0.5 mg/L Cd^{2} treatment. However, 0.8 mg/L Cd^{2} treatment promoted BaP accumulation in the cytosolic fraction. Cd^{2} may influence BaP distribution and accumulation by affecting protein content and AChE activity. Low Cd^{2} concentrations may induce AChE expression, enhancing the organism' s defense and metabolic capacity against xenobiotics, while high Cd^{2} concentrations may inhibit these protective mechanisms.

References

[1] Mielke HW, Wang GD, Gonzales CR, Powell ET, Le B, Quach VN. PAHs and metals in the soils of inner-city and suburban New Orleans, Louisiana, USA. *Environmental Toxicology and Pharmacology*, 2004, 18(3): 243-247.

[2] [Reference content indicates earthworms as soil pollution indicators and potential for enhanced remediation]

[3] Ping LF, Luo YM, Zhang HB, Li QB, Wu LH. Distribution of polycyclic aromatic hydrocarbons in thirty typical soil profiles in the Yangtze River Delta region, east China. *Environmental Pollution*, 2007, 147(2): 358-365.

[4] [Reference content on soil pollution evaluation indicators]

[5] Ministry of Environmental Protection of the People' s Republic of China. Ministry of Environmental Protection and Ministry of Land and Resources release national soil pollution status survey bulletin. [2016-07-10]. http://www.zhb.gov.cn/gkml/hbb/qt/201404/t20140417_270670.htm.

[6] Binelli A, Riva C, Cogni D, Provini A. Assessment of the genotoxic potential of benzo(a)pyrene and -dichlorodiphenyldichloroethylene in Zebra mussel (*Dreissena polymorpha*). *Mutation Research*, 2008, 649(1/2): 135-145.

[7] Juhasz AL, Naidu R. Bioremediation of high molecular weight polycyclic aromatic hydrocarbons: a review of the microbial degradation of benzo[a]pyrene. *International Biodeterioration & Biodegradation*, 2000, 45(1/2): 57-88.

[8] Tung EWY, Philbrook NA, Belanger CL, Ansari S, Winn LM. Benzo[a]pyrene increases DNA double strand break repair in vitro in vivo: a possible mechanism for benzo[a]pyrene-induced toxicity. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis*, 2014, 760: 64-69.

[9] Williams R, Hubberstey AV. Benzo(a)pyrene exposure causes adaptive changes in p53 and CYP1A gene expression in Brown bullhead (*Ameiurus nebulosus*). *Aquatic Toxicology*, 2014, 156: 201-210.

[10] Hamdi H, Benzarti S, Manusadžianas L, Aoyama I, Jedidi N. Bioaugmentation and biostimulation effects on PAH dissipation and soil ecotoxicity under controlled conditions. *Soil Biology and Biochemistry*, 2007, 39(8): 1926-1935.

[11] Wei BG, Yang LS. A review of heavy metal contaminations in urban soils, urban road dusts and agricultural soils from China. *Microchemical Journal*, 2010, 94(2): 99-107.

[12] Organization for Economic Cooperation and Development. No. 207: Earthworm, Acute Toxicity Tests. *OECD Guidelines for the Testing of Chemicals*, 1984.

[13] [Reference on effects of zinc pollution on earthworm cellulase activity]

[14] [Reference on effects of benzo[a]pyrene on earthworm cytochrome and antioxidant enzymes]

[15] Zhao ZY. [Thesis on combined cadmium and phenanthrene pollution effects on earthworms]. Shanghai Jiao Tong University, 2007.

[16] [Thesis on ecotoxicological effects of combined heavy metal and phenanthrene pollution on *Eisenia andrei* and *Fridericia bulbosa*]. Shanghai Jiao Tong University, 2008.

[17] Marques C, Pereira R, Gonçalves F. Using earthworm avoidance behaviour to assess the toxicity of formulated herbicides and their active ingredients on natural soils. *Journal of Soils and Sediments*, 2009, 9(2): 137-147.

[18] Wu SJ, Wu EM, Qiu LQ, Zhong WH, Chen JM. Effects of phenanthrene on the mortality, growth, and antioxidant system of earthworms *Eisenia fetida* under laboratory conditions. *Chemosphere*, 2011, 83(4): 429-434.

[19] Li LZ, Zhou DM, Peijnenburg WJGM, Wang P, Van Gestel CAM, Jin SY, Wang QY. Uptake pathways and toxicity of Cd and Zn in the *Eisenia fetida* earthworm. *Soil Biology and Biochemistry*, 2010, 42(7): 1045-1050.

[20] [Thesis on distribution of phenanthrene in earthworms and its effects on antioxidant defense systems]. Nanjing Agricultural University, 2013.

[21] Mekahli MN, Tine S, Menasria T, Amieur H, Salhi H. *In vitro* biomarker responses of earthworm exposed to herbicide sekatox and phosphate fertilizer. *Water, Air, & Soil Pollution*, 2016, 227(1): 1-8.

[22] Shi ZM, Xu L, Hu F. A hierarchical method for studying the distribution of phenanthrene in *Eisenia fetida*. *Pedosphere*, 2014, 24(6): 743-752.

[23] [Reference on effects of heavy metal cadmium on silkworm growth and enzyme activities]

[24] Gambi N, Pastoris A, Fabbri E. Acetylcholinesterase activity in the earthworm *Eisenia andrei* at different conditions of carbaryl exposure. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology*, 2007, 145(4): 678-685.

[25] Rao JV, Shilpanjali D, Kavitha P, Madhavendra SS. Toxic effects of profenofos on tissue acetylcholinesterase and gill morphology in a euryhaline fish, *Oreochromis mossambicus*. *Archives of Toxicology*, 2003, 77(4): 227-232.

[26] Hayes JD, Flanagan JU, Jowsey IR. Glutathione transferases. *Annual Review of Pharmacology and Toxicology*, 2005, 45: 51-88.

[27] [Reference on role of plant glutathione S-transferases in phytoremediation]

[28] [Reference on molecular biology of plant glutathione S-transferases]

[29] Velki M, Hackenberger BK. Biomarker responses in earthworm *Eisenia andrei* exposed to pirimiphos-methyl and deltamethrin using different toxicity

tests. *Chemosphere*, 2013, 90(3): 1216-1226.

[30] Łaszczyca P, Augustyniak M, Babczyńska A, Bednarska K, Kafel A, Migula P, Wilczek G, Witás I. Profiles of enzymatic activity in earthworms from zinc, lead and cadmium polluted areas near Olkusz (Poland). *Environment International*, 2004, 30(7): 901-910.

[31] Li LZ, Zhou DM, Wang P, Luo XS. Subcellular distribution of Cd and Pb in earthworm *Eisenia fetida* as affected by Ca ions and Cd-Pb interaction. *Ecotoxicology and Environmental Safety*, 2008, 71(3): 632-637.

[32] Contreras-Ramos SM, Álvarez-Bernal D, Dendooven L. *Eisenia fetida* increased removal of polycyclic aromatic hydrocarbons from soil. *Environmental Pollution*, 2006, 141(3): 396-401.

[33] [Thesis on ecotoxicological effects and mechanistic differences of heavy metals and organic pollutants on *Eisenia fetida*]. Nanjing Agricultural University, 2015.

[34] Gao YZ, Cao XZ, Kang FX, Cheng ZX. PAHs pass through the cell wall and partition into organelles of arbuscular mycorrhizal roots of ryegrass. *Journal of Environmental Quality*, 2011, 40(2): 653-656.

[35] Xiong WG, Ding XY, Zhang YM, Sun YX. Ecotoxicological effects of a veterinary food additive, copper sulphate, on antioxidant enzymes and mRNA expression in earthworms. *Environmental Toxicology and Pharmacology*, 2014, 37(1): 134-140.

[36] Mo XH, Qiao YH, Sun ZJ, Sun XF, Li Y. Molecular toxicity of earthworms induced by cadmium contaminated soil and biomarkers screening. *Journal of Environmental Sciences*, 2012, 24(8): 1504-1510.

[37] Yu S, Lanno RP. Uptake kinetics and subcellular compartmentalization of cadmium in acclimated and unacclimated earthworms (*Eisenia andrei*). *Environmental Toxicology and Chemistry*, 2010, 29(7): 1568-1574.

[38] Mosleh YY, Paris-Palacios S, Arnoult F, Couderchet M, Biagianti-Risbourg S, Vernet G. Metallothionein induction in aquatic oligochaete *Tubifex tubifex* exposed to herbicide isoproturon. *Environmental Toxicology*, 2004, 19(1): 88-93.

[39] [Thesis on defense mechanisms of *Sitobion avenae* under long-term stress]. Northwest A&F University, 2013.

[40] Tylko G, Banach Z, Borowska J, Niklińska M, Pyza E. Elemental changes in the brain, muscle, and gut cells of the housefly, *Musca domestica*, exposed to heavy metals. *Microscopy Research and Technique*, 2005, 66(5): 239-247.

[41] Stebbing ARD. Hormesis—The stimulation of growth by low levels of inhibitors. *Science of the Total Environment*, 1982, 22(3): 213-234.

[42] Pickett CB, Lu AY. Glutathione S-transferases: gene structure, regulation, and biological function. *Annual Review of Biochemistry*, 1989, 58: 743-764.

[43] Schreck E, Garet F, Gontier L, Treilhou M. Neurotoxic effect and metabolic responses induced by a mixture of six pesticides on the earthworm *Aporrectodea caliginosa nocturna*. *Chemosphere*, 2008, 71(10): 1832-1839.

[44] Lei F, Zhang L, Zhang YN, Zhang P, Jiang HY. Inhibition and recovery of biomarkers of earthworm *Eisenia fetida* after exposure to thiacloprid. *Environmental Science and Pollution Research*, 2015, 22(12): 9475-9482.

Note: Figure translations are in progress. See original paper for figures.