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Exposure to lethal levels of benzo[*a*]pyrene or cadmium trigger distinct protein expression patterns in earthworms (*Eisenia fetida*)

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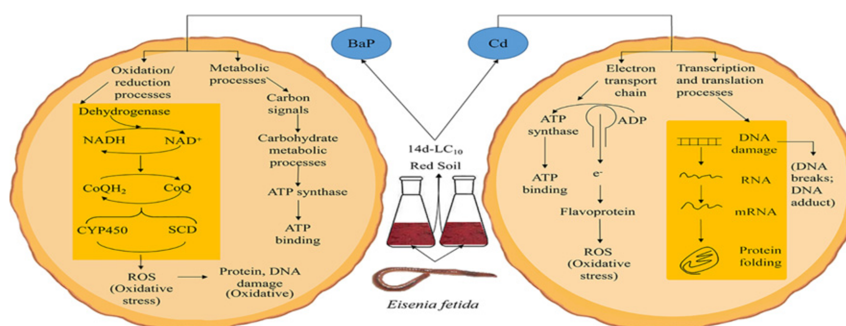
HIGHLIGHTS

- Different pollutants induce distinct patterns of protein expression in earthworms.
- mRNA expression does not correlate well with protein expression.
- Metallothionein gene expression was specifically induced by the Cd treatment.
- Worms subjected to the BaP or Cd treatments exhibited opposite patterns of CAT expression.

GRAPHICAL ABSTRACT



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ABSTRACT

Different pollutants induce distinct toxic responses in earthworms (*Eisenia fetida*). Here, we used proteomics techniques to compare the responses of *E. fetida* to exposure to the 10% lethal concentration (14d-LC_{10}) of benzo[*a*]pyrene (BaP) or cadmium (Cd) in natural red soil (China). BaP exposure markedly induced the expression of oxidation-reduction proteins, whereas Cd exposure mainly induced the expression of proteins involved in transcription- and translation-related processes. Furthermore, calmodulin-binding proteins were differentially expressed upon exposure to different pollutants. The calcium (Ca^{2+})-binding cytoskeletal element myosin was down-regulated upon BaP treatment, whereas the Ca^{2+}-binding cytoskeletal element tropomyosin-1 was up-regulated upon Cd treatment. Some proteins exhibited opposite responses to the two pollutants. For instance, catalase (CAT) and heat shock protein 70 were up-regulated upon BaP treatment and down-regulated upon Cd treatment. A significant ($p < 0.05$, one-way ANOVA with least-significant difference (LSD) test) increase in the level of reactive oxygen species (ROS) and CAT activity further showed that BaP mainly induces oxidative stress. Real-time PCR analysis showed that mRNA expression often did not correlate well with protein expression in earthworms subjected to Cd or BaP treatment. In addition, the expression of the gene encoding the protein metallothionein, which was not detected in the protein analysis, was induced upon Cd treatment, but slightly reduced upon BaP treatment. Therefore, BaP and Cd have distinct effects on the protein profile of *E. Fetida* with BaP markedly inducing ROS activity, and Cd mainly triggering genotoxicity.

Capsule summary: Distinct patterns of protein expression are induced in earthworms upon exposure to different pollutants; BaP markedly induces high levels of ROS, while Cd resultes in genotoxicity.

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1. Introduction

Soil is a major sink for organic and inorganic pollutants (Martínez et al., 2006; Wang et al., 2007). According to a national survey of soil contaminants in China (Ministry of Environmental Protection of the People's Republic of China, 2014), pesticides, such as dichlorodiphenyltrichloroethane (DDT) and hexachlorocyclohexane (HCH), polycyclic aromatic hydrocarbons (PAHs), and heavy metals are the principal pollutants in soil. Benzo[*a*]pyrene (BaP) is the PAH with the highest toxicity equivalent factor (TEF) (Duan et al., 2015) and cadmium (Cd) more frequently exceeded permissible limits in soil samples than any other toxic chemical and had the highest single pollution index (SPI). Although these toxic chemicals pose serious environmental risks, their ecotoxicological risks are unclear (Ge et al., 2016).

Earthworms (Annelida, Oligochaeta), which promote a healthy soil structure and play a key role in terrestrial food chains, are commonly used as bioindicators in standard bioaccumulation tests and ecotoxicological studies (Spurgeon et al., 2003). *Eisenia fetida* is an ideal model animal for assessing the toxicity of pollutants in soil due to its sensitivity to contaminants and ease of handling in the laboratory. Numerous studies have analyzed the effects of toxic chemicals on earthworms in contaminated soils, focusing on enzymatic responses, energy reserve responses, responses in neural impulse conductivity, changes in lysosomal membrane stability, and the formation of histopathological lesions (Scott-Fordsmand and Weeks, 2000; Maria et al., 2009; Panzarino et al., 2016). Inorganic pollutants (such as nonessential heavy metals) and organic pollutants (such as PAHs) could induce different responses in earthworms and, due to differences in their chemical properties, the toxic chemicals exhibit different patterns of accumulation and distribution once absorbed (Jager et al., 2003; Vijver et al., 2005; Chen et al., 2011; Sforzini et al., 2014).

Although bioassays measure the effects of pollutants on an organism, they do not reveal the biological mechanisms used by the organism to withstand toxicity. Due to technical limitations, little is known about the mechanisms that enable organisms to tolerate toxic chemicals. Emerging "omics"-based approaches have been used to investigate the genomes, transcriptomes, proteomes, and metabolomes of single organisms or even of mixed communities of organisms, and have given rise to new opportunities to decipher the molecular mechanisms underlying ecotoxicology in earthworms (Owen et al., 2008; Agbo et al., 2013; Asensio et al., 2013; Ji et al., 2013). Two studies using transcriptome analysis to compare the response mechanisms of earthworms to different pollutants and found that different contaminants induced the expression of different genes (Owen et al., 2008; Agbo et al., 2013). However, transcriptome analyses (i.e., studies of gene expression at the mRNA level) may not be sufficient to predict actual protein expression (Yan et al., 2005). Therefore, proteomics approaches, which monitor protein expression, might better elucidate the effects of exposure to toxic compounds on earthworms (Nabby-Hansen et al., 2001). By using the proteomics approaches, Wang et al. (2010) found that the defense-related protein, lysenin-related protein 2, was down-regulated, while the metabolism-related protein, glutamate dehydrogenase (GDH), was up-regulated in earthworms exposed to Cd. However, Wu et al. (2013) found that lysenin-related protein 2 was up-regulated and GDH was not significantly changed after exposure to the PAH phenanthrene (Phe). Therefore, earthworms may have complex responses to exposure to different contaminants and proteomics-based studies may elucidate these distinct response profiles.

It is important to consider the concentration of the toxic chemicals used when comparing the responses to different pollutants. Various methods are used to evaluate the toxicity of different pollutants. In some studies, the same concentration of different pollutants is used, whereas in others a series of concentrations, ranging from unexposed (controls) to just below the lethal level or the effective half-maximal concentration (EC50), is used (Owen et al., 2008; Lee et al., 2015). In this study, the concentrations of pollutants that produced the same level of lethality (i.e., 10% following a 14-d exposure) were used to examine the response mechanisms of earthworms to BaP or Cd exposure. Standard soils, including artificial soil and the natural standard soil LUFA 2.2, are commonly used in toxicity tests conducted by the Organisation for Economic Co-operation and Development (OECD, 1984). However, using standard soils can decrease the relevance of the results of environmental risk assessments (Duan et al., 2015). Thus, we decided to use natural red soil, which is widely distributed in China and is known to be contaminated with pollutants, to compare the responses of earthworms to BaP and Cd (Tao et al., 2008).

The primary goal of this study was to compare the protein expression of *E. fetida* exposed to the same lethal concentrations (14d–LC_{10}) of BaP or Cd. Two-dimensional electrophoresis (2-DE) and quantitative image analysis were used to identify sets of up- or down-regulated proteins. These profiles may provide insight into the different response mechanisms activated in earthworms following exposure to these toxic chemicals.

2. Materials and methods

2.1. Experimental design

Red soil was collected from Jiangxi Province in South China, which is one of the main food production areas of China. The physicochemical properties of the red soil were as follows: pH, 4.89; organic matter, 9.95 g/kg; clay content, 47%; C/N ratio, 5.81; and CEC, 9.76 cmol/kg. The soil was air dried and sieved through a 1 mm sieve. The soil was artificially polluted by sprinkling with BaP in acetone solution or Cd ($CdCl_2 \cdot 2.5H_2O$) in aqueous solution. Detailed mortality data for BaP and Cd are shown in Fig. S1. The concentrations and duration of the chemical treatments used in the experiments were 14 days at LC_{10} (i.e., 26 mg/kg of BaP or 37.5 mg/kg of Cd). Solutions of Cd in H_2O or H_2O alone (control) and BaP in acetone or acetone alone (control) were mixed thoroughly in the soil. The acetone was allowed to evaporate from the soil in a fume hood overnight and all of the soil was equilibrated for 7 days. Three replicates per treatment were performed.

E. fetida with clitella (i.e., adults) were purchased from an earthworm farm in Nanjing, China and weighed 0.3–0.6 g each. Before the experiment, the earthworms were incubated in clean red soil for 1 week at 20 ± 1 °C and then subjected to gut purging on moist filter paper for 24 h at 20 ± 1 °C. According to OECD (1984) guidelines, 10 earthworms were incubated in 100 g soil (at 70% of the maximum water holding capacity) for 14 d in 250 mL Erlenmeyer flasks at 20 ± 1 °C. After a 14-d exposure, the earthworms were transferred onto filter paper at 20 ± 1 °C for 2 d for gut purging. The *E. fetida* were then killed in liquid nitrogen and stored at -70 °C until analysis.

2.2. Protein extraction

Protein was extracted from the whole body of *E. fetida* using the TCA/acetone method as previously described (Wang et al., 2010). Briefly, earthworms from three replicates (two earthworms were collected from the same container, and the total weight was 2.5 g) were pulverized to a fine powder in liquid nitrogen for the protein and mRNA extraction on three independent occasions. The powder (1.5 g) was resuspended in 20 mL protein extraction buffer (acetone/20% TCA/0.1% DTT) and the homogenate was precipitated overnight at -20 °C. After centrifugation (12,000 x g for 30 min at 4 °C), the supernatants were collected for two-dimensional electrophoresis (2-DE) and the protein concentration was determined using the 2D–Quant Protein Assay Kit (which is based on the BCA method, GE Healthcare Biosciences). Protein samples were kept at -70 °C or directly loaded for isoelectric focusing (IEF).

2.3. IEF and SDS-PAGE

IPG strips (24 cm, nonlinear, pH 4–7, GE Healthcare Biosciences Immobiline DryStrips) were rehydrated overnight with 450 μL IEF (Isoelectric Focusing) buffer containing approximately 600 μg protein and focused using an Ettan IPGphor Multiphor III (GE Healthcare Biosciences) at 20 °C using the following program: 60 V for 2 h, 100 V for 3 h, 500 V gradient for 1 h, 1000 V gradient for 1 h, 3000 V gradient for 1 h, 5000 V gradient for 2 h, 8000 V gradient for 3 h, 10,000 V for 3 h, and 10,000 V for 60,000 Vh. After focusing, SDS-PAGE was conducted according to the manufacturer's instructions (GE Healthcare Biosciences). At least three gels were run per sample.

2.4. Image analysis and MALDI-TOF/TOF analysis

Protein spots were detected by CBB G-250 staining. After 2-DE, gels were stained using CBB solution (0.075% (*w*/*v*) CBB G-250, 10% (*w*/*v*) o-phosphoric acid, 17.5% $(NH_4)_2SO_4$, and 40% methanol) overnight. Then the gels were washed with deionized water until the gel background was clear.

The 2-D gels were scanned using an image ScannerIII (Amersham Biosciences). Image analysis was performed using PDQuest software v8.0 (Bio-Rad, USA). After volumetric quantification and matching of the protein bands, differences in protein content between the control and treated samples were analyzed using Student's *t*-test and calculated as the fold ratio. Data were from three biological replicates. A threshold of $p \leq 0.05$ and a fold change of ≥2 or ≤0.5 were used to identify significantly differentially expressed proteins.

Differentially expressed proteins were excised from the gels and proteins were digested in-gel using trypsin (Promega, Madison, USA) overnight at 37 °C. The digested proteins were extracted from the gel pieces with extraction buffer (67% ACN and 5% TFA). The digests were mixed with a saturated solution of CHCA in 50% ACN/0.1% TFA and spotted on a MALDI target plate. Peptide masses were measured using a MALDI-TOF/TOF Ultraflex Mass Spectrometer (Bruker Daltonics, Billerica, MA, USA). After MS/MS analysis, single peptide scores of >30 were considered identical. The identified proteins were functionally characterized using the gene ontology tool available at http://www.uniprot.org.

2.5. Total RNA extraction and quantitative RT-PCR analysis

Total RNA was extracted from 50 mg of whole earthworm tissue using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA). The extraction was repeated three times for each sample. The samples were treated with RNase-free DNase I (TaKaRa Biotechnology, Dalian, China) to remove DNA contamination. Then, cDNA was constructed using the PrimeScriptTM RT Reagent Kit (Perfect Real Time; TaKaRa Biotechnology). Primer pairs were designed using Primer Express v5.0 software or according to published references (Table 1).

Gene expression was assayed using an ABI PRISM 7900HT Fast Real Time PCR system. Quantitative real-time PCR (QRT-PCR) was performed using SYBR Premix Ex Taq TM II (Perfect Real Time; TaKaRa Biotechnology) with a two-step reaction. Each reaction was performed in a total volume of 25 μL in triplicate using the following PCR profile: denaturation at 95 °C for 30 s; 40 cycles of 95 °C for 5 s and 60 °C for 30 s; and melting curve analysis at 95 °C for 15 s, 60 °C for 30 s, and 95 °C for 15 s. The amplification efficiencies of the primers ranged from 93.2% to 103.9%, and the correlation coefficients (R^2) were 0.99. Then, the melting curve was analyzed for primer–dimer or nonspecific product accumulation after gene expression analysis. Finally, the fold changes in target gene expression levels were normalized to the level of β-actin and calculated using the $2^{-\Delta\Delta CT}$ method (Simon, 2003).

2.6. In vitro ROS and antioxidant enzyme activity assays

Three earthworms (collected from the same container) were pulverized to a fine powder in liquid nitrogen, and three replicates per sample were performed. Then, 0.15 g of powdered earthworm sample was homogenized on ice with phosphate buffer solution (PBS, pH 7.0) and centrifuged at 10,000 x g for 15 min at 4 °C and the supernatant was collected. ROS levels were measured immediately using the oxidation-sensitive fluorescent dye 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA), following the manufacturer's instructions (Jiancheng, China). To measure catalase (CAT) activity, 0.15 g of powdered earthworm sample was were homogenized on ice with 50 mmol L^{-1} Tris-sucrose buffer (pH 7.5) and centrifuged at 10,000 x g for 10 min at 4 °C and the supernatant was stored at −70 °C until analysis. The CAT activity was determined using the method described by Song et al. (2009) and Duan et al. (2015), which uses H_2O_2 solution as the substrate. CAT activity was calculated based on the decrease in ultraviolet absorption at 240 nm per unit time, which resulted from the decomposition of H_2O_2 by CAT in the sample. The ROS content and CAT activity were normalized to the protein level (Bradford, 1976) and then the relative fold changes were determined.

2.7. Statistical analysis

Statistical analyses were conducted with SPSS 20.0 (SPSS, Chicago, IL, USA). ROS content, total protein content, and CAT activity were calculated using one-way ANOVA and the least significant difference (LSD) was used to identify differences between the four treatments (i.e., treatments with BaP and Cd and their corresponding controls). Pearson's correlation test (two tailed) was performed to identify significant correlations between the variables. A *p*-value of <0.05 was considered statistically significant.

3. Results

3.1. Identification of proteins related to BaP or Cd stress

After 14 days of exposure to contaminated soil, earthworm samples containing 600 μg of protein were loaded onto IPG strips (Immobiline DryStrips). The average number of spots on each 2-DE gel was 300 (Fig. 1). The pI values of the protein spots ranged from 4 to 6, and the molecular masses ranged from 14 to 120 kDa. For the BaP treatment and acetone control (using a statistical significance level of $p < 0.05$ and a minimum protein expression increase or decrease of a 2-fold), 30 differential protein spots were found, among which 12 were down-regulated and 18 up-regulated in the BaP treatment compared to the

Table 1
List of primers used for quantitative RT-PCR analysis.

Gene	Spot No.	Gene symbol	Sense primer (5′–3′)	Antisense primer (5′–3′)	References
CAT	2403	DQ286713	GCGGCCCGAGACCACNCAYCARGT	GATCTGCTCCACCTCGGCRAARWARTT	Brulle et al., 2007
Hsp70	2121	DQ286711	TTTACCACCTACTCGGACAAC	TTGAGCTTCTCATCCTCGAC	Brulle et al., 2007
Myosin	8	1803425A	CAGAAGGGAGTGAAGCAG	AAGCCAATCAAACAAACG	In this study
Fibrinolytic enzyme	203	AAM73677	ATGGTTGGAGTCGGTGGA	TCTGGGCAGTTCAGTGGG	In this study
Gelsolin like	7505	Q8MPM1	AAAAGCACTCAGGACGAA	TACGCATCCAACTCAACG	In this study
MT		DQ286714	CGCAAGAGAGGGATCAACTT	CTATGCAAAGTCAAACTGTC	Brulle et al., 2007
β-Actin		DQ286722	TCTCCACCTTCCAGCAGATG	CGAAAAATGTCCTCCGCAAG	Brulle et al., 2007

acetone control. For the Cd treatment and water control, 35 differential protein spots were identified, among which 18 were down-regulated and 17 up-regulated in the Cd treatment compared to the control.

3.2. Effects of BaP and Cd on protein expression profiles

Forty-seven differentially expressed proteins were identified through MALDI-TOF/TOF-MS (Table S1). All MS/MS spectra were analyzed using MASCOT software and the NCBInr database. As the earthworm genome is only partially sequenced, the identified proteins may be derived from other species (i.e., a subset of the proteins identified were predicted or hypothetical proteins). BLASTp (http://www.ncbi.nlm.nih.gov/BLAST/) was further used to search for homologs of the unknown proteins (predicted and hypothetical proteins and others). Protein functions were analyzed using an online tool (http://www.uniprot.org). The accession numbers corresponding to the protein spots are shown in (Table S1).

The differentially expressed proteins were classified into the following five major groups based on their biological function (Table 2): (i) metabolism-related proteins, 34.62% in samples subjected to the BaP treatment and 34.78% in those subjected to the Cd treatment; (ii) stress response-related proteins, 36.42% in samples subjected to the BaP treatment and 17.39% in those subjected to the Cd treatment; (iii) transcription- and translation-related proteins, 3.85% in samples subjected to the BaP treatment and 21.74% in those subjected to the Cd treatment; (iv) predicted and hypothetical proteins, 26.92% in samples subjected to the BaP treatment and 21.74% in those subjected to the Cd treatment; (v) other proteins that could not be identified, 0% in samples subjected to the BaP treatment and 4.35% in those subjected to the Cd treatment.

As shown in Table 2 and Table S1, different proteins with the same functions showed differential expression patterns under the same treatment. For example, the enzymes dehydrogenase (AAD, spot 3111) and short-chain dehydrogenase (SCD, spot 9605), which play a role in oxidation/reduction, were down-regulated and up-regulated, respectively, upon BaP treatment. Moreover, different proteins performing the same functions were differentially expressed under the different pollutant treatments. For example, myosin (spot 8) was down-regulated in samples subjected to the BaP treatment, while tropomyosin-1 (spot 1503) was up-regulated in those subjected to the Cd treatment. Both myosin and tropomyosin-1 are involved in calcium regulation. Additionally, the same protein showed different responses to the different pollutants. For example, catalase (CAT, spot 2403) and heat shock protein 70 (Hsp70, spot 2121) were up-regulated in samples subjected to the BaP treatment and down-regulated in the Cd treatment.

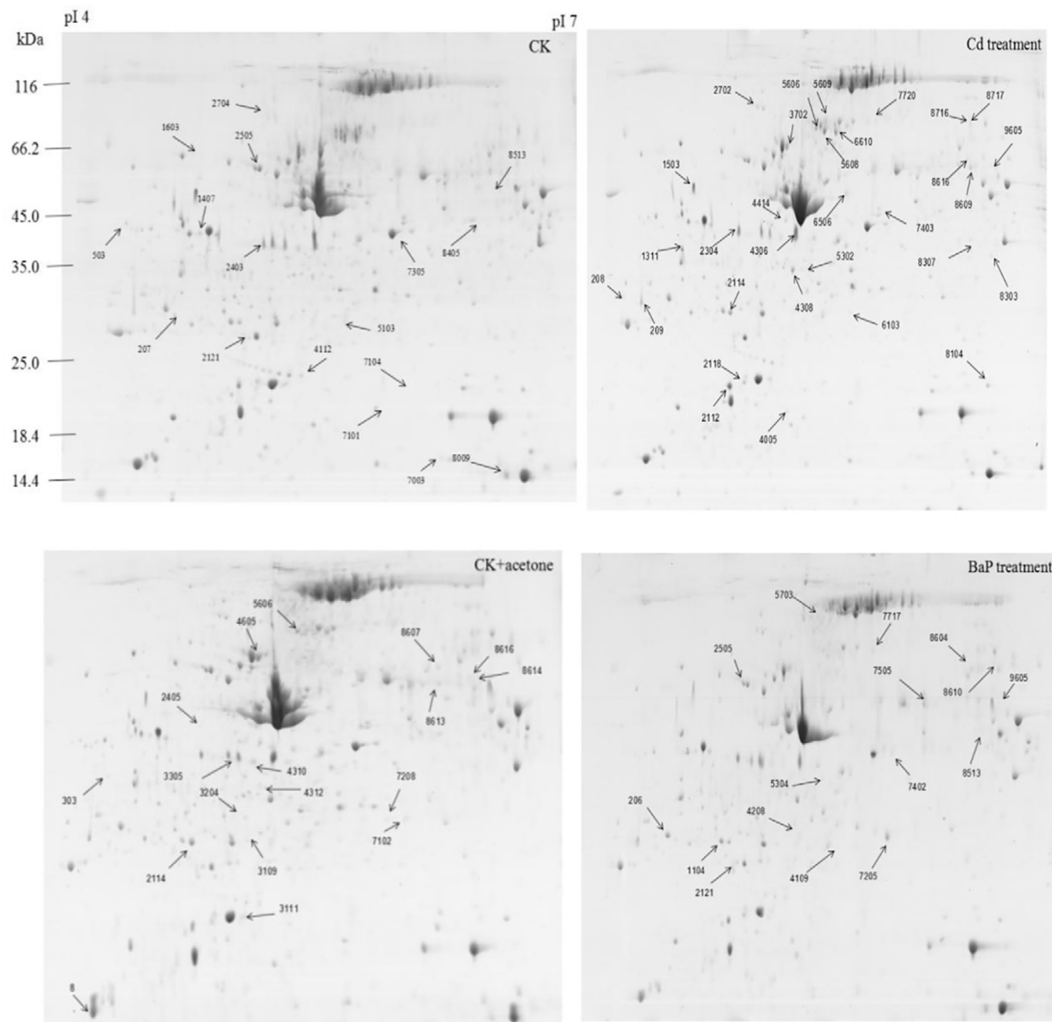

Fig. 1. CBB G-250 2-DE profiles of total *E. fetida* proteins. Proteins (600 μg) were separated onto 1-D (pH 4–7, 24 cm, nonlinear) IPG strips and 2-D 12% vertical slab gels. 2-DE maps of proteins extracted from the earthworms after 14 days of exposure to the water control, acetone control, Cd (37.5 mg/kg), and BaP (26.0 mg/kg) in red soil. Arrows represent significantly ($p < 0.05$) increased and decreased protein abundance (as compared with the controls) following Cd and BaP treatment. Two independent biological experiments were performed and yielded similar results.

Table 2
Functional classification of the proteins identified by MALDI-TOF/TOF-MS and found to be differentially regulated in the earthworm *E. fetida* after BaP and Cd exposure. The relative percentages of proteins in each category are shown.

Functional classification of the proteins	BaP treatment	Cd treatment
Metabolism	34.62%	34.78%
Stress response	34.62%	17.39%
Transcription and translation	3.58%	21.74%
Predicted and hypothetical proteins	26.92%	21.74%
Other	0	4.35%

3.3. Effects of BaP and Cd on gene and protein expression

To establish if the protein level and the corresponding mRNA level were correlated following exposure to BaP or Cd, we analyzed changes in CAT and Hsp70 protein and mRNA levels. In addition, myosin (spot 8), fibrinolytic enzyme component A partial (fibrinolytic enzyme, spot 206), and gelsolin-like protein 2 (gelsolin-like, spot 7505) were also selected due to their high MASCOT scores. Although metallothionein (MT) was not identified using 2-DE, it was selected due to its numerous reported functions related to heavy metals (Liang et al., 2011).

We found that mRNA expression did not always correlate with protein abundance (Fig. 2). The mRNA expression of CAT showed a similar trend as observed for its protein expression under the BaP or Cd treatment, while the mRNA expression of myosin did not correlate with its protein expression. For Hsp70 and fibrinolytic enzyme, the mRNA expression was negatively correlated with protein abundance under Cd treatment, but was positively correlated under BaP treatment. MT mRNA expression was elevated in samples subjected to the Cd treatment, but was decreased in those subjected to the BaP treatment relative to the respective controls.

3.4. Post-transcriptional effects of BaP and Cd

The levels of ROS in *E. fetida* exposed to BaP- or Cd-polluted soils are presented in Fig. 3A. The ROS level was not significantly changed ($p > 0.05$, one-way ANOVA with least-significant difference (LSD) test) in control samples treated with water or acetone. Significant increases ($p < 0.05$, LSD test) in the levels of ROS were observed in samples subjected to BaP treatment compared to other treatments. However, the ROS content was not significantly changed ($p > 0.05$, LSD test)in samples subjected to the Cd treatment compared to the water and acetone controls.

A significant decrease ($p < 0.05$, LSD test) in CAT activity was observed in samples subjected to the Cd treatment, while a significant increase ($p < 0.05$, LSD test) was found both in samples subjected to the BaP treatment (Fig. 3B).

4. Discussion

Xenobiotic detoxification is mediated by specialized enzyme systems in living organisms and can be classified into three phases: modification (i.e., primary metabolism), conjugation, and excretion (Goldstone et al., 2006). Primary metabolism-related proteins, which are involved in energy production and carbon metabolism, can function in an organism's adaptation to environmental stresses (Yan et al., 2006). The rpib family sugar-phosphate isomerase (RPIB family, spot 4312), which functions in carbohydrate metabolism and serves as a source of carbon signals, was down-regulated in samples subjected to BaP treatment (Park et al., 2009). This may have occurred because organic pollutants serve as carbon signals, which influence carbohydrate metabolism and, in turn, regulate ATP synthase. Under Cd treatment, the expression of the ATP synthase F1 subunit gamma (ATP synthase protein, spot 2704), which participates in ATP synthesis, was inhibited.

Phase II biotransformation is a detoxification pathway that produces polar metabolic products that are more readily excreted. Here, we

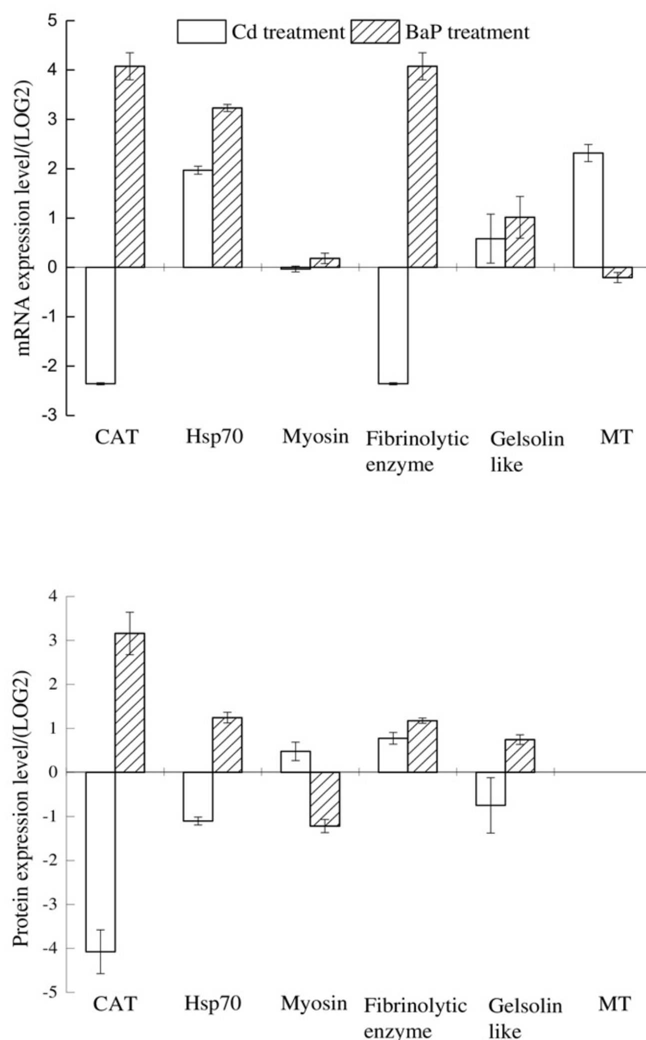

Fig. 2. Functional comparison of different genes at the mRNA and protein level in *E. fetida* exposed to BaP or Cd. QRT-PCR was performed using gene-specific primers (Table 1) and SYBR Green Real-Time Master Mix. The protein and mRNA $\log_2$ values of the ratio of Cd treatment to the water control, and BaP treatment to the acetone control are plotted. Relative gene expression was evaluated using the ΔΔCT method.

identified two proteins that were previously shown to be involved in the biotransformation of BaP, fibrinolytic enzyme component A (FIB, spot 206) and polyprenyl diphosphate synthase (PDDS, spot 8613). FIB, an important chemotherapeutic agent containing hydrophobic amino acid residues that can bind to hydrophobic pollutants, was up-regulated under BaP stress (Wang et al., 2014). The up-regulation of FIB might indicate that fibrinolytic enzymes are responsive to BaP stress. PDDSs belong to a large family of prenyltransferases, and are potential anticancer proteins involved in the transformation of certain drugs. The decrease in PDDS levels following BaP treatment might indicate that BaP increases the risk of cancer and inhibits the biosynthesis of ubiquinone (UQ) (Ernster and Dallner, 1995; Simonen et al., 2008; Zhang and Li, 2013). However, no proteins that influence the transferase activity were found in the earthworm samples subjected to the Cd treatment.

ATP-binding proteins, which are phase III detoxification enzymes and use the energy of ATP hydrolysis to absorb, accumulate, and excrete various toxic chemicals have distinct functions in the response to xenobiotics (Kumar et al., 2016). In this study, an ATP-binding protein (unnamed protein product, spot 8614) was decreased in *E. fetida* subjected to BaP treatment and another ATP-binding protein (glycerol-3-phosphate ABC transporter ATP-binding protein, spot 7305) was also decreased under Cd treatment.

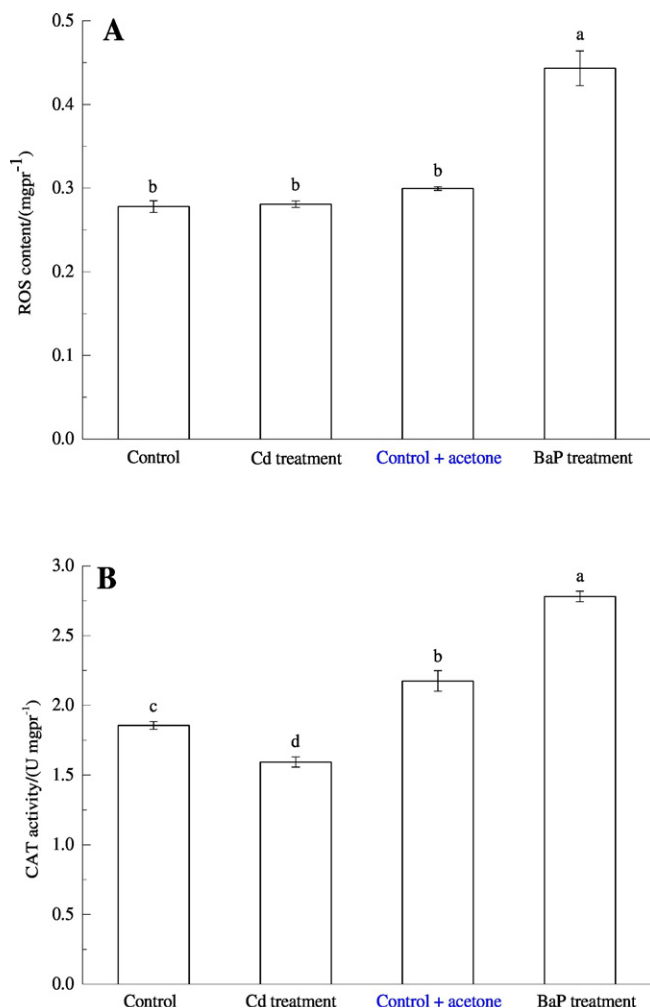

Fig. 3. Changes in ROS content (A) and CAT activity (B) following BaP or Cd treatment. Bars with different lowercase letters indicate statistically significant differences ($p < 0.05$, LSD test).

Calmodulin-binding proteins play vital roles in numerous biological processes; in addition to protecting against oxidative stress and neurotoxic effects, these proteins are important for cytoskeletal formation (Nunez et al., 2001; Sanders et al., 2015). Here, two proteins related to Ca^{2+} level regulation (myosin, spot 8 and tropomyosin-1, spot 1503) that are linked to actin-binding protein, were differentially expressed upon exposure to BaP or Cd (Ohtsuki et al., 1986). The reduction in myosin expression and down-regulation of the actin-binding protein (Piso0_001953, spot 7102) under BaP stress might inhibit cytoskeleton formation and result in cellular injury. Similar down-regulation of the calcium-regulated proteins and actin-binding proteins was reported in *E. fetida* subjected 2,2′,4,4′-tetrabromodiphenyl ether (BDE 47) stress (Ji et al., 2013). Tropomyosin-1 was up-regulated under Cd treatment. Furthermore, metals induce adverse neurotoxic effects through Ca^{2+} signaling (Sanders et al., 2015). N-synaptobrevin(N-syn, spot 2702), a neuronal calcium sensor protein that regulates the synaptic activity and plasticity of neuronal networks by stimulating Ca^{2+} signaling, was up-regulated by Cd stress (Xu et al., 2007).

Environmental contaminants induce oxidative stress and alter the cellular redox balance (Franco et al., 2009). ROS play dual roles as both the toxic by-products of normal cell metabolism and the regulatory molecules in stress perception and signal transduction (Hu et al., 2016). The high ROS levels accumulated in the BaP treatment might be due to the down-regulation of PDDS, which inhibits UQ biosynthesis (Ernster and Dallner, 1995; Zhang and Li, 2013). Several proteins that participate in oxidation/reduction or the regulation of redox homeostasis, such as the enzyme amino acid dehydrogenase (AAD, spot 3111), NAD(P)-binding domain protein (NDP, spot 3204), alpha-aminoadipate reductase-like protein (ALP, spot 3305), and cytochrome P450 (CYP450, spot 5606), were down-regulated under BaP treatment. Similar studies reported that Phe could disrupt glycolysis and reduce energy production, leading to an imbalance in redox homeostasis in earthworms, which could result in the production of ROS (Son et al., 2009; Gao et al., 2013). However, short-chain dehydrogenase (SCD, spot 9605) was up-regulated upon BaP treatment, probably because this enzyme oxidizes and thereby neutralizes PAHs (Zalina et al., 2016). Two proteins involved in oxidation/reduction were identified as undergoing changes in expression in earthworms treated with Cd; electron-transferring flavoprotein (ETF, spot 8616) was up-regulated, whereas pyridine nucleotide-disulfide oxidoreductase was down-regulated (Pyr_redox, spot 7003). Another study also suggested that heavy metals stimulate the electron transport chain, and thereby boost ROS production (Chen et al., 2003). Therefore, BaP could lead to the accumulation of ROS by creating an imbalance in redox homeostasis, while Cd influences the electron transport chain.

Sophisticated ROS depletion mechanisms, including the activation of antioxidant enzymes, were detected in the treated earthworms. Catalase (CAT, spot 2403), which eliminates H_2O_2 in cells, showed different expression patterns in response to the different pollutants. This result was in accordance with the changes in CAT activity measured in this study that it was up-regulated in the BaP treatment but down-regulated under Cd treatment(Fig. 3B). Furthermore, our previous study also showed that CAT activity was promoted by a series of BaP concentrations (1–500 mg/kg) in *E. fetida* (Duan et al., 2015). However, other studies reported that CAT activity was promoted by low doses (1–10 mg/kg) and inhibited by higher concentrations (50 mg/kg) of Cd in *E. Fetida* (Zhang et al., 2009; Asensio et al., 2013; Panzarino et al., 2016). Therefore, the concentration of the pollutant might determine whether CAT expression and activity are increased or decreased.

Gene expression is regulated at the transcriptional and translational levels. After BaP treatment, the deoR family transcriptional regulator (DEOR family, spot 8307), which is involved in transcriptional processes, was down-regulated. DEOR family proteins also affect carbohydrate metabolism (Yoshikiyo and Badal, 2008), similar to members of the RPIB family. After Cd treatment, four proteins involved in transcriptional processes, including the zinc ion-binding protein alanine–tRNA ligase AlaS (ALAS), spot 3702 and related to PRP11-pre-mRNA splicing factor (PRPM), spot 8513; RNA-binding protein (RNA-binding domain-containing protein; RBBC), spot 209; and DNA-binding protein (chromosome partitioning protein ParB; PARB), spot 8009, exhibited changes in abundance. DNA-binding, RNA-binding, and zinc ion-binding proteins are important regulators of transcription, and are crucial for various cellular processes, such as regulation of transcription, DNA replication, RNA splicing, and post-transcriptional regulation (Stawiski et al., 2003; Qin et al., 2016). The changes observed in the abundance of these proteins might suggest that Cd has an important effect on transcriptional processes. Similar research showed that Cd accumulation can lead to the breaks in double-stranded DNA, and zinc replacement in zinc ion binding proteins (Michalek et al., 2012; Qin et al., 2016). Wang et al. (2010) also showed that zinc ion-binding proteins were down-regulated by Cd stress in *E. fetida*. However, ALAS and RBBC were up-regulated by Cd stress. The differential regulation of different proteins in response to Cd stress in this study suggested that transcriptional processes are influenced by a complex mechanism in earthworms.

DNA damage is an indicator of toxic stress (Duan et al., 2015). Pollutants have been demonstrated to cause DNA damage via two pathways, through (i) the covalent binding of the toxic chemicals to DNA at specific bases or (ii) the stimulation of ROS production (Cai et al., 2016). In earthworms subjected to the BaP treatment in this study, the down-regulation of CYP450, which is a key enzyme in DNA adduct formation

suggested that stimulation of ROS was the primary pathway for BaP-mediated DNA damage (Table S1, Venier and Canova, 1996; Saint-Denis et al., 1999; Sforzini et al., 2012). This was further supported by the high level of ROS accumulation in *E. fetida* subjected to the BaP treatment (Fig. 3A). For the Cd treatment, no significant increase in ROS was found. Li et al. (2009) showed that Cd can induce significant DNA damage even at the lowest concentration (0.1 mg/kg) in *E. fetida*, and Cd can bind to DNA and perturb DNA methylation (Koo et al., 2011). Therefore, Cd may induce DNA damage through covalent binding to DNA.

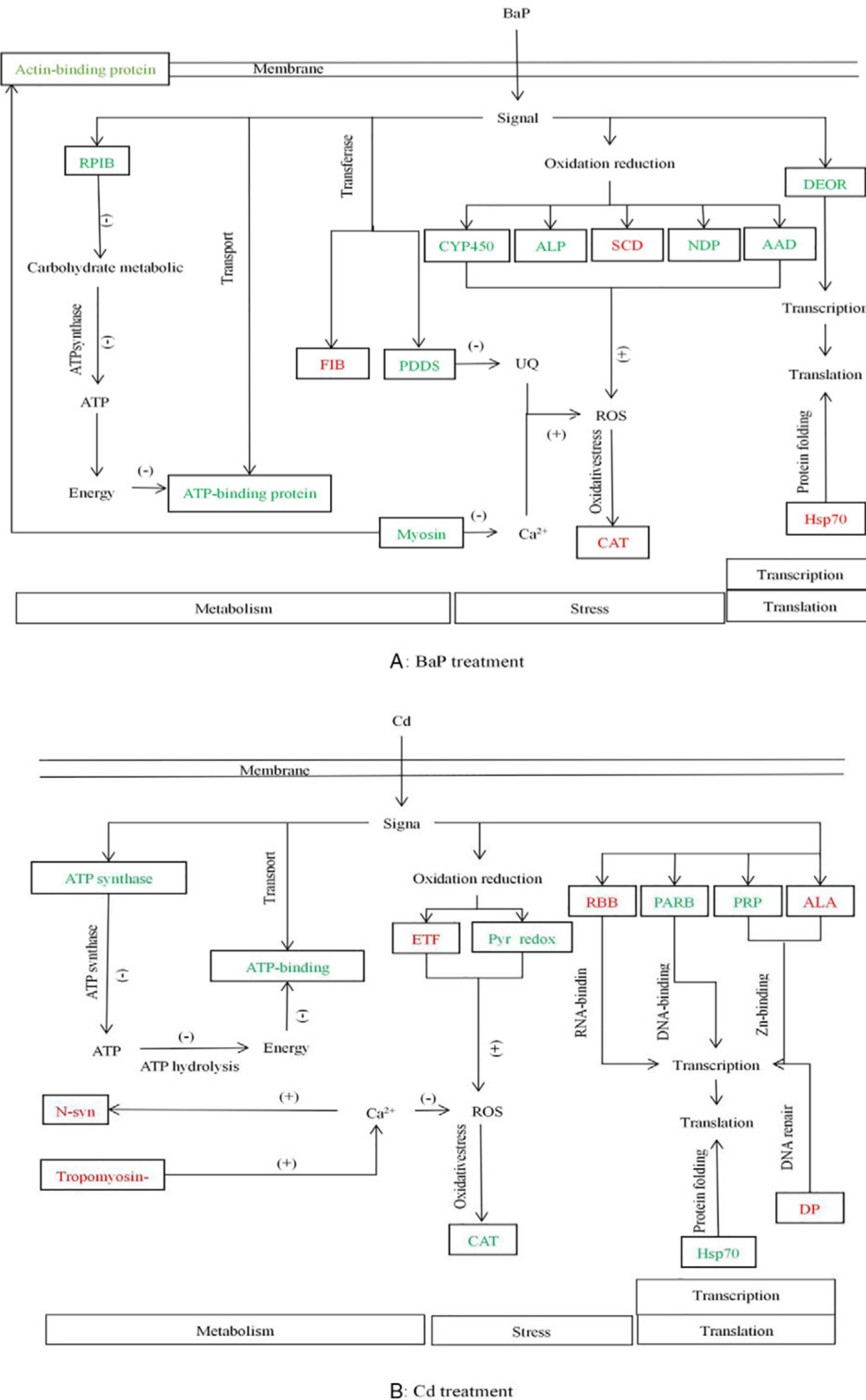

Fig. 4. Proposed working models of differentially expressed proteins involved in metabolism, stress, transcription, and translation after BaP (A) and Cd (B) treatment, based on using proteomics analysis. Proteins shown in red were up-regulated during BaP (A) and Cd (B) treatment, and those in green were down-regulated. (+) means promoted and (−) means inhibited. AAD, amino acid dehydrogenase, spot 3111; Actin-binding protein, piso0_001953, spot 7102; ALAS, alanine–tRNA ligase AlaS, spot 3702; ALP, alpha-aminoadipate reductase-like protein, spot 3305; ATP synthase protein, ATP synthase F1 subunit gamma, spot 2704; ATP-binding protein, unnamed protein product, spot 8614 (BaP treatment); ATP-binding protein, glycerol-3-phosphate ABC transporter ATP-binding protein, spot 7305 (Cd treatment); CAT, catalase, spot 2403; CYP450, cytochrome P450, spot 5606; DEOR, deoR family transcriptional regulator, spot 8307; DP, deoxyribodipyrimidine photolyase, spot 4306; ETF, electron transferring flavoprotein, spot 8616; FIB, fibrinolytic enzyme component A, spot 206; Hsp70, heat shock proteins 70, spot 2121; NDP, NAD(P)-binding domain protein, spot 3204; N-syn, n-synaptobrevin, spot 2702; PARB, chromosome partitioning protein ParB, spot 8009; PDDS, polyprenyl diphosphate synthase, spot 8613; PRPM, related to PRP11-pre-mRNA splicing factor, spot 8513; Pyr_redox, pyridine nucleotide-disulfide oxidoreductase, spot 7003; RBBC, RNA-binding domain-containing protein, spot 209; RPIB, rpib family sugar-phosphate isomerase, spot 4312; SCD, short-chain dehydrogenase, spot 9605; ROS, reactive oxygen species; UQ, ubiquinone.

Some reports have suggested that DNA repair genes might be involved in relieving the DNA damage, and DNA repair pathways are distinct for the different chemicals (Li et al., 2015; Lan et al., 2016). In our current study, we did not identify proteins that were directly involved in DNA repair following BaP treatment, which could be due to the high levels of ROS inhibiting the expression of DNA repair genes (Karran and Brem, 2016). By contrast, a deoxyribodipyrimidine photolyase with DNA repair functions (DP, spot 4306) was up-regulated in *E. fetida* exposed to the Cd treatment.

Heat shock protein 70 (Hsp70, spot 2121) is involved in protein translation and processing and may prevent the aggregation of denatured proteins and facilitate refolding in the presence of environmental contaminants (Kim et al., 2013). In our study, Hsp70 was up-regulated by BaP stress and down-regulated by Cd stress. Previous studies have also reported that Hsp70 was selectively induced by various environmental toxicants; copper decreased the expression of Hsp70 and naphthenic acid exposure increased the expression of Hsp70 (Fisker et al., 2013; Wang et al., 2015).

We also found that mRNA expression did not always coincide with protein expression under BaP or Cd treatment, except in the case of CAT (Fig. 2). The level of mRNA expression does not always correlate well with the level of protein expression due to posttranscriptional and posttranslational modifications and differences in the degradation rates of mRNA and proteins (Yan et al., 2005; Wang et al., 2010). Furthermore, MT, which binds to metal ions and is regarded as a biomarker of heavy metal pollution, cannot be detected by 2-DE as its molecular mass (7.59 kDa) is below the detection limit (14 kDa) (Wang et al., 2010). Our QRT-PCR data showed that MT gene expression was induced by the Cd treatment, but slightly suppressed in the BaP treatment (Fig. 2). These results are in accordance with the results of Agbo et al. (2013), which showed that MT expression was increased by Cd stress, but slightly reduced by BaP treatment.

Endogenous ROS can act as second messengers and may be essential participants in normal cell signaling (Briganti and Picardo, 2003). In addition, these molecules are an important metric of oxidative stress resulting from aerobic metabolism (Lim and Luderer, 2010). In this work, the ROS content of *E. fetida* was significantly increased ($p < 0.05$, LSD test) under BaP treatment, but not significantly changed under Cd treatment (Fig. 3A). As ROS levels were consistent in the water and acetone controls, BaP was the main reason for ROS accumulation. Numerous reports have shown that BaP induces the production of ROS by metabolic oxidation and causes oxidative damage to cells (Burdick et al., 2003; Tsuji et al., 2011). In agreement with these reports, our proteomics analysis also showed that proteins involved in oxidation/reduction were induced by BaP stress, and that down-regulation of PDDS and myosin inhibits UQ biosynthesis and disrupts Ca^{2+} homeostasis, respectively, which could lead to the accumulation of ROS (Table S1). By contrast, exposure to Cd increased the abundance of the calcium-regulated protein tropomyosin-1, the calcium-regulation protein, which could therefore increase the intracellular Ca^{2+} concentration and inhibit the accumulation of ROS (Fig. 4).

Pearson's correlation analysis showed that the content of ROS was positively correlated with CAT activity after BaP exposure ($r = 0.893$, $p < 0.05$). This result differed from the findings of another study, which found that the increased CAT activity reduced ROS levels, and thereby retained the normal functioning of cells (Tsuji et al., 2011). These differences might have resulted from the CAT activity becoming saturated and the concentration of ROS exceeding the CAT levels produced (Limon-Pacheco and Gonsebatt, 2009). After Cd exposure, the content of ROS was negatively correlated with CAT activity ($r = -0.126$, $p > 0.05$).

Considering the distinct protein expression patterns in earthworms subjected to different pollutants, a tool based on protein expression could be used to monitor pollutants (Wu et al., 2013). Of all the proteins examined, we found that the expression of CYP450 protein (−22 fold) was affected the most by BaP stress, while that of RBBC (+5.9 fold) was affected the most by Cd stress. These two proteins could potentially be used to monitor BaP and Cd pollution, respectively. In addition, BaP and Cd had opposite effects on the expression patterns of CAT at the gene and protein levels, and also on their activities. Thus, CAT might be a suitable indicator to distinguish between BaP and Cd pollution (Table S1, Asensio et al., 2013).

5. Conclusion

In summary, we found that earthworms respond to different pollutants via unique complex mechanisms. Proteomics analysis revealed that BaP exposure markedly induced oxidation/reduction proteins, which are involved in stress-related responses, whereas Cd exposure mainly induced proteins involved in transcription and translation. Some proteins exhibited opposite responses following exposure to BaP or Cd; for instance, CAT and Hsp70 were up-regulated under BaP treatment and down-regulated under Cd treatment. Furthermore, the significant increase in ROS levels and CAT activity observed demonstrated that BaP treatment induced oxidative stress. QRT-PCR analysis showed that MT expression was induced by Cd treatment, and that mRNA expression did not correlate well with the protein expression.

Author contribution statement

Lihao Zhang, Li Xu, and Huixin Li wrote the main manuscript text. Li Xu and Huixin Li were responsible for the experimental design and guidance. Lihao Zhang, Xiaochen Duan, and Nannan He contributed to earthworm cultivation, protein extraction, and 2-DE. Xu Chen and Jili Shi designed primers and conducted the QRT-PCR experiment. Lihao Zhang, Nannan He, and Weiming Li determined the CAT activity, total protein content, and ROS content. All authors reviewed the manuscript.

Additional information

The authors declare that they have no competing interests, as defined by Nature Publishing Group, or other interests that might be perceived to influence the results and/or discussion reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.scitotenv.2017.04.003.

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