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Accumulation, biotransformation, and multi-biomarker responses after exposure to arsenic species in the earthworm *Eisenia fetida*†

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Abstract: Earthworms (*Eisenia fetida*) were exposed to OECD soils contaminated with arsenite (29.3 mg kg^{-1}), arsenate (35.2 mg kg^{-1}), monomethylarsonate (342.5 mg kg^{-1}) and dimethylarsinate (373.0 mg kg^{-1}) for 64 days, respectively. The exposure concentration for the four arsenic species was set at one-tenth of 14d-LC_{50} in order to compare their toxicity. Eight biomarkers including superoxide dismutase, catalase, glutathione peroxidase, glutathione S-transferase, glutathione reductase, reduced glutathione, lipid peroxidation and metallothioneins were analyzed in the organisms. A multi-biomarker approach, integrated biomarker response (IBR) index, was adopted to summarize the multi-biomarker responses to a single value, reflecting the integrated stress of different arsenic species on the earthworm. Furthermore, total arsenic and arsenic speciation were analyzed in earthworm tissue to evaluate relationship between arsenic accumulation and biomarker responses at the subcellular level and to observe the role of arsenic biotransformation in the earthworm. The results showed that the toxicity of the four arsenic species was ranked as: arsenite ＞arsenate＞monomethylarsonate and dimethylarsinate. Although organic arsenics showed a low degree of biotoxicity, they could be turned into highly toxic inorganic arsenic under the effect of demethylation, which caused toxic effect on organisms. The biomarker responses indicated that a sub-lethal dose of both arsenite and arsenate could trigger the response of the antioxidant defense

system and cause oxidative damages when the protective capacity of the system was exhausted. Arsenic in earthworm could be detoxified during the process of biotransformation, where inorganic arsenics were converted into organic arsenics, which would then be excreted out. Based on the results, it was proved that different arsenic species showed different degrees of toxicity. Therefore, arsenic species should be differentiated in order to obtain accurate results in quality/risk assessment programs.

Key words: biomarker, earthworm, arsenite, arsenate, monomethylarsonate, dimethylarsinate

1. Introduction

Arsenic (As) is a pollutant widely distributed in the environment and can be easily found in detectable concentrations in all types of soils. Elevated levels of As in soils have already been reported in many literatures.[1-4] Certain changes in the physical and chemical properties in soils may lead to the transport, dispersion and accumulation of As in plants and animals, which could be passed on along the food chain to human beings as a final consumer.[5] As a toxic element that could affect essential functions of many human organs, As has been listed in a large number of quality standards and safety standards around the world. However, nearly all the As content described in these standards refers to total arsenic content, while different arsenic species are not differentiated.

Historical studies have proved that the toxicity of As element is predestined by its chemical species presented; thus the toxicity assessment only based on total arsenic content is far from enough.[6] For example, inorganic As is the number one toxin in the United States Environmental Protection Agency list of prioritized pollutants based on epidemiological data of human-beings, while the methylated As species such as monomethylarsonate (MMA) and dimethylarsinate (DMA) are less toxic, arsenobetaine (AsB), arsenocholine (AsC) and other arsenosugars are considered to be of non-toxicity.[7] In terms of two common inorganic arsenic species, the arsenite [As (III)] is generally considered to have more potent toxic properties than the arsenate [As (V)].[8] Therefore, when activities such as risk assessment and toxicity test were conducted for As element, the contents of different arsenic species in environmental should be taken into consideration.

Earthworms are sentinels for terrestrial systems due to their definitive ecological roles. In many parts of the world, earthworms are the principal organisms responsible for the mixture and translocation of soil constituents. Furthermore, earthworms also aid soil fertility by partially removing decomposed litter from the soil surface, ingesting it and transporting it to the subsurface layers.[9] When exposed to contaminated soils, earthworms can accumulate contaminants in the body and transfer pollutants to birds, small mammals, and other soil biota through the terrestrial food web.[10-12] These make them one of the most suitable bioindicator organisms for risk assessment in soil.[13] *Eisenia fetida* was chosen for this study due to the standardization of acute and chronic ecotoxicological assays. It has been considered a suitable model species and prescribed as test organism in previous studies.[14, 15]

Biomarkers are often applied in toxicity testing of environmental pollutants as indirect measurements of bioavailability.[16] Furthermore, they are key elements in the understanding of toxic mechanism underlying observed effects at individual level.[17] Biomarkers have been primarily used in earthworms experimentally exposed to polluted environments. However, the effects of As exposure on earthworm biomarkers have been little reported, let alone the effects of different arsenic species.[18-20] In addition, compared with the use of a single biomarker, the application of a battery of biomarkers may be more effective in evaluating the effects of contaminant exposure and assessing the environmental stress.[21] Therefore, a multi-biomarker approach, integrated biomarker response (IBR) index, was employed to summarize the multi-biomarker responses to a single value reflecting the integrated stress of different arsenic species on earthworm.[22]

In this study, earthworm *E. fetida* were exposed to OECD soils contaminated by four common arsenic species in environment including As(III), As(V), MMA and DMA, respectively. Seven kinds of oxidative stress biomarkers including superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione S-transferase (GST), glutathione reductase (GR), reduced glutathione (GSH) and lipid peroxidation (LPO), as well as metallothioneins (MTs) were analyzed in the organisms. The aim of the present study was to systematically investigate and compare the multi-biomarker responses of earthworm, *E. fetida*, to the four kinds of arsenic species in artificial

soil. Furthermore, As speciation in earthworms was characterized so as to determine the effects of As bioaccumulation and metabolism on the biomarkers.

2. Materials and methods

2.1. Earthworms and chemicals

The study protocol was approved by the Chinese Association for Laboratory Animal Science. Adult earthworms *E. fetida* with well-developed clitella were obtained from a local commercial supplier in Jinan, China, which were selected from a synchronized culture with the same age for every exposure group as well as the control group. The selected earthworms possessed a weight of 0.35 to 0.45 g and acclimated for 7 d to the artificial soil substrate prior to test.

Standard solutions of As(III) (1.011 μmol mL^{-1}), As(V) (0.233 μmol mL^{-1}), MMA (0.335 μmol mL^{-1}) and DMA (0.706 μmol mL^{-1}) were supplied by the China Standard Certification Center (CSC). Ultrapure water (18 MΩ), obtained by using a Milli-Q water purification system (Millipore, USA), was used throughout. All glassware was cleaned by using 10% (v/v) nitric acid (Merck KGaA, Germany), followed by multiple rinses with ultrapure water. Reagents used in biomarker assays were obtained from Sigma-Aldrich China Co. (Shanghai, China). All other chemicals used were analytical grade reagents obtained from Beijing Chemical Co. (Beijing, China).

2.2. Soil preparation

The artificial soil was prepared according to OECD guideline 207,[14] which was comprised (by dry weight) of 70% quartz sand, 20% kaolinite, and 10% finely ground sphagnum peat, with pH adjusted to 6.5 by addition of calcium carbonate.

2.3. Treatments

In order to compare the chronic toxicity of the selected arsenic species, the concentrations of spiked soil samples were designed based on 14 d median lethal concentration (LC_{50}) obtained in an artificial soil test following the OECD guideline 207.[14] Our previous research has found that the LC_{50} for As (III), As (V), MMA and DMA in the standard toxicity tests were 293, 352, 3425, 3730 mg kg^{-1}, respectively. Therefore, according to the limit value of As in Chinese Environmental Quality Standard for Soils (GB 15618-1995) and the concentration of As in typical polluted soils in

China,[23] the concentrations of spiked soils were designed as one-tenth of the 14 d LC_{50}, namely 29.3, 35.2, 342.5, 373.0 mg kg^{-1} for the four arsenic species.

Spiking solutions were prepared using standard solutions and added to four soil samples to satisfy soil As concentrations of 29.3 mg kg^{-1} As(III), 35.2 mg kg^{-1} As(V), 342.5 mg kg^{-1} MMA and 373.0 mg kg^{-1} DMA, respectively, as well as 70% water holding capacity. Each polyethylene plastic box (30×25×20 cm) was filled with 2000 g of As-spiked soil for 4 days prior to experimentation. The culture was maintained at 20 ^{o}C, 80% ambient humidity with a 12 h light/12 h dark cycle. The control test soils were prepared in a similar way with no contaminants added.

Before introduction into the soils, earthworms were rinsed with distilled water to remove adhering soils or particles and then blotted dry using tissue paper. Seventy mature earthworms with nearly equivalent masses were added into each box. Ten earthworms were removed from each box at t = 2, 4, 8, 16, 32 and 64 days following soil exposure. Five earthworms were used to determine the biomarkers, while five of them were utilized to analyze the As body burden in the tissue. An appropriate amount of arsenic free diet (6-10 g per box) was applied on the soil surface at the start of the experiment and was supplemented when consumed. The box was covered with a lid punched with holes to allow ventilation. During the exposure period, dead earthworms were removed immediately when found, and less than 10% individuals were dead after the 64-day period.

2.4. Sample pre-treatment and analysis of biomarkers

2.4.1. Preparation of tissue extract

Sampled earthworms of each experimental group for biomarker analysis were placed in petri dishes for voiding their gut (24 h at 15 ^{o}C and dark) and subsequently they were weighted. Earthworms were cooled on ice to facilitate dissection process. A sample of the body wall muscle (1.5-2.0 g wet weight) was taken and washed in distilled water to remove soil particles. Muscle samples were immersed in liquid nitrogen and stored frozen at -80 ^{o}C until analysis.

The whole procedure was conducted at 4 ^{o}C. Tissue samples from each exposure treatment and control were pooled and divided into two aliquots. For measurement of protein content and seven

oxidative stress biomarkers, the samples were homogenized (1:4, w/v) in chilled Tris-HCl buffer (20 mM, pH 7.8) by a dispersator. Next, homogenates were centrifuged at 10000 g at 4 ^{o}C for 15 min and the supernatant was carefully collected. For detection of MTs, the sample was homogenized (1:4, w/v) with cold Tris-HCl buffer (20 mM, pH 8.6) containing 0.5 M sucrose, 0.5 mM phenylmethylsulfonyl fluoride as an antiproteolytic agent, and 0.01% β-mercaptoethanol as a reducing agent. The homogenates were centrifuged at 25000 g at 4 ^{o}C for 20 min and the supernatant was used for MTs quantification.

2.4.2. Biomarker analysis

SOD activity was assayed by the method interpreted by McCord and Fridovich[24] and the absorption of the reduction in cytochrome c by O_2^- generated by xanthine oxidase/hypoxanthine system at 550 nm was measured. CAT activity was analyzed by utilizing the method described by Aebi[25] and measuring the decrease in absorbance at 240 nm because of the hydrogen peroxide consumption. SOD activity was expressed as U mg^{-1} of total protein concentration, while CAT activity was expressed as U g^{-1} of total protein concentration. GPx activity was measured by the method proposed by Hafeman et al.[26] and expressed as nmoles of GSH used by every milligram of protein per minute. GST activity was quantified by the method developed by Habig et al.[27] and expressed as nmol min^{-1} mg^{-1} protein. GR activity was determined according to the method described by Ramos-Martínez et al.,[28] which measures the oxidation of NADPH at 340 nm in the presence of oxidized glutathione and 0.1 M Na-phosphate buffer. The resulting data of GR activity was expressed in unit of U g^{-1} protein. GSH content (μmol g^{-1} protein) was determined by the fluorimetric method put forward by Hissin and Hilf.[29] LPO was quantified in terms of malondialdehyde (MDA) (nmol mg^{-1} protein) by the method described by Buege and Aust.[30] Protein concentration was measured spectrophotometrically by the method developed by Bradford[31] and consulting bovine serum albumin as a standard.

MTs content was determined using the spectrophotometric method of Viarengo et al..[32] Three volumes of absolute ethanol (-20 °C) were added to the supernatant resulting from ethanol/chloroform extraction to precipitate the MTs. Then the MT pellets were resuspended in

NaCl/HCl/EDTA to remove arsenic cations still bound to the MTs. After this Ellman reagent (pH 8.0 phosphate buffer containing DTNB) was added to the solution. The DTNB reacts with the thiol (-SH) groups on the MTs. Quantification of MTs was performed spectrophotometrically at 412 nm. Standard solutions of GSH (0-400 μM) were used for calibration. The MTs content was expressed as nmoles of -SH g^{-1} protein.

2.5. IBR index calculation

IBR was applied to the four experimental groups at each sampling time by combining the responses of the eight biomarkers in the earthworms (SOD, CAT, GPx, GST, GR, GSH, LPO and MTs) into an index according to Beliaeff and Burgeot,[22] which is accepted as a measurement of "stress".[22, 33-35]

To calculate the IBR values, data were standardized first. After this, the scores of all the biomarkers were expressed in the form of star plots. The basis of data processing of each biomarker was described as follows. (1) The mean and standard deviation (SD) for each sample was calculated. (2) Y_i value was calculated via the equation $Y_i = (X_i - m_i)/S_i$, where Y_i was the standardized value of a biomarker, X_i referred to the mean value of a biomarker for each sample, m_i and S_i represented the mean value and SD of a biomarker calculated for all the samples, respectively. (3) Z_i value was computed via the equation $Z_i = Y_i$ on the condition that a biomarker was induced in comparison with the control group, or via the equation $Z = -Y_i$ on the condition that a biomarker was inhibited. The minimum value (min_i) of Z_i for a biomarker was obtained for all the samples, and then the score (B_i) for a given sample was computed as $B_i = Z_i + |min_i|$, where $|min_i|$ was the absolute value.

The eight biomarkers were introduced to the IBR calculation. The respective eight scores for each sample (B_1–B_8) were expressed in the form of star plots. B_i represented the score of a biomarker for a sample, while B_{i+1} stood for the score of the next biomarker for the sample. The eight scores were arranged as a set. The IBR index for each sample was calculated as the area of the star plot where the scores were displayed:

$$IBR = \sum_{i=1}^{n} A_i$$

where A_i represented the triangular area represented by two consecutive biomarker scores (B_i, B_{i+1}) on the plot, and *n* stood for the number of biomarkers used in the IBR calculation. Since the biomarker arrangements on the star plots generated different IBR values,[33] all the sequences of the eight biomarkers were taken into account in this study and the average value of 2520 types of IBR values was calculated as the final value.

2.6. As analysis of earthworm tissue

The earthworms used to analyze As body burden in the tissue was placed on a moist filter paper for 48 h to allow defecation, and the filter paper was changed after 24 h. After rinsing the earthworms with distilled water, the samples were killed using liquid nitrogen and kept at -80 ^{o}C for 24 h. Earthworm samples were then pooled and dried by freeze drying at -40 ^{o}C for 72 h, ground with an agate pestle and mortar to fine powder.

For the total As content analysis, thirty milligrams of earthworm powder was transferred to a vial. Two milliliters of nitric acid was added to the vial and heated to 80 ^{o}C for 8 h. The extract was cooled, filtered through a 0.45 μm filter, diluted to 10 mL with distilled water, and then analyzed for the total As content using a cold vapor atom fluorescence spectrometry (AFS-920, Beijing Titan Instruments Co.). The quality assurance was checked by using a standard reference material (GSS-1) provided by the Institute of Geophysical and Geochemical Exploration (IGGE) of China. The results obtained for the standard reference material were in accordance with the certified value.

For the As speciation analysis, a microwave-assisted extraction method was used.[36] Twenty milligrams of finely ground earthworm sample was weighed directly in a PTFE microwave digestion vessel, and 10 mL of distilled water was added into the vessel. The sample was then digested in a high-pressure microwave system (XT-9900A, Xintuo analytical instruments Co., China). After cooling to room temperature, the extract was filtered through a 0.45 μm syringe filter. The final solutions were analyzed for As species by using a derivatization-gas chromatography method developed by Wang and Cui.[37] For quality control, matrix-spiked samples were used for each As species, and the recoveries of the four As species were 104-110%. The concentrations of total As and As species were expressed in the form of mg per kg of dry weight.

3. Results

3.1. Accumulation and biotransformation of As species by earthworms

The initial As concentration in the earthworms was 5.56 mg kg^{-1} dry weight, and the As content in the control group varied slightly between 4.62 and 6.29 mg kg^{-1} during the experimental period. The only As species detectable in the control group was As(V) with the concentration values ranging from 4.07 to 6.12 mg kg^{-1}.

Fig. 1 and Table S1 show the changes of the total arsenic content and the contents of the four arsenic species, to which the earthworms were exposed during the entire experimental period. For the earthworms of group I and group II that were exposed to soil spiked with As(III) and As(V), respectively, the total arsenic content showed a similar changing trend: both increased with the exposure time. For the earthworms of group III and group IV that were exposed to the soil added with MMA and DMA, respectively, the total arsenic content showed a changing trend which was quite different from that in the first two groups: both increased at the beginning and then decreased with the exposure time; the maximum values appeared on day 8.

For the contents of different arsenic species, the earthworms of group I were exposed to soil added with As(III), which was the species of the highest content and increased with the exposure time: rose gradually from 14.36 mg kg^{-1} on day 2 to 42.25 mg kg^{-1} on day 64. Furthermore, the content of As(V) in the earthworms also increased with the exposure time: rose from 6.13 mg kg^{-1} on day 2 to 25.49 mg kg^{-1} on day 32, yet slightly decreased to 24.10 mg kg^{-1} on day 64. The contents of MMA and DMA in the earthworms of group I were less than the contents of the two inorganic arsenics. As the exposure time increased, the contents of these two organic arsenics converted from undetectable to detectable and kept increasing.

The samples of group II were exposed to soil added with As(V), which was the species of the highest content and was accumulated during the whole exposure period. Compared with As(V), the content of As(III) was less and showed a continuous accumulation, except for the slight decrease on day 64. During the first eight days, the two organic arsenics were undetectable. At the later stage of the experiment, the content of MMA was detected as 3.22 mg kg^{-1} on day 16, 1.28 mg kg^{-1} on day

241 32 and 2.64 mg kg^{-1} on day 64, respectively, while the content of DMA increased gradually as the
242 exposure continued.

243 The samples of group III were exposed to soil added with MMA. Among the four arsenic
244 species, MMA and DMA showed a similar changing trend: both increased at first and then
245 decreased with the exposure time; besides, their maximum values appeared on day 8. The two
246 inorganic arsenics in earthworms increased slowly as the exposure time prolonged.

247 The samples of group IV were exposed to the soil added with DMA, which, among the four
248 arsenic species, was the only one that showed a distinct changing trend: increased first and then
249 decreased as the exposure continued and its maximum appeared on day 8. The contents of the two
250 inorganic arsenics increased slowly with the exposure time, which was similar to those of group III.
251 In the earthworms of group IV, however, MMA showed an irregular changing trend: its content was
252 8.22, 15.79, 18.10, 14.35, 18.04 and 11.73 mg kg^{-1} from day 2 to day 64, respectively.

253 **3.2. Biomarker responses**

254 Fig. 2 and Table S2 show the changing trend of eight biomarkers in the earthworms which
255 were exposed to the four arsenic species during the whole experimental period. It can be seen that
256 the eight biomarkers in the control group were basically stable during the 64-day experiment. In the
257 four experimental groups, however, the biomarkers showed different response characteristics. On
258 the whole, the changes of biomarkers in the earthworms exposed to inorganic arsenics were more
259 prominent than those exposed to organic arsenics. In the first two groups, the changes of most
260 biomarkers in the earthworms exposed to As(III) were more prominent than those exposed to
261 As(V).

262 For the earthworms of group I and group II, five out of the eight biomarkers showed similar
263 changing trend: increased first and then decreased as the exposure continued, including SOD, CAT,
264 GPx, GST and GR. Among the five biomarkers, the activities of SOD, CAT, GPx and GST were
265 inhibited at the later stage of the experiment. The content of GSH in the earthworms decreased first
266 and then greatly increased as the exposure time prolonged, and then decreased at the later stage.
267 Compared with the control group, the content of MDA was higher during the whole experiment,

and the degree of induction at the later stage of the experiment was higher than that at the early stage. The content of MTs increased with the exposure time during the whole experimental period.

For the earthworms of group III and group IV, six biomarkers, namely SOD, CAT, GPx, GR, GSH and MTs, showed a similar changing trend: no obvious changes could be found during the first 16 days but then somewhat increased in the later stage of the experiment. In these two groups, no marked changes in the GST activity and in the MDA content could be found during the whole experiment.

3.3. IBR calculation

The IBR index was applied to the eight biomarkers of the four exposure treatments. The values were calculated and displayed in Fig. 3 and Table S3. The IBR values of the earthworms in the experimental group exposed to As(III) were always higher than that in other groups. The IBR values of the earthworms in group II exposed to As(V) were lower than that in group I, but higher than those exposed to MMA and DMA. For the earthworms of group III and group IV, the IBR values were close to each other with little change during the whole experiment, and the maximum IBR values for the two groups appeared on day 64.

4. Discussion

Arsenic is widely found in many different chemical forms in the environment. As different arsenic species have different degrees of toxicity, recently many studies have been conducted for arsenic speciation in soil. Most of these studies showed that the trivalent and pentavalent arsenic species are the most common chemical forms of arsenic in soil samples,[38] while some suggested that organic arsenic takes a large proportion of the total arsenic in soil.[9, 39] Thomas et al.[40] determined As(III), As(V), MMA and DMA in a soil reference material containing 13.7 mg kg^{-1} of total As and found that only As(V) was detectable. The average concentration of As(V) was 10.5 mg kg^{-1}. As(V) was also the major compound in typical As-contaminated soils in Japan. Both of MMA and DMA were detected at lower levels, which was 5-88 μg kg^{-1} for MMA and 4-69 μg kg^{-1} for DMA, respectively.[41] Chatterjee and Mukherjee[42] collected soil samples on the ground of a chemical company producing Paris Green and arsenical pesticides. The water extractable arsenic

species in the soils contained 16.4 mg kg^{-1} As(III), 131 mg kg^{-1} As(V), 51.2 mg kg^{-1} MMA and 25.0 mg kg^{-1} DMA. Chappell et al.[43] determined As(III), As(V), and organic arsenic compounds in a contaminated soil with the total arsenic amount of 1.14 g kg^{-1} and found that the concentrations of the three arsenic species were 3 mg kg^{-1} for As(III), 942 mg kg^{-1} for As(V) and 40 mg kg^{-1} for organic arsenic, respectively. Hansen et al.[44] developed a system combined high-performance liquid chromatography (HPLC) and flame atomic absorption spectrometry (FAAS) to determine seven molecular forms of arsenic. The approach was applied in the analysis of arsenic species in the soil samples from a polluted land site. Only As(V), which showed an average concentration of 32 mg kg^{-1}, was found in the soil extracts. Yuan et al.[45] determined different arsenic species in several polluted soil samples and found that As(III) and As(V) were the major arsenic species in the soil samples resulting from irrigation by waste water. The concentrations of As(III) and As(V) were 0.59-0.72 mg kg^{-1} and 61.7-76.9 mg kg^{-1}, respectively.

In this paper, four common arsenic species in soil were selected and the recommended test species, *E. fetida*, was taken as the organism being tested to analyze the toxicity of different arsenic species by detecting several biomarker responses. In order to choose a proper exposure concentration, we conducted a pre-experiment to determine the value of 14d-LC_{50} of the four arsenic species, and one-tenth of which was taken as the dose to be added into the artificial soil. Then, four groups of earthworms were exposed to the soils spiked with As(III), As(V), MMA and DMA, respectively, to carry out a 64-day experiment on chronic toxicity. Given that the process of biotransformation would occur in the earthworms as the arsenic species were accumulated during the whole experimental period, the total arsenic content and arsenic species in the earthworms were therefore investigated.

Contaminants in soil were accumulated by earthworms mainly through ingestion and dermal contact in both the solid and aqueous phases. As the forms of arsenic in the soil depend on the amount of phosphorus, aluminium, iron and organic matter present, as well as pH, and the redox potential,[9] according to OECD guideline 207, the prepared artificial soil was used in this study to avoid interference from such factors. During the entire experiment, arsenic species in the soils were

detected in the same way (data not shown), and we found a basically unchanged concentration of the arsenic species in the four experimental groups during the first 16 days. On day 32 and 64, a small amount of As(V) was detected in the soil of group I, while a small amount of As(III) was detected in the soil of group II. For the soil added with organic arsenics, a small amount of As(V) and As(III) was detected on day 16, 32 and 64, indicating the occurrence of demethylation. When the speciation of arsenic in soil was changed, the accumulation of arsenic species in the earthworms would be influenced correspondingly.

A biotransformation pathway for arsenic through an earthworm has been proposed by Langdon et al.[9] and developed by Button et al.,[46] including four steps as follows: (1) As(V) is reduced to As(III);[47] (2) the MTs within the chloragogenous tissue is induced, followed by the formation of As(III)-thiol by complexing with the sulfur-rich protein;[48] (3) As(III) is methylated to MMA, followed by DMA;[46] (4) AsB is produced by a series of complex biochemical reactions of DMA.[49] An earthworm excretes AsB and other organic arsenics through mucus, casts and urine, which decreases the arsenic burden in the tissue. Therefore, the bioconcentration of As in the earthworms occurs when the accumulated As was sequestered in tissues and was not readily excreted.[50]

Many literatures have reported that earthworms can accumulate arsenic from contaminated soils.[15, 51, 52] In this study, there were marked elevations in the total As concentrations in the earthworms of group I and group II during the entire experimental period. For the two treatments, the total As levels in the earthworms exceeded the As concentration in the soil on the 4th day, indicating that As was readily bioconcentrated in *E. fetida*. As for the earthworms of group III and group IV, organic arsenics in an earthworm could be biologically transformed and excreted into the environment. Therefore, the total arsenic content decreased after the 8th day and did not become bioconcentrated during the whole experiment.

The analysis of arsenic species in the earthworms showed that the contents of the two inorganic arsenics in the earthworms of group I increased gradually as the exposure continued, and As(V) was mainly generated from the oxidation of As(III) in organisms. For earthworms of this group, the contents of MMA and DMA converted from undetectable to detectable, indicating that

the arsenic methylation was occurred in the earthworms. For earthworms of group II, high levels of As(III) in the tissue suggested that the first step of arsenic biotransformation, namely, the reduction of As(V), was occurred in the earthworms. Arsenic is only methylated in the As (III) form,[47] which can be biotransformed to decrease the toxicity by complexing with MTs. Therefore, the reduction of As(V) is a key step for earthworms to metabolize arsenics. The speciation analysis results corresponded well with a previous report which showed that As(V) was readily reduced to As(III) in the earthworms.[53] For the earthworms of group II, the contents of MMA and DMA also converted from undetectable to detectable, which indicated that the third step of arsenic biotransformation, namely, the methylation of inorganic arsenics, was occurred in the earthworms. For the earthworms of group III, elevated levels of DMA and MMA were detected, proving that MMA can be converted into DMA under the effect of methylation. Meanwhile, the gradually increased contents of As(III) and As(V) may come from the demethylation products of organic arsenics *in vitro* and *in vivo*. The analysis of the arsenic species in the earthworms of group IV indicated that DMA was the only species that showed high concentrations in the earthworms, while the contents of As(III) and As(V) were not increased obviously with the exposure time, indicating the fourth step of arsenic biotransformation, namely the formation of AsB. The process of demethylation which greatly increased the toxicity of arsenic was unlikely to happen in earthworms. All the results verified the arsenic biotransformation pathway as mentioned above.

In this study, only four arsenic species and the total arsenic were determined. The resulting data showed that the content of the total arsenic was higher than that of the sum of the four arsenic species. According to previous studies, such difference could represent the total content of AsB and the intermediate products of other organic arsenics.[54] For the first three experimental groups, such difference increased gradually as the experiment continued, suggesting that the toxic arsenic species in the earthworms were transformed into the nontoxic AsB and organic arsenics, which could decrease arsenic toxicity. For group IV, such difference increased first and then decreased as the experiment continued, indicating an excretion process of AsB. This could also be proved by the date acquired at the later stage of the experiment, during which the total arsenic content in the

earthworms became less accumulated.

In the past, the main indicators to study the toxicity on the earthworms were survival, growth, reproduction, behavior, pigmentation, etc. These biomarkers are often insensitive and usually respond to high levels of toxic chemicals. In contrast, molecular biomarkers are sensitive to response even under the effect of a low concentration of contaminants and are closely related to the toxicology of contaminants, which makes them more suitable for toxicology research. The antioxidant defense system includes many molecular biomarkers that have been widely used in toxicology researches.[55] Among the eight biomarkers adopted in this study, seven of them are oxidative stress markers, including five antioxidant enzymes and one non-enzymatic antioxidant in the antioxidant defense system as well as LPO, an indicator of oxidative damage. Furthermore, the biomarker of MTs which plays an important role in the pathway for arsenic detoxification was determined.

In general, the processes that generate and scavenge reactive oxygen species (ROS) in organisms are in a dynamic state of equilibrium. Exposure to hazardous chemical substances could stimulate the formation of ROS. In order to deal with the potential oxidative damage ensued, antioxidative defense mechanisms were developed in the organisms and antioxidant enzymes were usually induced. SOD catalyzes the transformation of superoxide radicals to H_2O_2, which is subsequently degraded into H_2O by CAT and GPx.[56] SOD and CAT act as important frontiers for defending against ROS toxicity.[55] In this study, the two biomarkers showed a similar response pattern. During the early stage of the experiment, the activities of both SOD and CAT in the earthworms of group I and group II were induced, indicating that the inorganic arsenic species induced the generation of superoxide radicals after entering into the earthworms. The SOD activity therefore needed to be enhanced to catalyze the superoxide radicals into H_2O_2, which further induced the CAT activity. However, under long-lasting contamination conditions, the antioxidant enzyme activities, such as SOD and CAT may be deactivated with an accumulation of oxidizing agent.[57] On day 32 and 64 of the experiment, the activities of both SOD and CAT in the earthworms of group I and group II were inhibited, exhibiting toxic effect on organisms with the long-term

accumulation of contaminants. Different from the first two groups, the activities of SOD and CAT in the earthworms of group III and group IV were changing slightly during the first sixteen days and induced at the later stage of the experiment. According to the arsenic speciation results in the earthworms, the accumulation of MMA and DMA did not cause obvious responses of SOD and CAT, which suggested that organic arsenics did not induce a large amount of ROS in the organisms. At the later stage of the experiment, the contents of the two inorganic arsenics gradually increased in the earthworms with an increase of the antioxidant enzyme activities, which implied that the toxic effect was caused by inorganic arsenics generated from the demethylation of MMA and DMA.

The metal contamination stress would also lead to the generation of organic hydroperoxides (ROOH), a species of ROS, which could be decomposed by GPx and GST, consume GSH and generate oxidized glutathione (GSSG) with an oxidation state simultaneously.[58] In this study, GPx and GST showed a similar response pattern, which indicated that they played a cooperative role in the process of clearing ROOH. During the early stage of the experiment, GPx and GST in the earthworms of group I and group II were induced, which indicated that the inorganic arsenics in the earthworms induced the generation of ROOH and therefore induced detoxification reactions catalyzed by the two enzymes. As the contaminants accumulated, the activities of GPx and GST decreased until they were inhibited, which suggested that the damages on the organisms exceeded the protective capacity of the antioxidant defense system.[59] Different from the first two groups, no obvious change in the GST activity was found in the earthworms of group III and group IV during the whole experiment, while the activity of GPx was induced only on day 32 and 64. The results implied that the high concentration of MMA and DMA did not induce the generation of a large amount of ROOH which was responsible for the oxidative stress response. At the later stage of the experiment, the induction of GPx was the same as that of SOD and CAT. Because another function of GPx is to catalytically degrade H_2O_2,[56] such induction exhibited the effect of GPx on eliminating hydrogen peroxide generated with the accumulation of inorganic arsenics in the earthworms of group III and group IV.

It has been widely accepted that GSH reduces As-mediated oxidative stress.[60] The mechanism

for such attenuation occurs in a double way. First, As^{3+} ions have a high affinity by the sulfhydryl groups and therefore GSH acts as arsenic scavenger. Second, As causes oxidative stress via ROS production, which is reduced by the action of antioxidant enzymes with the consumption of GSH. As a result of this reaction, the glutathione is oxidized to its disulfide form. In this study, the content of GSH in the earthworms of group I and group II decreased on day 2, which was due to over-utilization in order to challenge the prevailing oxidative stress. However, when the organisms consumed excessive GSH, more GSH would be synthesized as the adaptation to the environmental stress.[61] Therefore, the content of GSH in the earthworms in the first two groups rose greatly at the middle stage of the experiment, suggesting a pollutant-induced adaptive response. The decrease of the GSH content at the later stage could be attributed to the inhibition of antioxidant enzyme activities under the long-term contaminant stress. For the earthworms of group III and group IV, the content of GSH gradually increased with the exposure time and was greatly induced on day 64, which showed a changing trend similar to that of the inorganic arsenic contents. These results suggested that under the contamination of MMA and DMA, it was inorganic arsenics generated from demethylation, rather than organic arsenics itself, that induced the oxidative stress responses of the organisms.

By catalyzing GSSG to GSH, GR can maintain a suitable GSH/GSSG ratio in the presence of oxidative stress to maintain the –SH level in cells.[62] In this study, the GR activity in the earthworms of group I and group II was induced during the first sixteen days, while it lagged behind the GPx activity. This explained the decrease in GSH levels which was excessively consumed at the beginning of the experiment as well as explained the maximum value of GSH that appeared on day 16. At the later stage of the experiment, the GR activity in the two groups was decreased to the similar level of the control group, which was also related to the inhibition of the enzymatic activities under long-term contaminant stress. For the earthworms of group III and group IV, the GR activity gradually increased as the exposure continued, which was similar to the changing trend of GSH, indicating that the antioxidant enzyme activity could be increased due to the induction of oxidative stress as inorganic arsenics accumulated in the organisms.

Lipid peroxidation (LPO) is a biomarker for oxidative damage. MDA is a major oxidation product of peroxidized polyunsaturated fatty acids which are considered to be an important indicator of lipid peroxidation.[63] In this study, the content of MDA in the earthworms of group I and group II were always higher than those in the control group, indicating that the arsenics accumulated in the earthworms had induced oxidative toxicity which far exceeded the protective capacity of the antioxidant defense system and caused actual damages. Among the two groups, the earthworms of group I exposed to As(III) showed the highest content of MDA, which suggested that this arsenic species could cause the most serious toxic effect. In contrast, the content of MDA in the earthworms of group III and group IV varied slightly during the entire experiment, which indicated that the effective operation of the antioxidant defense system had prevented the oxidative damages. Large amounts of organic arsenic were accumulated in the earthworms of group III and group IV without inducing a great increase of LPO level, which also proved that the biotoxicity of both MMA and DMA was very low.

MTs have been widely used as specific biomarkers for metal and metalloid contamination. A study conducted by Morgan et al. suggested that there was a possibility of arsenic inducing MTs synthesis in earthworm chloragocytes.[64] The hypothesis was supported by Langdon et al., who found that arsenic could induce MTs expression and was sequestered by the sulfur-rich proteins in certain target cells and tissues of contaminated earthworms.[65] In this study, the content of MTs in earthworms were closely related to the contents of the two inorganic arsenics in the earthworms, especially associated with the content of As(III), which was in good agreement with previous studies.[64, 65] The earthworms of group I which accumulated the maximum amount of As(III) exhibited the highest content of MTs, indicating that the trivalent arsenic species can induce the generation of MTs before complexing with it. Although the formation of the complexation between MTs and As(III) was proved to decrease the toxicity of trivalent arsenic,[18] the response of the oxidative stress biomarkers in this study implied that the toxicity of arsenic to the earthworms cannot be eliminated by the only pathway. Arsenic accumulation always leads to the generation of ROS, which will induce the responses of several oxidative stress biomarkers. Therefore, arsenic

detoxification in an earthworm needs a joint effort from biotransformation, the complexation between As(III) and sulfydryl proteins (such as MTs) as well as from the response of antioxidant defense system which eliminates ROS.

The IBR index was considered as a practical tool that could be applied to examine the integrated stress responses of different contaminants by the combination of multi-biomarker responses to a single value. In this study, the IBR index was applied to compare the toxicity of different arsenic species comprehensively. The IBR index of the control group cannot be calculated according to the calculation rules. As a result, the IBR values of the four experimental groups at each sampling time point were obtained. As can be seen from the IBR values shown in Fig. 3 and Table S3, the toxicity of the four arsenic species was ranked as: As(III)＞As(V)＞MMA and DMA. Although there was a big difference of 14d-LC_{50}, the chronic toxicity of MMA and DMA on *E. fetida* had no great difference during the 64-day experiment. According to changing trend of several biomarkers in the earthworms, the responses of oxidative stress biomarkers and MTs were not greatly induced when large amounts of organic arsenic were accumulated. The results showed that MMA and DMA exhibited very low toxicity when the soil concentration was set at one-tenth of 14d-LC_{50}, and the oxidative stress was mainly caused by inorganic arsenics generated from the demethylation products of organic arsenics. As the concentrations of DMA and MMA in real environment are generally lower than those specified in this study, a small amount of organic arsenics can be considered as nontoxic in non-extreme cases. However, demethylation of these two organic arsenics could occur either in environment or in organisms to generate toxic inorganic arsenics.[9] In the pre-experiment, the exposure concentrations of DMA and MMA were ten times higher than those utilized in this experiment. As a result, half of the earthworms were dead on the 14th day, which might be caused by the toxic inorganic arsenics generated from the demethylation of DMA and MMA. In this sense, the MMA and DMA level should be considered as a reference for long-term monitoring programs. According to the value of 14d-LC_{50}, the spiked concentration of As(III) was close to that of As(V). Nevertheless, the trivalent arsenic was proved to be more toxic

than the pentavalent arsenic, which agreed with the results of previous toxicology researches.[39] In real environment, As(III) always coexists with As(V). Hence, it would not be accurate when only the total arsenic content is used for ecological risk assessment. In order to obtain reasonable results, the contents of different arsenic species should be determined and treated with different weights and assessment criteria. In further studies, the toxicology of the main arsenic species should be specially investigated to provide scientific evidences for accurate quality/risk assessment.

5. Conclusions

In conclusion, the responses of multi-biomarkers in *E. fetida* showed that the toxicity of four arsenic species was ranked as: As(III)＞As(V)＞MMA and DMA. The two organic arsenics showed low biotoxicity. However, they could be transformed into highly toxic inorganic arsenic under the effect of demethylation during long-term exposure, which generated toxic effects on organisms. The results of multi-biomarker responses indicated that a sub-lethal dose of both As(III) and As(V) could trigger the response of the antioxidant defense system and cause oxidative damages when the protective capacity of the system was exhausted. The detoxication of arsenic in the earthworm was achieved in the process of biotransformation, where the accumulated inorganic arsenics were methylated and synthesized into organic arsenics, which would then be excreted out. In real environment, the two major inorganic arsenic species, As(III) and As(V), show different degrees of toxicity. Therefore, arsenic species should be differentiated to get accurate results in the quality/risk assessment programs.

Conflict of interest

The authors declare that there are no conflicts of interest.

Acknowledgments

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References

1. L. L. Embrick, K. M. Porter, A. Pendergrass and D. J. Butcher, *Microchem. J.*, 2005, **81**, 117-121.
2. L. Yang and R. J. Donahoe, *Appl. Geochem.*, 2007, **22**, 320-341.
3. T. N. Hartley, A. J. Macdonald, S. P. McGrath and F. J. Zhao, *Environ. Pollut.*, 2013, **180**, 259-264.
4. J. C. Kwon, J.-S. Lee and M. C. Jung, *Appl. Geochem.*, 2012, **27**, 1020-1026.
5. J. Y. Kim, K. W. Kim, J. S. Ahn, I. Ko and C. H. Lee, *Environ. Geochem. Health*, 2005, **27**, 193-203.
6. Z. Gong, X. Lu, M. Ma, C. Watt and X. C. Le, *Talanta*, 2002, **58**, 77-96.
7. L. Liu, B. He, Z. Yun, J. Sun and G. Jiang, *J. Chromatogr. A*, 2013, **1304**, 227-233.
8. M. F. Hughes, *Toxicol. Lett.*, 2002, **133**, 1-16.
9. C. J. Langdon, T. G. Piearce, A. A. Meharg and K. T. Semple, *Environ. Pollut.*, 2003, **124**, 361-373.
10. J. Cotter-Howells, J. M. Charnock, C. Winters, P. Kille, J. C. Fry and A. J. Morgan, *Environ. Sci. Technol.*, 2005, **39**, 7731-7740.
11. J. Nahmani, M. E. Hodson and S. Black, *Environ. Pollut.*, 2007, **145**, 402-424.
12. D. J. Spurgeon and S. P. Hopkin, *Sci. Total Environ.*, 1996, **187**, 167-183.
13. N. W. Xiao, Y. Song, F. Ge, X. H. Liu and Z. Y. Ou-Yang, *Chemosphere*, 2006, **65**, 907-912.
14. OECD, *OECD Guidelines for the Testing of Chemicals*, Organization for Economic, 1984.
15. E. Fischer and L. Koszorus, *Pedobiologia*, 1992, **36**, 172-178.
16. R. Lanno, J. Wells, J. Conder, K. Bradham and N. Basta, *Ecotoxicol. Environ. Saf.*, 2004, **57**, 39-47.
17. V. E. Forbes, A. Palmqvist and L. Bach, *Environ. Toxicol. Chem.*, 2006, **25**, 272-280.
18. B. T. Lee and K. W. Kim, *Environ. Toxicol.*, 2008, **24**, 369-376.
19. M. Button, G. R. Jenkin, K. J. Bowman, C. F. Harrington, T. S. Brewer, G. D. Jones and M. J. Watts, *Mutat. Res.-Genet. Toxicol. Environ. Mutag.*, 2010, **696**, 95-100.
20. C. Anderson, P. Kille, A. Lawlor and D. J. Spurgeon, *Environ. Pollut.*, 2013, **172**, 200-207.
21. N. Aarab, O. Champeau, P. Mora, M. Daubeze, P. Garrigues and J.-F. Narbonne, *Biomarkers*, 2004, **9**, 258-270.
22. B. Beliaeff and T. Burgeot, *Environ. Toxicol. Chem.*, 2002, **21**, 1316-1322.
23. L. Zhao, Y. Xu, H. Hou, Y. Shangguan and F. Li, *Sci. Total Environ.*, 2014, **468**, 654-662.
24. J. M. McCord and I. Fridovich, *J. Biol. Chem.*, 1969, **244**, 6049-6055.

25. H. Aebi, Catalase, in: *H. U. Bergmeyer (Ed.), Methods of enzymatic analysis*, Chemic Academic Press Inc., Verlag, 1974, pp. 673-685.
26. D. G. Hafeman, Sunde, R.A., Hoekstra, W.G, *J. Nutr.*, 1974, **104**, 580-587.
27. W. H. Habig, M. J. Pabst and W. B. Jakoby, *J. Biol. Chem.*, 1974, **249**, 7130-7139.
28. J. I. Ramos-Martinez, T. R. Bartolomé and R. V. Pernas, *Comp. Biochem. Physiol. B*, 1983, **75**, 689-692.
29. P. J. Hissin and R. Hilf, *Anal. Biochem.*, 1976, **74**, 214-226.
30. J. A. Buege and S. D. Aust, *Methods Enzymol.*, 1978, **52**, 302-310.
31. M. M. Bradford, *Anal. Biochem.*, 1976, **72**, 248-254.
32. A. Viarengo, E. Ponzano, F. Dondero and R. Fabbri, *Mar. Environ. Res.*, 1997, **44**, 69-84.
33. K. Broeg and K. K. Lehtonen, *Mar. Pollut. Bull.*, 2006, **53**, 508-522.
34. G. Damiens, M. Gnassia-Barelli, F. Loquès, M. Roméo and V. Salbert, *Chemosphere*, 2007, **66**, 574-583.
35. F. P. Meng, Z. F. Wang, F. L. Cheng, X. P. Du, W. C. Fu, Q. Wang, X. Y. Yi, Y. F. Li and Y. Zhou, *Mar. Environ. Res.*, 2013, **85**, 64-75.
36. J. A. Brisbin and J. A. Caruso, *Analyst*, 2002, **127**, 921-929.
37. Z. F. Wang and Z. J. Cui, *Chin. Chem. Lett.*, 2016, DOI: 10.1016/j.cclet.2015.10.001.
38. R. Pongratz, *Sci. Total Environ.*, 1998, **224**, 133-141.
39. M. Bissen and F. H. Frimmel, *Acta Hydroch. Hydrob.*, 2003, **31**, 9-18.
40. P. Thomas, J. K. Finnie and J. G. Williams, *J. Anal. At. Spectrom.*, 1997, **12**, 1367-1372.
41. T. Takamatsu, H. Aoki and T. Yoshida, *Soil Sci.*, 1982, **133**, 239-246.
42. A. Chatterjee and A. Mukherjee, *Sci. Total Environ.*, 1999, **225**, 249-262.
43. J. Chappell, B. Chiswell and H. Olszowy, *Talanta*, 1995, **42**, 323-329.
44. S. H. Hansen, E. H. Larsen, G. Pritzl and C. Cornett, *J. Anal. At. Spectrom.*, 1992, **7**, 629-634.
45. C.-G. Yuan, B. He, E.-L. Gao, J.-X. Lü and G.-B. Jiang, *Microchimica Acta*, 2007, **159**, 175-182.
46. M. Button, G. R. T. Jenkin, C. F. Harrington and M. J. Watts, *J. Environ. Monit.*, 2008, **11**, 1484-1491.
47. K. J. Irgolic, Arsenic in the environment, in: *A. V. Xavier (Ed.), Frontiers in bioinorganic chemistry*, VCH, Weinheim and Deerfield Beach, 1986, pp. 399-408.
48. A. J. Morgan, C. Winters and A. Yarwood, *Cell Biol. Int.*, 1994, **18**, 911-914.
49. C. J. Langdon, A. A. Meharg, J. Feldmann, T. Balgar, J. Charnock, M. Farquhar, T. G. Piearce, K. T. Semple and J. Cotter-Howells, *J. Environ. Monit.*, 2002, **4**, 603-608.
50. A. A. Meharg, R. F. Shore and K. Broadgate, *Environ. Toxicol. Chem.*, 1998, **17**, 1124-1131.

51. A. Geiszinger, W. Goessler, D. Kuehnelt, K. Francesconi and W. Kosmus, *Environ. Sci. Technol.*, 1998, **32**, 2238-2243.
52. C. J. Langdon, T. G. Piearce, S. Black and K. T. Semple, *Soil Biol. Biochem.*, 1999, **31**, 1963-1967.
53. C. J. Langdon, T. G. Piearce, J. Feldmann, K. T. Semple and A. A. Meharg, *Environ. Toxicol. Chem.*, 2003, **22**, 1302-1308.
54. M. Button, M. M. Moriarty, M. J. Watts, J. Zhang, I. Koch and K. J. Reimer, *Chemosphere*, 2011, **85**, 1277-1283.
55. R. Wan, F. Meng, W. Fu, Q. Wang and E. Su, *Ecotoxicol. Environ. Saf.*, 2015, **111**, 78-85.
56. C. Cossu, A. Doyotte, M. C. Jacquin, M. Babut, A. Exinger and P. Vasseur, *Ecotoxicol. Environ. Saf.*, 1997, **38**, 122-131.
57. K. A. Modesto and C. B. R. Martinez, *Chemosphere*, 2010, **78**, 294-299.
58. B. J. Richardson, E. Mak, S. B. De Luca-Abbott, M. Martin, K. McClellan and P. K. Lam, *Mar. Pollut. Bull.*, 2008, **57**, 503-514.
59. X. Wang, H. Yang, G. Liu and Q. Wang, *Chin. J. Oceanol. Limnol.*, 2011, **29**, 981-989.
60. J. Ventura-Lima, P. B. Ramos, D. Fattorini, F. Regoli, L. Ferraz, L. M. de Carvalho and J. M. Monserrat, *Environ. Sci. Pollut. Res.*, 2011, **18**, 1270-1278.
61. R. van der Oost, J. Beyer and N. P. E. Vermeulen, *Environ. Toxicol. Pharmacol.*, 2003, **13**, 57-149.
62. E. Stephensen, J. Sturve and L. Förlin, *Comp. Biochem. Physiol., C: Toxicol. Pharmacol.*, 2002, **133**, 435-442.
63. A. Valavanidis, T. Vlahogianni, M. Dassenakis and M. Scoullos, *Ecotoxicol. Environ. Saf.*, 2006, **64**, 178-189.
64. A. J. Morgan, C. Winters, A. Yarwood and N. Wilkinson, *Scanning Microsc.*, 1994, **9**, 1041-1060.
65. C. J. Langdon, C. Winters, S. R. Stürzenbaum, A. J. Morgan, J. M. Charnock, A. A. Meharg, T. G. Piearce, P. H. Lee and K. T. Semple, *Environ. Sci. Technol.*, 2005, **39**, 2042-2048.

633 **Figure Legends**

634 **Fig. 1** Concentration (mg kg^{-1} dry wt) of four As species and total As in *E. fetida* following 64 days
635 exposure to As contaminated soils. Earthworms of group I, II, III, IV were exposed to arsenite
636 [As(III)], arsenate [As(V)], monomethylarsonate (MMA) and dimethylarsinate (DMA),
637 respectively.

638 **Fig. 2** Multi-biomarker responses in *E. fetida* following 64 days exposure to As contaminated soils.
639 Earthworms of group I, II, III, IV was exposed to As(III), As(V), MMA and DMA, respectively.

640 **Fig. 3** Integrated biomarker responses (IBR) values in *E. fetida* following 64 days exposure to As
641 contaminated soils. Earthworms of group I, II, III, IV was exposed to As(III), As(V), MMA and
642 DMA, respectively.

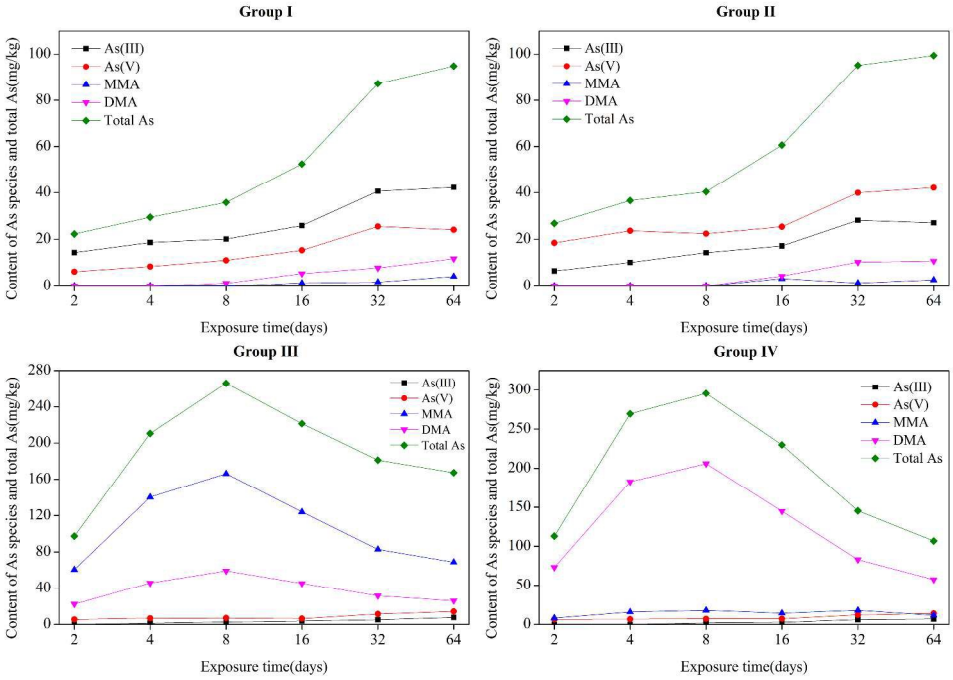
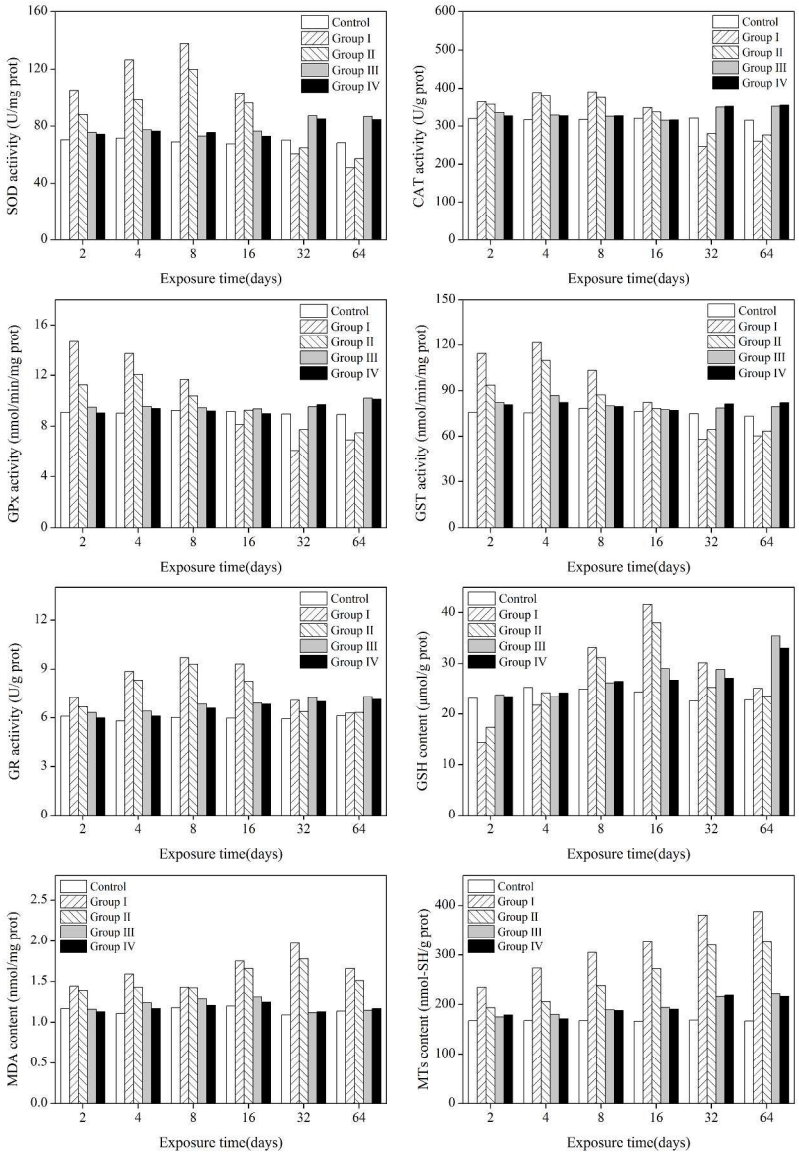


Fig. 1 Concentration (mg kg-1 dry wt) of four As species and total As in E. fetida following 64 days exposure to As contaminated soils. Earthworms of group I, II, III, IV were exposed to arsenite [As(III)], arsenate [As(V)], monomethylarsonate (MMA) and dimethylarsinate (DMA), respectively.



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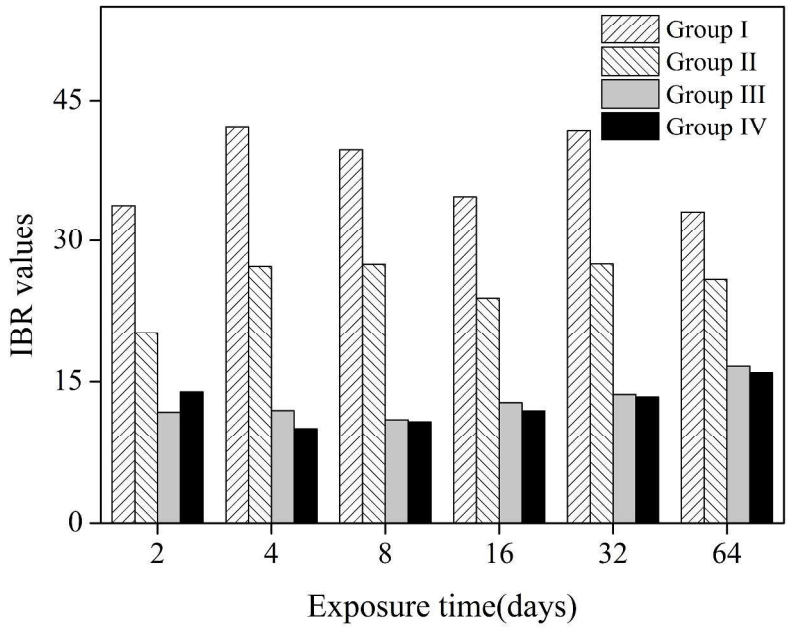

Fig. 3 Integrated biomarker responses (IBR) values in E. fetida following 64 days exposure to As contaminated soils. Earthworms of group I, II, III, IV was exposed to As(III), As(V), MMA and DMA, respectively.

Graphical Abstract

Accumulation, biotransformation, and multi-biomarker responses after exposure to arsenic species in the earthworm *Eisenia fetida*

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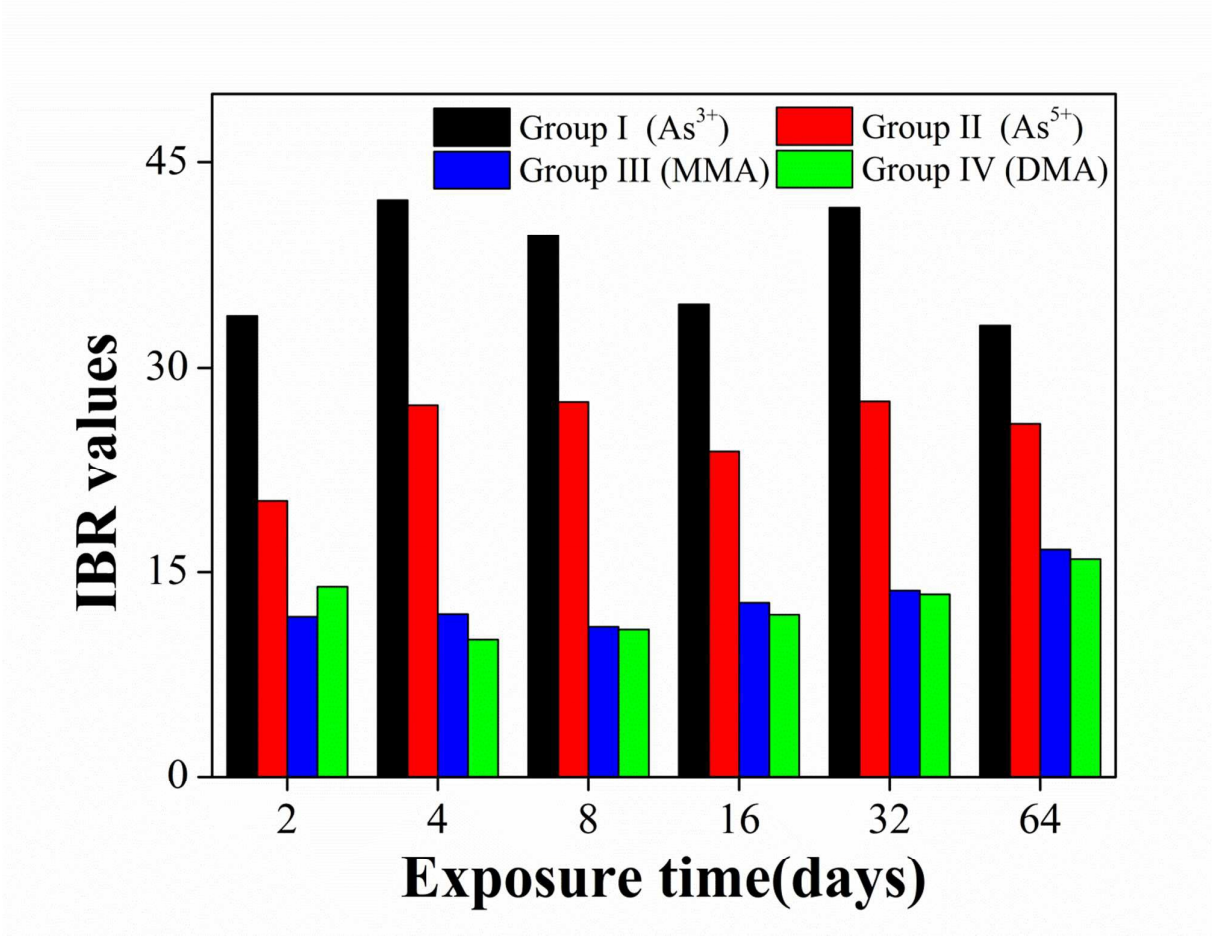

Integrated biomarker response (IBR) index was calculated to reflect the integrated stress of four arsenic species on earthworm *Eisenia fetida*.