Evaluation of joint toxicity of heavy metals and herbicide mixtures in soils to earthworms (Eisenia fetida)

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A B S T R A C T

It is widely acknowledged that a simplified and robust approach to evaluating the combined effects of chemical mixtures is critical for ecological risk assessment (ERA) of contaminated soil. The earthworm (Eisenia fetida) was used as a model to study the combined effects of polymetallic contamination and the herbicide siduron in field soil using a microcosm experiment. The responses of multiple biomarkers, including the activities of catalase (CAT), superoxide dismutase (SOD), glutathione reductase (GR) and acetylcholine esterase (AChE), the concentrations of glycogen, soluble protein (SP), malonaldehyde (MDA), and metallothionein (MT), and the neutral red uptake test (NRU), were investigated. Multivariate analysis, Principal Component Analysis (PCA) and Spearman’s Rank Correlations analysis (BVSTEP) revealed that the activities of AChE and CAT and the NRU content were the prognostic biomarkers capturing the minimum data set of all the variables. Internal Cd (tissue Cd) in earthworms was closely related to the health status of worms under combined contamination of heavy metals and siduron. The integrated effect (Eₘᵢₓ) calculated based on the activities of AChE and CAT and NRU content using the stress index method had significantly linear regression with internal Cd (p < 0.01). Eₘᵢₓ(10), Eₘᵢₓ(20), and Eₘᵢₓ(50) were then calculated, at 1.27, 1.63 and 2.71 mg/kg dry weight, respectively. It could be concluded that a bioassay-based approach incorporating multivariate analysis and internal dose was pragmatic and applicable to evaluating combined effects of chemical mixtures in soils under the guidance of the top-down evaluation concept of combined toxicity.

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Introduction

The phenomenon known as the combined effect, mixture toxicity, joint toxicity or cocktail effect, caused by a complex mixture of chemical contaminants in the environment, has been frequently stressed in recent years in reports, sci-
entific opinions and guidance by experts and scientists, especially in the EU and USA (USEPA, 2016; OECD, 2011; EC, 2012; EFSA, 2014). Current methods developed for determining combined effects are mostly based on the Mode of Action (MoA) of a contaminant, such as models of concentration addition (CA), independent action (IA) and their extended models such as the toxic unit (TU) model and toxic index (TI) model (Perrodin et al., 2011). However, all these models require the presence of substantial ecotoxicological databases (Backhaus and Faust, 2012); and, as suggested by Beyer et al. (2014), the CA and IA models are limited to use in laboratory-controlled exposure studies and have little feasibility regarding field data. One of the exposure-effect models in integrated ecotoxicology is critical to quantification of toxic effects; it is only applicable for single-chemical toxicity testing with a single effect (Jensen et al., 2000). Exposure-effect models for combined pollution have seldom been reported and used (Proctor et al., 2017). Therefore, a simplified and robust approach for assessment of combined effects is needed in ecological risk assessment.

It was revealed that the exposure of a mixture of contaminants to organisms could be related to bioavailability; e.g., either the competitive reactions for metal speciation in water/sediment or the competitive reactions at the biotic ligand (BL) (Nys et al., 2017). Researchers found that heavy-metal speciation or tissue-concentration-based models had more prediction value for mixture toxicity than total-concentration-based models (Thouft et al., 2017; Gogotapillai et al., 2017). Thus, the internal dose, which integrates all metal-metal interactions in the external environment, such as soil and uptake into the organism, may provide a more direct metric describing toxicity (Gogotapillai et al., 2017). It was reported by Cao et al. (2017) that the activities of cytochrome P450 3A4 (CYP3A4) and cytochrome P450 1A1 (EROD) had significant correlation with the bioaccumulation of metal. It has been widely reported by studies on critical body residues (CBR) that internal toxicity metrics were more credible and less variable than external toxicity metrics (Ma, 2005; Schmidt et al., 2011).

As a significant proportion of soil biomass, earthworms are regarded as “keystone species” in soil chemical, physical and biological functions (Rombke et al., 2005) and sensitive indicators of the adverse environmental impact (Lavelle et al., 2006; Sforzini et al., 2011). In earthworm ecotoxicology, there are a multitude of exposure biomarkers in earthworms that would be impacted by metals and herbicides, including the concentrations of glycogen, soluble protein (SP), malonaldehyde (MDA) and metallothionein (MT), the activities of catalase (CAT), superoxide dismutase (SOD), glutathione reductase (GR) and acetylcholine esterase (AChE), and the neutral red uptake test (NRU) (Holmstrup et al., 2011; Li et al., 2018a,b; Mosleh et al., 2003; Stanley and Preetha, 2016; Zhou et al., 2012). Boughattas et al. (2016) and Salvio et al. (2016) revealed that NRU was a significant biomarker, indicating that the lysozomal membrane stability would decrease significantly with the increase of contaminant levels. MT and AChE have been verified as exposure biomarkers specific to the exposure of heavy metals and organophosphate pesticides (Hagger et al., 2008). Despite the wide investigation of biomarker responses at the biochemical level, few have been used in real ecological risk assessment.

An integration system summarizing the responses of multiple biomarkers into a simple value or graph could represent an integrated biomarker effect and provide comprehensive information for environmental managers (Panzarino et al., 2016). Currently, two traditional approaches are often used to integrate and interpret multiple biomarker responses in terrestrial ecotoxicology. The first and foremost is the integrated index approach, which is calculated based on the reference deviation concept and calibrated by threshold values, weights and statistical significance (Piva et al., 2011). Though the integrated index approach, examples being the integrated biomarker response (IBR) and biomarker stress index (BSI), is mostly used in aquatic pollution (Beliaeff and Burgeot, 2002; Sanchez et al., 2013), it is equally suitable for assessing multi-biomarker responses in terrestrial organisms (Wang et al., 2017). The other is multivariate analysis, which can also integrate and interpret multi-biomarker responses according to principal component analysis (PCA), BVSTEP and so on. Prognostic biomarkers and health status may thus be identified and characterized (Sforzini et al., 2015).

Urban soil is the sink and source of multiple chemicals; heavy metals, agrochemicals, originating mainly from industrial activity, traffic emission and fertilizer application (Li et al., 2008a,b; Wei and Yang, 2010). It has been reported that Cu, Cd, Pb and Zn were the main heavy metal contaminants in soils of urban Greenland in Beijing (Wang et al., 2012). Siduron is a widely used pre-emergence herbicide for the control of annual grass weeds in newly sown or established lawns of some cool-season grasses in northern cities in China. Its residue has been detected in surface water (Kong et al., 2015) and soils (Gianessi and Anderson, 1996). Although siduron shows weak or slight toxicity to humans and aquatic organisms, it has been reported that siduron is toxic to soil microorganisms (Wang et al., 2017), high plants (Jiang et al., 2017) and earthworms (Uwizeyimana et al., 2016). Due to the wide application of siduron and extensive heavy metal contamination in urban soils, coexistence and interaction between the two chemicals would be expected to exist.

In this study, earthworms (Eisenia fetida) were exposed to three soils, which were collected from a reference site (N1) and two heavy-metal-contaminated sites (N2 and N3), subsequently spiked with siduron. Multiple biomarkers including CAT, SOD, GR, AChE, MDA, SP, NRU, MT and Glycogen in earthworms were analyzed after exposure to the mixture contaminated soils. With the guidance of the top-down evaluation concept of combined toxicity (Beyer et al., 2014), we hypothesized that there would be a stress-response relationship between causal toxicants bioaccumulated by earthworms and integrated effect values. The purpose of this study is to evaluate joint effects of an herbicide and field-aged heavy metals in soil on earthworms through testing the multi-biomarker system, and further develop an approach for assessment of the integrated effect of multi-chemical pollution in soils based on bioaccumulation by a combination of multivariate analysis and an integrated index approach.

1. Materials and methods

1.1. Soil sampling and chemical analysis

The studied soils were sampled from Nanguan Park (39°56′57″N, 116°29′39″E) which was built up on an ancient copper smelting plant of the Ming Dynasty (AD 1368–1644). A previous investigation found gradient levels of Cu, Cd, Pb and Zn in the soils, from clean to severe contamination (Wang et al., 2017). Three surface soils (0–20 cm) were collected, one from a reference site (N1) and the other two from sites with different levels of heavy metal contamination (N2 and N3). For each site, one composite soil sample was collected from five sub-samples within a 10 m × 10 m square. After being delivered to the lab, some of the soil was left to dry on the basis for chemical analysis and the rest of the fresh soil was passed through a 2 mm sieve and stored at 4°C for later toxicological testing.

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Soil pH (soil:water ratio = 1:2.5, W/V) and electric conductivity (EC) (soil:water ratio =1:5, W/V) were determined in distilled water after shaking for 30 min. Total soil carbon and nitrogen were analyzed using an Elemental analyzer (Elementar Vario ELIII, Hanau Germany). Soil organic carbon (SOC) was also determined using the Elemental analyzer (Elementar Vario ELIII, Hanau Germany) after digestion by 1 mol/L HCl for 24 hr (Wang et al., 2011). For analysis of trace elements (Cu, Cd, Pb and Zn), a 0.25 g sample of air-dried soil sieved to 100 mm mesh was digested with a mixture of 10 ml HCl (GR), 5 ml HNO₃ (GR), 3 ml HF (GR) and 3 ml HClO₄ (GR). The digested extracts were then dissolved with 2 ml HCl (1:1, V/V) and made up to 50 ml with deionized water for determination. The heavy metals Cu and Zn were detected by Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES, Prodigy7, USA). Cd and Pb were detected by Inductively Coupled Plasma Mass Spectroscopy (ICP-MS, NexION 300+, USA) after further diluting the extracts to 250 ml (Wang et al., 2012). For quality control, reference sample Geochemical Standard Soil GSS-1 was used during the digestion process.

For the determination of DTPA-extractable metal concentrations in soils, 10 g of air-dried soil sieved to 2 mm was added to a 50 mL mixture of 5 mmol/L DTPA and 10 mmol/L CaCl₂ and shaken for 3 hr. Then the sample was centrifuged and filtered. The supernatant was determined using Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES, Prodigy7, USA) (Wang et al., 2005).

1.2. Chemicals and reagents

The tested herbicide siduron (N-(2-methylcyclohexyl)-N’-Phenylurea) was brought from the institute of Ping An garden and plant protection technology in Zhengzhou, China. All other chemicals were of analytical grade for biochemical analysis of earthworms and guaranteed reagent for analyzing the trace elements.

1.3. Earthworm cultivation

In this study, E. fetida was selected as the tested animal because it has a short life cycle and is highly responsive to soil functioning, susceptible to contaminants and facile to acquire and cultivate (OECD, 1984). Earthworms for testing were purchased from Rui Feng farming factory (Beijing, China). After being delivered to the lab, earthworms were cultivated in artificial soil (OECD) with cattle manure (from Rui Feng farming factory) in a climate-controlled chamber with a temperature of 20°C and a humidity of 70% to 80%. The artificial soil was composed of 10% finely ground sphagnum peat, 20% kaolin clay and 70% industrial sand. Soil moisture was set to about 35% of dry weight by adding deionized water, and pH was adjusted to about 6.5 using calcium carbonate. In order to acclimate to laboratory conditions, earthworms were cultivated for at least one week before testing (OECD, 2004).

1.4. Toxicity test

The toxicity test for earthworms in the three studied soils (N1, N2 and N3) mixed with gradient levels of siduron was conducted as follows: (1) The tested concentration of siduron was set as suggested by Zhou et al. (2004) such that 1/50 of LC₅₀ was considered as the medium level in the test, which is about 50 mg/kg based on the LC₅₀ of 4044 mg/kg for earthworm mortality (E. fetida) (Jiang et al., 2019). The lowest tested concentration was one third of the medium level, 30 mg/kg corresponding to the field application amount of siduron in urban Greenland; and the highest test level was three times the medium level, 270 mg/kg. Thus, the final test concentrations of siduron were 0, 30, 90 and 270 mg/kg. Our previous study found that there was insignificant difference between the nominal concentrations and the real concentrations in the studied soils (Jiang et al., 2018). (2) Each of the three tested soils (N1, N2 and N3) was mixed with siduron to obtain 4 treatments with a gradient of siduron concentrations. Soil moisture was adjusted to approximately 25% of dry weight and stabilized for about two days before introduction of earthworms. Twelve earthworms (E. fetida) (350–500 mg) with well-developed clitellum were introduced to a 1 L beaker containing 600 g of soil spiked with a gradient level of siduron after gut content purging. The beaker was then covered with perforated plastic film as suggested in the OECD guidelines (OECD, 1984). Each treatment was repeated 4 times. Thus, altogether there were 48 beakers. (3) All the beakers were kept in the climate-controlled chamber with a temperature of 20°C and a humidity of 70%–80% for 12 hr light/12 dark per day. Moisture was maintained by re-weighing the test containers and adding deionized water every 3 and 4 days. Results of a pre-experiment showed that the interim responses of biomarkers for 14-day incubation were more sensitive than those for 28-day incubation. Thus, after incubating for 14 days, the experiment ended, and earthworms were removed from the microcosms. Earthworms were put on humid filter paper for 24 hr to purge the gut contents before later biomarker measurements. No deaths and insignificant changes in weights were observed throughout the exposure period.

1.5. Measurements of biomarker responses and internal dose

After purging the gut contents, 12 earthworms in each replicate were divided into 5 groups (2–3 earthworms for each group) for analysis of enzyme activities, MT, glycogen, NRU assays and internal heavy metals. The chemical analysis for each replicate was conducted separately.

For enzyme analysis, 2 or 3 earthworms were homogenized in iced buffer (1:9, W/V) (0.01 mol/L Tris–HCl, 0.1 mmol/L EDTA-2Na, 0.01 mol/L sucrose and 0.8% NaCl, pH 7.4) using a tissue homogenizer and centrifuged at 8000 r/min for 30 min. The supernatant was then centrifuged at 3000 r/min one more time for cleaning at 4°C. The final supernatant was collected and stored at −80°C for analysis of antioxidant enzymes (SOD, CAT and GR), lipid peroxidation (MDA), AChE and SP. Biomarker measurements were conducted using the thawed homogenate within 2 days.

SOD activity was determined according to the hydroxylamine method. One unit was defined as the amount of enzyme causing 50% inhibition of the reaction (Liu et al., 2010). CAT activity was determined based on the yellow MA-H₂O₂ complex produced by molybdenum acid (MA), a terminator of the complex hydrolysis reaction of hydrogen peroxide (H₂O₂) with CAT. GR was measured by the decrease of NADPH (Zhou et al., 2016). AChE was measured based on the Ellman method and the rate of acetylcholine hydrolysis was measured by spectrophotometer at 412 nm for 6 min (Ellman et al., 1961). The four assayed enzyme activities were expressed as U/mg protein. MDA was determined with thiobarbituric acid (TBA) by spectrophotometer at 532 nm and expressed as nmol/mg protein (Zhou et al., 2016). SP was detected by a spectrophotometer at 595 nm according to the Bradford method, and bovine serum albumin was used as the standard (Bradford, 1976).

For MT analysis, 2 or 3 earthworms in one replicate were homogenized in (1:9, W/V) ice-cold buffer (25 mmol/L buffer Tris–HCl, pH 7.4, 0.1 mmol/L phenylmethylsulfonyl fluoride and 0.5 mmol/L dithiothreitol). Homogenates were cen-
trifuged at 10,000 r/min at 4°C for 10 min for metallothionein analysis according to Eaton and Cherian (1991).

For glycogen analysis, 2 earthworms in each replicate were homogenized in (1.5, W/V) ice-cold 100 mMol/L phosphate buffer with pH of 7.2, then centrifuged at 10,000 r/min at 4°C for 10 min (Baghban et al., 2014). The supernatant was digested in 0.5 mol/L NaOH at 80°C for 3 hr. Amyloglucosidase was added to the digested mixture, which was further incubated for 2 hr at 37°C. The produced glucose in the mixture was then tested using a standard glucose kit (Nanjing Jiancheng Bioengineering Institute, China).

For NRU assays, 2 or 3 earthworms in one replicate were measured by the method suggested by Pizar et al. (2014). A non-invasive method was used to sequentially collect earthworm coelomocytes. One earthworm was put in a 1.5 mL tube with 1 mL extrusion fluid (5.0% ethanol and 95% saline, 2.5 mg/mL EDTA and 10 mg/mL of amucolytic agent (guaiacol glycerol ether), adjusted to pH 7.3) and soaked for 2 min. Then the earthworm was removed from the solution and the extruded cells in the solution were washed with phosphate buffer solution (PBS, pH 7.3) twice by centrifuging at 500 r/min for 3 min. Cells were then re-suspended in PBS. The cell viability was determined by the trypan blue exclusion test. Then coelomocytes were incubated in a 96-well microplate for 1 hr, and non-adherent cells were removed. The remaining cells were subsequently incubated with fresh neutral red solution (0.05% neutral red in PBS) for 1 hr. After being washed, cells were subjected to extraction solution (1% acetic acid, 50% ethanol). The absorbance of the extracts was determined by a microplate reader (SPECTRA max190, USA) at 540 nm. NRU values of treated earthworms were expressed as the percentage of the absorbance to that of the control without siduron addition (Asensio et al., 2013).

The last group per replicate was used to analyze the tissue concentration of heavy metals. 2 or 3 earthworms per replicate were placed in a 50 mL, pre-weighted glass beaker and dried to a constant weight at 80°C for 8 hr. After drying, the beaker was weighed again to obtain the dried weight, then a 15 mL mixture of HNO3 and HClO4 (4:1, V/V) was added and left to soak overnight. The mixture was then digested at 120°C for 2 hr; then the temperature was increased to 150°C until the solution became nearly transparent. After digestion, the solution was made up to 25 mL with distilled water and the concentrations of Cu, Cd, Pb and Zn were determined using Inductively Coupled Plasma Mass Spectroscopy (ICP-MS, NexION 300x, USA) (Zhou et al., 2013).

1.6. Data analysis

Two-way ANOVA and Duncan’s multiple comparison for biomarker responses, bioavailability and tissue concentration of heavy metals were conducted using Data Processing System software (DPS V7.55) to assess the differences among treatments with different levels of siduron and heavy metals.

It was suggested by Dagnino et al. (2007) that the health status of organisms could be ranked into five levels: Healthy (not stressed); Low stress; Medium stress; High stress; Pathological. The responses of biomarkers (individually or integrated together) were considered to be the basis for characterizing the health status of worms. In our work, we used the concept of health status suggested by Dagnino et al. (2007) and ranked the levels qualitatively by the grouping of treatments resulting from principal component analysis (PCA).

PCA and cluster analysis were conducted to reduce the dimensionality of multiple biomarkers of treatments and thus to visualize the dissimilarities among different treatments derived individually. Dissimilarity matrices are used to describe the ecology of larvae. There are altogether 108 values in this study (3 soils × 4 treatments of siduron × 9 selected biomarkers), which meets the requirement of minimum sample size (100 samples) for PCA (Gorsuch, 1983). To examine the suitability of the data for PCA, Kaiser–Meyer–Olkin (KMO) and Bartlett tests were performed. The significance of cluster analysis was tested using the analysis of similarity (ANOSIM). All the original data were normalized by logarithm before analysis.

In this work, prognostic biomarkers were identified by Spearman’s Rank Correlations analysis (BVSTEP). Each of the selected prognostic biomarkers was further plotted against the unhealthy component group from PCA to characterize the relationship between biomarker responses and the health status of worms.

The stress index (SI) under chemical stress was calculated based on the screened prognostic biomarkers, using the improved method suggested by Dagnino et al. (2008) and Wang et al. (2017) (Eqs. (1) and (2)).

\[
RSR = \frac{|BR_t - BR_c|}{BR_c}
\]

(1)

where, RSR refers to the relative stress response, BRt and BRc refer to the biomarker responses of treatments and the corresponding controls, respectively.

In order to remove the variation associated with natural conditions and to rigorously represent the stress caused by contaminants, SI was then estimated in terms of the range of RSR relative to the Negligible Impairment Threshold (Th1) and Relevant Impairment Threshold (Th2) (Eq. (2)). All SI values were thus normalized and ranged from 0 to 1.

\[
SI = \frac{0}{RSR - Th1} \quad Th1 < RSR < Th2
\]

\[
SI = \frac{RSR - Th2}{Th2 - Th1} \quad Th1 < RSR < Th2
\]

(2)

where, SI is stress index; RSR refers to the relative stress response; Th1 is 0.2; and Th2 is 1.0 or 0.8 according to whether the response is increasing or decreasing, respectively (Dagnino et al., 2008).

The integrated index, BSI was estimated for each treatment based on the SI of each biomarker as follows (Eq. (3)).

\[
BSI = \frac{\sum SI \times W_i}{\sum W_i}
\]

(3)

where, BSI refers to integrated index; SIi refers to the stress index of biomarker i; Wi refers to the weighting for biomarker i. As suggested by Piva et al. (2013), the weighting for normal effect or exposure biomarkers such as SOD, CAT, CR, GP, MT and glycogen content was 1.0; the weighting was 1.2 for biomarkers with adverse effects such as MDA, while it was 1.5 for biomarkers reflecting impairment at high biological levels, such as AChE and NRU. Then the combined effect value was calculated by Eq. (4):

\[
E_{mix} = BSI \times 100\%
\]

(4)

where, \( E_{mix} \) (in % effect) is the effect of the mixture; BSI refers to the integrated index.

2. Results

2.1. Selected properties of tested soils

Selected soil properties of N1, N2 and N3 are shown in Table 1. There were significantly differences in the concentrations of both total and DTPA-extracted Cd, Zn, Pb and Cu among the three soils (p<0.01) with a gradient of N3>N2>N1. However,
Fig. 1 – Multivariate analysis of biomarker responses of earthworms in treatments of the three studied soils (N1, N2, N3) with the addition of different levels of siduron. (a) Principle component analysis (PCA) superimposed with cluster analysis and the analysis of similarity (ANOSIM) of clusters, siduron, heavy metal contamination; (b-d) plots between the first principle component (PC1) and responses of prognostic biomarkers acetylcholine esterase (AChE), catalase (CAT) and neutral red uptake test (NRU).

Table 1 – Selected physicochemical properties of studied soils and the concentrations of Zn, Cu, Pb and Cd.

<table>
<thead>
<tr>
<th>Soil properties</th>
<th>N1</th>
<th>N2</th>
<th>N3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zn (mg/kg)</td>
<td>418</td>
<td>817</td>
<td>3623</td>
</tr>
<tr>
<td>Cu (mg/kg)</td>
<td>139</td>
<td>267</td>
<td>1342</td>
</tr>
<tr>
<td>Pb (mg/kg)</td>
<td>45.9</td>
<td>78.6</td>
<td>221</td>
</tr>
<tr>
<td>Cd (mg/kg)</td>
<td>0.194</td>
<td>0.295</td>
<td>0.763</td>
</tr>
<tr>
<td>Pn</td>
<td>1.3</td>
<td>2.5</td>
<td>10.7</td>
</tr>
<tr>
<td>Zn-DTPA (mg/kg)</td>
<td>50.3</td>
<td>103</td>
<td>393</td>
</tr>
<tr>
<td>Cu-DTPA (mg/kg)</td>
<td>19.6</td>
<td>32.9</td>
<td>128</td>
</tr>
<tr>
<td>Pb-DTPA (mg/kg)</td>
<td>6.5</td>
<td>11.1</td>
<td>36.2</td>
</tr>
<tr>
<td>Cd-DTPA (mg/kg)</td>
<td>0.065</td>
<td>0.095</td>
<td>0.179</td>
</tr>
<tr>
<td>K (%)</td>
<td>1.79</td>
<td>1.70</td>
<td>1.58</td>
</tr>
<tr>
<td>Na (%)</td>
<td>1.43</td>
<td>1.32</td>
<td>1.24</td>
</tr>
<tr>
<td>Ca (%)</td>
<td>3.50</td>
<td>3.44</td>
<td>3.60</td>
</tr>
<tr>
<td>C (%)</td>
<td>2.03</td>
<td>2.42</td>
<td>2.15</td>
</tr>
<tr>
<td>N (%)</td>
<td>0.087</td>
<td>0.106</td>
<td>0.074</td>
</tr>
<tr>
<td>SOC (%)</td>
<td>1.26</td>
<td>1.88</td>
<td>1.41</td>
</tr>
<tr>
<td>pH</td>
<td>8.42</td>
<td>8.21</td>
<td>8.46</td>
</tr>
<tr>
<td>EC (µS/cm)</td>
<td>1440</td>
<td>1287</td>
<td>1276</td>
</tr>
</tbody>
</table>

Pn: Nemero index; SOC: soil organic carbon; EC: electric conductivity.

The percentages in brackets represent the ratio of the DTPA-extracted heavy metal concentration to the total concentration.

Slight difference in the ratios of DTPA-Zn, Cu, Pb and Cd to the total heavy metals were shown among different soils. The ratio of DTPA-Cd was much higher than the rest, indicating the greater mobility of Cd in soils compared with the other three heavy metals. According to the soil environmental quality standards (MEE, 2018), the risk screening values for Cd, Zn, Cu and Pb are 0.6, 300, 100 and 170 mg/kg, respectively. Thus, Zn and Cu in N2 were both more than 2 times the risk screening values, while Cu and Zn in N3 were both more than 10 times the risk screening values. Moreover, the concentrations of Cd and Pb in N3 also exceeded the risk screening values. The comprehensive heavy-metal contamination at the three sites ranged from 1.3 to 10.7 according to the Nemero index (Pn) and exhibited the gradient N3>N2>N1. Soil N1 could be considered as the reference.

Other selected physicochemical properties of soils as shown in Table 1 were similar among all the three studied sites. Soil pH values were all above 8, showing alkalinity, and SOC ranged from 1.26% to 1.88%.

2.2. Changes of biomarker responses and tissue heavy metals among treatments

All the tested biomarker responses showed significant differences among the three studied soils, as well as with the addition of siduron (Appendix A, Fig. S1 and Table 2). As shown by ANOVA (Table 2), the tissue concentrations of heavy metals in earthworms, except for Zn, also showed significant differences among the three studied soils (p<0.01). The treatments of siduron significantly affected the activities of CAT and AChE, and concentrations of MDA, SP, NRU and MT in earthworms (p<0.01). Though the differences in DTPA-extracted heavy metals among different treatments of siduron were not significant (data not shown, p>0.05), significant difference in the tissue concentration of Cd in earthworms among different treatments of siduron was observed (p<0.01). However, the tissue concentrations of Cu, Zn and Pb in earthworms among
Table 2 – ANOVA of biomarker responses and tissue concentrations of heavy metals in earthworms among treatments.

<table>
<thead>
<tr>
<th>Variables</th>
<th>Soil</th>
<th>Siduron</th>
<th>Siduron × Soil</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAT</td>
<td>-0.01&lt;sup&gt;a&lt;/sup&gt;</td>
<td>-0.01</td>
<td>-0.01</td>
</tr>
<tr>
<td>SOD</td>
<td>-0.01</td>
<td>0.089</td>
<td>-0.01</td>
</tr>
<tr>
<td>GR</td>
<td>-0.01</td>
<td>0.068</td>
<td>-0.01</td>
</tr>
<tr>
<td>AChE</td>
<td>-0.01</td>
<td>-0.01</td>
<td>-0.01</td>
</tr>
<tr>
<td>MDA</td>
<td>-0.01</td>
<td>-0.01</td>
<td>-0.01</td>
</tr>
<tr>
<td>SP</td>
<td>-0.01</td>
<td>-0.01</td>
<td>-0.01</td>
</tr>
<tr>
<td>NRU</td>
<td>-0.05</td>
<td>-0.01</td>
<td>0.170</td>
</tr>
<tr>
<td>MT</td>
<td>-0.01</td>
<td>-0.01</td>
<td>0.098</td>
</tr>
<tr>
<td>Glycogen</td>
<td>-0.01</td>
<td>0.221</td>
<td>0.126</td>
</tr>
<tr>
<td>Tissue-Cu</td>
<td>-0.01</td>
<td>0.799</td>
<td>0.710</td>
</tr>
<tr>
<td>Tissue-Cd</td>
<td>-0.01</td>
<td>-0.01</td>
<td>-0.01</td>
</tr>
<tr>
<td>Tissue-Pb</td>
<td>-0.01</td>
<td>0.660</td>
<td>0.441</td>
</tr>
<tr>
<td>Tissue-Zn</td>
<td>0.109</td>
<td>0.264</td>
<td>0.779</td>
</tr>
</tbody>
</table>

CAT: catalase; SOD: superoxide dismutase; GR: glutathione reductase; AChE: acetylcholine esterase; MDA: malonaldehyde; SP: soluble protein; NRU: neutral red uptake test; MT: metallothionein.

<sup>a</sup> Denotes significant difference in biomarker responses and tissue concentrations among treatments at the 0.01 level.

different treatments of siduron did not change significantly (p>0.05).

Significantly joint effects of siduron and heavy metals were observed for the activities of CAT, SOD, GR and AChE, and the concentrations of MDA and SP, and tissue Cd. However, significantly joint effects of siduron and soils (N1, N2, N3) were observed for the contents of NRU, MT and Glycogen. In terms of the CBR concept (Ma, 2005), it can be deduced that Cd in soils may be the active heavy metal triggering the joint effect on earthworms exposed to combined contamination of heavy metals and siduron.

2.3. Prognostic biomarker screening based on health status analysis of earthworms under stress of multiple heavy metals and siduron

PCA was conducted to integrate and interpret treatments of the three soils with the addition of different levels of siduron, based on biomarker responses. It was demonstrated that the KMO was 0.588 (above 0.5) and the Bartlett test was significant (p<0.01), indicating that PCA was efficient in this work. It was shown by further superimposing the results with cluster analysis that all the treatments could be divided into four clusters (groups) (p<0.01, Fig. 1a). It was also suggested that heavy metal concentrations (p<0.01) rather than different concentrations of siduron (p>0.05) were the determinative factor in the division. Group 1 included treatments of soil N1 with siduron concentration ranging from 0 to 90 mg/kg and of soil N2 with siduron concentration of 0 and 30 mg/kg. Group 2 included all the treatments of soil N3 with and without siduron addition. Group 3 included N2 treated with 270 mg/kg siduron. Group 4 included N1 and N2 treated with 270 and 90 mg/kg siduron, respectively.

The two principal components (PC1 and PC2) extracted from PCA were responsible for 59.7% and 19.3% of the total variance, respectively. As shown in Fig. 1a, the left-hand side of PC1 represented the most heavily contaminated (unhealthy) treatments, while the right-hand side the cleanest (healthy) treatments. Thus, Group 1 could be considered as healthy, while Group 2 as unhealthy. The health status of the remaining two groups was between healthy and unhealthy.

Fig. 2 – Changes of tissue Cu (a), Cd (b) and Pb (c) in soil N1, N2 and N3 with the addition of siduron ranging from 0 to 270 mg/kg. dw: dry weight.

Extraction of key biomarkers was conducted using BVSTEP. It was shown that the activities of AChE and CAT and NRU were significantly correlated to each other (R²=0.947, p<0.01), and their combination could be used to capture the full minimum data set (MDS) of all the biomarker responses. Plots between PC1 and the prognostic biomarkers, i.e. the activities of AChE and CAT, and NRU (health status space), are shown in Fig. 1b, c, and d, respectively. Significant linear relationships were found between the three prognostic biomarkers and PC1. It was suggested that healthy earthworms had high AChE activity and lysosomal membrane stability (NRU) and low CAT activity, while unhealthy earthworms exhibiting low AChE activity and lysosomal membrane stability (NRU) and high CAT activity.

2.4. Bioavailability of heavy metals under combined stress of heavy metals and siduron in soils

As shown in Fig. 2, the tissue concentrations of Pb, Cd and Cu in earthworms in soil N1, N2 and N3 generally corre-
sponded to the heavy metal concentrations in soil. The addition of siduron at different levels did not change tissue Pb and Cu in earthworms in the three studied soils significantly (Fig. 2a and c), which was consistent with ANOVA (Table 2). The addition of siduron changed the tissue Cd in earthworms in soil N3 significantly (Fig. 2b, Table 2). Tissue Cd in soil N3 without the addition of siduron and with the addition of 270 mg/kg siduron was significantly higher than that in soil with the addition of 30 and 90 mg/kg siduron (p<0.05). However, little change in tissue Cd was observed for earthworms in soils N1 and N2 with the increase of siduron addition.

Based on ANOVA, Cd was suggested to be a key factor triggering joint effects on earthworms exposed to combined contamination with heavy metals and siduron. Furthermore, PCA results showed that there were four groups altogether, which were divided based on health status. Thus, the tissue concentration of Cd in earthworms, which varied significantly with the change of treatments, was chosen as a causal toxicant. A comparison of the tissue concentration of Cd among the four PCA groups is shown in Fig. 3. Group 1, deemed healthy, had the lowest average tissue Cd, while unhealthy Group 2 had the highest average tissue Cd. Groups 3 and 4 had tissue Cd at median levels.

2.5. Effect evaluation under combined contamination by heavy metals and siduron

When the tissue concentration of Cd in earthworms was chosen as the exposure indicator for the combined pollution of heavy metals and siduron, the dose-response relationships between the tissue Cd concentration and the responses of the three prognostic biomarkers AChE, CAT and NRU were plotted. As demonstrated in Fig. 4a–c, with the increase in tissue Cd concentration, the activity of AChE and NRU content decreased linearly (p<0.01), while CAT activity increased linearly (p<0.01).

The integrated effect of the combined pollution of heavy metals and siduron here, $E_{\text{mix}}$, was calculated based on the three prognostic biomarkers AChE, CAT and NRU. As suggested by PCA, treatments of soil N1 without siduron addition were the healthiest regarding these parameters. Therefore, treatments of soil N1 without siduron addition were chosen as the reference in calculating SI. The $E_{\text{mix}}$ increased linearly with the increase of the tissue concentration of Cd in earthworms (Fig. 4d). Based on the simulated linear regression equation (Fig. 4d), $E_{\text{mix}(10)}$, $E_{\text{mix}(20)}$, and $E_{\text{mix}(50)}$, referring to 10%, 20% and 50% effect, were 1.27, 1.63 and 2.71 mg/kg dry weight (dw), respectively.

3. Discussion

3.1. Tissue residues of heavy metals in earthworms under combined pollution by multiple heavy metals and the herbicide siduron

Earthworms can accumulate metals mainly by ingestion of metals bound to soil components and direct dermal uptake of dissolved ions (Becquer et al., 2005). Meanwhile, earthworms can regulate, detoxify and excrete excess heavy metals from their tissue, such as the posterior alimentary canal (PAC), for Cd, Pb and Zn (Kamitani and Kaneko, 2007). Studies reported that homeostatic control of the essential element Zn was much more efficient (Morgan and Morgan, 1990), and the uptake, equilibration and excretion of Zn was faster than that of non-essential elements (Nahnani et al., 2007). That may be the reason that the differences in tissue Zn among the treatments of the three soils were insignificant.

Pb and Cu tend to be bound with soil components and thus less available to earthworms, especially through dermal uptake, while Cd is more mobile in soils and thus more available to earthworms. Dermal uptake of Cd was deemed to be as important as ingestion uptake for earthworms, because water-extractable and -exchangeable Cd were closely related to bioaccumulation by the earthworms (Becquer et al., 2005). Siduron can make earthworms weak, and its deleterious effects at higher concentrations on earthworms were inflammation, lesions, and sometimes loss of posterior segments of the worms (Uwizeyimana et al., 2018). Besides,
the low bioavailability of Pb and Cu in soils limited their uptake by earthworms. Therefore, the addition of siduron did not change the accumulation of Pb and Cu in earthworms significantly. However, high availability of Cd, especially in soil N3, made the accumulation of Cd in earthworms sensitive to the stress of siduron. For example, the tissue Cd decreased at 30 and 90 mg/kg siduron because the stress of siduron made the earthworms weak and thus decreased the ingestion uptake of Cd. Water-extractable and -exchangeable Cd were closely related to bioaccumulation by the earthworms. However, 270 mg/kg siduron increased the accumulation of Cd, because dermal damage caused by inflammation and lesions etc. increased the permeability of the biological membrane of earthworms, as reported in our previous work, and thus increased the dermal uptake of Cd greatly.

3.2. The screening of prognostic biomarkers using PCA and BVSTEP

In this study, multivariate analysis followed by application of an integrated index was used to screen sensitive biomarkers and then evaluate the combined effects of heavy metals and siduron. Little has been reported on combining those two methods together, even though both are widely used due to their advantages in screening critical impacting factors and evaluating integrated effects of multiple stresses.

Multivariate analyses such as PCA and BVSTEP were the most-used tools for screening prognostic biomarkers (Sforzini et al., 2011). Allen and Moore (2004) summarized that PCA could be used to screen critical molecular or cellular biomarkers related to the overall health of organisms under a contaminated environment. It was also found that lysosomal membrane stability could be a prognostic indicator for earthworm health status under the stress of environmentally realistic concentrations of benzo[a]pyrene (B[a]P) and tetrachlorodibenzo-p-dioxin (TCDD) in OECD soil, according to multivariate analysis in studying a battery of biomarkers (Sforzini et al., 2015). In our study, the activities of AChE and CAT, and NRU were screened by BVSTEP analysis to capture the full minimum data set (MDS) of all the biomarker responses. Moreover, multivariate analysis could also be used to distinguish the discrimination among treatments with different exposure levels (Park et al., 2015). Gomes et al. (2015) used three-dimensional PCA to discriminate the effect of six pesticides on the earthworm E. fetida and observed significant differences. Sforzini et al. (2017) used PCA and hierarchical cluster analysis for evaluating the toxic effects of Cr on the immunocytes of earthworms, and found that exposure-time and pollutant-concentration were the two dependent factors determining the discrimination of clusters. In our study, a total of four clusters were grouped among the treatments of the three soils with or without siduron addition, depending on heavy metal contamination levels and siduron concentrations.

The integrated stress index is capable of integrating and interpreting multiple biomarker responses quantitatively (Sforzini et al., 2015). Panzarino et al. (2016) integrated multi-biomarker responses of earthworms at biochemical and molecular levels using a rank-based biomarker index to assess Cd ecotoxicity. Sforzini et al. (2011) further developed a spreadsheet-based Expert System of Classification using an algorithm integration procedure to integrate multi-biomarkers of earthworms at biological levels ranging from molecular to organism level.
The advantages of the combination of multivariate analysis and integrated index approaches can be summarized as follows: (1) the integration of prognostic biomarkers closely related to pollution exposure can reduce the interference of biomarkers irrelevant to the chemicals; (2) natural alterations independent of contaminants can be calibrated by the introduction of thresholds; (3) the introduction of weightings of biomarkers makes the risk assessment more rigorous.

3.3. Dose-response relationship in assessment of combined toxicity

Quantitative evaluation of effects is a preliminary step in ERA. Both substance-based and matrix-based approaches have to develop Risk Factors (RF) based on exposure values and non-effect values (Perrodin et al., 2011). Substance-based approaches such as CA and IA only take quantitative account of effects if they are additive. For matrix-based approaches (bioassay approaches), by contrast, effects are usually quantified based on the percentage of the matrix causing an effect on the target organisms, which makes the approaches too site-specific and expensive to be applied in the field.

The concept of top-down evaluation of mixture effects, e.g., Toxicity Identification and Evaluation (TIE) and Effect Directed Analysis (EDA), uses biological responses to direct the identification of the causal chemical in mixtures (Beyer et al., 2014), and thus determines the dose-response relationship between the exposures (concentrations of chemical mixtures) and their effects (joint toxicity of the chemical mixtures). It was suggested by Li et al. (2018a,b) that the incorporation of bioavailability into that cause-effect based approach should be highly recommended. It was also reported that the use of internal dose integrated all interactions of chemical mixtures, and thus was more related to toxicity in organisms. In this work, we screened out the causal toxicant (Cd) responsible for the joint effect of heavy metals and siduron using ANOVA (Table 2), and then developed a dose-response relationship between internal Cd in earthworms and the integrated effect value ($E_{\text{int}}$). The significance of the regression at $p<0.01$ (Fig. 4d) suggested the validity of predicting $E_{\text{int}}$ based on tissue Cd, while the value of $R^2$, at 0.407, revealed that there was an uncertainty of approximately 60% if tissue Cd was used. The missing determination of internal siduron in earthworms might be the reason leading to high uncertainty in this work, though the integration of external siduron concentration into multiple regression did not increase the $R^2$ value of the dose-response relationship (data not shown).

4. Conclusions

A microcosm experiment on the effects of combined contamination of heavy metals and siduron on earthworms was conducted in this work to develop a bioassay-based approach to evaluating the joint toxicity of chemical mixtures in soils. It could be concluded in this work that: (1) The activities of AChE, CAT and NRU content in earthworms were prognostic biomarkers which captured the full minimum data set of all the biomarker responses. Internal Cd in earthworms was identified as the key causal toxicant in the mixtures; (2) Four groups were delineated among all the treatments based on health status of the tested earthworms. Internal Cd in earthworms increased with the change in status from healthy to unhealthy; (3) A significant dose-response relationship between concentrations of the studied chemical mixtures, and the internal toxicity of the chemical mixtures was determined based on internal Cd and $E_{\text{int}}$, though uncertainty as high as 60% was involved. Finally, the missing determination of the internal siduron concentration in earthworms might be the reason leading to high uncertainty in this work. Our work suggested that a bioassay-based approach incorporating multivariate analysis and the internal dose concept was useful to evaluate the combined effects of chemical mixtures in soils under a top-down evaluation concept.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A Supplementary data

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jes.2020.03.055.

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