Electronic Supplementary Information (ESI)

for

Assessment of toxic effects of thallium on the earthworm *Eisenia fetida* using the Biomarker Response Index

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Table S1

Basic physical and chemical properties of soil.

Soil physicochemical parameters	Value
pH	7.25
specific conductance	120.5 μS cm^{-1}
cation exchange capacity	7.21 cmol kg^{-1}
organic matter	14.2 g kg^{-1}
percentage of saturated water content	41%
thallium concentration in soil	not detected
Soil particle size proportions	
Sand	35.25%
Silt	53.03%
Clay	11.72%

Table S2

Thallium concentration in soil.

Nominal soil thallium concentrations (mg kg^{-1})	Soil thallium concentration measurements (mg kg^{-1})
0	not detected
2.5	2.2 ± 1.4
20	16.7 ± 2.1
60	55.6 ± 4.5

Table S3

Microwave digestion heating program.

Order number	Temperature/℃	Warming time/min	Holding time/min
1	indoor temperature～120	6	3
2	120～150	8	10
3	150～180	8	30

Table S4

Sequences of primers used for real-time qPCR.

Gene	Accession number	Primer	Amplification efficiency
β-actin	GU177854	F: TCCATCGTCCACAGAAAG R: AAATGTCCTCCGCAAGCT	99.4%
Hsp70	GU177858	F: CCAAGGACAACAACCTGCTC R: CGGCGTTCTTCACCATTC	100%
MT	AJ236886	F: TGAAAAGTGAGTGCTTGCCG R: CACAGCACCCCTTCTTGCAT	99.3%
ANN	GU177859	F: TTTCTTCCGCCTGCTTTG R: ACCGACCTACCACCGACA	98.3%

Table S5

The data supporting this article

Biomarker	Exposure concentration (mg kg^{-1})	Day 7	Day 28	Day 56
SOD Acivity （U mg^{-1} prot）	0	139.18±6.47	155.92±5.2	148.48±6.58
	2.5	150.01±3.37	154.04±4.27	130.4±7.09
	20	161.27±7.43	137.17±5.14	118.92±5.88
	60	179.2±3.59	112.02±8.44	122.56±8.54
CAT Acivity （U mg^{-1} prot）	0	10.61±0.45	10.58±0.53	10.1±0.67
	2.5	12.06±0.81	9.28±0.66	9.77±0.59
	20	14.26±1.14	11.22±0.58	7.12±0.56
	60	13.09±0.81	7.83±0.72	6.92±0.64
GST Acivity （U mg^{-1} prot）	0	40.55±1.41	45.3±2.5	46.23±1.63
	2.5	45.13±2.86	46.02±2.62	41.35±2.27
	20	55.36±1.26	37.55±2.44	33.86±2.08
	60	65.95±4.06	37.92±1.29	28.92±1.91
MDA Content （nmol mg^{-1} prot）	0	1.3±0.06	1.49±0.05	1.54±0.11
	2.5	1.44±0.1	1.71±0.1	1.88±0.08
	20	1.38±0.07	1.83±0.08	2.34±0.23
	60	1.47±0.08	2.12±0.21	2.71±0.23
8-OHdG Content （ng L^{-1}）	0	22.33±0.86	21.93±0.86	19.54±0.41
	2.5	23.92±1.33	23.82±1.01	20.21±0.81
	20	27.06±1.93	28.66±1.29	25.73±1.17
	60	26.06±0.5	32.27±0.82	26.85±1.1
Relative expression level of *Hsp70* gene	0	1.04±0.07	1.1±0.04	1.05±0.06
	2.5	1.23±0.07	1.34±0.07	1.34±0.07
	20	1.46±0.05	1.74±0.07	1.93±0.08
	60	1.73±0.08	1.93±0.08	2.42±0.19
Relative expression level of *MT* gene	0	0.98±0.05	1.05±0.08	1±0.07
	2.5	1.29±0.11	1.18±0.05	1.22±0.11
	20	1.31±0.08	1.5±0.07	1.52±0.05
	60	1.59±0.1	1.72±0.09	1.92±0.08
Relative expression level of *ANN* gene	0	1.11±0.06	1.39±0.08	1.08±0.09
	2.5	1.28±0.09	1.12±0.07	0.73±0.03
	20	1.32±0.03	0.89±0.06	0.53±0.03
	60	1.08±0.07	0.65±0.05	0.39±0.02

Text S1

The 0.5 g of soil was placed in the digestive tube, then 5 mL of concentrated HNO_3, 3 ml of hydrofluoric acid, and 3 ml of hydrogen peroxide were added and rinsed according to the heating procedure (Table S3). The rinsing solution was cooled to room temperature and transferred to a 25 mL volumetric flask, and the solution was diluted with 1% nitric acid and then passed through a 0.45 μm membrane to be measured. Thallium (Tl) standards were obtained from Tanmo Technology Company (Jiangsu, China). Graphite furnace atomic absorption spectrophotometry (Thermo Fisher Scientific, ICE 3500) was used for the analysis. Tl content in soil was expressed as μg g^{-1} (wet weight). Tl recovery was determined by adding a known amount of Tl standard to the samples and the average recovery was 104.17 ± 0.11%.

Text S2

Methods for the determination of biomarkers of oxidative stress

1. Homogenizing solution preparation

Earthworm samples were mixed with a PBS buffer solution containing KH_2PO_4 and K_2HPO_4 (0.1 mol L^{-1}, pH = 7.4) in a ratio of 1:9 (weight : volume). The resulting mixture was homogenized using a mechanical homogenizer while being cooled under ice-water bath conditions. The homogenate was then centrifuged at 4,000 rpm for 10 minutes. The supernatant was collected for biomarker analysis.

2. Calculation of protein content

Protein determination was performed by Caulmers Brilliant Blue method. In brief, when the $-NH^{3+}$ group of protein molecule encounters the brownish-red Caulmers Brilliant Blue colorant, the anion on the Caulmers Brilliant Blue dye combines with the protein $-NH^{3+}$ to make the solution turn blue, and the protein content can be calculated by measuring the absorbance.[1] The assay was carried out according to the instructions of TP Kit (Catalog No. A045-2) from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Briefly, 0.05 mL of homogenized supernatant was taken, added to the working solution, mixed, and allowed to stand for 10 min, and the absorbance value was measured at 595 nm. The protein content was calculated following the instructions from the manufacturer.

3. Superoxide dismutase (SOD)

SOD activity was measured using the WST-1 method, which generates a water-soluble dye by reacting WST-1 with superoxide anion. The assay was carried out

according to instructions provided by the SOD Assay Kit (catalog no. A001-3) from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). UV absorbance readings at 450 nm were taken with a UV-2600 spectrophotometer (Shimadzu, Kyoto, Japan), and the SOD activity of each sample was subsequently calculated.

4. Catalase (CAT)

CAT was measured by the ammonium molybdate method. Briefly, the decomposition of H_2O_2 by CAT can be rapidly stopped by adding ammonium molybdate. The remaining H_2O_2 then reacts with the ammonium molybdate to form a yellowish complex, which is measured at 405 nm to calculate the activity of CAT.[2] The CAT activity of each sample was determined according to the instructions of the CAT Assay Kit (Catalog No. A007-1-1) from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). UV absorbance was measured at 405 nm using a UV-2600 spectrophotometer (Shimadzu, Kyoto, Japan). CAT activity was calculated according to the formula provided by the manufacturer.

5. Glutathione S-transferase (GST)

The activity of GST was performed using the instructions of the GST Assay Kit (Catalog No. A004-1-1) from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Briefly, 0.1 mL of homogenate supernatant was taken and mixed with the working solution. The mixture was then centrifuged, and the supernatant was collected as the supernatant of the color development reaction. Subsequently, 2 mL of the color reaction supernatant was taken and reacted with the application solution. The absorbance at 412 nm was measured to calculate the GST activity of each sample.

6. Malondialdehyde (MDA)

MDA was determined by the TBA method. The method utilizes the fact that MDA in the degradation products of lipid peroxidation can condense with thiobarbituric acid (TBA) to form a red product with a maximum absorption peak at 532 nm.[3] The assay was performed according to the instructions of the MDA Kit (Catalog No. A003-1) from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Briefly, 0.2 mL of homogenized supernatant was introduced into the working solution, thoroughly mixed, and subsequently subjected to a 40-minute heating at 95°C in a water bath. Following this, the sample was cooled using running water and centrifuged at a speed of 3,500 to 4,000 revolutions per minute for 10 minutes. The extracted supernatant is measured at a wavelength of 532 nanometers. The MDA content was calculated following the instructions of the manufacturer.

7. 8-hydroxy-2'-deoxyguanosine (8-OHdG)

8-OHdG content was tested by kit procedure. First, the tissue homogenate was added to the purified antibodies of 8-OHdG. After thoroughly washing and drying the plate, horseradish peroxidase (HRP)-conjugated 8-OHdG antibody reagent was added. After washing and drying, 3,3',5,5'-tetramethyl benzidine (TMB) was added to develop color at 37 °C for 25 min. The color depth of the solution was positively correlated with the 8-OHdG content. Finally, 1 M sulfuric acid was added to stop the reaction. The OD value was determined to be 450 nm.

Text S3

The BRI index calculation method

The Biomarker Response Index (BRI) is calculated comprehensively through three steps, summarizing four Biological Health Statuses (BHS). Firstly, the alteration level (*AL*) for each biomarker is calculated using equation (1):

$$AL = (BR_t - BR_c) / BR_c \quad (1)$$

where *BRt* and *BRc* refer to biomarker responses of exposure and control treatments, respectively.

Secondly, weights are assigned based on the mechanism of action of each biomarker. For biomarkers such as SOD, CAT, and GST, which do not directly reflect toxicity, a weight of 1.0 is assigned. Biomarkers related to specific adverse effects, such as MDA, are assigned a weight of 1.2, and 8-OHdG, which is associated with oxidative DNA damage, is given a weight of 1.5. Biomarkers related to genetic damage, such as Hsp70, MT, and ANN, are assigned a weight of 1.0.

Thirdly, the Biomarker Response Index (BRI) is calculated by integrating the values using equation (2).

$$BRI = \sum(S_n \times W_n) / \sum W_n \quad (2)$$

In this context, S_n represents the score of each biomarker, while W_n denotes the corresponding weight.

Ultimately, the BRI value is classified into four Biological Health Status (BHS) levels according to the categorization by Hagger *et al*.,[4] ranging from 1.0 to 2.5 (severe alterations), 2.51 to 2.75 (major alterations), 2.76 to 3.00 (moderate alterations), and 3.01 to 4.00. Due to the broad range of the last interval, it is further subdivided into 3.01 to 3.75 (minor alterations) and 3.76 to 4.00 (normal response with no change).

References

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