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Influence of multi-walled carbon nanotubes and fullerenes on the bioaccumulation and elimination kinetics of phenanthrene by geophagous earthworms (*Metaphire guillelmi*)

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Supporting information

Number of tables: 4

Number of figures: 7

Table S1. Selected physicochemical properties of carbonaceous nanomaterials.

Samples	Elemental composition (%)				Ash (%)	(O+N)/C	SA (m^2/g)	Pore volume (cm^3/g)	
	C	H	N	O				V_{mic}	$V_{mes} + V_{mac}$
C_{60}	99.65	0.18	0.21	0	0	0.002	1.75	0.021	0.005
MW50	96.73	0.26	0.29	0.07	2.65	0.006	77.10	0.054	0.696
MW8	90.33	0.57	0.22	4.83	4.05	0.044	388.18	0.226	0.705

SA: surface area; V_{mic}: micropore volume; $V_{mes} + V_{mac}$: sum of meso- and macro- pore volumes.
The C, H, N contents were given as the mean (n = 2); O content was calculated by mass balance. The SAs of samples were derived from N_2 sorption-desorption isotherms using the multipoint BET method. The meso- and macro- pore volumes were obtained from desorption isotherms using the BJH model; the micropore volume was calculated using the Dubinin-Radushkevich (DR) model with $P/P_0 \leq 0.05$.

Sorption experiment. Sorption isotherms of phenanthrene to soil, C_{60}, MW50 and MW8 were obtained using a batch equilibrium technique at room temperature (25 ± 1 °C). Given the high surface area of MW8 and MW50, a small amount of these materials was used to reach comparatively low solid-to-liquid ratio. Specifically, 0.4 mg MW8 and 0.8 mg MW50 were added to 100 and 40 mL vials, respectively, which were then amended with background solution to achieve the minimum headspace. For soil and C_{60}, 2.5 mg and 1.5 mg were added to 8 mL vial, and the background solution was added to leave a minimum headspace. The background solution (pH = 7.0) contained 0.01 mol/L $CaCl_2$ to maintain a constant ionic strength and 200 mg/L NaN_3 to inhibit microbial activity. The volume fraction of methanol in the test solution of each vial with phenanthrene added was controlled so as to be less than 0.1% (v/v) to avoid co-solvent effects. All vials were lined with aluminum foil, sealed with Teflon screw caps, and then placed on a rotary shaker to mix for 7 days. Our preliminary experiments showed that sorption equilibrium was reached within 5 days. After mixing, the vials were centrifuged at 3000 rpm for 30 min and the supernatant was filtered through 0.2 μm anodic alumina membrane (Whatman International, Germany). No sorption of phenanthrene to the filter during the filtration was detected. The pH of supernatant was found to be unchanged relative to that of the initial solution. The equilibrium phenanthrene concentrations were determined by HPLC as described in the main text. In sorbent-free controls, mass loss of the tested compound throughout the experiment was less than 2%. Consequently, the sorbed amount of the tested compound on various sorbents was calculated from the difference between the initial and equilibrium aqueous concentrations. The

sorption isotherms of phenanthrene by soil and carbonaceous nanomaterials were fitted with logarithmic form of the Freundlich model as below:

$$\log Q = \log K_f + n \log C_e$$

where Q and C_e are equilibrium solid (mg kg^{-1}) and liquid phase (mg L^{-1}) concentrations, respectively. K_f is sorption coefficient ((mg/kg)/(mg/L)^n), and n is the linearity index of sorption isotherms.

Table S2. Freundlich model parameters for sorption isotherms of phenanthrene by carbonaceous materials

compound	sorbent	log K_f [b]	n	R^2	K_d [a]	
					0.01S_w [c]	0.1S_w [c]
Phenanthrene	soil	3.095±0.025[d]	0.781±0.039[e]	0.980	3320	2010
	C_{60}	3.524±0.042	0.858±0.061	0.961	6290	4540
	MW50	4.548±0.025	0.326±0.017	0.979	715000	151000
	MW8	5.523±0.102	0.472±0.040	0.946	3520000	1040000

[a] K_d: distribution coefficient (L/kg), $K_d = Q/C_e = K_f \cdot C_e^{n-1}$; [b] K_f ((mg/kg)/(mg/L)^n); [c] S_w: water solubility (mg/L); [d] Standard error of log K_f; [e] Standard error of n.

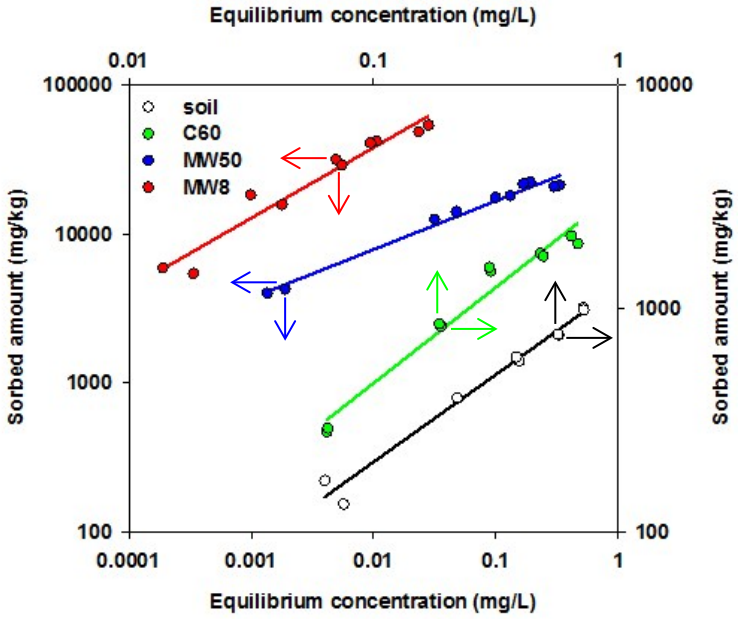

Fig. S1. Sorption isotherms of phenanthrene by soil, C_{60}, MW50 and MW8. Isotherms on soil and C_{60} refer to the top X axis and right Y axis.

Determination of MWCNT uniformity in soil using ^{13}C labeling. ^{13}Clabeled MWCNTs (outer diameter > 50 nm; 10-20 μm in length; > 95% purity) were synthesized via chemical vapor deposition of $^{13}CH_4/CH_4$ (v/v=1:20) as the feedstock gas and Ni/Al_2O_3 as the catalyst by Chengdu Organic Chemistry Co. Ltd., Chinese Academy of Sciences. The ^{13}C abundance of the MWCNTs was 5.44 ± 0.02% (n = 3) and the total C content was 96.21 ± 5.99% (n = 3) measured by isotope ratio mass spectroscopy (IRMS, MAT 253, Thermo Fisher Scientific, USA). One hundred grams of air-dried soil were added to a glass bottle (250 mL), and was then amended with 100 mg of ^{13}C-MWCNTs (dry powder) to yield a concentration of 1000 mg kg^{-1} dry soil. The bottles were sealed and thoroughly mixed on a rotary shaker at 45 rpm for 72 h. After homogenization, six soil samples of 2 g each were randomly withdrawn from the bottle, ground and passed through a 200 mesh sieve. The δ-^{13}C value of the sample was measured by IRMS and expressed as the following equation:

$$\delta\text{-}^{13}\mathrm{C} = \frac{^{13}\mathrm{C}/^{12}\mathrm{C}_{\mathrm{sample}} - ^{13}\mathrm{C}/^{12}\mathrm{C}_{\mathrm{standard}}}{^{13}\mathrm{C}/^{12}\mathrm{C}_{\mathrm{standard}}} \times 1000$$

where $^{13}C/^{12}C_{standard}$ (0.01118) is theratio of ^{13}C to ^{12}C in the reference material PDB (fossilized calcite standard); $^{13}C/^{12}C_{sample}$ is the ratio of ^{13}C to ^{12}C in the sample that can be obtained as δ-^{13}C value and the $^{13}C/^{12}C_{standard}$ is known. As a result, the concentration of ^{13}C-MWCNTs in soil was calculated according to the total C content in the sample and the ^{13}C abundance in the labeled MWCNTs that had been measured by IRMS.

Table S3. The concentration and uniformity of ^{13}C-MWCNTs in soil after homogenization.

	δ-^{13}C	$^{13}C/^{12}C$	C (%)	^{13}C-MWCNTs (mg/kg soil) Mean ± SD
^{13}C-MWCNTs amended soil	17.817	0.011379	10.39	1065.41 ± 69.95
	16.728	0.011367	10.32	
	8.350	0.011274	10.47	
	9.121	0.011282	10.44	
	11.331	0.011307	10.44	
	17.971	0.011381	10.39	

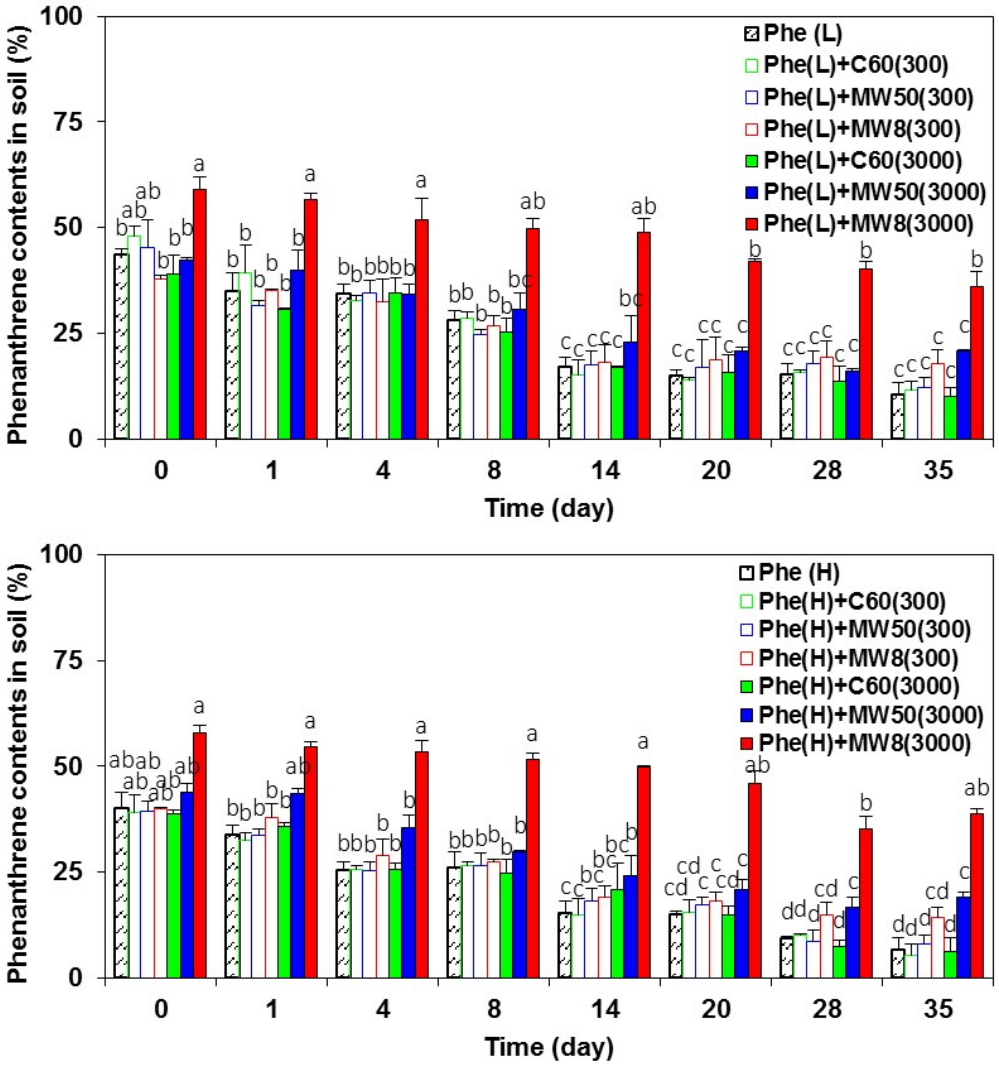

Fig. S2. The residual concentrations of phenanthrene in soil in L and H systems during 35 d exposure. Phe (L) and Phe (H) indicate soil spiked with low and high dose of phenanthrene alone. C_{60} (300), MW50 (300) and MW8 (300) indicate soil amended with 300 mg kg^{-1} of these CNMs; C_{60} (3000), MW50 (3000) and MW8 (3000) indicate soil amended with 3000 mg kg^{-1} of these CNMs. Column values with the same letter are not significantly different (one-way ANOVA with Tukey's multiple comparison test, $p < 0.05$). The statistical analysis was done on the data across all the sampling periods.

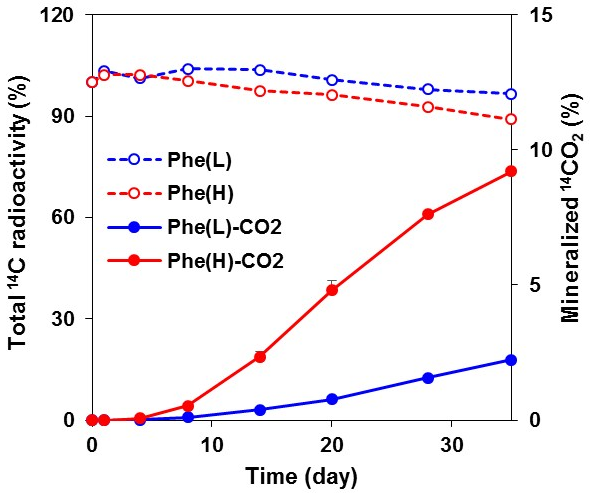

Fig. S3. Total ^{14}C radioactivity (dotted line) and cumulative release of $^{14}CO_2$ (solid line) mineralized by the indigenous microbial communities in soil spiked with a low (L) and high (H) dose of phenanthrene.

Total ^{14}C radioactivity as a function of time in earthworm-free soil. Five grams of the air-dried soil was added to 40 mL amber vials, and then were spiked with 125 µL non-labeled phenanthrene dissolved in acetone (80 mg L^{-1}) for the L system and 100 µL (1000 mg L^{-1}) for the H system, respectively. The initial phenanthrene concentrations in soil were 1.40 and 16.39 mg kg^{-1} in the L and H systems, respectively, as measured by the method described in the main text. Twenty microliters of ^{14}C-phenanthrene (9.34 mg L^{-1}, 0.107 MBq mL^{-1}) dissolved in methanol were added to the vial using a microsyringe to yield a radioactivity of 0.0115 µCi g^{-1} dry soil; the chemical concentration was 0.037 mg kg^{-1} dry soil. The vials were left open and placed in the fume hood for 0.5 h to evaporate the solvent, and were then sealed and thoroughly mixed on a rotary shaker at 45 rpm for 72 h. After homogenization, the soil was moistened with 2 mL deionized water and equilibrated for 12 h, and then was stirred with a stainless-steel spoon. All treatments were run in triplicate and the vials were incubated in the same chamber as the worm experiment. After 1, 4, 8, 14, 20, 28, 35 d, approximately 0.4 g soil was sampled from each vial, and was freeze-dried at -50 °C for 72 h. About 0.3 g soil was combusted at 900 °C in a biological oxidizer (OX-500; Zinsser Analytic, Germany). The $^{14}CO_2$ was

absorbed by 15 mL of alkaline cocktail Oxysolve C-400 (Zinsser Analytic, Germany) and the radioactivity was determined using a liquid scintillation counter (LS 6500, Beckman Coulter, USA).

Mineralization of ^{14}C-labeled phenanthrene by soil indigenous microorganisms. To better understand the fate of phenanthrene in soil, mineralization of ^{14}C-phenanthrene was evaluated using a respirometer as described in our previous study (Yang et al., 2010). Briefly, 5 g soil spiked with ^{14}C labeled and non-labeled phenanthrene was prepared using the same method as described in the total ^{14}C radioactivity experiment. The respirometer was made by attaching a 2.0-mL GC vial under the screw cap of a 40 mL amber vial. The GC vial was amended with 1.5 mL of 1 M NaOH solution to capture the $^{14}CO_2$ mineralized by indigenous soil microorganisms. All respirometers were incubated in the chamber with the same condition as worm exposure experiment. After the same time intervals, the NaOH solution was sampled and mixed with 4 mL cocktail prior to radioactivity measurement with liquid scintillation counter (LSC). The mineralized percentage was calculated as the ratio of the radioactivity of $^{14}CO_2$ to that of the total ^{14}C in soil. All respirometers were run in triplicate.

Total ^{14}C extractability in soil with hexane/acetone (v/v=1:1). With regard to the rapid loss of total phenanthrene during 12 h equilibrium, we hypothesized that most of the compound was retained in soil and not extractable by organic solvent. The soil spiked with ^{14}C labeled and non-labeled phenanthrene as described above. After the same time intervals, approximately 1.0 g soil was sampled from each vial. The freeze-dried soil (0.5 g) was extracted with 20 mL of hexane/acetone (v/v=1:1) using the same microwave-assisted reaction system as described in Section 2.5 of the main text. After filtration, the extract was condensed to approximately 1 mL with a vacuum rotary evaporator, and then mixed with 4 mL cocktail to determine the radioactivity of the extract. The extracted residues were collected and combusted at 900 °C in a biological oxidizer; the $^{14}CO_2$ was captured by 15 mL of alkaline cocktail Oxysolve C-400 and the radioactivity was also determined by LSC. The total extracted ^{14}C fractions are given as percentage of the initial values.

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127 **Table S4.** Total ^{14}C radioactivity extracted with hexane/acetone (v/v=1:1) from soil.

Day	Phenanthrene (L)[a]			Phenanthrene (H)[a]		
	DPM[b]		Extractability (%)	DPM[b]		Extractability (%)
	Extracts	Residues	Mean ± SD	Extracts	Residues	Mean ± SD
0[c]	4458.75	6977.79	42.03±1.55	5039.50	7060.10	44.80±1.60
	4805.00	8444.56		5481.00	9130.64	
	4616.00	7084.72		4153.75	4868.70	
1	3920.50	7035.44	35.92±1.83	4140.00	5536.65	39.63±1.61
	3914.25	8524.79		4609.50	9000.79	
	3671.25	7325.09		3940.00	5372.15	
4	3790.75	9378.00	31.26±1.80	4280.25	8404.50	34.66±1.66
	3536.50	9054.50		4094.25	10689.54	
	3310.25	9157.05		4155.75	9203.30	

128 [a]: Soil amended with a low and high dose of phenanthrene; [b] Radioactivity values measured by liquid
129 scintillation counter; [c] the time after 12 h equilibrium.

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135 **Fig. S4.** Compact and granular aggregates formed after 7 d exposure with earthworms.

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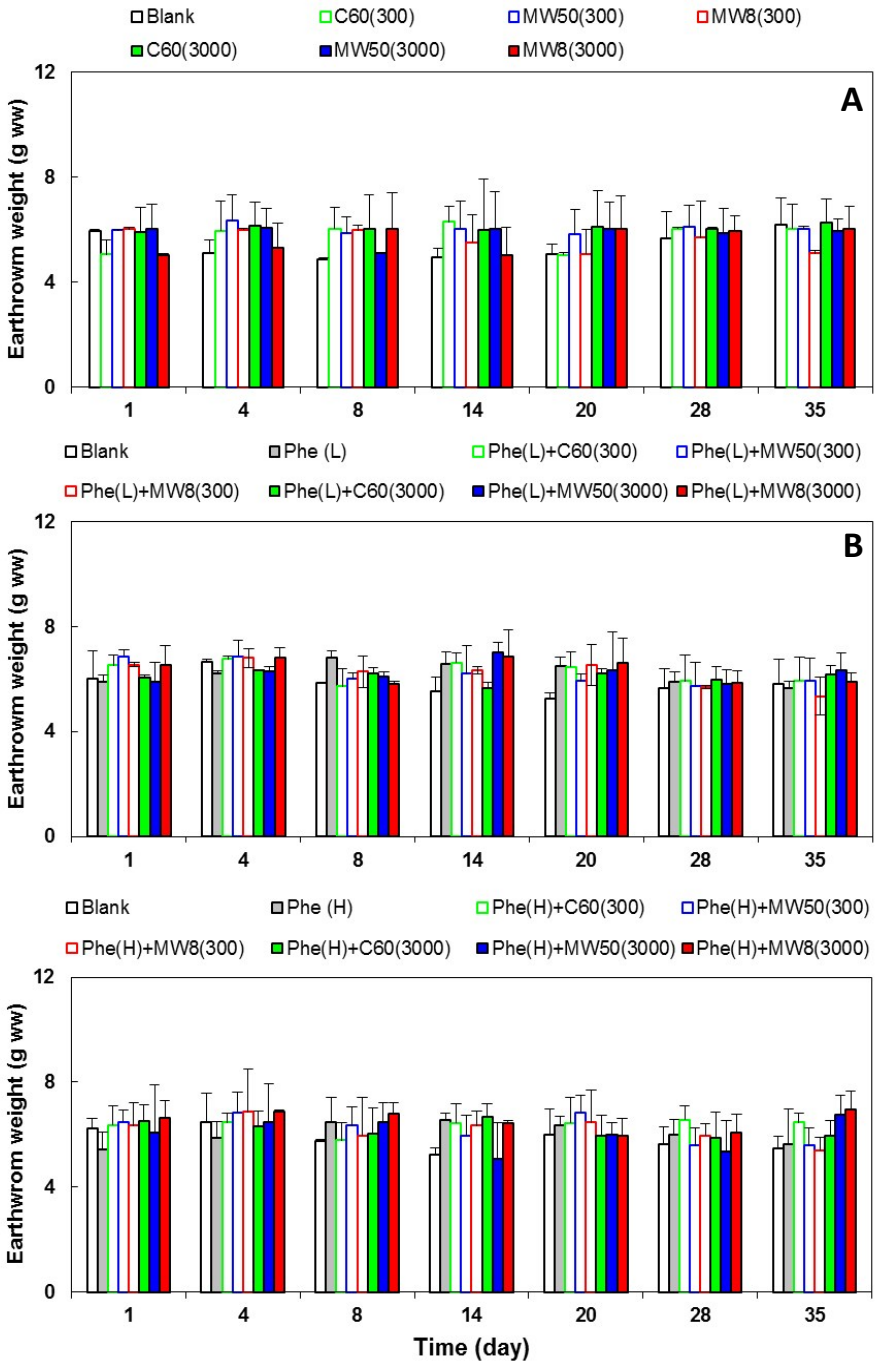


137 **Fig. S5.** Biomass of earthworms upon amendment with C_{60}, MW50 and MW8 alone (A); CNMs co-
138 exposure with low-dose phenanthrene (B); CNMs co-exposure with high-dose phenanthrene (C)
139 during 35 d exposure. Blank indicates soil without any amendment. Phe (L) and Phe (H) indicate soil
140 spiked with low and high dose of phenanthrene alone. C_{60} (300), MW50 (300) and MW8 (300)
141 indicate soil amended with 300 mg kg^{-1} of these CNMs; C_{60} (3000), MW50 (3000) and MW8 (3000)
142 indicate soil amended with 3000 mg kg^{-1} of these CNMs. There were no instances of statistically
143 significant differences as a function of treatment across the sampling period.
144

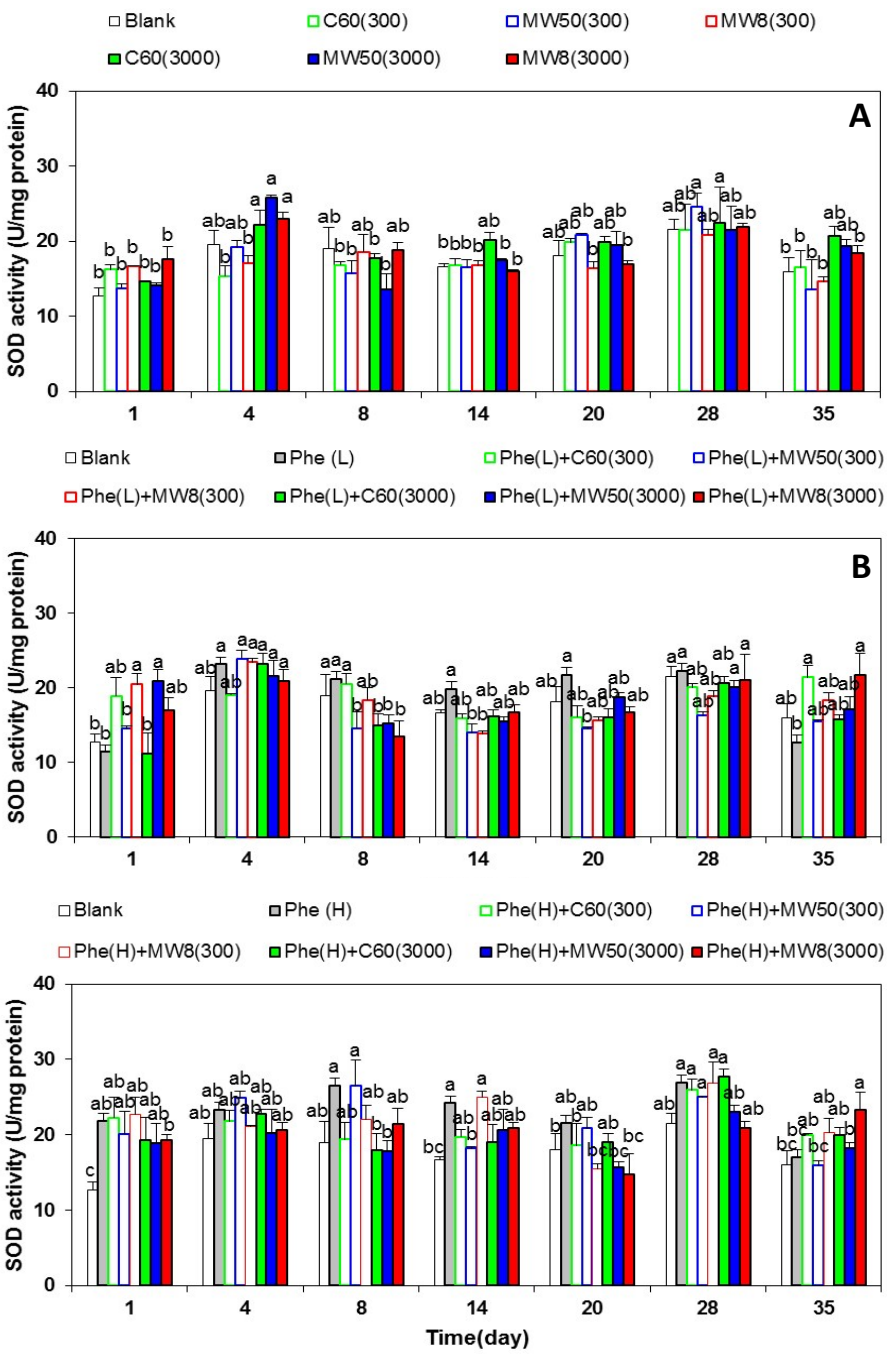
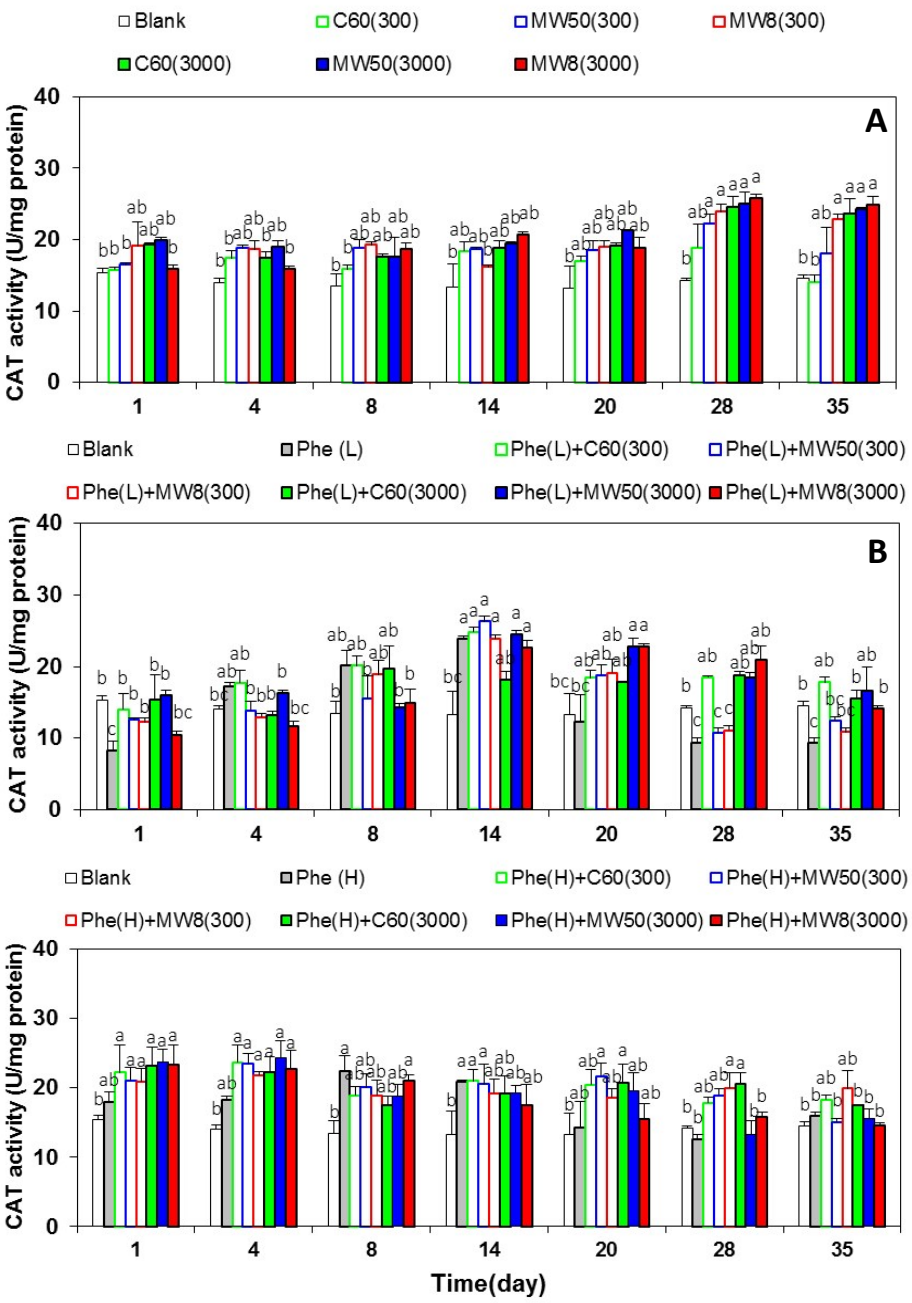


Fig. S6. SOD activities in earthworms upon amendment with C_{60}, MW50 and MW8 alone (A); CNMs co-exposure with low-dose phenanthrene (B); CNMs co-exposure with high-dose phenanthrene (C) during 35 d exposure. Blank indicates soil without any amendment. Phe (L) and Phe (H) indicate soil spiked with a low and high dose of phenanthrene alone. C_{60} (300), MW50 (300) and MW8 (300) indicate soil amended with 300 mg kg^{-1} of these CNMs; C_{60} (3000), MW50 (3000) and MW8 (3000) indicate soil amended with 3000 mg kg^{-1} of these CNMs. Columns with the same letter are not significantly different (one-way ANOVA with Tukey's multiple comparison test, $p < 0.05$). For each panel, the statistical analysis was done on the data from all of the sampling points.



153 **Fig. S7.** CAT activities in earthworms with the amendment of C_{60}, MW50 and MW8 alone (A);
154 CNMs co-exposure with low-dose phenanthrene (B); CNMs co-exposure with high-dose phenanthrene
155 (C) during 35 d exposure. Blank indicates soil without any amendment. Phe (L) and Phe (H) indicate
156 soil spiked with a low and high dose of phenanthrene alone. C_{60} (300), MW50 (300) and MW8 (300)
157 indicate soil amended with 300 mg kg^{-1} of these CNMs; C_{60} (3000), MW50 (3000) and MW8 (3000)
158 indicate soil amended with 3000 mg kg^{-1} of these CNMs. Columns with the same letter are not
159 significantly different (one-way ANOVA with Tukey's multiple comparison test, $p < 0.05$). For each
160 panel, the statistical analysis was done on the data from all of the sampling points.
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Anti-oxidative stress analysis. On each sampling day, two earthworms were removed from each container, rinsed with deionized water, wiped with filter paper and weighed into a 50 mL plastic centrifuge tube. The tube was immersed in liquid nitrogen, and then amended with cold physiological saline (1:10, w/v) and thoroughly mixed using an IKA T10 homogenizer (IKA, Germany). The homogenate was centrifuged at 4000 rpm and 4 °C for 15 min, and the supernatant was sampled for the biochemical assays. SOD, CAT activities and protein content in the supernatant were determined using the diagnostic reagent kits purchased from Jiancheng Bioengineering Institute (Nanjing). All absorbances were measured with a UV-VIS spectrometer (Lambda 35, PerkinElmer). SOD activity was determined at 550 nm following McCord and Fridovich (1969), which is based on the measurement of the inhibition of the reduction rate of cytochrome c by the superoxide radical. The activity was expressed as U/mg protein, with one U of SOD corresponding to the quantity of enzyme that caused 50% inhibition of cytochrome c reduction. CAT activity was evaluated by monitoring the residual H_2O_2 absorbance at 405 nm (Goth, 1991). The activity was expressed as U/mg protein. One U of CAT is the enzyme that decomposes 1 mM of hydrogen peroxide per min.

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