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Impact of Polystyrene Microplastic Carriers on the Toxicity of Pb^{2+} towards Freshwater Planarian *Dugesia japonica*

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Section I

Adsorption isotherms and kinetics of Pb^{2+} ions on PS MPs

The adsorption isotherms experiments were carried out by varying initial heavy metal concentrations in 75-mL flasks containing 20 mg of MPs. The initial heavy metal Pb concentrations were 0.1, 1, 10, 10, 20, and 50 mg/L. Similar to the operations of adsorption kinetics experiments, MPs were removed from the mixtures by filtration after continuously shaking for 144 h at 200 rpm at 20 °C. Then, the equilibrium concentrations of heavy metals in the medium were determined by inductively coupled plasma mass spectrometry (ICP-MS, 7900, Agilent Technologies) for the description of adsorption isotherms.

The adsorption kinetics of Pb^{2+} ions on PS-MPs were investigated based on batch experiments. Firstly, the standard Pb^{2+} solutions were diluted to 50 mg/L using DI water medium. After that, 50 mg of PS-MPs was added into a 75-mL glass flask filled with 20 mL of 50 mg/L Pb^{2+} solution. The aggregation behavior of particles in aquatic environments largely depends on water chemistry, and the pH value of the medium might affect the toxicity of MPs by changing its stability [1]. Herein, the pH of mixtures was adjusted to 7.0 with 0.05 M NaOH and 0.1 M HCl, in order to avoid the possible influence of water chemistry parameters on the adsorption results and to study the adsorption behaviors of MPs for metal ions in this fixed solution. After that, the mixtures were then placed in a centrifuge and shaken at 200 rpm at 20 °C. Then 5 mL of mixtures were collected and filtered through a Millipore filter (0.22 μm pore size, Millex-GP, Millipore, France) after predetermined time intervals (0, 1/6, 1/2, 1, 2, 4, 8, 12, 18, 24, 36, 48, 60, 72, 96, 120, and 144 h). The filtered samples collected from each flask were stored at 4 °C for further ICP-MS analysis. The possible losses of Pb^{2+} ions during the experiment were considered by setting the solutions without MPs as blank control. Three replicates were set up for each treatment. The adsorption capacity at the equilibrium (q_e) was calculated with the following equation: $q_e=(C_0-C_e) * V/W$, where C_0 is the initial Pb^{2+} concentrations (mg/L), C_e is the remaining Pb^{2+} concentrations (mg/L) in filtrate, V is the volume (L) of the mixtures, and W is the

mass (g) of the MPs.

Assessments of Post-Exposure Locomotion Velocity (pLMV)

The observation started at 30 s after placement of the planarians at the center of the grid and lasted for 8 mins. The planarian's *pLMV* was determined by the number of grid lines that the planarian crossed or re-crossed over an observation period of 8 min.

Whole-mount in situ hybridization (WISH)

DNA templates for *Djpiwi-A* were prepared as previously described by Xie, et al. [2]. In brief, *Djpiwi-A* probe primers (F: 5'-AAGAGAGATAGGAAGACTGCG-3' and R: 5'-GATCACTAATACGACTCACTATAGGGAAGAGAGATAGGAAGACTGCG-3') were designed, synthesized and labeled with digoxigenin (DIG) using an in vitro labeling kit (Roche, Switzerland). Purified templates were *in vitro* transcribed using DIG-labeling mix (Roche Diagnostics GmbH, Mannheim, Germany) to obtain RNA probes labeled with DIG. Planarians with appropriate sizes (~ 0.5 cm) were selected and washed in individual dishes on ice. 20 planarians were treated with 5% N-acetyl-L-cysteine for 5 min until they were fully stretched and then fixed for 20 min at room temperature in 4% paraformaldehyde. Subsequently, the planarians were washed with PBS containing 0.1% Triton X-100 (PBST) and hatched in Reduction buffer (50 mM DTT, 1% NP-40, 0.5% SDS in PBS) for 15 min at 37℃. The planarians were dehydrated with 100% and 50% methanol before bleaching (1:5, 30% H_2O_2: methanol) at room temperature under light irradiation. The planarians were permeabilized with proteinase K (10 mg/mL) for 10 min and then fixed again with 4% paraformaldehyde for 20 min at room temperature. Afterwards, acetylation was performed by adding triethanolamine before pre-hybridization (50% formamide, 5×SSC, 1 mg/mL yeast RNA, 1%Tween-20, 100 mg/mL heparin, 5 mM DTT, 1×Denhardt's in PBST) at 53℃ for 2-4 h and hybridization (10% dextran sulfate in pre-hybridization solution) at 53℃ for 28-36 h. The planarians were washed several times in washing buffer I (pre-hybridization solution), II (Pre-hybridization solution: 2×SSC1:1), III (2×SSC), and IV

(0.2×SSC) successively and incubated with an anti-DIG antibody (1:1000, Roche) overnight. The color was developed using NBT and BCIP (1:50, Roche) for a few hours until signals were visible. Finally, the planarians were washed with 100% and then 50% ethanol to eliminate nonspecific background staining. All samples were observed with a Nikon SMZ 1500 stereomicroscope (Nikon, Japan).

Table S1. Formula of washing buffers used for *in situ* hybridization.

Name	20×SSC（mL）	Formamide deionized (mL)	10% SDS (mL)	Tween-20 (μL)	PBS (mL)	Time (min) and temperature of hybridization
Wash I	2.5	5	0.5	5	1.995	20'×3, 53℃
Wash II	1	5	0.4	5	3.595	5'×3, 53℃ 15'×2, 37℃
Wash III	1	/	/	10	8.99	20'×3, 37℃
Wash IV	0.1	/	/	10	9.89	20'×1, RT

Whole-mount immunofluorescence

After PS-MPs and Pb^{2+} ions suspensions exposure, the nervous system of the regenerative planarian fragments was labeled by integration of immunofluorescence using anti-SYNORF1 (DSHB, Iowa City, IA, USA, a mouse monoclonal antibody specific for synapsin). Similarly, the mitotic cells were labeled with anti-phospho-Histone H3 (Ser10) antibody (Millipore, Billerica, MA, USA). The experimental method was developed based on Xie et al [2]. The samples were killed using 2% HCl and fixed in 4% paraformaldehyde at 4 °C for 2 h. Then, the planarians were dehydrated with 100% methanol and bleached with hydrogen peroxide overnight. The next day, the samples were rehydrated with 70%, 50%, 30% Methanol solutions and finally in 1×PBST, respectively, and blocked with 10% skim milk for 6 h. Subsequently, the samples were incubated overnight at 4 °C with anti-SYNORF1 antibodies (diluted 1:200 in 1× PBST) or anti-H_3P antibodies (diluted 1:200 in 1× PBST). After rinsing in 1×

PBST, samples were blocked with 10% skim milk for 6 h and incubated overnight with goat anti-rabbit IgG/FITC antibody (diluted 1:100 in 1× PBST) or anti-mouse IgG/Cy3 antibody (diluted 1:2000 in 1× PBST) at 4 °C. Finally, samples were rinsed with 1× PBST and examined. Fluorescence images of intact and regenerative planarians after PS and Pb^{2+} ions suspensions exposure were collected with a ZEISS LSM 900 Confocal Laser Scanning Microscope (ZEISS Microscopy, Germany).

Elemental Mapping by Synchrotron-based XRF measurement

Pb localization patterns in the planarian's tissues were mapped using micro-X-ray fluorescence (μ-XRF). After treating with PS and Pb^{2+} ions suspensions for 2 days, the fresh planarians were washed thoroughly and separately stuck to the 3M tape. The μ-XRF micro spectroscopy experiment was performed at 4W1B beamline, Beijing synchrotron Radiation Facility (BSRF), the ring storage energy of which is 2.5 GeV with current intensity from 150 to 250 mA. The incident X-ray energy was monochromatized by a multilayer reflector and was focused down to 20×50 μm by the polycapillary lens. The sample was held on a precision motor-driven stage, and the two-dimensional mapping was acquired by step mode with 50 μm step. The X-ray fluorescence emission lines were detected by a Si (Li) solid-state detector with a live time of 30 s. The data were analyzed using PyMca package.

Lipid Peroxidation and Enzyme Assays

In order to analyze the antioxidative enzymes, 5 ~ 6 planarians (with total weight around 100 mg, each groups contains 30 planarians) were homogenized in PBS on ice using a tissue grinder (Tiangen, OSE-Y20, Beijing, China) with a pestle (Tiangen, OSE-Y001, Beijing, China). The homogenates were centrifuged at 12,000 *g* and 4 ℃ for 10 min. The supernatant was collected as homogenized samples were stored at −80 °C for enzyme assay and lipid peroxidation measurements. The protein content in the supernatant was determined by the Bradford method and all measurements were normalized to protein content. The level of lipid peroxidation was determined using the thiobarbituric acid (TBA) assay which is based on the production of malondialdehyde (MDA). The MDA content in the planarians was

measured using an MDA assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's instructions. The level of lipid peroxidation was expressed as MDA level by ultraviolet visible spectrophotometry (UV-Vis) analysis at 532 nm. TBA-reactive substances were expressed as nmol of MDA/mg of total protein.

The supernatant of the tissue homogenate was used to measure the enzyme activities. The activities of GPx, and GST were determined using the Glutathione Peroxidase (GPx) assay kit, Glutathione S-transferase (GST) (Nanjing Jiancheng Bioengineering Institute, Nanjing, China), respectively, according to the manufacturer's instructions. The absorbance values were measured by UV-Vis at 412 nm, 420 nm and 412 nm, respectively. The enzyme activities were expressed as U/mg of total protein.

The contents of GSH and GSSG (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) were measured using the total glutathione in supernatant which was determined according to the manufacturer's instructions. GSH in the supernatant was removed with the GSH removal reagent (supplied by the manufacturer). After reacting for 1 h at 25 °C, the concentration of the oxidized form of GSH (GSSG) was determined. The concentrations of total glutathione and GSSG were measured and calculated by UV-Vis analysis at 412 nm. The results were corrected for protein levels and were reported in μmol GSH/L protein and μmol GSSG/L protein.

Real-time quantitative PCR analysis

Real time quantitative real-time PCR was used to monitor the quantitative expression of the oxidative stress- (*Cu-Zn SOD*, *gpx, gst*), ATP synthesis- (*nak*) cell cycle- (*cdc23*) and apoptosis-associated genes (*p53*, *bcl2*, *bax*, *caspase-3*, *caspase-7*) after 48 h of treatment with PS-MPs, PS-MPs and Pb^{2+} ions co-exposure suspensions. Total RNA from planarians was extracted using TRIzol Reagent (Invitrogen, Carlsbad, USA) and cDNAs used for PCR were synthesized with a ReverTra Ace qPCR RT Kit (TOYOBO, Shanghai, China). qPCR was performed using a Power 2×SYBR real-time PCR premixture (BioTeke, China) in a LightCycler 480 PCR system (Roche, Switzerland). The *β-actin* gene was used as the internal reference gene. The PCR

primers used were listed in **Table S2**. The PCR reaction had the following steps: 50°C enzyme activation for 2 min, 95 °C initial denaturation for 10 min, 40 cycles of 95 °C for 15 s, and 50 °C for 60 s for plate reader, and a melting curve analysis from 50 to 95 °C. Data were analyzed with the comparative Ct method ($2^{-\triangle\triangle Ct}$).

Table S2. Primers used in SYBR quantitative RT-PCR analysis

Gene name	Function	Primer sequences used for qRT-PCR (5'-3')
β-actin	Cytoskeleton protein	ACACCGTACCAATCTATG GTGAAACTGTAACCTCG
gpx	Detoxification	AAGCAAGAGCCACAAAC GGATATTCGGATTGGTC
gst	Detoxification	CATTGGCTTATGGGTGAA CTTCGCAATAGGCTCCA
p53	Apoptosis	CGTGTGCCCATTACTTTC TGCTAACATTGCCTACCC
nak	ATP synthesis	GGACATTGACTCGCATAG CACGAGCCAATCGCATC
Cu-Zn SOD	Detoxification	GGCATTATTACACGAATC TTCCACGATAACAGGTTT
bcl2	Apoptosis	GATGCACGGGAAAGAGGA GAGCACCATTTCAATAGACCA
bax	Apoptosis	AACTCTGATTCTTCGGATTG CAGCCACCATGTTGAAAT
caspase-3	Apoptosis	CTCCACAATTACCCAATA TCATCACAGAGTGCCTTA
caspase-7	Apoptosis	TCATCCAAGCTTGTCGTGGA ATAGCGCTTGAATATACCAA
cdc23	Cell cycle	CATAATCCCAACAGTCAC TTCTTCATAAACAGTCCC

ELISA for Caspase-3 and 8-OHdG activities

Caspase-3 and 8-OHdG were determined using a standard ELISA kit (Jiangsu Jingmei

Biotechnology Co., Ltd, Jiangsu, China) following the manufacturer's instructions. The standard working solution was added to the first two columns: each concentration of the solution was added in duplicate, in side-by-side wells (50 µL for each well). Samples were added to the other wells (50 µL for each well). 100 µL of HRP Conjugate working solution was added to each well and covered with the plate sealer provided in the kit and then incubated for 60 minutes at 37°C. Every effort was made to avoid touching the inside wall and causing foaming. The solution was aspirated from each well, and 350 µL of wash buffer added to each well and allowed to soak for 1–2 minutes, after which the solution was aspirated from each well, and then the wells were patted dry against clean absorbent paper. The wash step was repeated five times. Then 50 µL of Substrate Reagent A and B were added to each well and covered with a new plate sealer then incubated for another 15 minutes at 37°C without light. After this, 50 µL of Stop Solution was added to each well. The value of the light density of each well was determined with a microplate reader set to 450 nm.

Section II Results

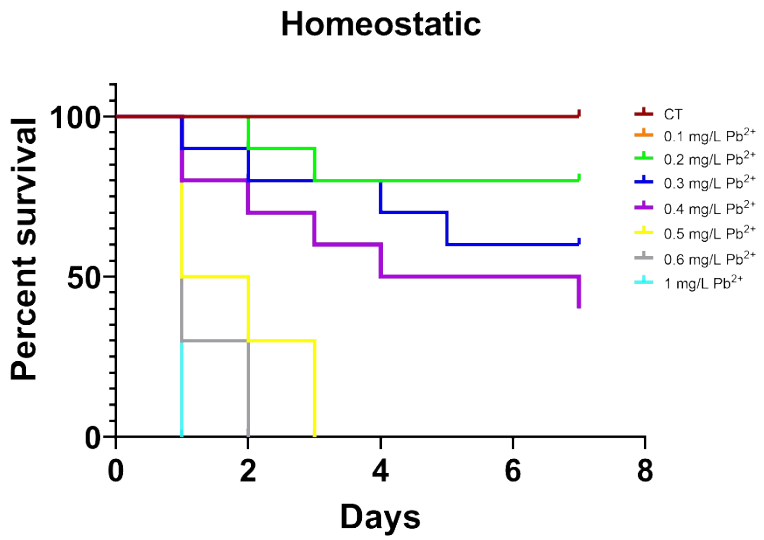

Fig. S1 Acute toxicity endpoints caused by Pb^{2+} ions suspensions on homeostatic planarians.

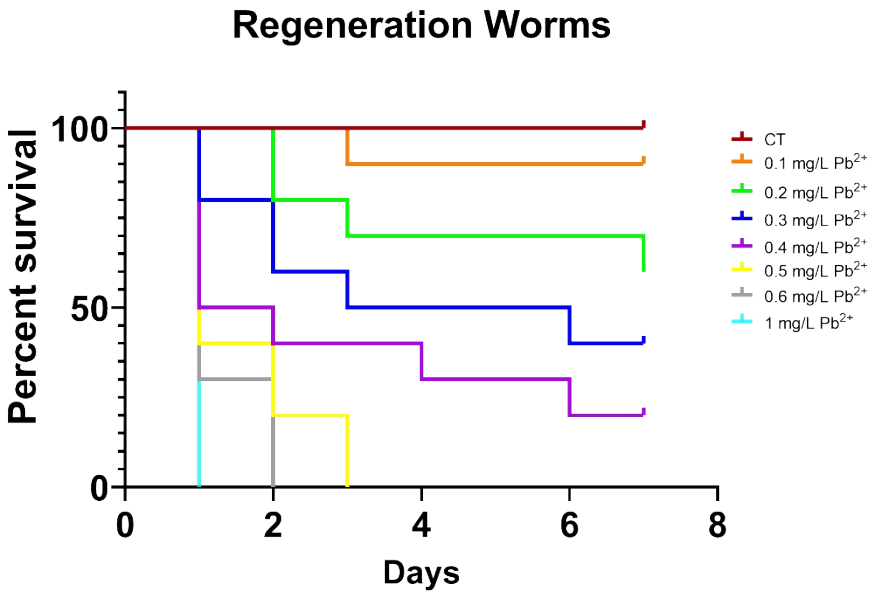

Fig. S2 Acute toxicity endpoints caused by Pb^{2+} ions suspensions on regeneration planarians.

Table. S3 LC_{50} values for the acute toxicity caused by PS MPs, PS-Pb^{2+}, and Pb^{2+} ions suspensions exposure to the homeostatic planarians.

Treatments	1st	2nd	3rd	4th	5th	6th	7th
PS MPs (mg/L)	-	-	-	-	-	-	-
Pb^{2+} (mg/L)	0.524	0.451	0.427	0.411	0.395	0.383	0.357
PS-Pb^{2+} (mg/L)	0.492	0.405	0.387	0.332	0.319	0.298	0.285

Table. S4 LC_{50} values for the acute toxicity caused by PS MPs, PS-Pb^{2+}, and Pb^{2+} ions suspensions exposure to the regeneration planarians.

Treatments	1st	2nd	3rd	4th	5th	6th	7th
PS MPs (mg/L)	-	-	-	-	-	-	-
Pb^{2+} (mg/L)	0.452	0.405	0.345	0.302	0.285	0.277	0.269
PS-Pb^{2+} (mg/L)	0.436	0.389	0.328	0.279	0.255	0.236	0.221

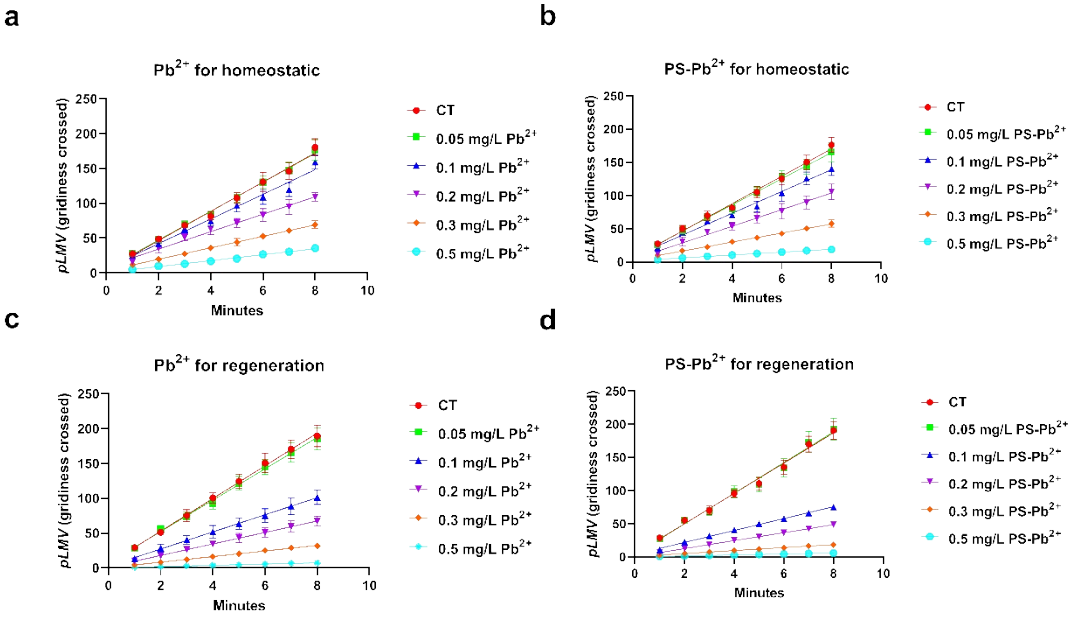

Fig. S3 Effect of Pb^{2+} and PS-Pb^{2+} suspensions on the planarians' motility at 7 days. Pb^{2+} suspensions on the homeostatic planarians' motility (**a**). PS-Pb^{2+} suspensions on the homeostatic planarians' motility (**b**). Pb^{2+} suspensions on the regeneration planarians' motility (**c**). PS-Pb^{2+} suspensions on the regeneration planarians' motility (**d**). *pLMV* was quantified as the number of gridlines crossed or re-crossed over an 8-min. This was plotted as cumulative crosses *vs*. time. Thirty animals per experimental condition were used, and each experiment was repeated three times. The results are presented as the mean ± SD, one-way ANOVA.

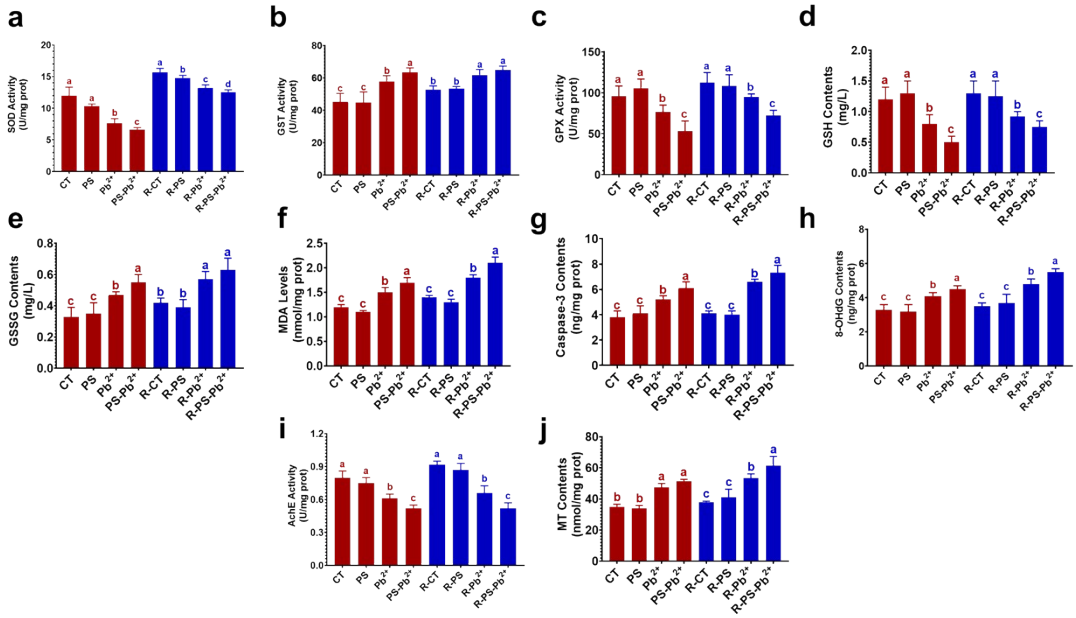

Fig. S4 PS-MPs regulates the antioxidant defense system and cell damage of planarians. SOD (**a**), GST (**b**), GPx (**c**), GSH (**d**), GSSG (**e**), and MDA levels (**f**) in planarians after exposure to PS-MPs and Pb^{2+} ions suspensions for 48 h. Contents of caspase-3 (**g**), 8-OHdG (**h**), AchE activity (**i**) and MT contents (**j**) in planarians affected by PS-MPs and Pb^{2+} ions suspensions treatments. Different

letters stand for statistical differences between treatments at $p < 0.05$. R stands for regeneration.

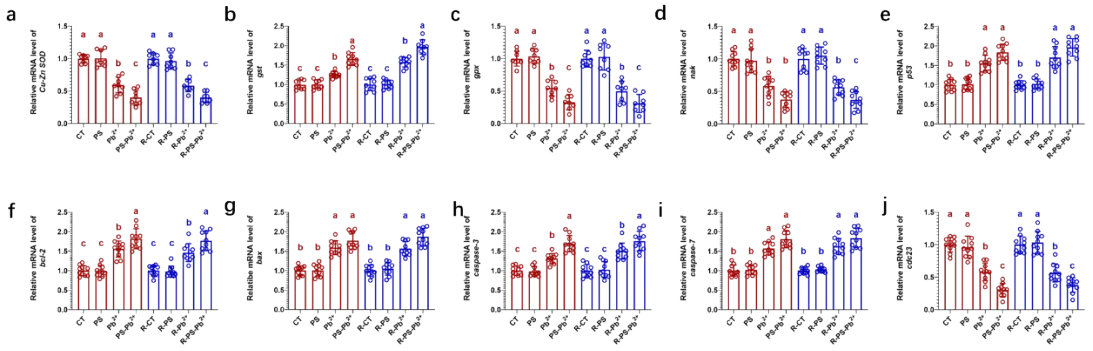

Fig. S5 The effect of PS MPs and PS-Pb^{2+} suspensions on relative mRNA expression levels of gene *Cu-Zn SOD* (a), *gst* (b), *gpx* (c), *nak* (d), *p53* (e), *bcl-2* (f), *bax* (g), *caspase-3* (h), *caspase-7* (i), and *cdc23* (j) exposed for 48 h. Different letters stand for statistical differences between treatments at $p < 0.05$. R stands for regeneration.

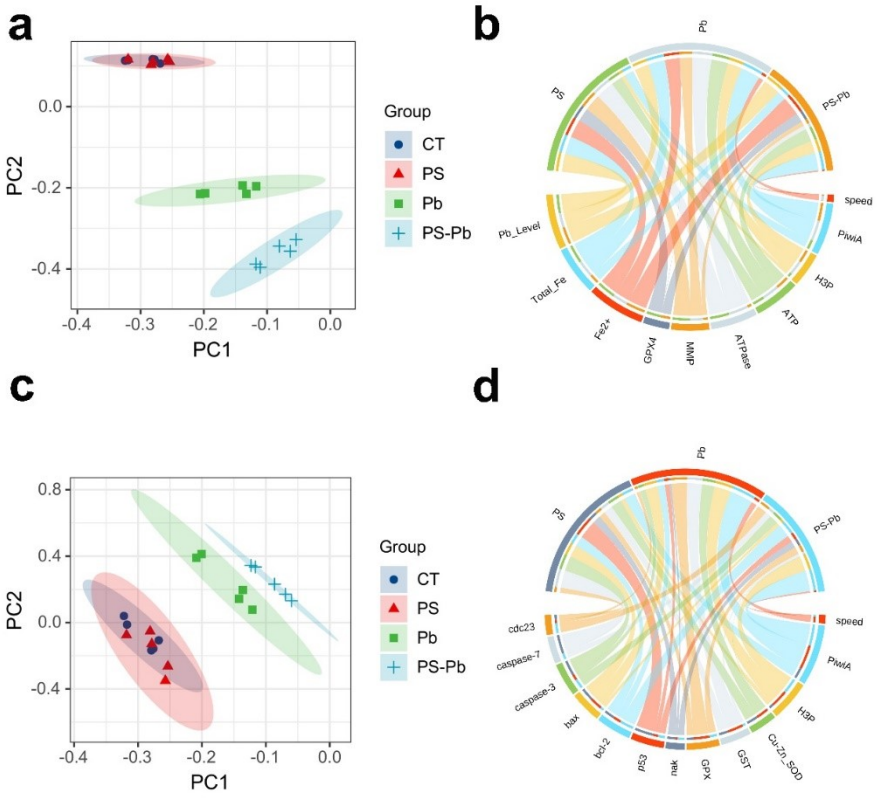

Fig. S6 PS-MPs regulate the mitochondrial function relative biomarkers and oxidative stress-relative genes of homeostatic planarians. Biplot originating from PCA integrating mitochondrial function relative biomarkers parameters and qPCR analyses (**a**, **c**). Correlation between PS, Pb and PS-Pb and the biomarker of homeostatic planarian based on Pearson correlation analysis for mitochondrial function and oxidative stress-related genes (**b**, **d**).

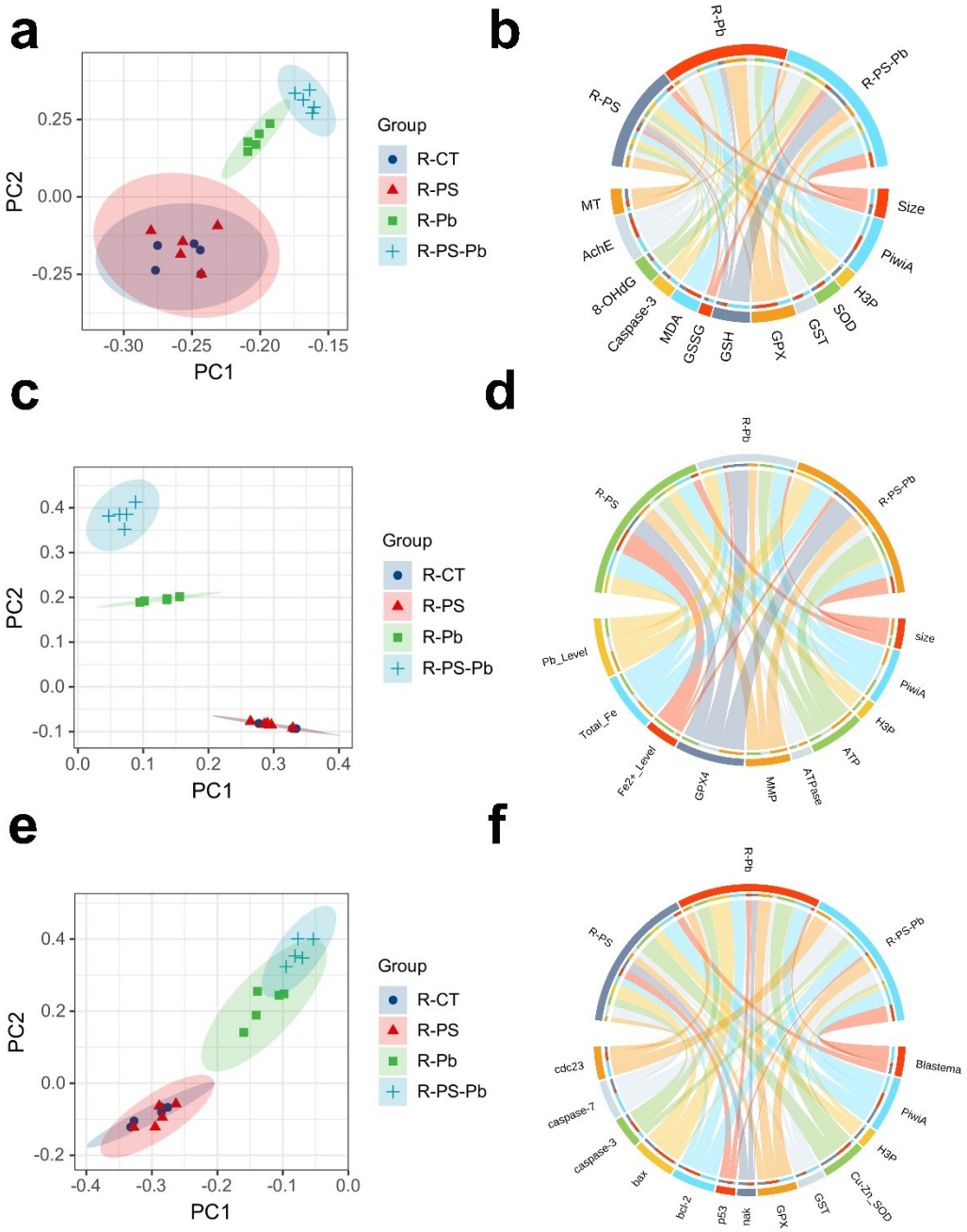

Fig. S7 PS-MPs regulate the oxidative stress-related enzymatic or non-enzymatic activities and genes, mitochondrial function relative biomarkers of regeneration planarians. Biplot originating from PCA integrating oxidative stress relative, mitochondrial function relative biomarkers parameters and qPCR analyses (**a**, **c**, **e**). Correlation between PS, Pb and PS-Pb and the biomarker of regeneration planarians based on Pearson correlation analysis for oxidative stress-related enzymatic and/or non-enzymatic activities, genes and mitochondrial function (**b**, **d**, **f**).

References

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