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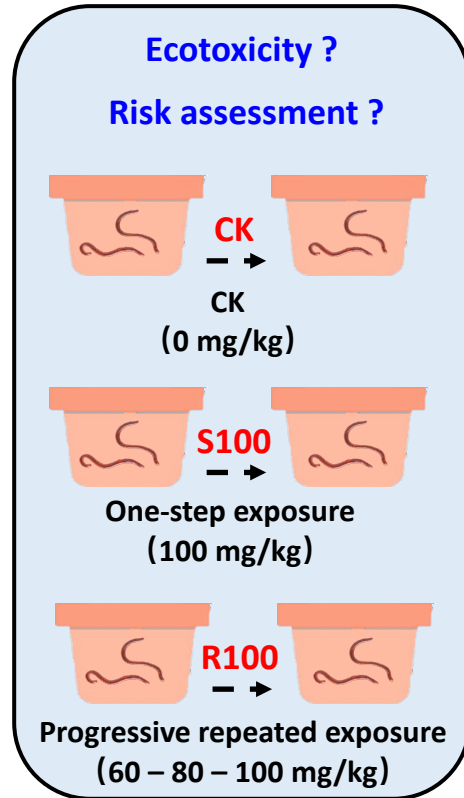
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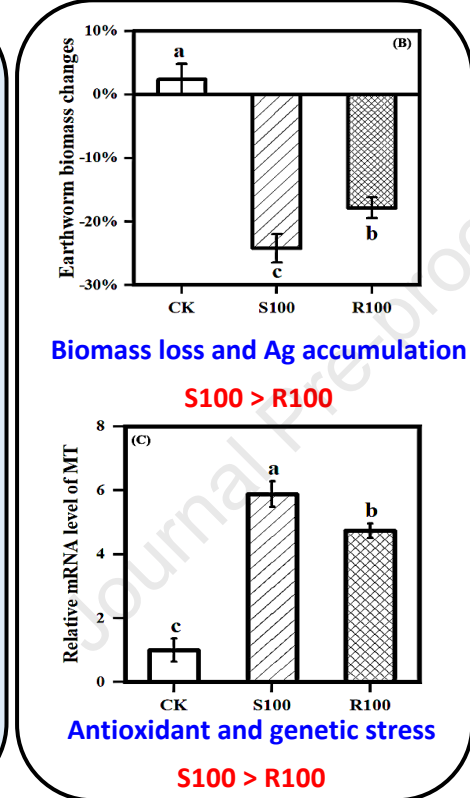
Caide Huang: Conceptualization, Methodology, Writing-original draft. **Xu Feng:** Methodology. **Shizhong Yue:** Writing - review & editing. **Li Jia:** Writing - review & editing. **Kun Wang:** Writing - review & editing. **Wenhao Zhou:** Methodology. **Yuhui Qiao:** Conceptualization, Funding acquisition, Supervision.

Treatments



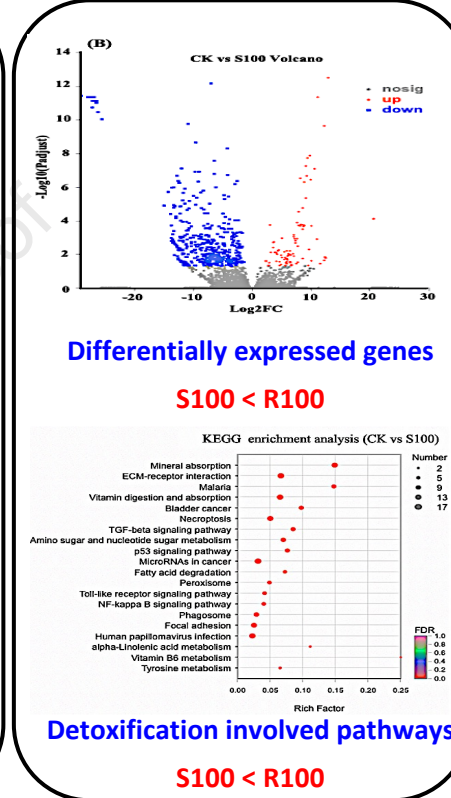
Different exposure patterns

Biomarkers



R100: less ecotoxicity

Transcriptome



R100: greater adaptation

1 **Impact of progressively cumulative exposure of AgNPs on earthworms (*Eisenia***
2 ***fetida*) and implication for eco-toxicological risk assessment**

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18

19 **Abstract**

20 Hazardous pollutants released into the real environment mostly own long-lasting
21 cumulative characteristics and have progressively negative impacts on organisms,
22 which are always neglected in laboratory toxicological tests. Here in this study, the
23 different ecotoxicity of Ag nanoparticles (AgNPs) on earthworm *Eisenia fetida* was
24 compared via various endpoints and transcriptional sequencing between the 28-day
25 progressively repeated (from 60 to 80, final 100 mg/kg) and one-step (directly to 100
26 mg/kg) exposure. The results showed that earthworms under progressively repeated
27 exposure showed significantly less biomass loss and reproductive inhibition, as well as
28 lower Ag bioaccumulation (15.6 mg/kg) compared with one-step exposure (17.9
29 mg/kg). The increases in enzyme activities (superoxide enzyme and catalase) and gene
30 expression (metallothionein) also implied higher antioxidant and genetic toxicity in
31 one-step exposed earthworms compared with those from progressively repeated
32 exposure. Furthermore, the transcriptomic analysis identified 582 and 854 differentially
33 expressed genes in the treatments of one-step and repeated exposure respectively
34 compared with the control group. The results of pathway annotation and classification
35 suggested similar enrichments of damage induction but different in toxic stress
36 responses, whereas earthworms from repeated exposure possessed more detoxification-
37 related pathways like translation and multicellular organismal processes. This study
38 innovatively took into account the impacts of processive exposure occurring in the real
39 environment and elucidated distinctions of toxicity and adaptation caused by different

exposure patterns, which provided the theoretical basis for real risk identification under the framework and guidance of traditional toxicology, also the implication for the improvement of eco-toxicological risk assessment.

Capsule: Earthworms under progressively repeated exposure to AgNPs showed greater fitness, detoxification, and adaptive changes compared with one-step exposure ones.

Keywords: Earthworms; transcriptome; toxicity; detoxification; risk assessment

1. Introduction

Nowadays, conventional and emerging pollutants were continuously released into aquatic and terrestrial ecosystems owing to the growing intensification of anthropological activities (Chen et al., 2022; Wu et al., 2022). Nanomaterials, as the new widespread-used technologies, have been involved in various industrial and agricultural applications (Lalau et al., 2020; Makama et al., 2016). Silver nanoparticle (AgNP) is one of the most extensive branches of nanomaterials and has been detected in lots of environmental soils and waters, owing to the quantity uses like textiles, water purification, makeups, surface antifouling, household products, and even food packaging (Luo et al., 2022; Tortella et al., 2020). A previous overview study estimated approximately 500 tons per year for global usage, and also reported the high environmental concentrations in nature fields (from $\mu\text{g/kg}$ to mg/kg) (Ghobashy et al., 2021). AgNPs with 0-100 nm dimensions can enter into food chains and induce adverse effects on non-target soil biotas, such as arthropods and earthworms (Chinnathambi et

al., 2023; Kwak et al., 2016; Vishwakarma et al., 2020). Some existing studies reported such AgNP transformation and consequent eco-toxicological risks on plants and microbes through the bio-amplification and bioaccumulation effects (Brinkmann et al., 2020; Das et al., 2018a). Therefore, more related investigations on ecotoxicological tests can help to fully understand the actual environmental risks of AgNPs.

In fact, most of the environmental pollutants, including the AgNPs, were released into the real ecosystems through progressive cumulation over a long-term period. Generally, most contaminated fields showed persistent, repetitive, and chronic pollution features and plots with long-lasting exposure histories were also easier as candidates for field survey studies (Huang et al., 2020a; Sun et al., 2019; Wang et al., 2018a). Contrarily, laboratory one-time acute tests always applied the higher chemical concentrations to obtain the adverse life-cycle consequences and used the data to extrapolate the impacts of long-term exposures, however, such exposure was easier to ignore the biological adaptations during the cumulative stress responses (Liu et al., 2019; Zhang et al., 2016). Organisms under increasing chronic exposure may induce different impacts and costs from those under one-time acute stress, and simultaneously exhibit greater fitness and resistance after the acclimation and even adaption (Huang et al., 2022; Wamucho et al., 2020). Therefore, the related one-step exposure studies may cause the underestimation or overestimation of real risks without the consideration of exposure backgrounds (Spurgeon et al., 2020). As such, studies are needed on comparing and understanding the difference of eco-toxicity and adaptation in organisms

under different exposure patterns, as well as the underlying biological or molecular mechanisms, which can help to obtain more evidence for accurate real risk evaluations.

Earthworms possess the largest biomass of all soil invertebrates and are regarded as “ecological engineers” due to their significant roles in soil improvement (Huang et al., 2021a). In addition, earthworms are also the sentinels and nature indicators of soil health, as earthworms *Eisenia fetida*, *Lumbricus terrestris*, and *Aporrectodea caliginosa* are always used as the model organisms for toxicological risk assessments (Morgan et al., 2013; Morgan et al., 2007). Presently, several test protocols, including OECD, ISO guidelines, and their variants support most earthworm toxicological bioassays, by monitoring the survival, individual and biological alternations to visually evaluate the toxicity and risk (ISO, 2012; OECD, 1984, 2010). Similarly, most of the guidelines recommend earthworm tests with one-time or one-step exposure under the lab microcosm conditions (Hu et al., 2016). Although these existing guidelines provided possibility and time-saving protocols for lab tests, based flexible improvements and modifications are still necessary considering the changing pollutants, exposure environments, and biological exposure patterns for more accurate assessments of real ecological risks of long-term and field-related impacts (Huang et al., 2022; Spurgeon et al., 2020; Sydow et al., 2017).

In this study, the 28-day progressively repeated exposure patterns were applied to assess the adverse impacts of AgNPs on earthworm *E.fetida* compared with one-step exposure. It was hypothesized that earthworms under progressively increased-

concentration exposure could show lower adverse ecotoxicity than those under one-step exposure due to the greater adaptability acquired during repeated exposure. The specific objectives were: (1) to compare earthworm survival, growth, reproduction, and Ag bioaccumulation under two different exposure patterns, (2) to determine the antioxidant (enzyme activities) and genetic (gene expression) stress and toxicity in earthworms under AgNPs exposure, (3) to investigate the pathways of AgNPs toxicity and detoxification in earthworms via transcriptional sequencing. The present study could contribute to the understanding and improvement of eco-toxicological risk assessment based on the framework and guidance of traditional toxicology.

2. Materials and methods

2.1 Test soils, earthworms and AgNPs

Using traditional OCED artificial soils as the exposure substrate in the field of nano-toxicology was very common and even frequently appeared in recent peer-reviewed studies (Bakr et al., 2023; Gao et al., 2023). Therefore, the OECD soil was also applied in this study according to the standard earthworm toxicity guideline (OECD, 1984). Specific ingredients composed of 70% air-dried quartz sand (size within 50-200 μm), 20% kaolinite clay, and 10 % ground sphagnum peat. Calcium carbonate was used to adjust the soil pH to 6.0 ± 0.5 and the moisture was adjusted with distilled water (Garcia-Velasco et al., 2017).

Earthworm *E.fetida* was used as the model organism in this study, which was

purchased from an earthworm farm around named “Dadi Julong Biotechnology Co., Ltd., Beijing, China”. Matured worms with fully developed clitellum and 300-500 mg weights were selected for further exposure, and the species was identified and confirmed by COI sequencing in our previous study (Huang et al., 2020b). Prior to the exposure, all earthworms were directly acclimated in the non-spiked OECD soils for one week and their biomasses showed non-observed changes prior and post to the acclimation (Huang et al., 2021b).

Uncoated AgNPs (CAS No.7440-22-4) with 99.9% purity, 20 nm powder size and 0.50 g/cm³ density were purchased from Shanghai Pantian Nano Materials Co., Ltd., China for soil spiking and earthworm exposure.

2.2 Progressively repeated exposure and comparative experiment designs

AgNPs concentration (up to 100 mg/kg) used in this study was following previous peer literature (Choi and Park, 2015; Khalil, 2016; Makama et al., 2016), actually higher than normal values of contaminated fields but this amount can obviously induce adverse impacts on earthworm fitness that easier to study the toxicity difference. To ensure the complete spiking and mixing, certain amounts of powder AgNPs were dissolved with pure acetone solution and stirred quickly to prevent aggregation, then completely homogenized with OECD soils to obtain the experiment’s expected concentrations (60, 80, and 100 mg/kg). Spiked soils were finally kept in a fume hood for 72 h to allow the acetone fully evaporate and avoid interference and then aged for one week in incubated conditions (Velicogna et al., 2017). Besides, the control group

dealt with the same acetone and ageing operations to reduce the potential solvent impacts on toxicity evaluation.

The progressively repeated exposures were established to simulate the scenes of gradually increased cumulation in natural fields (Fig.1). Specifically, for repeated treatments (named R100), earthworms were firstly exposed to 60 mg/kg AgNPs contaminated soils on day 0 (at least the starting dose that can induce toxicity referred to above references and pre-tests), progressively transferred to 80 mg/kg on day 7, and eventually transferred to 100 mg/kg on day 14 and exposed until the 28-day experiment ended (soils for day 7 and day14 transform were in advance spiked and aged as above). Besides, the one-step exposure (named S100) was also set up to conduct the comparison. Differently, earthworms were directly exposed to the soils that initially spiked with 100 mg/kg AgNPs, and all earthworms were kept constant exposure within 28 days. Finally, non-spiked OECD soils were applied as the pure control (named CK) for earthworm incubation, and three experimental replicates for each treatment. Considering the transfer activities may cause artificial disturbance and soil property changes for earthworms, thus the control and one-step exposure groups also followed the above transformations but ensured no changes in soil AgNP contents (Liu et al., 2019; Zhang et al., 2016).

After 24-h depuration, 10 individual earthworms were washed, weighed, and then introduced into each box (length \times width \times height: 175 \times 120 \times 65 mm) with 500 g clean or contaminated soils on day 0 (triple replicates), then all treatments were

incubated in an artificial climate chamber under 22 ± 2 °C, 60% relative humidity and the photoperiod of 12 h light and 12 h darkness. During the exposure, a total of 15 g (5 g \times 3 times, every week) air-dried cow manure was added to the soil surface to provide nutrition for earthworms (Huang et al., 2021b).

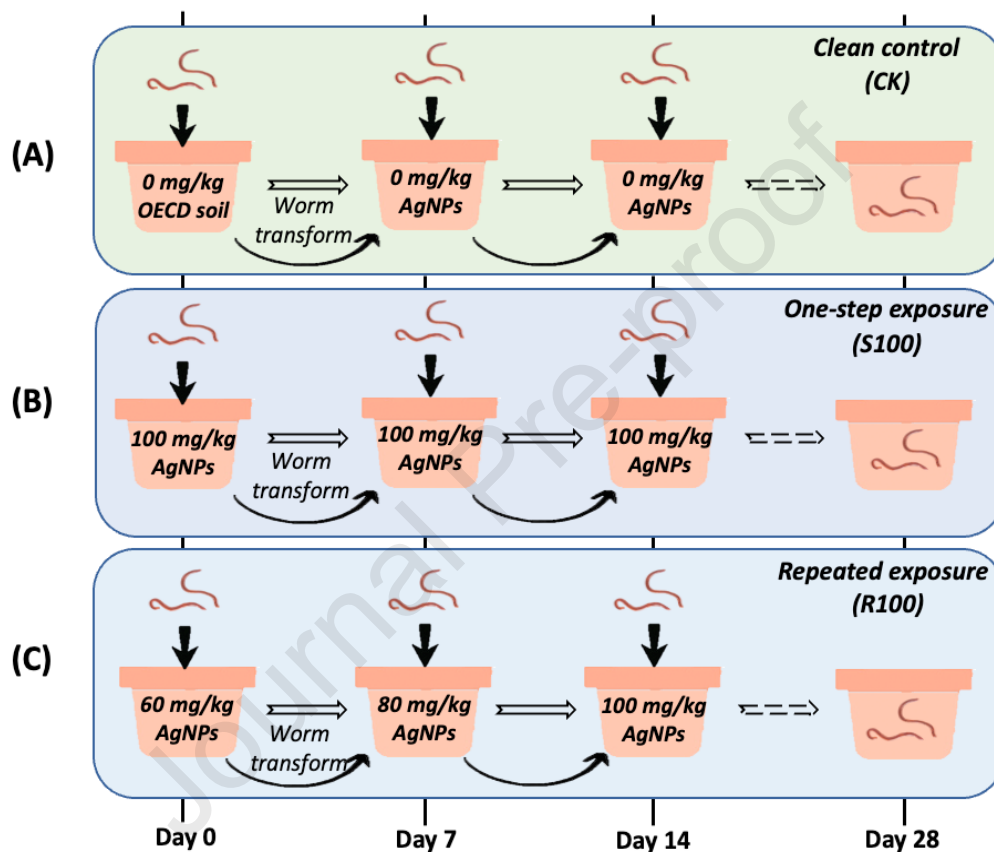


Fig.1. The conceptual framework and experimental design of progressively repeated and compared exposure of AgNPs on earthworm *E. fetida*. (A) the control treatment with the un-contaminated OECD soils; (B) one-step exposure, constantly exposed to 100 mg/kg AgNPs contaminated soils; (C) progressively repeated exposure, exposed to contaminated soils with a progressively increased gradient of AgNPs, from 60, 80, and finally to 100 mg/kg.

2.3 Earthworm survive, growth, reproduction and Ag bioaccumulation

All lived earthworms were collected from each pot after 28-day exposure, depurated for 24 h, weighed, then quick-frozen with liquid nitrogen, and stored in a -80 °C freezer for further analysis. Earthworm survival rates and growth were calculated by comparing the number and biomass of earthworms before and after AgNPs exposure. Earthworm reproduction was evaluated by cocoon numbers that were counted after the wet-sieving.

Whole earthworms were ground into tissue powder after the freeze-drying in a freeze dryer (TF-FD-18S, Shanghai, China), then digested with a mixture of HNO₃-H₂O₂ in an electric hot plate digester for Ag analysis. Filtered digest liquid was determined by an inductively coupled plasma-mass spectrometry (ICP-MS; 7700ce, Agilent Technologies, USA). The authenticated reference substance (Shrimp powder, GBW10050) was used to ensure the analysis quality, and the overall recovery deviation was less than 5% (Wang et al., 2018b; Yue et al., 2021).

2.4 Earthworm antioxidant enzyme activities and gene expressions

The superoxide enzyme (SOD) and catalase (CAT), as some of the most commonly used antioxidant enzymes, were chosen to assess the antioxidant toxicity and response of earthworms. The complete worms were ground with liquid nitrogen, weighted the tissue and added the relative amount (1:9) of prepared saline to obtain the 10% tissue homogenate, then extracted and analyzed the protein contents using the test kits. The

SOD and CAT activities were also determined under 560 and 240 nm absorbance also using the tissue and enzyme kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) following the manufacturer's specifications (Huang et al., 2020b).

Detoxification-related metallothionein gene (MT) and reproductive-related annetocin precursor gene (ANN) were selected to evaluate the detoxification response and genetic damage. Simply, three randomly-selected full earthworms from each treatment were used to ground into powder, and then extract total RNA through commercial kits. The expressions of MT and ANN were determined by qRT-PCR after the purity detection and RNA reverse-transcribed. Primer sequences are shown in Table S1 and detailed PCR procedures have been listed in Text S1 and ever reported in our previous study (Huang et al., 2022).

2.5 Transcriptomic analysis of earthworm *E.fetida*

Transcriptomic sequencing was used to identify the differentially expressed genes (DEGs) induced by different exposure patterns. Earthworm tissue RNA and cDNA were isolated and synthesized, respectively as above, and then constructed the non-reference sequencing library by purifying and end-repairing. An Illumina Novaseq 6000 platform (Illumina, Inc., USA) was used to sequence the qualified library and generate the paired-end reads. After sequencing, all reads were screened for quality control in fastp V0.19.5, and the clean reads were successfully aligned using Trinity V2.8.5 (sequencing meets the analysis requirements, and all quality control results were performed in Table S2). The threshold of $p\text{-value} < 0.05$ and $a | \log_2 (\text{fold change}) | >$

1 further confirmed such genes with significant expressed discrepancies. Finally, the significant enrichment of DEGs of pathways was performed and annotated by the Kyoto Encyclopedia of Genes and Genomes (KEGG) and Gene Ontology (GO) databases (Qiao et al., 2022; Zhao et al., 2022).

2.6 Statistical analysis

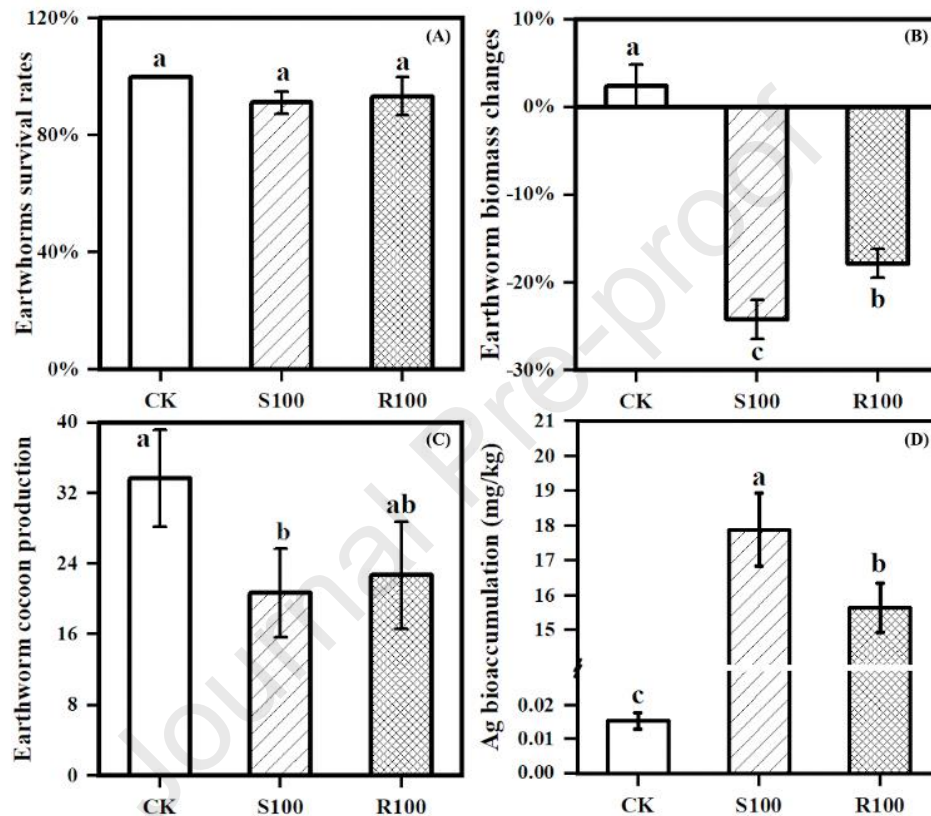
IBM SPSS 24.0 was used for statistical analysis, and one-way analysis of variance (ANOVA) and Tukey's honest tests were performed to check the significant differences among the normality data from different exposure treatments. Data were expressed as means \pm standard deviations while $P \leq 0.05$ represents the significant differences. Transcriptome data were analyzed by the interactive data-mining system on the online Majorbio Cloud Platform (www.majorbio.com). Tables and figures were completed in Excel 2016 and Origin 2020.

3. Results

3.1 Effects of AgNPs on earthworm fitness and Ag bioaccumulation

The survival rates, biomass changes, reproduction, and Ag bioaccumulation are shown in Fig 2. AgNPs induced slight death in some pots of step and repeated exposure, but no significance was found among different treatments (Fig 2A; $P > 0.05$). Earthworms under treatment S100 showed the greatest 24.2% biomass loss, which was significantly higher than 17.8% of R100 earthworms (Fig 2B; $P \leq 0.05$). The mean number of cocoons in treatment CK, S100, and R100 was 33.7, 20.7, and 22.6

237 respectively, which suggested a significant decrease after AgNPs exposure (Fig 2C; P
 238 $\leq 0,05$). Compared with R100 earthworms, earthworms under one-step exposure
 239 bioaccumulated more soil Ag, which showed an increase from 15.6 to 17.9 mg/kg (Fig
 240 2D; $P \leq 0,05$).



241
 242 **Fig.2.** The survives, biomass, reproduction, and Ag bioaccumulation of earthworm
 243 *E.fetida* under progressively repeated and one-step exposures. (A) earthworm survival
 244 rates; (B) earthworm biomass changes; (C) mean number of earthworm cocoons in each
 245 pot; (D) Ag bioaccumulation in earthworm tissues. Bars represent means \pm standard
 246 deviation (SD) and different letters (a-c, *) indicate the significant differences ($P \leq 0.05$)
 247 among different treatments.

248

249 *3.2 Effects of AgNPs on earthworm antioxidant and gene expression*

250 The activities of SOD and CAT enzymes in earthworms are shown in Fig 3A, B.

251 For SOD, the activities were significantly increased after the AgNPs exposure, which

252 enhanced by 54.4% and 43.7%, respectively (Fig 3A; $P \leq 0,05$). CAT activities also

253 indicated a similar increasing trend, which was 1.32, 4.22, and 3.96 U/mgprot for

254 treatment CK, S100, and, R100, respectively (Fig 3B).

255 The relative expressions of MT and ANN genes were displayed in Fig 3C, D. The

256 MT gene was significantly up-regulated because of the AgNPs exposure, which raised

257 from 1 to 5.88 and 4.74 for S100 and R100 earthworms compared with the control

258 group, respectively (Fig 3C; $P \leq 0,05$). Besides, a significant difference was also

259 observed between treatment S100 and R100 owing to the distinct exposure patterns.

260 The ANN expressions were significantly inhibited by AgNPs, which decreased by 82.7%

261 and 85.7% for earthworms under S100 and R100 exposures, respectively (Fig 3D; $P \leq$

262 0,05).

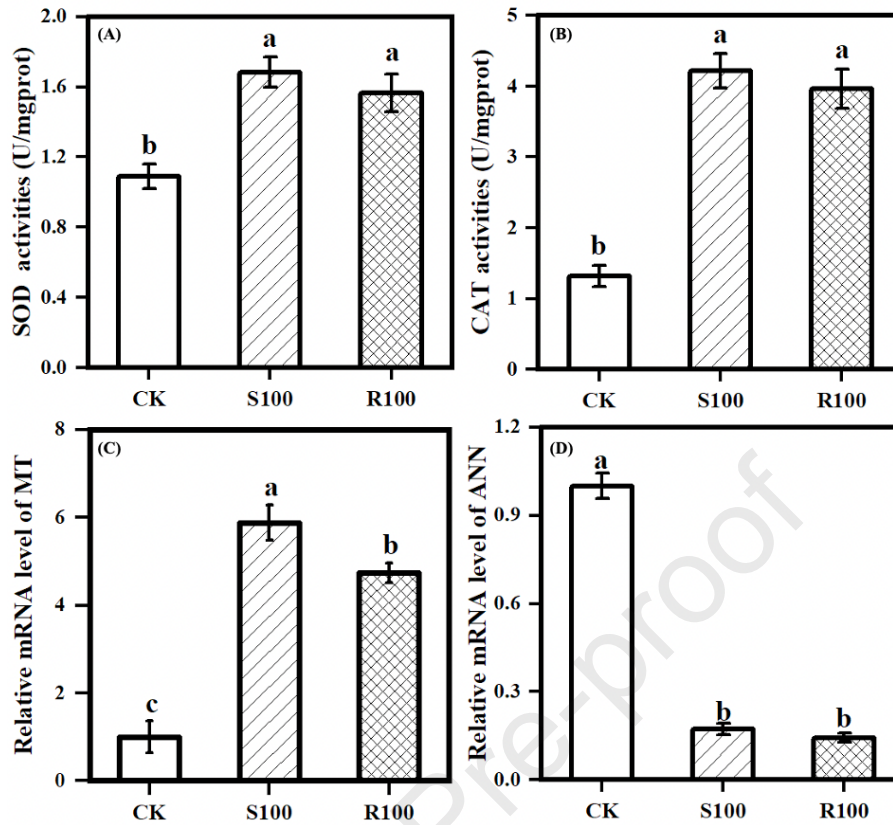


Fig.3. The antioxidant enzyme activities and gene expression of earthworm *E.fetida* under progressively repeated and one-step exposures. (A-B) SOD and CAT activities; (C-D) relative mRNA expressions of MT (metallothionein) and ANN (annetocin precursor) genes. Bars represent means \pm standard deviation (SD) and different letters (a-c) indicate the significant differences ($P \leq 0.05$) among different treatments.

3.3 Results and quality of transcriptomic sequencing

The transcriptomic sequencing for 9 earthworm samples generated an average of 51,198,327 clean reads with 96.8% and 91.6% high-quality reads for Q20 and Q30 values, respectively (Table S2). A total of 170,533 unigenes were identified in the

current study, and the Venn diagram exhibited that 6783, 9799, and 4183 unigenes individually belonged to treatment CK, S100, and R100, respectively. Besides, 27,964 unigenes, which accounted for 46.6% were shared by all these three groups (Fig S1).

A total of 582 and 854 DEGs were identified here for treatment S100 and R100, respectively, compared with the control group (Fig 4A). The volcano figures demonstrated that for S100 worms 93 and 489 genes were upregulated and downregulated, respectively (Fig 4B). Besides, there were 399 and 455 genes significantly upregulated and downregulated for the earthworms from repeated-exposure treatments (R100), respectively (Fig 4C).

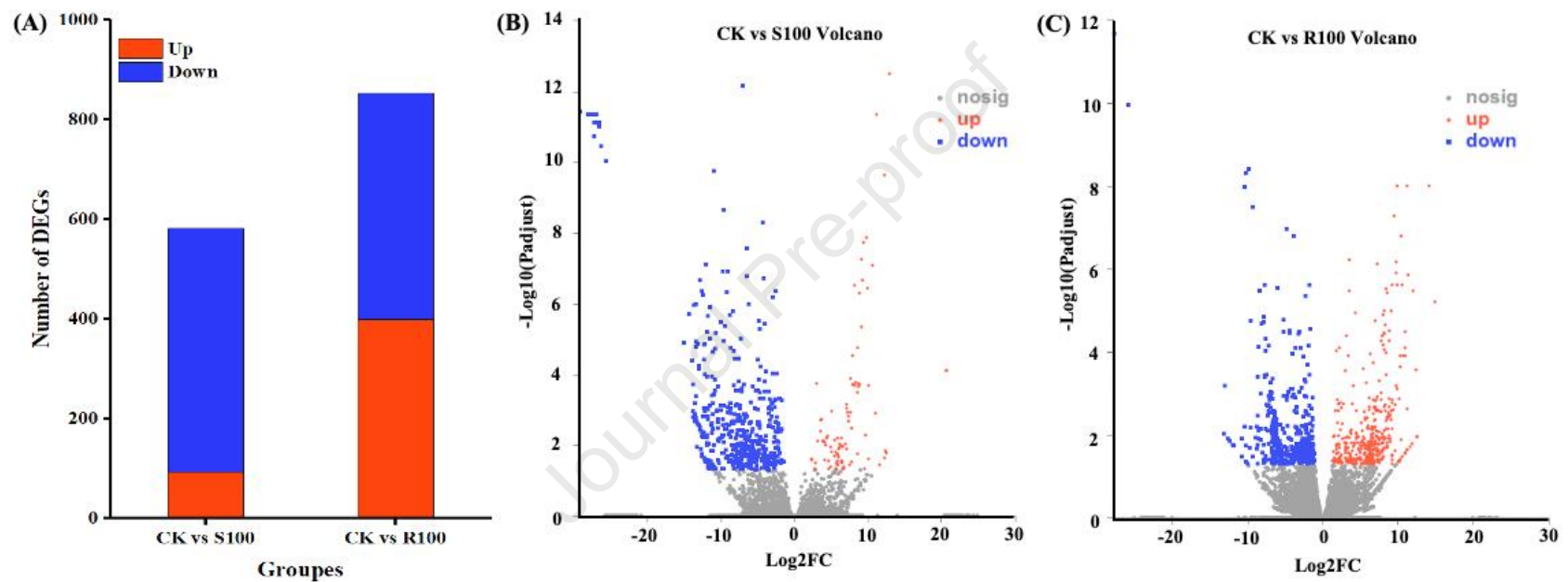
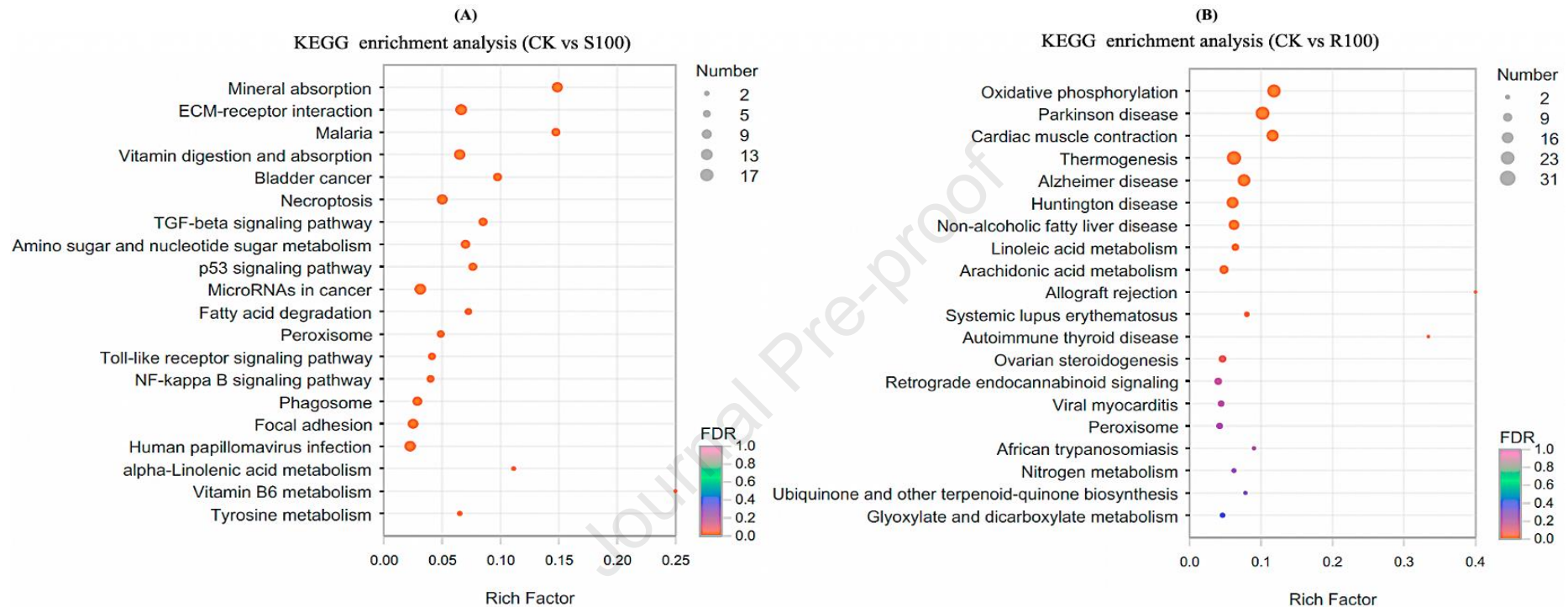


Fig.4. Volcanic figures of DEGs in earthworm *E.fetida* after the 28-day repeated and step AgNPs exposures compared with the control group. (A) statistic results of DEGs; (B) one-step exposure compared with the control; (C) progressively repeated exposure compared with the control.

288 3.4 Pathway annotation of DEGs in earthworms

289 The top 20 significantly KEGG-enriched pathways are annotated and listed in Fig
290 5, and different DEGs enrichments were shown in S100 and R100 earthworms.
291 Specifically, the KEGG annotation analysis showed that vitamin metabolism and
292 mineral absorption were the most enriched pathways for S100 earthworms after step
293 AgNPs exposure (Fig 5A). The pathways of allograft rejection, autoimmune thyroid
294 diseases, and oxidative phosphorylation were most significantly enriched by
295 earthworms after repeated exposure (Fig 5B).

296

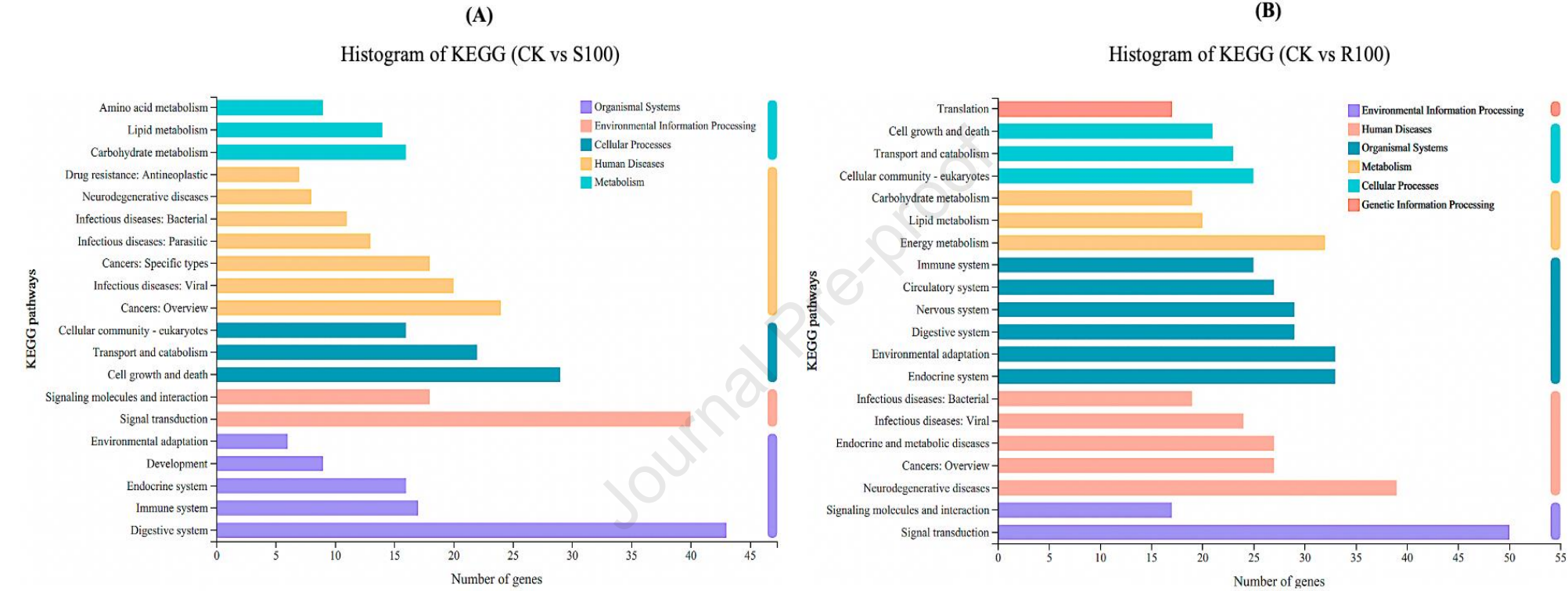


297 **Fig.5.** KEGG pathway enrichment analysis of DEGs in earthworm *E.fetida* after the 28-day repeated and step AgNPs exposures compared with

298 the control group. (A) one-step exposure compared with the control; (B) progressively repeated exposure compared with the control.

All the pathways were classified into 6 different categories, which were associated with the organism system, environmental information processing, cellular processes, human diseases, metabolism, and genetic information processing (Fig 6). For S100 worms, most of the DEGs were classified into the category of human diseases (101), like the pathways of drug resistance, neurodegenerative and infectious diseases (Fig 6A). 176 DEGs were classified by organism system involving the immune system and environmental adaptation, which accounted for most of the six categories. R100 worms also possessed another unique category of genetic information processing, which was translation related compared with S100 worms (Fig 6B). Moreover, the GO annotations indicated similar classifications between S100 and R100 worms, whereas R100 worms owned more pathways of biological processes, such as the multicellular organismal and stimulus responses (Fig S2).

311



312 **Fig.6.** KEGG pathway classifications of DEGs in earthworm *E.fetida* after the 28-day repeated and step AgNPs exposures compared with the

313 control group. (A) one-step exposure compared with the control; (B) progressively repeated exposure compared with the control.

4. Discussion

4.1 Better fitness of earthworms under progressively repeated exposure

In this study, the results showed that 100 mg/kg AgNPs significantly inhibited the growth and reproduction of earthworms. These findings were in agreement with the study of Bami et al. (2017), which reported that the biomasses of endogeic earthworm *Allolobophora chlorotica* were significantly decreased under 125 mg/kg AgNPs exposure and 100% of mortality happened when the concentration was over 250 mg/kg. Similarly, Schlich et al. (2013) found that the reproduction of earthworm *Eisenia Andrei* showed 50% of inhibition when AgNPs concentration was 80 mg/kg ($EC_{50} = 80$ mg/kg). Unlike the soil metals or liquid chemicals, lower bioavailability and bioaccessibility of AgNPs possibly induce delayed lethal effects, and that also might be the explanation for non-significant results of earthworm deaths in this study (Brinkmann et al., 2022; Khodaparast et al., 2021). Silver nanoparticles can be transferred into earthworm tissues through epidermal absorption and intestine digestion due to burrowing and chewing activities. Besides, silver ion (Ag^+), as one of the most toxic metal cations, could induce toxicity and adverse impacts by the processes of dissolution, oxidation, and also an imbalance of osmotic pressure (Schlich et al., 2013; Shoults-Wilson et al., 2011). Therefore, less biomass loss and more cocoon production of earthworms under repeated cumulative exposure indicated their slighter body burden and toxicity, compare with those from one-step exposure treatment.

Ag nanoparticles and their Ag^+ could induce the rapid production of reactive oxygen species and thus interfere with the acceleration of antioxidant processes (Ahn et al., 2014; Jiang et al., 2014). As such, the activities of SOD and CAT were significantly enhanced after the 28-day exposure to AgNPs, which showed increasing antioxidant toxicity (Fig 3). SOD and CAT are both universal enzymes, SOD can catalyze the superoxide conversion into oxygen and hydrogen peroxide, while CAT catalyzes the decomposition of hydrogen peroxide to water and oxygen (Huang et al., 2021b). Except for SOD and CAT, many published papers also reported other sensitive antioxidant biomarkers, like malondialdehyde (MDA), glutathione S-transferase (GST), and lipid peroxidation (LPO), and it could be concluded that the most of enzyme activities were significantly enhanced with the increase in AgNPs concentrations (Bakr et al., 2023; Li et al., 2022; Singh et al., 2022). In this study, the same concentration (100 mg/kg) was applied in these two different exposure patterns, however, higher Ag bioaccumulation in S100 earthworms could also help to explain their elevated enzyme activities compared with R100 worms.

Furthermore, the mRNA endpoints disclosed the difference in genetic toxicity between S100 and R100 earthworms. MT is a low-molecular-weight cysteine-rich peptide and is ubiquitously expressed in different organisms, which plays a key role in ROS removal and cell oxidative protection (Lavradas et al., 2016; Li et al., 2020). Here the results of MT gene expression suggested larger genetic stress and toxicity of AgNPs in S100 worms. The relative expression of ANN genes was significantly down-

regulated owing to the toxic AgNPs, which was in correspondence with the results of earthworm cocoon production (Fig 3). ANN in earthworms is an oxytocin-related and reproductive peptide, which had been proven and considered a sensitive biomarker for reproductive evaluation (Mo et al., 2012; Ricketts et al., 2004).

4.2 Transcriptional mechanisms behind the earthworm AgNP toxicity and adaptation

Earthworms under repeated exposure initially suffered less toxic body burden and might have more time and chances of recovering and coordinating the biological processes for detoxification and adaptive responses considering the gradually increased contamination. The process of toxicity and detoxification of AgNPs in earthworms was complex as well as the result of various genes working together, and therefore transcriptional sequencing played a crucial and comprehensive role in mechanism analysis (Li et al., 2022; Qiao et al., 2022). After exposure to AgNPs, a large number of genes were up-regulated or down-regulated whether in step-exposure or repeated-exposure treatments, which implied many functional genes were involved in stress response (Fig 4). Most of the DEGs were associated with pathways of carbon metabolism, energy conversion, and disease induction. Similar results were reported by Zhao et al. (2022), which demonstrated the pathways of vitamin metabolism and mineral absorption were significantly enriched in earthworm *Metaphire guillelmi* after 44 days of exposure to Pipolymyxin. The antioxidant response was known as a primary defence for those organisms under stress to protect cells and macromolecules from oxidative damage (Hu et al., 2016), whereas enough energy and material were essential

like minerals and vitamins. For example, Sun et al. (2021) validated the extra addition of vitamin C alleviated the antioxidant and protected earthworm *E.fetida* from the toxic damage of polycyclic aromatic hydrocarbons.

The pathway of oxidative phosphorylation (OXPHOS) was significantly enriched in R100 earthworms compared with S100 ones. OXPHOS is a typical metabolic pathway that efficiently synthesizes ATP using the energy released from oxidized nutrients. The OXPHOS inhibition was regarded as one of the major toxic pathways for the impairments of AgNPs (Saleeb et al., 2020), while this potentially explains the results of elevated OXPHOS process and toxic tolerance in earthworms under repeated exposure. Besides, the extracellular matrix (ECM) receptor interaction, a significantly enriched pathway in S100 earthworms, was possibly related to AgNPs toxicity. ECM is a connective network assembled by fibrous glycoproteins and plays a significant role in the maintenance of tissue structures, while the ECM interactions are responsible for the cell activities like differentiation, proliferation, and apoptosis (Bao et al., 2019; Zhu et al., 2021). Relatedly, a recent study also reported that differential genes in earthworms were also significantly enriched in ECM pathways after exposure to 25 and 50 mg/kg flupyradifurone-contaminated soils (Qiao et al., 2022).

The classifications of enriched pathways revealed the biological processes in S100 and R100 worms after exposure to AgNPs (Fig 6 and Fig S2). As the results of KEGG pathways, the classification of diseases associated with infectious diseases (bacterial and viral) and neurodegenerative diseases, was similarly distributed in S100 and R100

earthworms, which implied the adverse effects of AgNPs exposure including the inflammation and neurodegeneration. Some previous studies also reported consistent pathway enrichments in earthworms exposed to microplastics and alumina nanoparticles, while they attributed these results to oxidative stress, a vicious cycle of autocrine, and neurotoxicity (Dong et al., 2019; Li et al., 2021). Differently, compared with the S100 group, R100 worms showed a unique categorization of genetic information processing mainly related to translation, which was composed of 17 genes with the functions of protein export, mRNA surveillance pathway, and protein processing in the endoplasmic reticulum. The above results possibly owing to the less toxicological stress and greater resistance in earthworms under repeated exposure, while the elevated pathways enrichments of signal transductions and multicellular organismal processes also could help to explain (Markad et al., 2016; Qiao et al., 2022).

4.3 Implication of different exposure patterns for eco-toxicological risk assessments

The “fight-or-flight” stress responses are the determinants for the survival and health of organisms under unfavourable conditions (Hajdu et al., 2021). Such responses originated from the trade-offs between toxicity and adaptations, including multicellular alterations, biological detoxifications, damage elimination, and even genetic adjusting (Hajdu et al., 2021; Huang et al., 2022). The biggest difference with one variable here was the AgNPs exposure pattern, with one keeping the same concentration and another exposing to an increasing gradient of concentrations. For step-exposure earthworms, the high concentration of AgNPs induced more adaptation cost and body burden or

somewhat directly impaired certain biological functions and processes. Specifically, step-exposure worms lived under 100 mg/kg for two more weeks, and such processes might induce higher individual and genetic toxicity, which thus limited the adaptability of S100 earthworms. Contrarily, repeated-exposure worms suffered less impact and cost at the beginning and had more chances to train and maintain the adaptive changes with the increase of external pressure, and that was also the reason that R100 worms showed greater fitness in the present study (Liu et al., 2019). Similarly, organisms under laboratory acute exposure also showed larger toxicological stress than those from real contaminated fields, because higher tolerance could be achieved through natural selections including increased defence and adaptation mechanisms like metallothionein (Mirmonsef et al., 2017; Spurgeon et al., 2020).

Generally, with the increasing complexity of the pollutants in environments, traditional toxicological guidelines exist some scope limits and make it difficult to meet more comprehensive studies. For instance, the OECD organization proposed and developed the guidelines from NO.207 (1984) about the acute toxicity of a single pollutant to NO. 296 (2018) regarding the risk assessments of combined pollutants, in order to standardize the emerging studies of chemical mixtures (Pereira et al., 2018; Terekhova, 2022). However, there is no universal guideline or protocol at present for repeated exposure involved in risk assessments. Most of the laboratory tests were established to simulate the real contamination and biological exposure but ignored the repeated, cumulative and long-lasting are among the most significant characteristics of

actual polluted fields (Liu et al., 2019; Zhang et al., 2016). In fact, this study revealed the difference in toxicity and adaptation between one-step exposure and repeated exposure patterns, which probably cause a deviation in the assessment of real chemical risks.

In addition, even though our study highlighted the importance of exposure patterns, some improvements still needed to be stated. Further studies can extend the exposure period to months or even generations to investigate the mechanisms and continuation of adaptative changes. The method of multiple interval sampling should be applied to monitor the detailed development, and toxicokinetic and toxicodynamic can also help to underlie the related mechanisms (Short et al., 2021). Besides, in this study the exposure concentration was indeed established higher than field contaminations to obtain the toxicity effects, however, the dose-response curves should be achieved by setting more exposure concentrations so as to confirm the margins between reversibility and irreversibility. It is also interesting to explore AgNP potential impacts on the ecosystem between toxic and non-toxic doses when using as friendly agricultural materials, like the popular plant promoters (Das et al., 2018b). Additional data and biomarker results can help to discover the connections between one-step exposure and progressive exposure, and achieve reliable risk estimation and calculation by constructing equations, linear or multiple relationships. Continuing to carry out the verification experiments on field soils or *in-situ* contaminated soils will also help to reach the goal of standard improvement.

Conclusion

This study innovatively took into account the impacts of processive exposure occurring in the real environment. In summary, the results revealed that different AgNP patterns of one-step and progressively repeated treatments induced the difference in toxicity and biological adaptation in earthworms. Earthworms under repeated exposure performed less toxicity and better adaptation than one-step exposed worms, including the regulation and coordination of biomass, AgNP bioaccumulation, antioxidant system and gene detoxification. Future studies could extend the exposure period and increase the sampling times to explore related mechanisms and long-term effects, and more consideration should be given to the real risks under repeated exposure considering the cumulative environmental pollutants. The present study was conducted based on the traditional toxicological guidelines, as the improvement and supplement, contributing to the understanding of chemical risk assessments under real environmental conditions.

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Conflict of interest

There are no conflicts to declare.

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Highlights

- (1) Progressive and one-step exposure of AgNPs on earthworms were performed.
- (2) Progressive exposure worms showed greater fitness and less Ag bioaccumulation.
- (3) Less antioxidant and genetic stress and toxicity for progressively exposed worms.
- (4) Transcriptomics revealed the difference in pathways of toxicity and detoxication.
- (5) One-step exposure induced larger toxicity than progressively repeated exposure.

Declaration of interests

☒ 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.

☐ The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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