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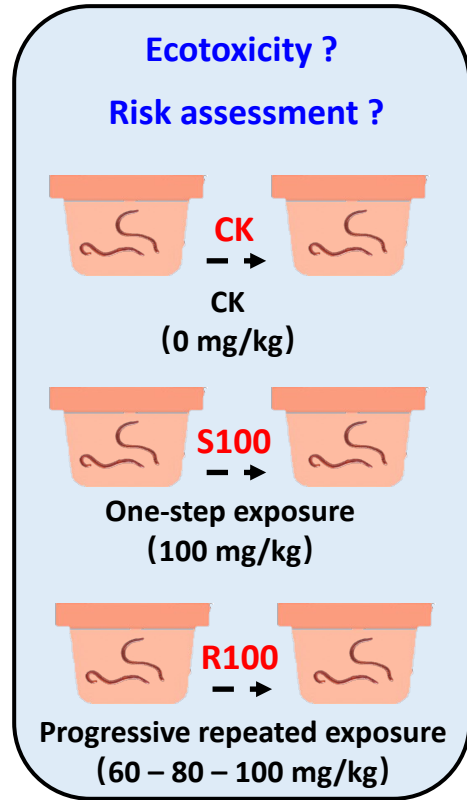
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### **Author contribution statement**

**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.

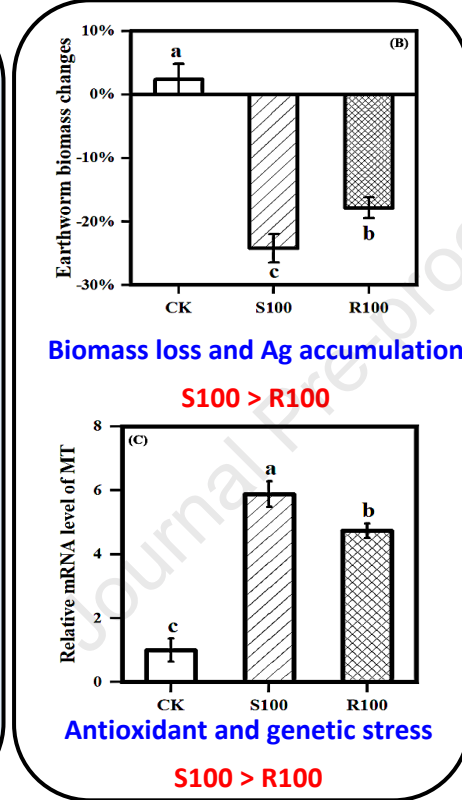
Journal Pre-proof

Treatments



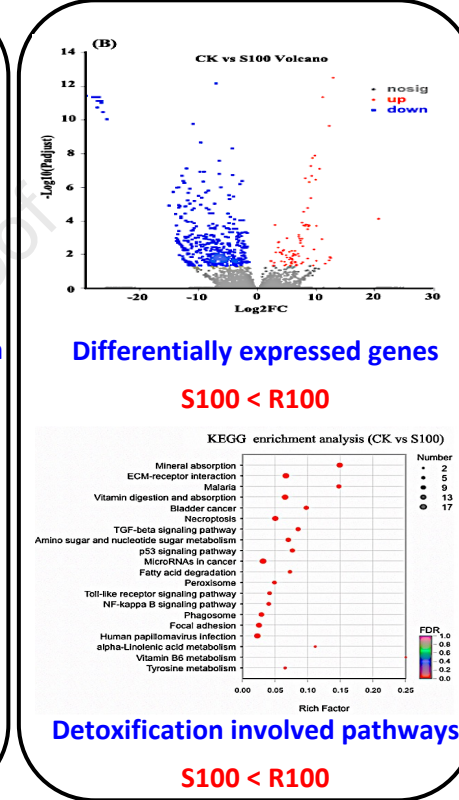
Different exposure patterns

Biomarkers



R100: less ecotoxicity

Transcriptome



R100: greater adaptation



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

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

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

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

## 46 1. Introduction

47 Nowadays, conventional and emerging pollutants were continuously released into  
48 aquatic and terrestrial ecosystems owing to the growing intensification of  
49 anthropological activities (Chen et al., 2022; Wu et al., 2022). Nanomaterials, as the  
50 new widespread-used technologies, have been involved in various industrial and  
51 agricultural applications (Lalau et al., 2020; Makama et al., 2016). Silver nanoparticle  
52 (AgNP) is one of the most extensive branches of nanomaterials and has been detected  
53 in lots of environmental soils and waters, owing to the quantity uses like textiles, water  
54 purification, makeups, surface antifouling, household products, and even food  
55 packaging (Luo et al., 2022; Tortella et al., 2020). A previous overview study estimated  
56 approximately 500 tons per year for global usage, and also reported the high  
57 environmental concentrations in nature fields (from  $\mu\text{g}/\text{kg}$  to  $\text{mg}/\text{kg}$ ) (Ghobashy et al.,  
58 2021). AgNPs with 0-100 nm dimensions can enter into food chains and induce adverse  
59 effects on non-target soil biotas, such as arthropods and earthworms (Chinnathambi et

60 al., 2023; Kwak et al., 2016; Vishwakarma et al., 2020). Some existing studies reported  
61 such AgNP transformation and consequent eco-toxicological risks on plants and  
62 microbes through the bio-amplification and bioaccumulation effects (Brinkmann et al.,  
63 2020; Das et al., 2018a). Therefore, more related investigations on ecotoxicological  
64 tests can help to fully understand the actual environmental risks of AgNPs.

65 In fact, most of the environmental pollutants, including the AgNPs, were released  
66 into the real ecosystems through progressive cumulation over a long-term period.  
67 Generally, most contaminated fields showed persistent, repetitive, and chronic pollution  
68 features and plots with long-lasting exposure histories were also easier as candidates  
69 for field survey studies (Huang et al., 2020a; Sun et al., 2019; Wang et al., 2018a).  
70 Contrarily, laboratory one-time acute tests always applied the higher chemical  
71 concentrations to obtain the adverse life-cycle consequences and used the data to  
72 extrapolate the impacts of long-term exposures, however, such exposure was easier to  
73 ignore the biological adaptations during the cumulative stress responses (Liu et al.,  
74 2019; Zhang et al., 2016). Organisms under increasing chronic exposure may induce  
75 different impacts and costs from those under one-time acute stress, and simultaneously  
76 exhibit greater fitness and resistance after the acclimation and even adaption (Huang et  
77 al., 2022; Wamucho et al., 2020). Therefore, the related one-step exposure studies may  
78 cause the underestimation or overestimation of real risks without the consideration of  
79 exposure backgrounds (Spurgeon et al., 2020). As such, studies are needed on  
80 comparing and understanding the difference of eco-toxicity and adaptation in organisms



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

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

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

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

## 111 **2. Materials and methods**

### 112 *2.1 Test soils, earthworms and AgNPs*

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

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

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

129 Uncoated AgNPs (CAS No.7440-22-4) with 99.9% purity, 20 nm powder size and  
130 0.50 g/cm<sup>3</sup> density were purchased from Shanghai Pantian Nano Materials Co., Ltd.,  
131 China for soil spiking and earthworm exposure.

## 132 *2.2 Progressively repeated exposure and comparative experiment designs*

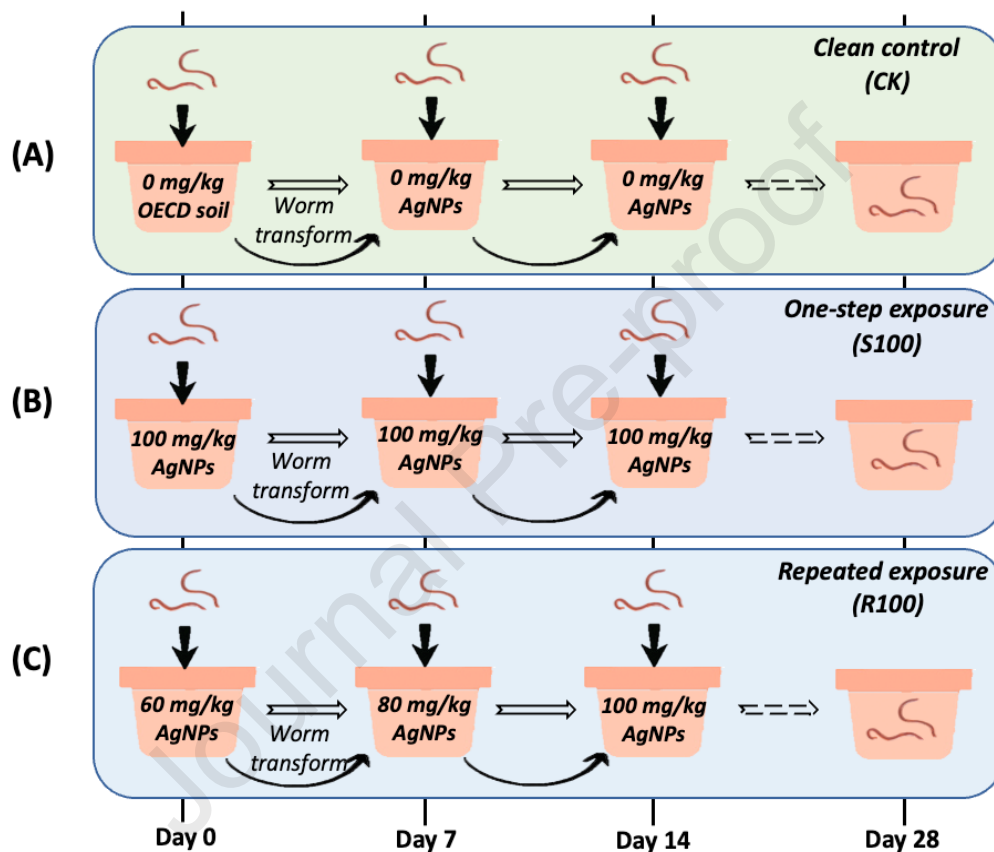
133 AgNPs concentration (up to 100 mg/kg) used in this study was following previous  
134 peer literature (Choi and Park, 2015; Khalil, 2016; Makama et al., 2016), actually  
135 higher than normal values of contaminated fields but this amount can obviously induce  
136 adverse impacts on earthworm fitness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

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

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

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

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



168

169 **Fig.1.** The conceptual framework and experimental design of progressively repeated  
 170 and compared exposure of AgNPs on earthworm *E.fetida*. (A) the control treatment  
 171 with the un-contaminated OECD soils; (B) one-step exposure, constantly exposed to  
 172 100 mg/kg AgNPs contaminated soils; (C) progressively repeated exposure, exposed to  
 173 contaminated soils with a progressively increased gradient of AgNPs, from 60, 80, and  
 174 finally to 100 mg/kg.

175

### 176 *2.3 Earthworm survive, growth, reproduction and Ag bioaccumulation*

177 All lived earthworms were collected from each pot after 28-day exposure,  
178 depurated for 24 h, weighed, then quick-frozen with liquid nitrogen, and stored in a -  
179 80 °C freezer for further analysis. Earthworm survival rates and growth were calculated  
180 by comparing the number and biomass of earthworms before and after AgNPs exposure.  
181 Earthworm reproduction was evaluated by cocoon numbers that were counted after the  
182 wet-sieving.

183 Whole earthworms were ground into tissue powder after the freeze-drying in a  
184 freeze dryer (TF-FD-18S, Shanghai, China), then digested with a mixture of HNO<sub>3</sub>-  
185 H<sub>2</sub>O<sub>2</sub> in an electric hot plate digester for Ag analysis. Filtered digest liquid was  
186 determined by an inductively coupled plasma-mass spectrometry (ICP-MS; 7700ce,  
187 Agilent Technologies, USA). The authenticated reference substance (Shrimp powder,  
188 GBW10050) was used to ensure the analysis quality, and the overall recovery deviation  
189 was less than 5% (Wang et al., 2018b; Yue et al., 2021).

### 190 *2.4 Earthworm antioxidant enzyme activities and gene expressions*

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

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

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

### 207 *2.5 Transcriptomic analysis of earthworm E.fetida*

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

217 1 further confirmed such genes with significant expressed discrepancies. Finally, the  
218 significant enrichment of DEGs of pathways was performed and annotated by the  
219 Kyoto Encyclopedia of Genes and Genomes (KEGG) and Gene Ontology (GO)  
220 databases (Qiao et al., 2022; Zhao et al., 2022).

## 221 2.6 Statistical analysis

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

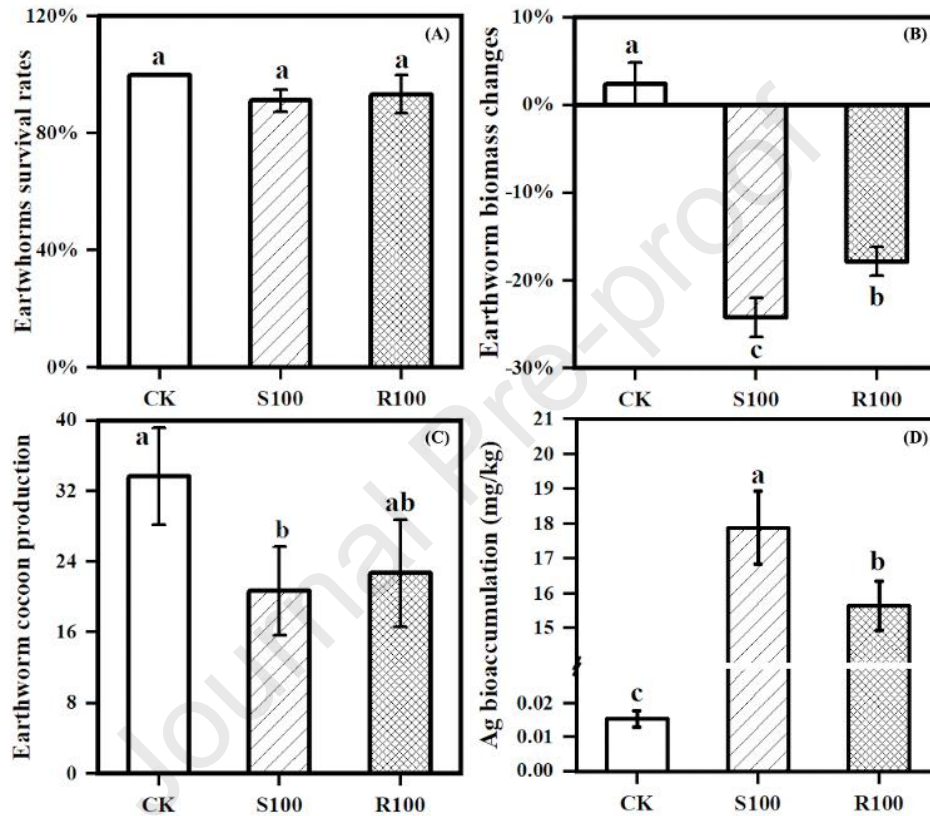
## 229 3. Results

### 230 3.1 Effects of AgNPs on earthworm fitness and Ag bioaccumulation

231 The survival rates, biomass changes, reproduction, and Ag bioaccumulation are  
232 shown in Fig 2. AgNPs induced slight death in some pots of step and repeated exposure,  
233 but no significance was found among different treatments (Fig 2A;  $P > 0,05$ ).  
234 Earthworms under treatment S100 showed the greatest 24.2% biomass loss, which was  
235 significantly higher than 17.8% of R100 earthworms (Fig 2B;  $P \leq 0,05$ ). The mean  
236 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

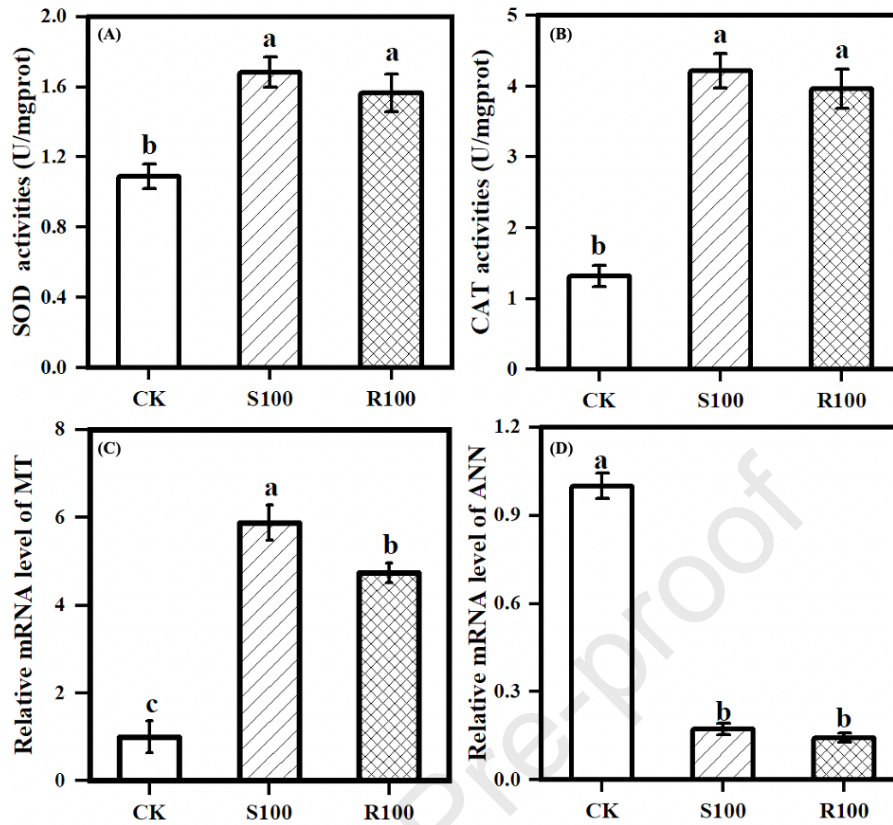
258 group, respectively (Fig 3C;  $P \leq 0,05$ ). Besides, a significant difference was also

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).



263

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

269

### 270 3.3 Results and quality of transcriptomic sequencing

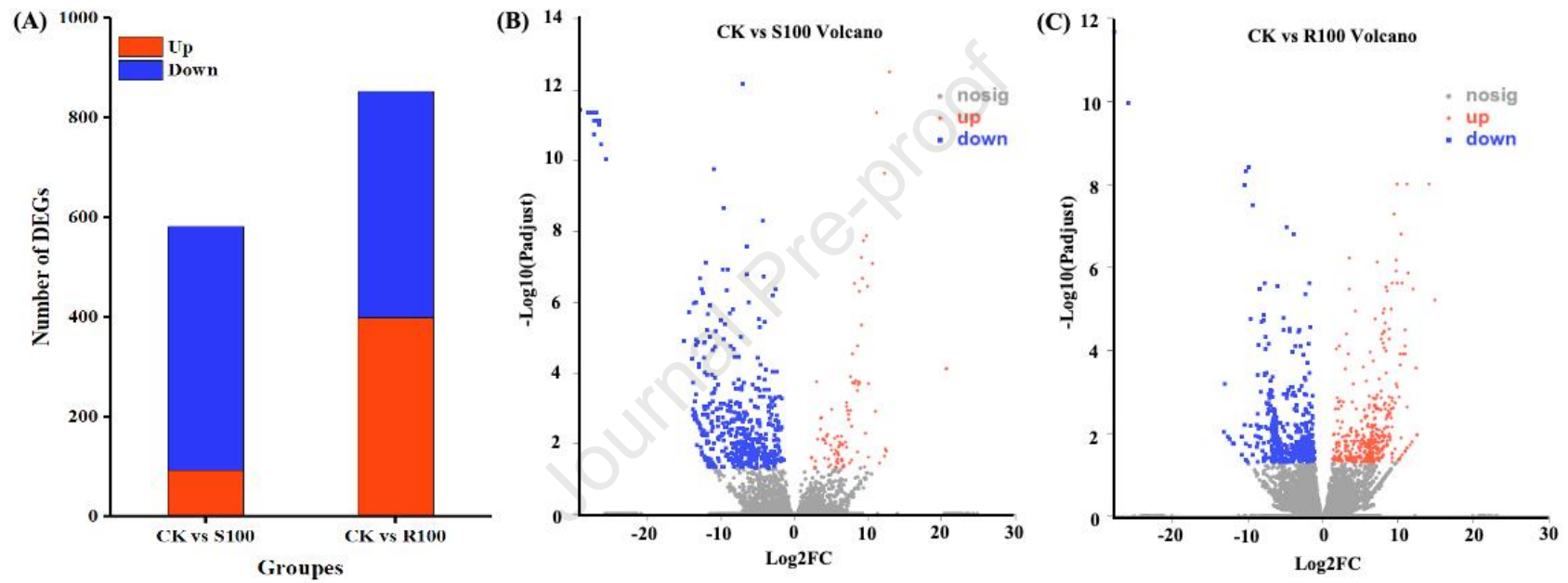
271 The transcriptomic sequencing for 9 earthworm samples generated an average of  
 272 51,198,327 clean reads with 96.8% and 91.6% high-quality reads for Q20 and Q30  
 273 values, respectively (Table S2). A total of 170,533 unigenes were identified in the

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

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

283

284



285

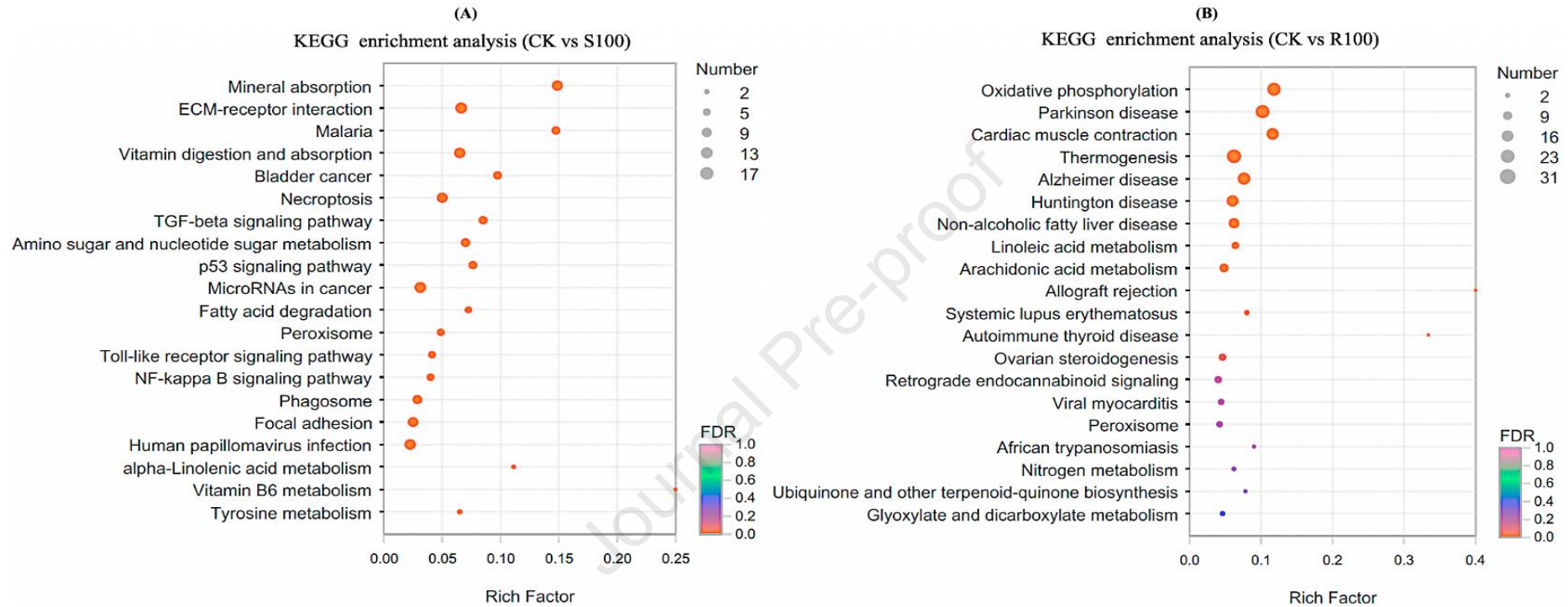
286 **Fig.4.** Volcanic figures of DEGs in earthworm *E.fetida* after the 28-day repeated and step AgNPs exposures compared with the control group. (A)

287 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



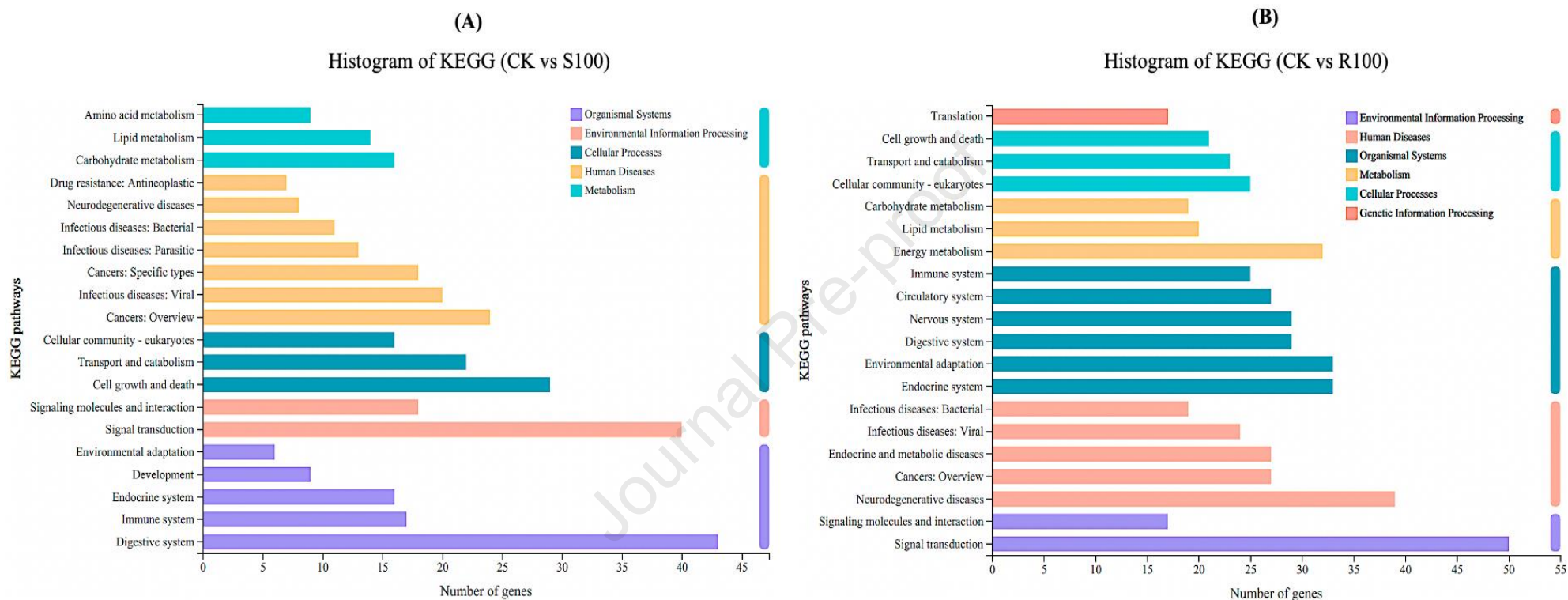
297 **Fig.5.** KEGG pathway enrichment analysis of DEGs in earthworm *E.fetida* after the 28-day repeated and step AgNPs exposures compared with

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

299 All the pathways were classified into 6 different categories, which were associated  
300 with the organism system, environmental information processing, cellular processes,  
301 human diseases, metabolism, and genetic information processing (Fig 6). For S100  
302 worms, most of the DEGs were classified into the category of human diseases (101),  
303 like the pathways of drug resistance, neurodegenerative and infectious diseases (Fig  
304 6A). 176 DEGs were classified by organism system involving the immune system and  
305 environmental adaptation, which accounted for most of the six categories. R100 worms  
306 also possessed another unique category of genetic information processing, which was  
307 translation related compared with S100 worms (Fig 6B). Moreover, the GO annotations  
308 indicated similar classifications between S100 and R100 worms, whereas R100 worms  
309 owned more pathways of biological processes, such as the multicellular organismal and  
310 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.

## 314 4. Discussion

### 315 4.1 Better fitness of earthworms under progressively repeated exposure

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

334 Ag nanoparticles and their Ag<sup>+</sup> could induce the rapid production of reactive  
335 oxygen species and thus interfere with the acceleration of antioxidant processes (Ahn  
336 et al., 2014; Jiang et al., 2014). As such, the activities of SOD and CAT were  
337 significantly enhanced after the 28-day exposure to AgNPs, which showed increasing  
338 antioxidant toxicity (Fig 3). SOD and CAT are both universal enzymes, SOD can  
339 catalyze the superoxide conversion into oxygen and hydrogen peroxide, while CAT  
340 catalyzes the decomposition of hydrogen peroxide to water and oxygen (Huang et al.,  
341 2021b). Except for SOD and CAT, many published papers also reported other sensitive  
342 antioxidant biomarkers, like malondialdehyde (MDA), glutathione S-transferase (GST),  
343 and lipid peroxidation (LPO), and it could be concluded that the most of enzyme  
344 activities were significantly enhanced with the increase in AgNPs concentrations (Bakr  
345 et al., 2023; Li et al., 2022; Singh et al., 2022). In this study, the same concentration  
346 (100 mg/kg) was applied in these two different exposure patterns, however, higher Ag  
347 bioaccumulation in S100 earthworms could also help to explain their elevated enzyme  
348 activities compared with R100 worms.

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

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

#### 359 4.2 Transcriptional mechanisms behind the earthworm AgNP toxicity and adaptation

360 Earthworms under repeated exposure initially suffered less toxic body burden and  
361 might have more time and chances of recovering and coordinating the biological  
362 processes for detoxification and adaptive responses considering the gradually increased  
363 contamination. The process of toxicity and detoxification of AgNPs in earthworms was  
364 complex as well as the result of various genes working together, and therefore  
365 transcriptional sequencing played a crucial and comprehensive role in mechanism  
366 analysis (Li et al., 2022; Qiao et al., 2022). After exposure to AgNPs, a large number of  
367 genes were up-regulated or down-regulated whether in step-exposure or repeated-  
368 exposure treatments, which implied many functional genes were involved in stress  
369 response (Fig 4). Most of the DEGs were associated with pathways of carbon  
370 metabolism, energy conversion, and disease induction. Similar results were reported by  
371 Zhao et al. (2022), which demonstrated the pathways of vitamin metabolism and  
372 mineral absorption were significantly enriched in earthworm *Metaphire guillelmi* after  
373 44 days of exposure to Pipolymyxin. The antioxidant response was known as a primary  
374 defence for those organisms under stress to protect cells and macromolecules from  
375 oxidative damage (Hu et al., 2016), whereas enough energy and material were essential

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

379 The pathway of oxidative phosphorylation (OXPHOS) was significantly enriched  
380 in R100 earthworms compared with S100 ones. OXPHOS is a typical metabolic  
381 pathway that efficiently synthesizes ATP using the energy released from oxidized  
382 nutrients. The OXPHOS inhibition was regarded as one of the major toxic pathways for  
383 the impairments of AgNPs (Saleeb et al., 2020), while this potentially explains the  
384 results of elevated OXPHOS process and toxic tolerance in earthworms under repeated  
385 exposure. Besides, the extracellular matrix (ECM) receptor interaction, a significantly  
386 enriched pathway in S100 earthworms, was possibly related to AgNPs toxicity. ECM is  
387 a connective network assembled by fibrous glycoproteins and plays a significant role  
388 in the maintenance of tissue structures, while the ECM interactions are responsible for  
389 the cell activities like differentiation, proliferation, and apoptosis (Bao et al., 2019; Zhu  
390 et al., 2021). Relatedly, a recent study also reported that differential genes in  
391 earthworms were also significantly enriched in ECM pathways after exposure to 25 and  
392 50 mg/kg flupyradifurone-contaminated soils (Qiao et al., 2022).

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

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

#### 409 *4.3 Implication of different exposure patterns for eco-toxicological risk assessments*

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

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

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

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

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



**460 Conclusion**

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

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**477 Conflict of interest**

478 There are no conflicts to declare.

479

480 **Reference**

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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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