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1           **Accumulation, speciation and localization of silver nanoparticles in the earthworm *Eisenia fetida***

2  
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31 ABSTRACT

32

33 The use of silver nanoparticles (AgNPs) in agriculture and many consumer products has led to  
34 significant release of Ag in the environment. Although Ag toxicity in terrestrial organisms has been studied  
35 extensively, very little is known about the accumulation capacity and coping mechanisms of organisms in Ag-  
36 contaminated soil. In this context, we exposed *Eisenia fetida* earthworms to artificial OECD soil spiked with a  
37 range of concentrations of Ag (AgNPs or AgNO<sub>3</sub>). The main aims were to (1) identify the location and form of  
38 accumulation of Ag in the exposed earthworms and (2) better understand the physiological mechanisms involved  
39 in Ag detoxification. The results showed that similar doses of AgNPs or AgNO<sub>3</sub> did not have the same effect on  
40 *E. fetida* survival. The two forms of Ag added to soil exhibited substantial differences in speciation at the end of  
41 exposure, but the Ag speciation and content of Ag in earthworms were similar, suggesting that biotransformation  
42 of Ag occurred. Finally, 3D images of intact earthworms obtained by X-ray micro-computed tomography  
43 revealed that Ag accumulated preferentially in the chloragogen tissue, coelomocytes and nephridial epithelium.  
44 Thus, *E. fetida* bioaccumulates Ag, but a regulation mechanism limit its impact in a very efficient manner. The  
45 location of Ag in the organism, the competition between Ag and Cu, and the speciation of internal Ag suggest a  
46 link between Ag and the thiol-rich proteins that are widely present in these tissues, most probably  
47 metallothioneins, which are key proteins in the sequestration and detoxification of metals.

48

49 Keywords

50 Silver, nanomaterials, earthworm, accumulation, speciation, X-ray absorption spectroscopy, X-ray micro-  
51 computed tomography

52

## 53 1. INTRODUCTION

54 Due to advances in nanotechnology and the increasing use of nanomaterials, metallic silver  
55 nanoparticles (AgNPs) are an emerging contaminant in the terrestrial environment (McGillicuddy et al., 2017).  
56 The incorporation of AgNPs in consumer products is increasing due to their unique properties, particularly their  
57 antimicrobial effects (Vance et al., 2015). Most silver (Ag) release occurs in municipal wastewater, and  
58 wastewater treatment plants allow efficient sequestration of Ag in sewage sludge (Kaegi et al., 2011). However,  
59 the Ag species trapped in these biosolids are subsequently spread on agricultural soil when sludge is recycled as  
60 fertilizer (Usman et al., 2012). A number of studies have shown that metallic Ag is transformed mostly into  
61 silver sulfide (Kaegi et al., 2013; Ma et al., 2014) and silver bound to thiols in the sewage system. This  
62 transformation strongly affects the behavior of Ag in the environment (Levard et al., 2012; Pradas del Real et al.,  
63 2017).

64 Another potential environmental exposure scenario in terrestrial ecosystems is the use of Ag as a  
65 nanopesticide or nanofertilizer via the direct application of metallic Ag to agricultural soils. Ag has bactericidal,  
66 fungicidal, insecticidal and herbicidal properties, and AgNPs have high inhibitory activity against crop  
67 pathogens (Chhipa, 2019; Khan and Rizvi, 2017). Moreover, AgNPs positively impact root elongation and the  
68 general growth of cultivated plants (Chhipa, 2019) when applied at concentrations between 1 and 200 ppm,  
69 depending on the plant species. However, at these concentrations, AgNPs are toxic to a variety of organisms.

70 The toxicological effects of AgNPs are quite well documented and include numerous impacts on soil  
71 microflora, flora and soil invertebrates (Courtois et al., 2019). The potential transfer of Ag in plants has received  
72 more attention (Yan and Chen, 2019) than transfer of Ag in animals. Most studies in animals have focused on  
73 life traits and protein changes in exposed animals (Yu et al., 2013). Although accumulation of Ag could be an  
74 important vector of the transfer of this metal in the trophic chain, studies of the underlying mechanisms are  
75 scarce.

76 In soil ecotoxicology, earthworms are widely studied based on their key role in most continental  
77 ecosystems and importance in the soil macrofauna. Earthworms participate in the maintenance of soil structure  
78 and fertility. In addition to enriching the soil with organic matter available for plants, they aerate the soil and  
79 promote water penetration by forming galleries during their burrowing activity (Bernard et al., 2010; Carbonell  
80 et al., 2009). Earthworms are highly consumed by birds, snakes, insectivorous mammals and rodents, especially  
81 during tillage. Consequently, accumulated contaminants can quickly move to upper trophic levels.

82 Studying Ag accumulation/defense mechanisms requires comprehensive knowledge of Ag distribution  
83 and speciation. In the present study, we investigated the accumulation, localization, and speciation of Ag  
84 (presented as NM-300K AgNPs or AgNO<sub>3</sub>) in earthworms using several X-ray techniques. The main objective  
85 was to better understand how earthworms cope with Ag soil contamination in the context of silver  
86 pesticide/fertilizer use. For this purpose, *Eisenia fetida* earthworms were exposed to artificial OECD soil  
87 contaminated with a range of AgNP concentrations for 4 weeks. The effect of ionic Ag (AgNO<sub>3</sub>) was  
88 investigated as a positive control. Biomass and mortality were followed, and Ag accumulation, speciation and  
89 localization in earthworms were measured. Finally, the accumulation mechanisms are discussed.

90

## 91 **2. MATERIALS AND METHODS**

### 92 *2.1 Test species*

93 Genetically identified *Eisenia fetida* earthworms (Homa et al., 2015) from the laboratory breeding  
94 facility (LGCgE, University of Lille) were fed cow manure *ad libitum*. Adult earthworms, clitellated or not, were  
95 randomly selected and introduced into the microcosms after being weighed individually. The earthworms  
96 weighed 296 mg on average (min: 104 mg, max: 751 mg, mean standard deviation: 95 mg).

97

### 98 *2.2. Soil*

99 Artificial soil was prepared for this experiment according to OECD guideline n° 207 (OECD, 1984) and  
100 contained 10% sphagnum peat moss, 20% kaolin clay and 70% quartz sand. The soil pH was adjusted with  
101 calcium carbonate to  $6 \pm 0.5$ . Five weeks before adding the contaminant, 16.5 kg of soil was moistened with 6  
102 liters of demineralized water. When the earthworms were added to the soil, water represented 27% of the weight  
103 of the wet soil.

104

### 105 *2.3. Silver species*

106 The standard reference material Ag-NM300K from the European Commission Joint Research Centre  
107 (JRC) was used as the AgNP source and was fully characterized in a previous work (Klein et al., 2011).  
108 Commercial NM300K-NPs were kindly provided by the Fraunhofer Institute for Molecular Biology and Applied  
109 Ecology IME. Each bottle contained 2 g of NM300K diluted in dispersant with a volume of 2 mL. These  
110 metallic nanoparticles (NPs) were spherical and not coated and were dispersed in polyoxyethylene glycerol  
111 trioleate and polyoxyethylene sorbitan mono-laurate (dispersant) with a nominal silver content of 10.2% by

112 weight. Ninety-nine percent of the particles had a nominal size below 20 nm. Transmission electron microscopy  
113 indicated a mean size of  $17 \pm 8$  nm. Smaller NPs of approximately 5 nm were also present (Mendes et al., 2015).

114  $\text{AgNO}_3$  solution was also prepared for comparison with exposure to Ag in ionic form. Silver nitrate salt  
115 ( $\text{AgNO}_3$ ) was dissolved in sterile distilled water. The AgNPs and  $\text{AgNO}_3$  solution were diluted with ultrapure  
116 water to obtain a final Ag concentration of  $2 \text{ mg mL}^{-1}$ .

117

#### 118 *2.4. Experimental scheme (earthworm exposure)*

119 Earthworms in microcosms (with OECD artificial soil) were exposed to a range of Ag forms and  
120 concentrations. Four types of soil mixtures corresponding to 2 controls and 2 exposed conditions were prepared:  
121 control (soil only), dispersant (soil spiked with dispersant solution), AgNPs (soil spiked with AgNP solution) and  
122  $\text{AgNO}_3$  (soil spiked with  $\text{AgNO}_3$  solution) (see Sup. Inf 1). Four different Ag concentrations, C1, C2, C3 and  
123 C4, were used for the AgNP and  $\text{AgNO}_3$  microcosms:  $30 (\pm 20)$ ,  $70 (\pm 10)$ ,  $120 (\pm 15)$  and  $280 (\pm 40) \text{ mg kg}^{-1}$   
124 (dry matter), respectively (these concentrations were chosen based on mortality rates reported in Garcia-Velasco  
125 et al. (2016) and Gomes et al. (2015)). Four different volumes of NM300K dispersant were used in the dispersant  
126 microcosms, which served as controls. These volumes were named D1, D2, D3 and D4 and corresponded to the  
127 dispersant volumes added in the AgNP microcosms for C1, C2, C3 and C4, respectively. Thus, a total of 13  
128 microcosm conditions were established in triplicate. Ten earthworms were introduced in each microcosm, with a  
129 total of 390 earthworms. The exposure lasted 4 weeks. No food was added to the initial soil or during exposure.

130

#### 131 *2.5. Analyses*

132 *Life traits.* Survival and biomass were measured. Biomass was followed by comparing the masses of the  
133 groups of organisms before and after exposure, and the results were expressed as the percentage loss.

134

135 *Metal concentrations in soils and accumulation in earthworms.* Immediately before exposure (T0),  
136 unexposed earthworms from the breeding facility were sacrificed to measure the metal concentrations present in  
137 the organisms. After exposure, earthworms were collected from each microcosm and placed in 1% agar for 24  
138 hours for depuration (i.e. to remove the gut content). Then, the earthworms were sacrificed by freezing for at  
139 least 48 hours and freeze-dried. The organisms were reduced to powder using liquid nitrogen and mineralized by  
140 digestion in acid medium (using  $\text{HNO}_3$ ,  $\text{H}_2\text{SO}_4$  and  $\text{HCl}_4$  in a ratio of 10:2:3) as described by Bernard et al.  
141 (2010).

142 Soil samples were collected at the beginning (T<sub>0</sub>) and at the end of exposure (T<sub>f</sub> = 4 weeks). These samples were  
143 freeze-dried and ground with a mortar and a pestle. For mineralization, 300 mg of sample was digested in 7 mL  
144 of concentrated HNO<sub>3</sub> using a Berghof microwave digestion system (speed wave MWS-2 microwave pressure  
145 digestion). The solutions obtained (mineralized earthworms and soils) were analyzed by ICP-OES (inductively  
146 coupled plasma-optical emission spectrometry) (Varian 720-ES, USA). The following classically studied metals  
147 were quantified: arsenic (As), chromium (Cr), cadmium (Cd), copper (Cu), nickel (Ni), lead (Pb), zinc (Zn) and  
148 Ag.

149

#### 150 *Localization of silver in exposed earthworms using X-ray 3D imaging*

151 Two earthworms were imaged in 3D using X-ray micro-computed tomography (micro-CT): one non-exposed  
152 sample and one sample exposed to AgNPs (AgNP-C3) collected after 4 weeks in the dispersant and AgNP  
153 microcosms, respectively.

154 *Sample preparation.* After 24 hours of depuration in 1% agar, the earthworms were first anesthetized on ice and  
155 fixed for 16 hours in ice-cold 4% paraformaldehyde in 0.1 M phosphate buffer. They were then dehydrated by  
156 soaking in a graded series of ethanol solutions (from 30% vol to 100% vol.) and subjected to supercritical point  
157 drying (Leica EM CPD300°). In this drying process, ethanol is replaced with liquid CO<sub>2</sub>, which avoids the  
158 creation of damaging surface tension forces associated with drying by bringing the liquid in the sample to the gas  
159 phase without crossing the liquid-gas phase boundary. The dried samples were finally placed in polyimide tubing  
160 (Kapton).

161 *3D image acquisition.* 3D imaging of the earthworms was performed with a microXCT-400 X-ray microscope  
162 (Zeiss). High-resolution scans were acquired at 40 kV and 250 μA. A total of 2501 projections were collected  
163 through 360° rotation with an exposure time of 20 s per projection. A 20x magnification optical objective was  
164 selected to achieved an isotropic voxel of 0.9 μm and a field-of-view (FOV) of 0.9x0.9x0.9 mm<sup>3</sup>. The FOV was  
165 centered at the lower end of the earthworm (rings 9 and 10 for the control and exposed earthworms, respectively)  
166 and included the coelomic cavity and the nephridia epithelium. The position of the FOV was selected from pre-  
167 visualization scans of the entire earthworm with lower spatial resolution (Supporting information 2). Volume  
168 reconstruction was performed with XMReconstructed-Parallel Beam-9.0.6445 software using a filtered back  
169 projection algorithm.

170 *3D image analysis.* Avizo 8.0 software (Hillsboro, OR, USA) was used for the visualization, processing, and  
171 analysis of the reconstructed dataset. The procedure developed in Chaurand et al. (2018) to isolate metal-based

172 NPs was followed. Briefly, images of exposed and non-exposed (control) samples were compared after  
173 histogram x-axis normalization (i.e. colormap normalization). The histogram represents the X-ray attenuation in  
174 each voxel (expressed as an arbitrary gray scale value, GSV) of the analyzed volume as a function of the number  
175 of voxels for each GSV (intensity). The histogram x-axis was normalized using air as an internal standard. After  
176 the normalization step, the brilliant voxels in the image of the exposed sample that were not identified in the  
177 image of the control sample were attributed to the presence of Ag by thresholding (Supporting information 3).

178  
179 *Speciation of silver in soils and earthworms.* Silver speciation in soil and earthworms was determined  
180 by X-ray absorption near-edge structure (XANES) spectroscopy, which permits the determination of the local  
181 atomic environment (speciation) of targeted atoms present in complex media. Silver K-edge (25.51 keV)  
182 XANES spectra were acquired at the European Synchrotron Radiation Facility (ESRF, France) on the FAME  
183 beamline (BM30b) with Si(220) monochromator crystals (Proux et al., 2005). Prior to analysis, the earthworm  
184 samples were lyophilized, ground and pressed into 5-mm pellets. Spectral acquisition was performed at liquid  
185 helium temperature to avoid sample evolution under the beam. Measurements were carried out in fluorescence  
186 mode using a 30-element Canberra Ge solid-state detector. Each spectrum was the sum of at least three scans. A  
187 set of model compounds including metallic AgNPs, AgNO<sub>3</sub>, Ag<sub>2</sub>S, AgCl, Ag-thiocarbamate (Ag-thio), and Ag-  
188 humic acid (Ag-HA) was run in transmission mode. Normalization data reduction and linear combination fitting  
189 (LCF) were performed according to standard methods using Athena software (Ravel and Newville, 2005). The  
190 residual factor of LCF was calculated according to the formula  $R = \frac{\sum(\text{exp} - \text{fit})^2}{\sum(\text{exp})^2}$ , where the sums are  
191 over the data points in the fitting region. At each step of the fitting, an additional reference spectrum was added  
192 if the following two conditions were true: the R factor decreased by 20% or more and the additional reference  
193 had a contribution equal to or higher than 10% among Ag species.

194  
195 *2.6. Statistical analysis*

196 For biomass, mortality and metal content in earthworms, the majority of the data did not follow a  
197 normal distribution, and the variances were not homogeneous (Shapiro, Liliefors and Bartlett tests). Thus,  
198 Sheirer-Ray-Hare non-parametric tests and post-hoc tests based on ranks were used. For data following a normal  
199 distribution with homogeneous variances, ANOVA tests and Tuckey post-hoc tests were used. Correlation  
200 matrices (based on the Kendall method) were constructed. Tests were performed using the R package (R Core  
201 Team, 2018).



202

### 203 3. RESULTS

204 *Metal quantities in soil.* At the initial time point, the concentrations of As, Cr, Cu, Ni, Pb and Zn were  
205 1.08 (standard deviation 0.34), 1.05 (s.d. 0.30), 5.31 (s.d. 1.22), 0.62 (s.d. 0.38), 10.13 (s.d. 2.11) and 5.16 (s.d.  
206 0.66) mg kg<sup>-1</sup>, respectively. The concentration of Cd was below the detection limit. The Ag concentrations in the  
207 microcosms are shown in Table 1. As expected, there was no significant difference in Ag doses between the  
208 AgNP and AgNO<sub>3</sub> microcosms at each concentration (C1, C2, C3 and C4). Only very low concentrations of Ag  
209 were detected in the control and dispersant microcosms. One control microcosm appeared to have been very  
210 slightly contaminated by accident, but its Ag concentration remained negligible compared with the exposure  
211 conditions.

212 The concentrations of other metals were very low compared with Ag, which suggests that these metals would not  
213 hinder the accumulation of Ag by earthworms.

214

215 *Life traits.* A survival rate of 100% was observed in all of the control, dispersant and AgNP microcosms  
216 (Fig. 1). In the AgNO<sub>3</sub> microcosms, dose-dependent mortality was observed, with 6.7% mortality at the lowest  
217 concentration and 100% mortality at the highest concentration. During the 4 weeks of exposure, the earthworms  
218 lost weight in all microcosms, including the controls. Thus, the loss of weight cannot be linked to Ag  
219 contamination. Biomass data for earthworms in the AgNO<sub>3</sub>-C4 microcosm are absent due to total mortality.

220

221 *Metals bioaccumulation: silver.* At the end of exposure, the Ag content in earthworms varied among the  
222 different treatments (Fig. 2). Silver was not detected in organisms in the control microcosms. In the presence of  
223 Ag, earthworms accumulated between 2.8 and 9.9 mg kg<sup>-1</sup> (average 5 mg kg<sup>-1</sup> of dry matter). The accumulation  
224 of Ag in the earthworms exposed to Ag was independent of the form of Ag (NPs or ionic) or the Ag dose in the  
225 microcosm. Data for earthworms in the AgNO<sub>3</sub>-C3 and AgNO<sub>3</sub>-C4 microcosms are absent because the  
226 insufficient quantity of material available for analysis due to significant mortality.

227

228 *Metals bioaccumulation: other metals.* No significant variations of metal quantities in earthworm bodies  
229 were observed for Cd, Cr, Ni, Pb and Zn compared with the corresponding control. A single significant  
230 difference in As concentration was observed between the AgNO<sub>3</sub>-C2 microcosm and its control (Supporting  
231 information 2). The Cu concentration in earthworms was similar in all treatments without Ag (Fig. 3). In the

232 AgNO<sub>3</sub>-C2 microcosm (no results were obtained for the AgNO<sub>3</sub>-C3 and AgNO<sub>3</sub>-C4 microcosms because of  
233 earthworm mortality), there was a decrease in the Cu concentration, but this difference was not significant  
234 compared with the control. However, in the AgNP microcosms, earthworms accumulated significantly less Cu  
235 (approximately two times less) compared with the controls.

236 In summary, Ag was the only metal present in greater concentrations in earthworms exposed to AgNPs  
237 conditions than in those under control conditions.

238  
239 *Localization of Ag in earthworms:* Brilliant voxels (i.e. voxels exhibiting high X-ray absorption) were  
240 observed in the micro-CT volume of exposed earthworms (AgNP-C3, with 109.57 (± 8.05) mg kg<sup>-1</sup> of AgNPs).  
241 Although this imaging technique cannot identify the source of these brilliant voxels, they were not observed in  
242 the non-exposed earthworm volume (Dis-D3, in dispersant) and can therefore be attributed to Ag accumulation  
243 areas by thresholding (Fig. 4) due to the absence of differences in bioaccumulation for other elements with high  
244 densities (metals). Thresholding provides the distribution of these areas of brilliant voxels (colored in red) in the  
245 whole scanned volume. Ag accumulation areas/spots were observed around the digestive tract, in the coelomic  
246 cavity, in free cells in the coelomic cavity (coelomocytes) and in the nephridial epithelium (Fig. 4).

247  
248 *Speciation of silver.* Ag speciation in OECD soil after 4 weeks of incubation depended on the initial  
249 form (NPs or ionic). Ag initially spiked as AgNPs remained mainly metallic, but approximately 15% became  
250 complexed with natural organic thiols (Fig. 5). Ag initially spiked as AgNO<sub>3</sub> was linked with humic acid (52%)  
251 and organic thiols (33%), and approximately 15% was in metallic form. Regardless of the exposure scenario  
252 (AgNPs or AgNO<sub>3</sub>), the speciation of Ag accumulated in earthworms was similar and consisted of Ag bound to  
253 thiols (Fig. 5).

254

255

#### 256 **4. DISCUSSION**

257

258 OECD soil is a simplified matrix for evaluating the effects of medium- and long-term exposure in a soil  
259 naturally deprived of many metals and other contaminants. In the present study, the use of OECD soil allowed  
260 the effects of added Ag and the underlying mechanisms to be explored under simplified experimental conditions.  
261 To prevent the possible ingestion of additional metals, no food was added to the medium. Use of this medium

262 was therefore appropriate for the main objective of our work, which was to locate the sites of Ag accumulation  
263 by micro-CT.

264 High AgNP concentrations did not affect the life traits of *E. fetida* earthworms. The weight loss  
265 observed in the AgNPs microcosms was similar to that observed under control conditions and was due to a lack  
266 of food. OECD soil is poor in organic matter and nutrients, and food was not provided during the experiment. By  
267 contrast, dose-dependent toxicity of AgNO<sub>3</sub> resulting in weight loss and mortality was observed. Thus, the  
268 toxicity of Ag<sup>+</sup> was stronger than that of AgNPs, consistent with previous observations of *E. fetida* in both  
269 artificial (Diez-Ortiz et al., 2015a; Gomes et al., 2015; Heckmann et al., 2011) and natural soils (Novo et al.,  
270 2015). Higher toxicity of ionic Ag compared with AgNPs has been reported for many plants and animal species  
271 (Courtois et al., 2019), and there is a consensus that the toxicity of Ag is mainly due to its ionic form. We  
272 recognize that the starvation of the earthworms may have interfered with the results presented here. In the  
273 presence of optimal food, the effects of the two forms of Ag might be exacerbated or reduced.

274 Despite the differences in toxicity observed between the treatments, in all conditions with Ag (ionic or  
275 NPs), the mean bioaccumulation by earthworms in the body was 4 to 5 mg kg<sup>-1</sup> (dry matter). Thus, the form of  
276 Ag (NPs or ionic) had no influence on the amount of Ag bioaccumulation. In earthworms, metal accumulation is  
277 related not only to food intake but also to dermal absorption of dissolved ions (Vijver et al., 2003). Because of  
278 the differences in Ag speciation in soil, one might expect Ag linked to organic matter (humic acids) to be  
279 metabolized more readily than thiolated Ag. When combined with the dermal absorption of Ag<sup>+</sup> ions, this  
280 increased metabolism could explain the higher toxicity of AgNO<sub>3</sub>. However, (Diez-Ortiz et al., 2015b; Garcia-  
281 Velasco et al., 2016) showed that Ag is mainly internalized by soil ingestion. Our bioaccumulation results show  
282 that Ag can enter the earthworm body. Ag may also pass through the epidermis as dissolved Ag<sup>+</sup> ions. Unrine et  
283 al. (2008) demonstrated that dermal absorption of Au nanoparticles occurs in earthworms. However, as  
284 mentioned previously, dermal absorption is not the main route of metal internalization.

285 Contradictory results were reported by Shoults-Wilson et al. (2010) and Bourdineaud et al. (2019), who  
286 showed that *E. fetida* in artificial soil accumulated two to fifteen times more silver when exposed to AgNO<sub>3</sub>  
287 compared with AgNPs. Interestingly, these authors used AgNPs that were two to three times larger (between 50  
288 and 60 nm) than the NM-300K AgNPs used in this study, which may have hindered the dermal absorption and  
289 metabolism of AgNPs. Supporting this hypothesis, Unrine et al. (2008) showed that the internalization of  
290 metallic NPs (Zn and Au) in *E. fetida* decreased with increasing NP size.

291 The Ag concentration in the soil did not influence Ag bioaccumulation by earthworms, which suggests  
292 that a very efficient regulation mechanism limits the internal content of Ag even when the environmental  
293 concentration is very high. A similar phenomenon (plateau and regulation) was reported by Coutris et al. (2011),  
294 who observed rapid Ag excretion from *E. fetida* after the end of exposure.

295 Regardless of the original form of Ag in the microcosms, the bioaccumulated Ag in earthworms was  
296 always bound to organic thiols. However, in OECD soil, even after 4 weeks of incubation, Ag speciation differed  
297 greatly between the AgNP and AgNO<sub>3</sub> microcosms, indicating biotransformation of Ag by earthworms. Since a  
298 regulation/excretion mechanism limits the Ag content in the body, it is likely that earthworms release Ag after  
299 biotransformation. Thus, soil organisms like earthworms might change the speciation of Ag in the environment  
300 and, consequently, its availability.

301 3D images of entire earthworms obtained by micro-CT showed that Ag (originating from AgNPs) was  
302 stored and/or transiting in chloragogenous tissue, coelomocytes and the nephridial epithelium. A similar result  
303 was obtained by Diez-Ortiz et al. (2015b) by X-ray chemical analysis (micro-XRF) of the internal distribution in  
304 transverse sections: Ag was observed in the gut wall, liver-like chloragogenous tissue and nephridia. These cells  
305 and organs are related to immunity and detoxification functions. Chloragogenous tissue covers the outer part of  
306 the intestine and is considered to have a liver-like function. For instance, the chloragogenous tissue accumulates  
307 wastes produced by digestion and can sequester metals (Lapied et al., 2010; Morgan and Morgan, 1993; Vijver  
308 et al., 2004). Moreover, chloragogenous tissue plays a role in earthworm immunity (Fischer, 1993).  
309 Coelomocytes are immune cells involved in the elimination of foreign bodies by phagocytosis and encapsulation  
310 (Garcia-Velasco et al., 2017), and at least some coelomocytes are derived from chloragocytes (Hamed et al.,  
311 2002). Nephridia are organs involved in osmoregulation and excretion (Davidson et al., 2013).

312 In the present study, accumulation of Ag was concomitant with a decrease in Cu accumulation. Cu is an  
313 essential metal that is specifically stored by metallothioneins (MTs). MTs, stress proteins that bind essential and  
314 non-essential metals through thiolated bonds, participate in the homeostasis of essential metals such as Zn and  
315 Cu as well as non-essential metals such as Cd or mercury (Hg) (Demuyne et al., 2006; Vijver et al., 2004).  
316 Interestingly, in earthworms, MTs are preferentially but not exclusively localized in the epithelial cells of the  
317 intestine, chloragogenous tissue, coelomocytes and nephridia (Morgan et al., 2004). According to the  
318 localization of Ag observed by micro-CT, its internal speciation (linked to a thiolated molecule), and competition  
319 with Cu, Ag is probably bound by MTs in earthworms. The same hypothesis was proposed by Baccaro et al.  
320 (2018), who also observed that bioaccumulated Ag was related to sulfur. Furthermore, in mice, Ag can bind to

321 MT with higher affinity than Cu (Sugawara and Sugawara, 1984). Consequently, Ag probably displaces Cu from  
322 MT. Moreover, Hayashi et al. (2013) and Curieses Silvana et al. (2017) observed changes in the expression of  
323 genes encoding MTs in *E. fetida* exposed to AgNPs and AgNO<sub>3</sub> (in natural and OECD soil, respectively).  
324 Therefore, it seems that MTs have a role in the detoxification mechanisms of Ag. Taken together, these results  
325 suggest a pathway for the absorption, detoxification and excretion of Ag.

326 In summary, Ag is probably mainly taken up by ingestion, absorbed by the gut and at least temporarily  
327 stored in chloragogenous tissue before detoxification. For excretion, Ag must be transferred from chloragocytes  
328 to the nephridia. Two mechanisms can be proposed. First, MT-metal complexes are discharged from the  
329 coelomic cavity and then excreted by the nephridia. This mechanism is supported by the work of Nordberg  
330 (1989) and Morgan et al. (2004), who described the capacity of MTs linked to metals to enter excretory organs in  
331 mammals and earthworms. Second, Ag could be transferred to coelomocytes in the coelomic cavity and stored,  
332 inducing the gene encoding MTH (Brulle et al., 2008). Transfer of Ag into coelomocytes can occur via two  
333 pathways: transformation of chloragocytes that have stored Ag into free coelomocyte cells or release of metal-  
334 bound MT into the coelomic cavity by chloragocytes and subsequent uptake by coelomocytes. In the event of  
335 excessive ingestion of Ag that can be not managed conventionally by storage in proteins and excretion, another  
336 mechanism might help limit the levels of Ag in the body. For example, Roubalová et al. (2018) showed that  
337 when earthworms are confronted by aggression, earthworms can expel coelomic fluid with coelomocytes via the  
338 dorsal pores. This mechanism would quickly remove from the body a large amount of metals trapped in the  
339 coelomocytes.

340

341 **Legends of table, figures and supplementary information**

342

343 *Table 1: Silver content in the microcosms at the initial time point (mean in mg kg<sup>-1</sup> of dry matter). The results*  
344 *were obtained by ICP analysis. Standard deviations are in parentheses.*

345 *Figure 1: A. Mean percentage of weight loss of the earthworm groups in the microcosms between the beginning*  
346 *and end of the experiment. B. Mean percentage of earthworm survival in the microcosms between the beginning*  
347 *and end of the experiment. The concentrations C1, C2, C3 and C4 correspond to mean concentrations of AgNO<sub>3</sub>*  
348 *and AgNPs of 33 (± 16), 71 (± 8), 117 (± 11) and 277 (± 24) mg kg<sup>-1</sup> (dry matter) (the values were not*  
349 *significantly different between the two Ag sources). Asterisks (\*) indicate statistically important differences*  
350 *between the Ag treatment and control. Lowercase letters in green indicate significant differences in biomass*  
351 *between the 4 doses of Ag (for one form of Ag). Lowercase letters in blue indicate significant differences in*  
352 *survival between the 4 doses of Ag (for one form of Ag). Uppercase letters in yellow indicate significant*  
353 *differences in biomass between the two forms of Ag (NPs or ionic), taking into account all concentrations. 'NA'*  
354 *indicates that biomass data were not available due to total mortality.*

355 *Figure 2: Ag content in earthworm bodies (mg kg<sup>-1</sup>). "AgNPs" corresponds to microcosms with silver*  
356 *nanoparticles. "AgNO<sub>3</sub>" corresponds to microcosms with silver nitrate. The concentrations C1, C2, C3 and C4*  
357 *correspond to 33 (± 16), 71 (± 8), 117 (± 11) and 277 (± 24) mg kg<sup>-1</sup> (dry matter) (the mean concentrations of*  
358 *AgNO<sub>3</sub> and AgNPs were not significantly different). Stars (\*) indicate significant differences from the associated*  
359 *control without silver. Lowercase letters in orange indicate significant differences in metal content between the 4*  
360 *concentrations (C1, C2, C3 and C4) of Ag (AgNPs and AgNO<sub>3</sub> were not compared). Uppercase letters in yellow*  
361 *indicate significant differences in Ag content between the 2 forms of Ag (NPs or ionic), taking into account all*  
362 *concentrations. 'NA' indicates that data were not available due to significant mortality.*

363 *Figure 3: Cu contents in earthworm bodies (mg kg<sup>-1</sup>). "AgNPs" corresponds to microcosms with silver*  
364 *nanoparticles. "AgNO<sub>3</sub>" corresponds to microcosms with silver nitrate. The concentrations C1, C2, C3 and C4*  
365 *correspond to 33 (± 16), 71 (± 8), 117 (± 11) and 277 (± 24) mg kg<sup>-1</sup> (dry matter) (mean concentrations of*  
366 *AgNO<sub>3</sub> and AgNPs, which were not significantly different). Stars (\*) indicate significant differences from the*  
367 *associated control without Ag. Lowercase letters in purple indicate significant differences in metal contents*  
368 *among the 4 doses of Ag (for one form of Ag). Uppercase letters in yellow indicate significant differences in Cu*  
369 *content between the 2 forms of Ag (NPs or ionic), taking into account all concentrations. Lowercase letters in*

370 black indicate significant differences in Cu content between the 2 forms of Ag at one given concentration. 'NA'  
371 indicates that data were not available due to significant mortality.

372 Figure 4: Examples of 2D orthoslices extracted from the reconstructed volume of (A) non-exposed earthworms  
373 (Dis-D3) and (B, C, D) exposed earthworms (AgNP-C3). The digestive tract (d), free cells in the coelomic cavity  
374 (coelomocytes) (c) and the nephridial epithelium (n) are indicated. The pixels colored in red in (B, C, D) in  
375 dotted circles are brilliant voxels isolated by thresholding and associated with Ag. These brilliant pixels are not  
376 observed in (A). 1 px = 0.9  $\mu\text{m}$ .

377 Figure 5: A. Linear combination fitting (LCF) of the XANES spectra of the samples collected at different time  
378 points (dotted lines) and experimental spectra (solid lines) of soil and earthworms after 4 weeks of exposure to  
379 AgNPs and AgNO<sub>3</sub>. The curves for the samples are colored as follows: dark green, soil spiked with AgNO<sub>3</sub>; dark  
380 blue, soil spiked with AgNPs; light green, earthworms exposed to AgNO<sub>3</sub>; light blue, earthworms exposed to  
381 AgNPs. B. XANES spectra of the model compounds used for LCF. Ag-HA (in yellow) was used as a proxy for Ag  
382 complexed to natural organic matter (humic acids). AgNPs (in grey) corresponds to the initial NM300K AgNPs  
383 used for the experiment. Ag-thiol (in purple) was used as a proxy for Ag bound to an organic thiol. Ag<sub>2</sub>S (in  
384 orange) corresponds to silver sulfide (acanthite mineral).

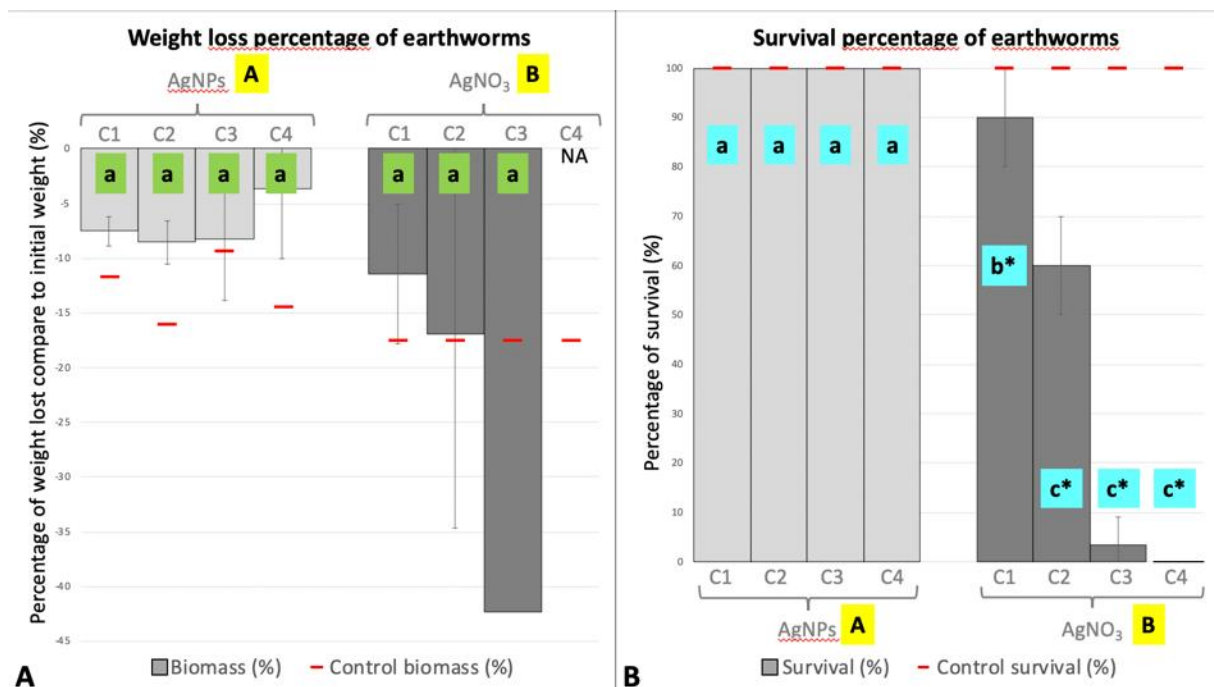
385 Sup. Inf. 1: Scheme of the experimental design. "Control" corresponds to the microcosm without any inputs.  
386 "AgNPs" corresponds to silver nanoparticles. "Dis" corresponds to dispersant. "AgNO<sub>3</sub>" corresponds to silver  
387 nitrate. The concentrations C1, C2, C3 and C4 correspond to 33 ( $\pm$  16), 71 ( $\pm$  8), 117 ( $\pm$  11) and 277 ( $\pm$  24) mg  
388 kg<sup>-1</sup> of Ag (dry matter) (the mean concentrations of AgNO<sub>3</sub> and AgNPs were not significantly different). The  
389 volumes D1, D2, D3 and D4 correspond to the volumes of dispersant added to the microcosms. Dispersant was  
390 added in the same amount as in the corresponding AgNPs microcosms, that is, 1.599, 2.666, 5.331 and 10.662  
391 mL.

392 *Sup. Inf. 2: Metal contents in earthworms (mean in mg kg<sup>-1</sup>). The results were obtained by ICP analysis.*  
 393 *“Control” corresponds to the microcosm without silver addition. “AgNPs” corresponds to silver nanoparticles.*  
 394 *“Dis” corresponds to dispersant. “AgNO<sub>3</sub>” corresponds to silver nitrate. The concentrations C1, C2, C3 and C4*  
 395 *correspond to 33 (± 20), 71 (± 10), 117 (± 15) and 277 (± 45) mg kg<sup>-1</sup> (dry matter) (the mean concentrations of*  
 396 *AgNO<sub>3</sub> and AgNPs were not significantly different). Stars (\*) indicate significant differences between the*  
 397 *condition with Ag and the associated control without Ag. Standard deviations are in parentheses.*

398 *Sup. Inf. 3: 3D imaging of an earthworm by micro-CT. (top) Selection of FOV for high-resolution micro-CT*  
 399 *scan. (bottom) 3D image analysis procedure for isolating Ag accumulation areas (normalization and*  
 400 *thresholding step).*

Concentration	Control microcosms	Dispersant	AgNPs	AgNO <sub>3</sub>
C1 (or D1)	1.95 (1.23)	0.69 (0.97)	26.33 (10.63)	39.53 (20.66)
C2 (or D2)		0.61 (1.06)	70.50 (8.15)	71.03 (8.71)
C3 (or D3)		0.18 (0.31)	109.57 (8.05)	124.30 (7.59)
C4 (or D4)		0.00 (0.00)	262.93 (27.87)	290.90 (10.81)

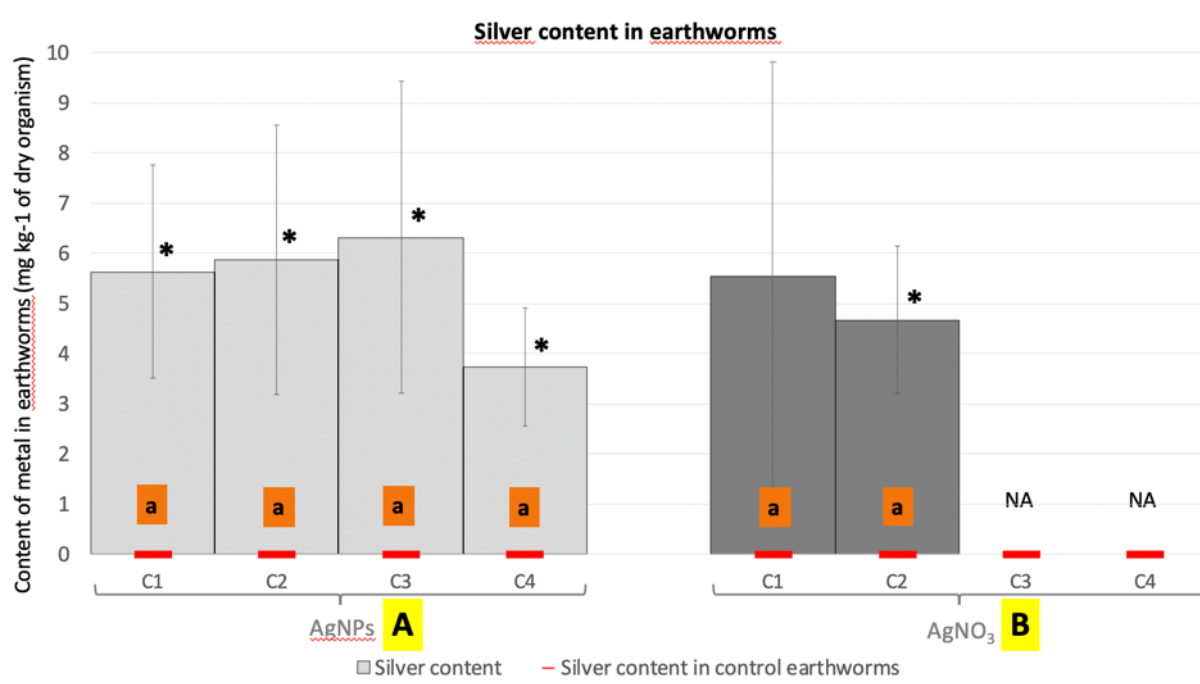
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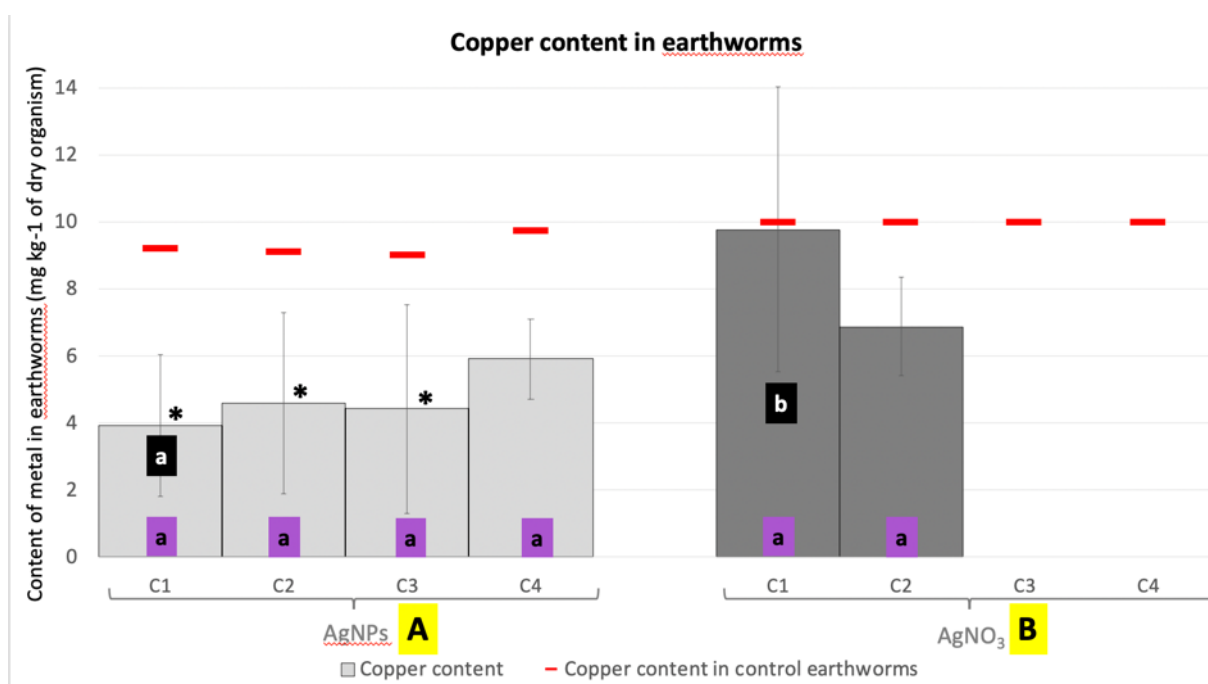
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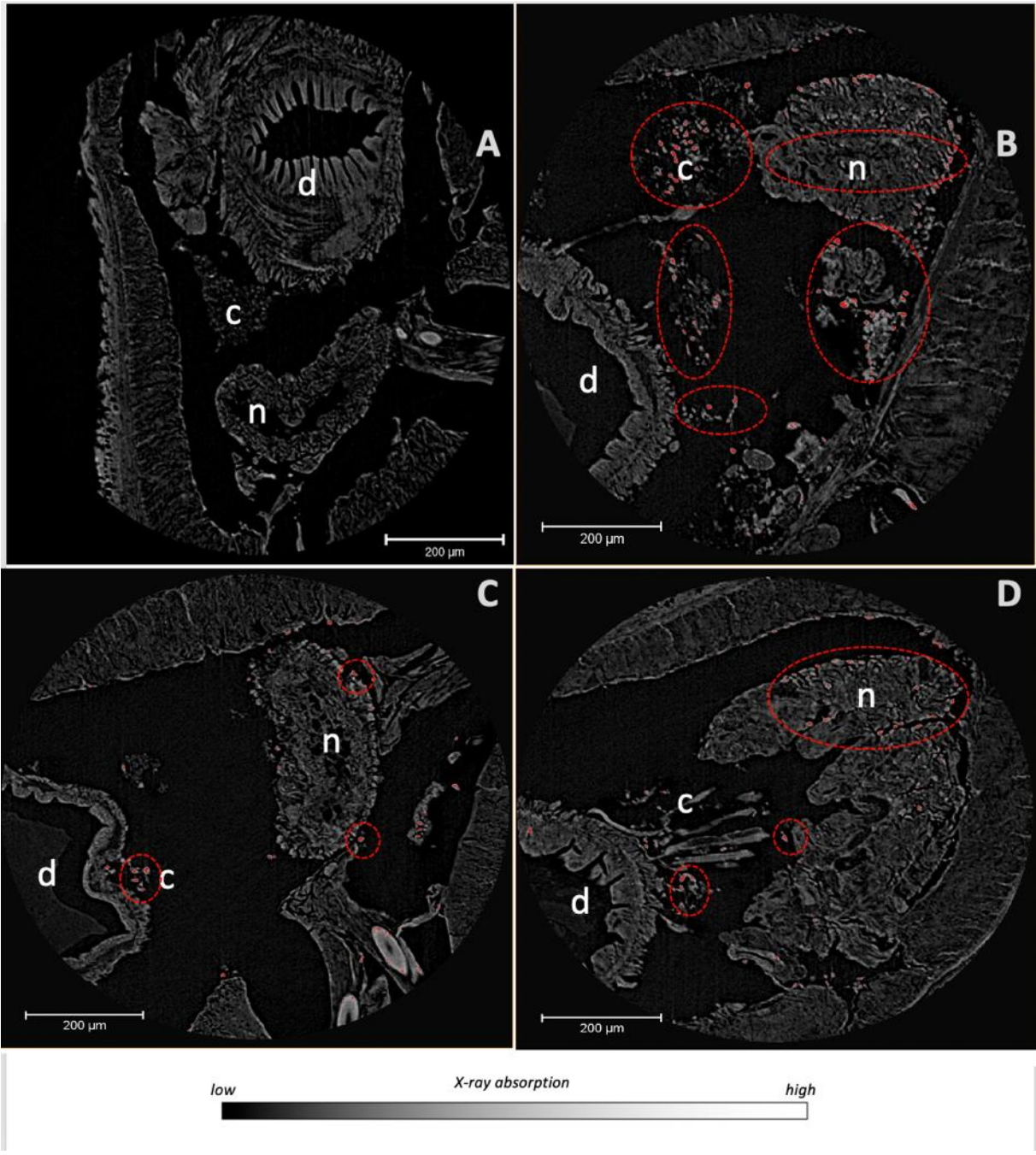
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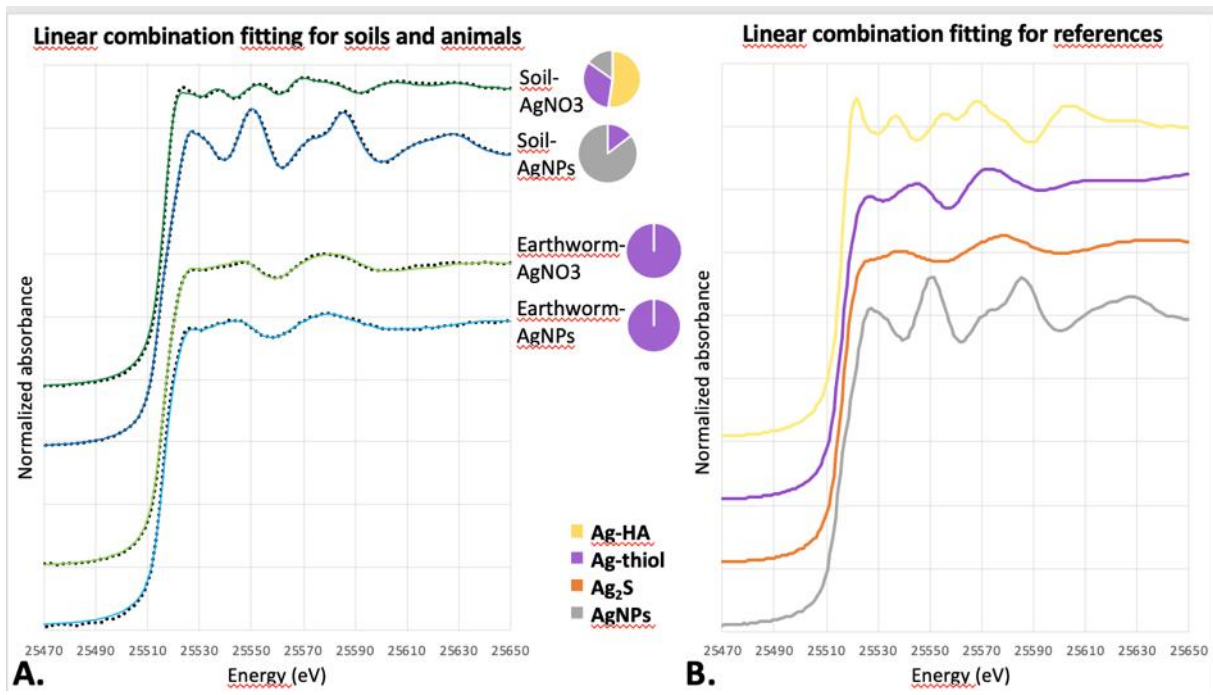
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412 **References**

- 413 Baccaro, M., Undas, A.K., de Vriendt, J., van den Berg, J.H.J., Peters, R.J.B., van den Brink, N.W., 2018.  
414 Ageing, dissolution and biogenic formation of nanoparticles: how do these factors affect the uptake  
415 kinetics of silver nanoparticles in earthworms? *Environ. Sci. Nano* 5, 1107–1116.  
416 <https://doi.org/10.1039/C7EN01212H>
- 417 Bernard, F., Brulle, F., Douay, F., Lemièrre, S., Demuyneck, S., Vandebulcke, F., 2010. Metallic trace element  
418 body burdens and gene expression analysis of biomarker candidates in *Eisenia fetida*, using an  
419 “exposure/depuration” experimental scheme with field soils. *Ecotoxicol. Environ. Saf.* 73, 1034–1045.  
420 <https://doi.org/10.1016/j.ecoenv.2010.01.010>
- 421 Bourdineaud, J.-P., Štambuk, A., Šrut, M., Radić Brkanac, S., Ivanković, D., Lisjak, D., Sauerborn Klobučar, R.,  
422 Dragun, Z., Bačić, N., Klobučar, G.I.V., 2019. Gold and silver nanoparticles effects to the earthworm  
423 *Eisenia fetida* – the importance of tissue over soil concentrations. *Drug Chem. Toxicol.* 1–18.  
424 <https://doi.org/10.1080/01480545.2019.1567757>
- 425 Brulle, F., Cocquerelle, C., Wamalah, A.N., Morgan, A.J., Kille, P., Leprêtre, A., Vandebulcke, F., 2008.  
426 cDNA cloning and expression analysis of *Eisenia fetida* (Annelida: Oligochaeta) phytochelatin synthase  
427 under cadmium exposure. *Ecotoxicol. Environ. Saf.* 71, 47–55.  
428 <https://doi.org/10.1016/j.ecoenv.2007.10.032>
- 429 Carbonell, G., Pro, J., Gómez, N., Babín, M.M., Fernández, C., Alonso, E., Tarazona, J.V., 2009. Sewage sludge  
430 applied to agricultural soil: Ecotoxicological effects on representative soil organisms. *Ecotoxicol.*  
431 *Environ. Saf.* 72, 1309–1319. <https://doi.org/10.1016/j.ecoenv.2009.01.007>
- 432 Chaurand, P., Liu, W., Borschneck, D., Levard, C., Auffan, M., Paul, E., Collin, B., Kieffer, I., Lanone, S., Rose,  
433 J., Perrin, J., 2018. Multi-scale X-ray computed tomography to detect and localize metal-based  
434 nanomaterials in lung tissues of in vivo exposed mice. *Sci. Rep.* 8, 1–11.  
435 <https://doi.org/10.1038/s41598-018-21862-4>
- 436 Chhipa, H., 2019. Applications of nanotechnology in agriculture. *Methods Microbiol.* 115–142.
- 437 Courtois, P., Rorat, A., Lemiere, S., Guyoneaud, R., Attard, E., Levard, C., Vandebulcke, F., 2019.  
438 Ecotoxicology of silver nanoparticles and their derivatives introduced in soil with or without sewage  
439 sludge: A review of effects on microorganisms, plants and animals. *Environ. Pollut.* 253, 578–598.  
440 <https://doi.org/10.1016/j.envpol.2019.07.053>
- 441 Coutris, C., Hertel-Aas, T., Lapied, E., Joner, E.J., Oughton, D.H., 2011. Bioavailability of cobalt and silver  
442 nanoparticles to the earthworm *Eisenia fetida*. *Nanotoxicology* 6, 186–195.  
443 <https://doi.org/10.3109/17435390.2011.569094>
- 444 Curieses Silvana, P., García-Velasco, N., Urionabarrenetxea, E., Elena, S.M., Bilbao, E., Di Marzio Walter, D.,  
445 Soto, M., 2017. Responses to silver nanoparticles and silver nitrate in a battery of biomarkers measured  
446 in coelomocytes and in target tissues of *Eisenia fetida* earthworms. *Ecotoxicol. Environ. Saf.* 141, 57–  
447 63. <https://doi.org/10.1016/j.ecoenv.2017.03.008>
- 448 Davidson, S.K., Powell, R., James, S., 2013. A global survey of the bacteria within earthworm nephridia. *Mol.*  
449 *Phylogenet. Evol.* 67, 188–200. <https://doi.org/10.1016/j.ympev.2012.12.005>
- 450 Demuyneck, S., Grumiaux, F., Mottier, V., Schikorski, D., Lemièrre, S., Leprêtre, A., 2006. Metallothionein  
451 response following cadmium exposure in the oligochaete *Eisenia fetida*. *Comp. Biochem. Physiol.*  
452 *Toxicol. Pharmacol. CBP* 144, 34–46. <https://doi.org/10.1016/j.cbpc.2006.05.004>
- 453 Diez-Ortiz, M., Lahive, E., George, S., Ter Schure, A., Van Gestel, C.A.M., Jurkschat, K., Svendsen, C.,  
454 Spurgeon, D.J., 2015a. Short-term soil bioassays may not reveal the full toxicity potential for  
455 nanomaterials; bioavailability and toxicity of silver ions (AgNO<sub>3</sub>) and silver nanoparticles to  
456 earthworm *Eisenia fetida* in long-term aged soils. *Environ. Pollut.* 203, 191–198.  
457 <https://doi.org/10.1016/j.envpol.2015.03.033>
- 458 Diez-Ortiz, M., Lahive, E., Kille, P., Powell, K., Morgan, A.J., Jurkschat, K., Van Gestel, C.A.M., Mosselmans,  
459 J.F.W., Svendsen, C., Spurgeon, D.J., 2015b. Uptake routes and toxicokinetics of silver nanoparticles  
460 and silver ions in the earthworm *Lumbricus rubellus*. *Environ. Toxicol. Chem.* 34, 2263–2270.  
461 <https://doi.org/10.1002/etc.3036>
- 462 Fischer, E., 1993. The myelo-erythroid nature of the chloragogenous-like tissues of the annelids. *Comp.*  
463 *Biochem. Physiol. A Physiol.* 106, 449–453. [https://doi.org/10.1016/0300-9629\(93\)90237-X](https://doi.org/10.1016/0300-9629(93)90237-X)
- 464 Garcia-Velasco, N., Gandariasbeitia, M., Irizar, A., Soto, M., 2016. Uptake route and resulting toxicity of silver  
465 nanoparticles in *Eisenia fetida* earthworm exposed through Standard OECD Tests. *Ecotoxicology* 25,  
466 1543–1555. <https://doi.org/10.1007/s10646-016-1710-2>
- 467 Garcia-Velasco, N., Peña-Cearra, A., Bilbao, E., Zaldibar, B., Soto, M., 2017. Integrative assessment of the  
468 effects produced by Ag nanoparticles at different levels of biological complexity in *Eisenia fetida*  
469 maintained in two standard soils (OECD and LUFA 2.3). *Chemosphere* 181, 747–758.  
470 <https://doi.org/10.1016/j.chemosphere.2017.04.143>

471 Gomes, S.I.L., Hansen, D., Scott-Fordsmand, J.J., Amorim, M.J.B., 2015. Effects of silver nanoparticles to soil  
472 invertebrates: Oxidative stress biomarkers in *Eisenia fetida*. *Environ. Pollut.* 199, 49–55.  
473 <https://doi.org/10.1016/j.envpol.2015.01.012>

474 Hamed, S.S., Kauschke, E., Cooper, E.L., 2002. Cytochemical Properties of Earthworm Coelomocytes Enriched  
475 by Percoll, in: *A New Model for Analyzing Antimicrobial Peptides with Biomedical Applications.*,  
476 NATO Science Series. <https://doi.org/10.3923/ijzr.2005.74.83>

477 Hayashi, Y., Heckmann, L.-H., Simonsen, V., Scott-Fordsmand, J.J., 2013. Time-course profiling of molecular  
478 stress responses to silver nanoparticles in the earthworm *Eisenia fetida*. *Ecotoxicol. Environ. Saf.* 98,  
479 219–226. <https://doi.org/10.1016/j.ecoenv.2013.08.017>

480 Heckmann, L.-H., Hovgaard, M.B., Sutherland, D.S., Autrup, H., Besenbacher, F., Scott-Fordsmand, J.J., 2011.  
481 Limit-test toxicity screening of selected inorganic nanoparticles to the earthworm *Eisenia fetida*.  
482 *Ecotoxicology* 20, 226–233. <https://doi.org/10.1007/s10646-010-0574-0>

483 Homa, J., Rorat, A., Kruk, J., Cocquerelle, C., Plytycz, B., Vandenbulcke, F., 2015. Dermal exposure of *Eisenia*  
484 *andrei* earthworms: Effects of heavy metals on metallothionein and phytochelatin synthase gene  
485 expressions in coelomocytes. *Environ. Toxicol. Chem.* 34, 1397–1404. <https://doi.org/10.1002/etc.2944>

486 Kaegi, R., Voegelin, A., Ort, C., Sinnet, B., Thalmann, B., Krismer, J., Hagendorfer, H., Elumelu, M., Mueller,  
487 E., 2013. Fate and transformation of silver nanoparticles in urban wastewater systems. *Water Res.* 47,  
488 3866–3877. <https://doi.org/10.1016/j.watres.2012.11.060>

489 Kaegi, R., Voegelin, A., Sinnet, B., Zuleeg, S., Hagendorfer, H., Burkhardt, M., Siegrist, H., 2011. Behavior of  
490 Metallic Silver Nanoparticles in a Pilot Wastewater Treatment Plant. *Environ. Sci. Technol.* 45, 3902–  
491 3908. <https://doi.org/10.1021/es1041892>

492 Khan, M.R., Rizvi, T.F., 2017. Application of Nanofertilizer and Nanopesticides for Improvements in Crop  
493 Production and Protection, in: Ghorbanpour, M., Manika, K., Varma, A. (Eds.), *Nanoscience and Plant–*  
494 *Soil Systems, Soil Biology.* Springer International Publishing, Cham, pp. 405–427.  
495 [https://doi.org/10.1007/978-3-319-46835-8\\_15](https://doi.org/10.1007/978-3-319-46835-8_15)

496 Klein, C.L., Stahlmecke, B., Romazanov, J., Kuhlbusch, T.A.J., Van Doren, E., De Temmerman, P.-J., Mast, J.,  
497 Wick, P., Krug, H., Locoro, G., Hund-Rinke, K., Kördel, W., Friedrichs, S., Maier, G., Werner, J.,  
498 Linsinger, T., Gawlik, B.M., Comero, S., Institute for Health and Consumer Protection, European  
499 Commission, Joint Research Centre, Institute for Environment and Sustainability, Institute for  
500 Reference Materials and Measurements, 2011. NM-Series of representative manufactured  
501 nanomaterials: NM-300 silver characterisation, stability, homogeneity. Publications Office,  
502 Luxembourg.

503 Lapiéd, E., Moudilou, E., Exbrayat, J.-M., Oughton, D.H., Joner, E.J., 2010. Silver nanoparticle exposure causes  
504 apoptotic response in the earthworm *Lumbricus terrestris* (Oligochaeta). *Nanomed.* 5, 975–984.  
505 <https://doi.org/10.2217/nmm.10.58>

506 Levard, C., Hotze, E.M., Lowry, G.V., Brown, G.E., 2012. Environmental Transformations of Silver  
507 Nanoparticles: Impact on Stability and Toxicity. *Environ. Sci. Technol.* 46, 6900–6914.  
508 <https://doi.org/10.1021/es2037405>

509 Ma, R., Levard, C., Judy, J.D., Unrine, J.M., Durenkamp, M., Martin, B., Jefferson, B., Lowry, G.V., 2014. Fate  
510 of Zinc Oxide and Silver Nanoparticles in a Pilot Wastewater Treatment Plant and in Processed  
511 Biosolids. *Environ. Sci. Technol.* 48, 104–112. <https://doi.org/10.1021/es403646x>

512 McGillicuddy, E., Murray, I., Kavanagh, S., Morrison, L., Fogarty, A., Cormican, M., Dockery, P., Prendergast,  
513 M., Rowan, N., Morris, D., 2017. Silver nanoparticles in the environment: Sources, detection and  
514 ecotoxicology. *Sci. Total Environ.* 575, 231–246. <https://doi.org/10.1016/j.scitotenv.2016.10.041>

515 Mendes, L.A., Maria, V.L., Scott-Fordsmand, J.J., Amorim, M.J.B., 2015. Ag Nanoparticles (Ag NM300K) in  
516 the Terrestrial Environment: Effects at Population and Cellular Level in *Folsomia candida*  
517 (Collembola). *Int. J. Environ. Res. Public Health* 12, 12530–12542.  
518 <https://doi.org/10.3390/ijerph121012530>

519 Morgan, A.J., Stürzenbaum, S.R., Winters, C., Grime, G.W., Aziz, N.A.A., Kille, P., 2004. Differential  
520 metallothionein expression in earthworm (*Lumbricus rubellus*) tissues. *Ecotoxicol. Environ. Saf.* 57,  
521 11–19. <https://doi.org/10.1016/j.ecoenv.2003.08.022>

522 Morgan, J.E., Morgan, A.J., 1993. Seasonal changes in the tissue-metal (Cd, Zn and Pb) concentrations in two  
523 ecophysiologicaly dissimilar earthworm species: pollution-monitoring implications. *Environ. Pollut.*  
524 82, 1–7. [https://doi.org/10.1016/0269-7491\(93\)90155-H](https://doi.org/10.1016/0269-7491(93)90155-H)

525 Nordberg, G.F., 1989. Modulation of metal toxicity by metallothionein. *Biol. Trace Elem. Res.* 21, 131.  
526 <https://doi.org/10.1007/BF02917245>

527 Novo, M., Lahive, E., Díez-Ortiz, M., Matzke, M., Morgan, A.J., Spurgeon, D.J., Svendsen, C., Kille, P., 2015.  
528 Different routes, same pathways: Molecular mechanisms under silver ion and nanoparticle exposures in  
529 the soil sentinel *Eisenia fetida*. *Environ. Pollut.* 205, 385–393.  
530 <https://doi.org/10.1016/j.envpol.2015.07.010>

531 OCDE, 1984. Ver de terre, essai de toxicité aigüe, Ligne directrice N°207, Ligne directrice de l'OCDE pour les  
532 essais de produits chimiques, OCDE, Paris.

533 Pradas del Real, A.E., Vidal, V., Carrière, M., Castillo-Michel, H., Levard, C., Chaurand, P., Sarret, G., 2017.  
534 Silver Nanoparticles and Wheat Roots: A Complex Interplay. *Environ. Sci. Technol.* 51, 5774–5782.  
535 <https://doi.org/10.1021/acs.est.7b00422>

536 Proux, O., Biquard, X., Lahera, E., Menthonnex, J.-J., Prat, A., Ulrich, O., Soldo, Y., Trévisson, P., Kapoujyan,  
537 G., Perroux, G., Taunier, P., Grand, D., Jeantet, P., Deléglise, M., Roux, J.-P., Hazemann, J.-L., 2005.  
538 FAME : A new beamline for X-ray absorption investigations of very-diluted systems of environmental,  
539 material and biological interests. *Phys. Scr.* 115, 970–973.  
540 <https://doi.org/10.1238/Physica.Topical.115a00970>

541 R Core Team, 2008. R: A language and environment for statistical computing. R Foundation for Statistical  
542 Computing, Vienna, Austria. URL <https://www.R-project.org/>

543 Ravel, B., Newville, M., 2005. ATHENA, ARTEMIS, HEPHAESTUS: data analysis for X-ray absorption  
544 spectroscopy using IFEFFIT. *J. Synchrotron Radiat.* 12, 537–541.  
545 <https://doi.org/10.1107/S0909049505012719>

546 Roubalová, R., Płytycz, B., Procházková, P., Navarro Pacheco, N.I., Bilej, M., 2018. Annelida: Environmental  
547 Interactions and Ecotoxicity in Relation to the Earthworm Immune System, in: Cooper, E.L. (Ed.),  
548 *Advances in Comparative Immunology*. Springer International Publishing, Cham, pp. 933–951.  
549 [https://doi.org/10.1007/978-3-319-76768-0\\_27](https://doi.org/10.1007/978-3-319-76768-0_27)

550 Shoults-Wilson, W.A., Reinsch, B.C., Tsyusko, O.V., Bertsch, P.M., Lowry, G.V., Unrine, J.M., 2010. Effect of  
551 silver nanoparticle surface coating on bioaccumulation and reproductive toxicity in earthworms (*Eisenia fetida*). *Nanotoxicology* 5, 432–444. <https://doi.org/10.3109/17435390.2010.537382>

552 Sugawara, N., Sugawara, C., 1984. Comparative study of effect of acute administration of cadmium and silver  
553 on ceruloplasmin and metallothionein: Involvement of disposition of copper, iron, and zinc. *Environ.*  
554 *Res.* 35, 507–515. [https://doi.org/10.1016/0013-9351\(84\)90157-9](https://doi.org/10.1016/0013-9351(84)90157-9)

555 Unrine, J., Bertsch, P., Hunyadi, S., 2008. Bioavailability, Trophic Transfer, and Toxicity of Manufactured  
556 Metal and Metal Oxide Nanoparticles in Terrestrial Environments, in: *Nanoscience and*  
557 *Nanotechnology*. John Wiley & Sons, Ltd, pp. 345–366. <https://doi.org/10.1002/9780470396612.ch14>

558 Usman, K., Khan, S., Ghulam, S., Khan, M.U., Khan, N., Khan, M.A., Khalil, S.K., 2012. Sewage Sludge: An  
559 Important Biological Resource for Sustainable Agriculture and Its Environmental Implications. *Am. J.*  
560 *Plant Sci.* 03, 1708–1721. <https://doi.org/10.4236/ajps.2012.312209>

561 Vance, M.E., Kuiken, T., Vejerano, E.P., McGinnis, S.P., Hochella, M.F., Rejeski, D., Hull, M.S., 2015.  
562 Nanotechnology in the real world: Redeveloping the nanomaterial consumer products inventory.  
563 *Beilstein J. Nanotechnol.* 6, 1769–1780. <https://doi.org/10.3762/bjnano.6.181>

564 Vijver, M.G., Van Gestel, C.A.M., Lanno, R.P., Van Straalen, N.M., Peijnenburg, W.J.G.M., 2004. Internal  
565 metal sequestration and its ecotoxicological relevance: a review. *Environ. Sci. Technol.* 38, 4705–4712.  
566 <https://doi.org/10.1021/es040354g>

567 Vijver, M.G., Vink, J.P.M., Miermans, C.J.H., van Gestel, C.A.M., 2003. Oral sealing using glue: a new method  
568 to distinguish between intestinal and dermal uptake of metals in earthworms. *Soil Biol. Biochem.* 35,  
569 125–132. [https://doi.org/10.1016/S0038-0717\(02\)00245-6](https://doi.org/10.1016/S0038-0717(02)00245-6)

570 Yan, A., Chen, Z., 2019. Impacts of Silver Nanoparticles on Plants: A Focus on the Phytotoxicity and  
571 Underlying Mechanism. *Int. J. Mol. Sci.* 20, 1003. <https://doi.org/10.3390/ijms20051003>

572 Yu, S., Yin, Y., Liu, J., 2013. Silver nanoparticles in the environment. *Env. Sci Process. Impacts* 15, 78–92.  
573 <https://doi.org/10.1039/C2EM30595J>

574  
575

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