

## Article

# Effects of silver nanoparticles on survival, biomass change and avoidance behaviour of the endogeic earthworm *Allolobophora chlorotica*

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1 **Effects of silver nanoparticles on survival, biomass change and avoidance behaviour of the**  
2 **endogeic earthworm *Allolobophora chlorotica***

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22 **Abstract**

23 Increasing commercial application of silver nanoparticles (Ag NP) and subsequent presence in  
24 wastewater and sewage sludge has raised concerns regarding their effects in the aquatic and  
25 terrestrial environment. Several studies have employed standardised acute and chronic  
26 earthworm-based tests to establish the toxicological effects of Ag NP within soil. These  
27 studies have relied heavily on the use of epigeic earthworm species which may have limited  
28 ecological relevance in mineral soil. This study assessed the influence of Ag NP (uncoated 80  
29 nm powder) and AgNO<sub>3</sub> on survival, change in biomass and avoidance behaviour in a soil  
30 dwelling (endogiec) species, *Allolobophora chlorotica*. Earthworms were exposed for 14 days  
31 to soils spiked with Ag NP or AgNO<sub>3</sub> at 0, 12.5, 25, 50 and 100 mg kg<sup>-1</sup> either separately for  
32 survival and biomass measurement, or combined within a linear gradient to assess avoidance.  
33 Avoidance behaviour was shown to provide the most sensitive endpoint with an observable  
34 effect at an Ag NP / AgNO<sub>3</sub> concentration of 12.5 mg kg<sup>-1</sup> compared with 50 mg kg<sup>-1</sup> for  
35 biomass change and 100 mg kg<sup>-1</sup> for survival. Greater mortality was observed in AgNO<sub>3</sub>  
36 (66.7%) compared with Ag NP-spiked soils (12.5%) at 100 mg kg<sup>-1</sup>, attributed to increased  
37 presence of silver ions. Although comparison of results with studies employing *Eisenia fetida*  
38 and *Eisenia andrei* suggest that the *A. chlorotica* response to Ag NP is more sensitive, further  
39 research employing both epigeic and endogiec earthworms under similar experimental  
40 conditions is required to confirm this observation.

41

42 **Keywords :** *Allolobophora chlorotica*; Avoidance behaviour; Ecotoxicology; Linear gradient;  
43 Silver nanoparticles

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## 49 Introduction

50 Use of engineered nanomaterials has expanded rapidly in the last decade. Proven  
51 antimicrobial properties of silver nanoparticles (Ag NP) have resulted in their use in a range  
52 of products including clothes, cosmetics, food packaging and medical devices (Rayner et al.,  
53 2010) with Ag NPs constituting almost 25% of nanomaterials used in commercial products  
54 (Vance et al., 2015). This has led to an increased Ag NP presence in wastewater and sewage  
55 sludge (biosolids), with the latter spread onto agricultural fields as a fertilizer and organic  
56 amendment in many countries (European Commission, 2016; United States Environmental  
57 Protection Agency, 2016). Recent studies have suggested that the application of biosolids to  
58 soils is a major contaminant pathway which has increased concerns on the effects of  
59 nanomaterials in the terrestrial environment (Kwak and An, 2015).

60

61 The presence of nanomaterials in the environment is difficult to quantify and has led to a  
62 reliance on modelling Predicted Environmental Concentrations (PECs). Sun et al. (2014)  
63 predicted levels of Ag NP in biosolids in the EU ranging between 0.01 and 0.08 mg kg<sup>-1</sup>, with  
64 annual increases in sludge-treated soil of 0.08 to 0.65 µg kg<sup>-1</sup>. While current predicted figures  
65 may indicate low concern, accumulation in the environment seems inevitable. This has led to  
66 a focus on the toxicological effects of Ag NP on aquatic and terrestrial species, such as  
67 earthworms.

68

69 Silver ions (Ag<sup>+</sup>) are one of the most toxic forms of heavy metal (Ratte, 1999) and it is  
70 suggested that Ag NP toxicity in earthworms results mainly from the release of such ions  
71 through dissolution / oxidation (Shoults-Wilson et al., 2011a). However, it is also recognised  
72 that physico-chemical properties, including size, shape and coating can influence Ag NP  
73 toxicity (Makama et al., 2016). For example, Schlich et al. (2013) reported that coated Ag NPs  
74 (utilised in an experiment by Shoults-Wilson et al., 2011b) were ten times less toxic to  
75 earthworms than uncoated Ag NPs. Exposure to Ag NPs is known to induce production of  
76 reactive oxygen species and cause oxidative stress in several organisms (e.g. *Caenorhabditis*  
77 *elegans*, Ahn et al., 2014). Gomes et al. (2015) demonstrated that Ag NPs caused oxidative  
78 stress in the earthworm *Eisenia fetida*, where effects were partially attributed to release of  
79 Ag<sup>+</sup> ions, but it was also determined that specific “particle effects” could not be excluded. It

80 has been suggested that, in a “natural soil environment”, soil type may be a more important  
81 factor determining toxicity than particle properties (Shoults-Wilson et al., 2011b). In soil, a  
82 range of environmental factors including dissolved oxygen levels, humidity, pH and  
83 concentration of organic compounds (Reidy et al., 2013) can influence Ag NP stability. For  
84 example, humic acid may cause the formation of Ag NP through reduction of Ag<sup>+</sup> ions (Akaighe  
85 et al., 2011) and increase Ag NP mobility (Sagee et al., 2012).

86 In ecological assessment of soil, earthworms are considered sentinel organisms (Butt and  
87 Lowe, 2010). A recent review of the ecotoxicological effects of nanomaterials on earthworms  
88 by Kwak and An (2015) showed, from 2008 to 2015, that thirty-six nano-toxicological studies  
89 utilising earthworms were reported. Seventy-two percent (n =26) of these were performed  
90 with the epigeic species *E. fetida* as recommended in OECD (1984), USEPA (2012) and ISO (ISO  
91 17512-1:2008, ISO 11268-1:2012) standardised acute and chronic tests. Studies have  
92 investigated a range of different parameters/endpoints including mortality (Shoults-Wilson  
93 et al., 2011a), reproduction (Shoults-Wilson et al., 2011b; Schlich et al., 2013; van der Ploeg  
94 et al., 2014), growth (Shoults-Wilson et al., 2011a), uptake (Coutris et al., 2011; Diez-Ortiz et  
95 al., 2015), molecular response (Tsyusko et al., 2012; Hayashi et al., 2013, Novo et al., 2015)  
96 and avoidance behaviour (Shoults-Wilson et al., 2011c; Velicogna et al., 2016). However, the  
97 continued use of epigeic species in ecotoxicology is questioned (Spurgeon et al., 2003; Lowe  
98 and Butt, 2007). Epigeic species do not inhabit the soil and have a limited distribution often  
99 directly associated with surface organic matter, and as a result have limited ecological  
100 relevance when assessing soil quality. Therefore, the use of soil dwelling species (endogeic  
101 and anecic) is increasingly advocated (Spurgeon et al., 2003; Svendsen et al., 2005; Suthar et  
102 al., 2008). In addition, Suthar et al. (2008) suggested that endogeic species are more  
103 susceptible to soil contaminants.

104 Several studies have suggested that earthworm avoidance of contaminants may be a more  
105 sensitive endpoint than more established biological parameters such as mortality, growth or  
106 reproduction (e.g. Shoults-Wilson et al., 2011c). A standardized avoidance test (ISO 17512-1,  
107 2008) was developed to assess the impact of test substances on avoidance behaviour utilising  
108 epigeic species *E. fetida* and *E. andrei*. This standard details methods for both a two- and a  
109 six-section test. However, the latter is rarely used as it is difficult to establish and Schaefer  
110 (2003) questioned the validity of the test because earthworm movement was restricted to

111 directly adjacent chambers. More recently, Lowe et al. (2016) developed an avoidance test  
112 that provided a linear pollution gradient within rectangular mesocosms. This design is easier  
113 to establish than the six-section test and allows for a larger range of concentrations than the  
114 two-section design, with the use of soil dwelling earthworms species as test organisms.

115 The main aim of this study was to assess the influence of Ag NP on a geophagous, soil dwelling  
116 earthworm, namely, *Allolobophora chlorotica*. The work also sought to provide further  
117 information on the mechanism of toxicity (the role of nanoparticles) by including comparative  
118 assessment with silver nitrate (AgNO<sub>3</sub>). Earthworm survival, change in biomass and silver  
119 uptake were determined in soil spiked with either Ag NP or AgNO<sub>3</sub> concentrations, and  
120 avoidance behaviour was recorded in a linear concentration gradient.

## 121 **Materials and Methods**

122 The endogeic earthworm *Allolobophora chlorotica* was selected because it is relatively  
123 common in the UK (Sims and Gerard, 1999) and has been the subject of a number of  
124 ecological/life cycle studies (e.g. Lowe and Butt, 2008). Moreover, this species has previously  
125 been used in ecotoxicology tests (Homa et al., 2010). Adult (clitellate) and sub-adult  
126 individuals of similar biomass were selected from stock culture at the University of Central  
127 Lancashire.

### 128 *Experiment 1: Uptake, survival and change in biomass of A. chlorotica under a range of Ag* 129 *NP concentrations*

130 In this initial, developmental experiment, the effect of ten Ag NP treatments (0, 1, 5, 10, 50,  
131 125, 250, 500, 750 and 1000 mg kg<sup>-1</sup>) was assessed on *A. chlorotica* survival and change in  
132 biomass over a 14-day period. The substrate selected for use in this study was Kettering loam  
133 (composition: Clay 24%, Silt 18%, Sand 58%, Organic content 6.72%, pH 6.8), obtained from  
134 Boughton Loam Ltd. Kettering loam has been widely used in earthworm research (e.g. Ellis *et*  
135 *al.*, 2010; Lowe et al., 2016) and is recommended for culture of soil dwelling species (Lowe  
136 and Butt, 2005). Uncoated, spherical 80 nm Ag NP powders (99.9 % purity) were purchased  
137 from GetNanoMaterials, Saint-Cannat, France. Particle size distribution was confirmed from  
138 manufacture certification. The soil was spiked with Ag NPs following the ISO11268-1:2012  
139 recommendation for testing insoluble substances. A 500 g dry mass of each Ag NP soil

140 treatment was established. Ag NP was first added (in given amounts to achieve the desired  
141 concentrations, e.g. 0.5 g of Ag NP for the 1000 mg kg<sup>-1</sup> dose) to 50 g of oven-dried quartz  
142 sand (≤ 2 mm) in a 50 ml sealed plastic centrifuge tube and agitated for 1 h using a laboratory  
143 flask shaker. The Ag NP and sand mixture was then added to the other dry constituents (see  
144 below) in a 25 l plastic container. A plastic rod was used to thoroughly mix the experimental  
145 substrate and water gradually added. The final substrate was composed of 10% by mass of  
146 the nanoparticle and sand mixture, 88% dried Kettering loam and 2% dried and sieved organic  
147 matter (horse manure). The substrate was rewetted to a moisture content of 25% (Lowe and  
148 Butt, 2005) and 450 g (wet weight) of each Ag NP treatment placed in an opaque plastic 750  
149 ml volume container. Earthworms had their mass determined and 5 individuals were added  
150 to the surface of each treatment (this initial experiment had no replication). Each container  
151 was covered with a lid, pierced with a mounted needle to allow ventilation, and kept in  
152 constant darkness in a temperature-controlled incubator at 15 °C. Treatments were sampled  
153 after 7 and 14 days, at which point earthworm survival was recorded, individuals washed,  
154 carefully blotted dry and mass re-determined. At the end of the experiment, three samples  
155 of each soil treatment were air-dried for 24 h and pH assessed using a Hanna pH meter in  
156 accordance with ISO 10390: 2005.

157

158 *Experiment 2: Ag NP / AgNO<sub>3</sub> avoidance response and effects of exposure on survival and*  
159 *change in biomass in A. chlorotica*

160 *A. chlorotica* were exposed to 5 concentrations (0, 12.5, 25, 50 and 100 mg kg<sup>-1</sup> – selected  
161 from results of experiment 1) - of either Ag NP or AgNO<sub>3</sub> powder (99.9+% ultra-pure, Alfa  
162 Aesar) in sequential linear gradients (to assess avoidance) and separately in mesocosms (to  
163 assess survival and change in biomass).

164 The linear gradients (Figure 1) were created in opaque plastic containers (0.6 m x 0.13 m x 0.1  
165 m), filled to a depth of 0.085 m with soil spiked with either Ag NP or AgNO<sub>3</sub> (n=5 replicates of  
166 each). The 5 Ag NP/AgNO<sub>3</sub> treatments were established sequentially in equal soil volumes  
167 (0.12 m x 0.13 m x 0.085 m) within the container. The spiked soil was prepared (a single batch  
168 of 9.5 kg dry weight for each dose) using the same protocol as Experiment 1 with the  
169 exception that the 50 g sand and Ag NP / Ag NO<sub>3</sub> component was mixed with the remainder

170 of the sand (900 g) in a sealed plastic bag before being added to the other constituents. Plastic  
171 spacers (cut with a laser to the dimensions of the container) initially separated each treatment  
172 in the linear gradient. Adult (clitellate) *A. chlorotica* had their mass determined and a single  
173 individual was placed on the surface at the centre of each gradient section. Once all  
174 earthworms had burrowed into the substrate, the spacers were removed and the containers  
175 covered with cling film, pierced with a mounted needle to allow ventilation and kept in  
176 darkness in a temperature-controlled incubator at 15 °C for 14 days. After this period, spacers  
177 were re-inserted and earthworm positions established by destructive sampling. Any  
178 earthworms cut by a spacer were recorded as half retrieved from each section adjacent to  
179 the spacer. The linear gradient methodology was adapted from Lowe et al. (2016). The 14  
180 day experimental period is significantly longer than the 2 day period utilised in standardised  
181 avoidance tests and allowed individual earthworms to move throughout the soil gradient.

182

183 In addition, three *A. chlorotica* were placed in circular (300 ml) clear plastic pots containing  
184 250 g of soil, individually spiked with the same concentrations of either Ag NP or AgNO<sub>3</sub> used  
185 in the linear gradient: 0, 12.5, 25, 50 and 100 mg kg<sup>-1</sup> (prepared as previously described). After  
186 earthworm introduction, the pots were maintained in the same environmental conditions as  
187 described for Experiment 1 with five replicates for each treatment (the 0 mg kg<sup>-1</sup> treatment  
188 serving as a control for both types of silver). Individual earthworm biomass was recorded at  
189 day 0 and 14. Soil pH and concentration of total silver present in earthworms and in soil was  
190 recorded after 14 days as described for Experiment 1.



191



192 *Figure 1. Linear gradient mesocosm (0.6 m x 0.13 m x 0.1 m) with nominal Ag NP / AgNO<sub>3</sub>*  
193 *concentrations (mg kg<sup>-1</sup>) used in the Experiment 2 avoidance test. Each treatment occupied*  
194 *an equal soil volume (0.12 m x 0.13 m x 0.085 m).*

195

#### 196 *Soil and Earthworm analysis*

197 At the end of the experiments, the concentration of total silver in soil treatments and  
198 earthworms was analysed using an Inductively Coupled Plasma Mass Spectrometer (ICP-MS)  
199 (X Series, Thermo Electron Corporation). For silver analysis of soil, 3 samples from each soil  
200 treatment were oven-dried at 80 °C for 24 h and a known mass of dried soil (approx. 0.1 g)  
201 placed in microwave vessels for digestion. After adding 10 mL of 70% nitric acid (Analytical  
202 reagent grade, Fisher), the vessel contents were digested using a microwave (Ethos EZ,  
203 Microwave digestion system, Milestone). Samples were gradually heated to 120 °C and after  
204 a 10 min plateau, allowed to cool for 30 min before dilution by a factor of 100 with ultra-pure  
205 water and ICP-MS analysis. The instrument was recalibrated after each run with a multi-  
206 element standard solution (Fluka Analytical). For tissue analysis, earthworms were kept for  
207 48 h on moist filter paper to void gut contents. Individuals were then “snap-frozen” at - 80 °C  
208 for 1 h and placed for 24 h in a freeze-dryer (Scanvac). Samples were ground and total silver  
209 concentration analysed by ICP-MS following the same protocol as described for soil. Only  
210 earthworms alive at the end of the experiments were assessed for Ag concentration because  
211 of difficulties associated with post-mortem removal of soil present in the digestive tract of  
212 dead earthworms.

#### 213 *Statistical analysis*

214 Statistical analyses were performed with Minitab software (Version 17). One-way ANOVA  
215 followed by a Tukey post-hoc test was used to analyse biomass results in the first experiment.  
216 In experiment 2, a Kruskal Wallis test, followed by a post hoc test executed using Mann  
217 Whitney with a Bonferroni correction, was used to analyse differences in earthworm location  
218 in the linear gradient. Welch’s One-way ANOVA followed by a Games-Howell post hoc test  
219 was used to analyse earthworm biomass results (at day 0 and day 14) across the 5 separate  
220 Ag NP/AgNO<sub>3</sub> concentrations. A Welch’s 2 sample t-test was used to analyse differences in

221 earthworm biomass between day 0 and day 14 in each Ag NP / AgNO<sub>3</sub> concentration and  
 222 between each concentration level for Ag NP and AgNO<sub>3</sub> at day 14. A Student's 2 sample-t-test  
 223 was also used to analyse differences in uptake of total Ag by earthworms in Ag NP and AgNO<sub>3</sub>  
 224 treatments at 12.5, 25, 50 and 100 mg kg<sup>-1</sup>. Where parametric tests have been applied, the  
 225 Anderson-Darling Normality test and Levene's test for equality of variances were used to  
 226 validate that test assumptions had been met. For all statistical tests a significance level of 5%  
 227 (p = 0.05) was applied.

## 228 Results

### 229 *Experiment 1: Uptake, survival and change in biomass of A. chlorotica under a range of Ag* 230 *NP concentrations*

231 After 14 days, 100% survival was recorded in Ag NP concentrations 0-50 mg kg<sup>-1</sup>, 60% survival  
 232 in 125 mg kg<sup>-1</sup> and 0% survival at concentrations of 250-1000 mg kg<sup>-1</sup> (Table 1) with dead  
 233 earthworms mainly located on the substrate surface. Significant decreases in *A. chlorotica*  
 234 biomass were recorded at 50 and 125 mg kg<sup>-1</sup> (p < 0.05) but could not be assessed at Ag NP  
 235 concentrations above 125 mg kg<sup>-1</sup> due to earthworm mortality (see Figure 2).

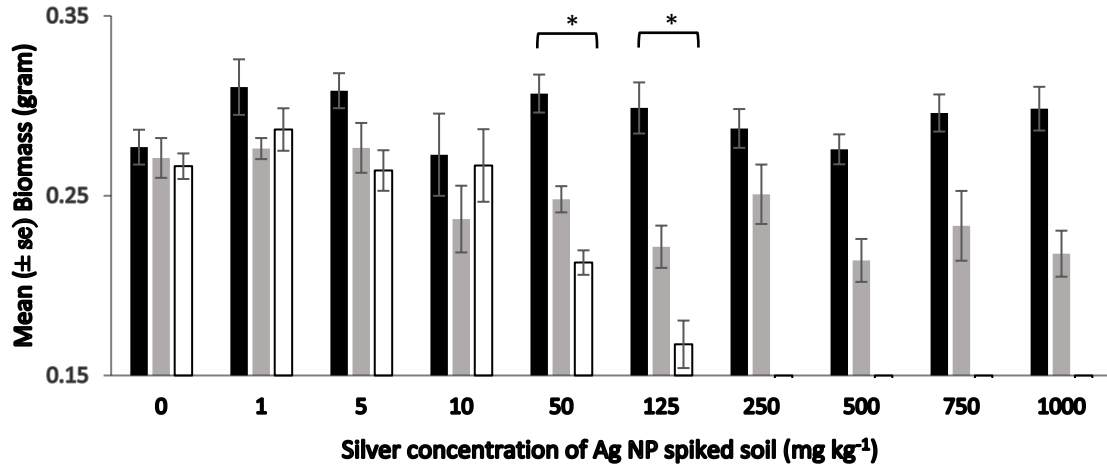
236 Total silver concentration in earthworms increased with silver concentration in the soil (Table  
 237 1).

238 *Table 1. Total silver (Ag) concentration (mg kg<sup>-1</sup>) in experimental soil and earthworm tissue*  
 239 *(± Standard error), soil pH and earthworm survival after 14 days exposure to Ag NP.*

[Ag] Nominal concentration (mg kg <sup>-1</sup> )	Mean [Ag] concentration in soil (mg kg <sup>-1</sup> )	Soil pH	Mean [Ag] concentration in <i>A. chlorotica</i> (mg kg <sup>-1</sup> )	% Survival
0	0.38 ± 0.24	8.11 ± 0.1	0	100
1	2.24 ± 0.92	8.03 ± 0.07	0	100
5	6.57 ± 0.33	8.13 ± 0.03	0.04 ± 0.01	100
10	7.68 ± 0.62	7.88 ± 0.01	0.06 ± 0.01	100

50	41.44 ± 2.62	7.85 ± 0.03	1.36 ± 0.17	100
125	132.98 ± 26.18	7.78 ± 0.03	1.83 ± 0.35	60
250	310.38 ± 8.88	7.97 ± 0.01	-	0
500	528.4 ± 13.56	7.92 ± 0.06	-	0
750	564.9 ± 23.83	7.86 ± 0.03	-	0
1000	1156.6 ± 77.02	8.01 ± 0.04	-	0

240



241

242 *Figure 2. Mean (± standard error) biomass of A. chlorotica in Ag NP spiked soil after 0 (black*  
 243 *bar), 7 (grey bar) and 14 (white bar) days. Asterisks indicate a significant difference in biomass*  
 244 *between day 0, day 7 and day 14 for each Ag NP concentration (p < 0.05).*

245

246 *Experiment 2: Ag NP / AgNO<sub>3</sub> avoidance response and effects of exposure on survival and*  
 247 *change in biomass in A. chlorotica*

248 The pH of soils spiked with Ag NP and AgNO<sub>3</sub> was between 7.77 and 8.07 (Table 2). Recorded  
 249 Ag concentrations in soils spiked with Ag NP and AgNO<sub>3</sub> were comparable with expected  
 250 concentrations (based on experimental design) and suggest that both substances were  
 251 homogenously distributed throughout the experimental substrate (Table 2).

252

253 *Table 2. Total silver (Ag) concentration (mg kg<sup>-1</sup>) (± Standard error) in soil spiked with Ag NP*  
 254 *and AgNO<sub>3</sub> and associated pH.*

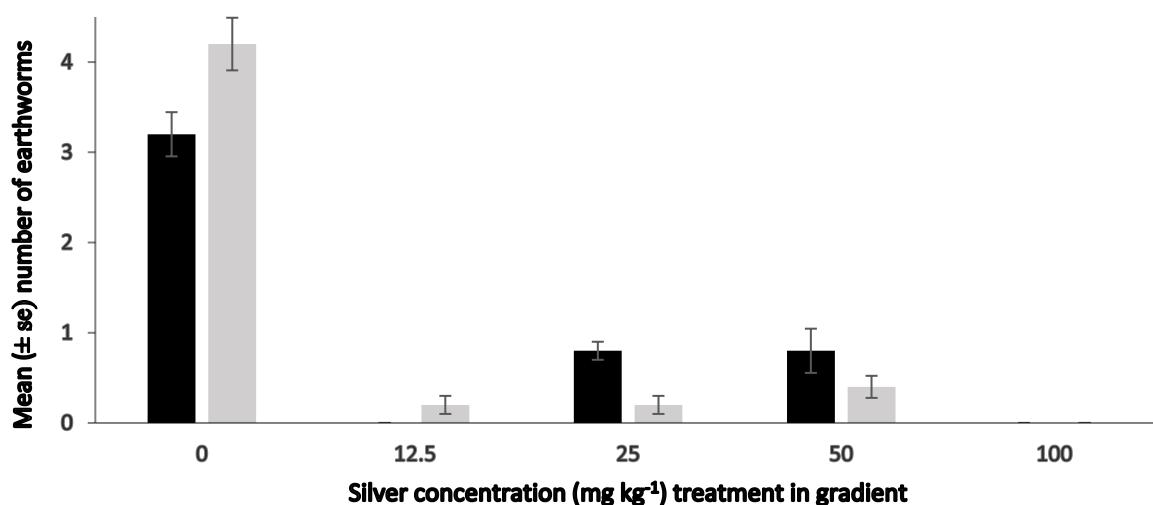
[Ag] Nominal Concentration (mg kg <sup>-1</sup> )	Mean [Ag] concentration in soil spiked with AgNP (mg kg <sup>-1</sup> )	pH	Mean [Ag] concentration in soil spiked with AgNO <sub>3</sub> (mg kg <sup>-1</sup> )	pH
0	0	7.77 ± 0.05	0	7.77 ± 0.05
12.5	8.36 ± 1.02	7.89 ± 0.02	7.19 ± 0.83	8.02 ± 0.03
25	15.55 ± 0.72	7.77 ± 0.03	18.26 ± 1.78	8.07 ± 0.01
50	37.99 ± 1.94	7.89 ± 0.01	35.27 ± 1.84	7.97 ± 0.01
100	85.39 ± 5.84	7.99 ± 0.02	81.94 ± 11.33	7.93 ± 0.05

255

256 After the avoidance test, one earthworm was not recovered. At 14 days the majority of  
 257 earthworms in both Ag NP and AgNO<sub>3</sub> treatments were retrieved from the 0 mg kg<sup>-1</sup> section  
 258 of the gradient (64 and 84% respectively) and no individuals were located in the 100 mg kg<sup>-1</sup>  
 259 section (Figure 3). There was no significant difference in the distribution of earthworms in the  
 260 Ag NP and AgNO<sub>3</sub> gradients.

261 There was a significant difference in earthworm numbers located in each section of both Ag  
 262 NP (p = 0.001) and AgNO<sub>3</sub> (p = 0.002) gradients. However, a post hoc multiple comparison test  
 263 showed no significant difference between each level of the gradient (results associated with  
 264 the conservative approach of the statistical analysis).

265



266

267 Figure 3. Mean ( $\pm$  standard error) number of *A. chlorotica* recorded in each section of Ag NP  
 268 (black bar) and AgNO<sub>3</sub> (grey bar) gradients after 14 days.

269 Note: There was an overall significant difference in earthworm numbers present in each  
 270 section of the Ag NP ( $p=0.001$ ) and AgNO<sub>3</sub> ( $p = 0.002$ ) gradients but the conservative nature  
 271 of the post-hoc Mann Whitney with Bonferroni correction statistic did not allow for significant  
 272 differences between concentrations to be stated.

273

274 In the pots, 100% survival was recorded at Ag NP and AgNO<sub>3</sub> concentrations equal to or  
 275 below 50 mg kg<sup>-1</sup> after 14 days (Table 3). However, at 100 mg kg<sup>-1</sup> survival was reduced to  
 276 87.5 and 33.3% in AgNP and AgNO<sub>3</sub> spiked soils respectively.

277

278 Table 3. Mean biomass of *A. chlorotica* in soil spiked with either Ag NP or AgNO<sub>3</sub> at day 0  
 279 and day 14, silver (Ag) concentration in earthworm tissues and earthworm survival after 14  
 280 days.

[Ag] Nominal concentration (mg kg <sup>-1</sup> )	Mean Biomass ( $\pm$ s.e.) day 0	Mean Biomass ( $\pm$ s.e.) day 14	Mean [Ag] concentration ( $\pm$ s.e.) in <i>A. chlorotica</i> (mg kg <sup>-1</sup> )	% Survival
Ag NP				
0	0.17 $\pm$ 0.01	0.21 $\pm$ 0.01 <sup>*a</sup>	0.00	100
12.5	0.18 $\pm$ 0.01	0.19 $\pm$ 0.01 <sup>ab</sup>	0.26 $\pm$ 0.03	100
25	0.17 $\pm$ 0.02	0.18 $\pm$ 0.02 <sup>abc</sup>	0.60 $\pm$ 0.05	100
50	0.19 $\pm$ 0.01	0.15 $\pm$ 0.01 <sup>*bc</sup>	0.92 $\pm$ 0.08	100
100	0.19 $\pm$ 0.01	0.13 $\pm$ 0.01 <sup>*c</sup>	1.50 $\pm$ 0.12	87.5
AgNO <sub>3</sub>				

0	0.17 ± 0.01	0.21 ± 0.01 <sup>*A</sup>	0.00	100
12.5	0.17 ± 0.02	0.17 ± 0.02 <sup>AB</sup>	0.32 ± 0.03	100
25	0.17 ± 0.01	0.13 ± 0.01 <sup>B@</sup>	1.13 ± 0.10	100
50	0.16 ± 0.01	0.11 ± 0.01 <sup>*B@</sup>	1.50 ± 0.09	100
100	0.17 ± 0.01	0.12 ± 0.01 <sup>*B</sup>	1.67 ± 0.15	33.3

281 \* denotes a significant difference in mean biomass day 0 to day 14. At day 14 mean biomass figures  
 282 that do not share a letter (lower case for Ag NP and capitals for AgNO<sub>3</sub>) are significantly different. @  
 283 denotes a significant decrease in mean biomass in AgNO<sub>3</sub> compared with Ag NP at the same nominal  
 284 Ag concentrations.

285

286 At day zero, there was no significant difference ( $p = 0.757$ ) in mean earthworm biomass across  
 287 all Ag NP and AgNO<sub>3</sub> concentrations ( $n = 9$  manipulations). However, at day 14, significant  
 288 differences in mean earthworm biomass were recorded in both Ag NP and AgNO<sub>3</sub> treatments  
 289 (see table 3). In soil spiked with Ag NP, mean biomass at 50 and 100 mg kg<sup>-1</sup> was significantly  
 290 lower than at 0 mg kg<sup>-1</sup>. In soil spiked with AgNO<sub>3</sub>, mean biomass at 25, 50 and 100 mg kg<sup>-1</sup>  
 291 was significantly lower than at 0 mg kg<sup>-1</sup>. Observed differences in biomass in the 5 Ag  
 292 concentrations in the two treatments can be directly associated with changes in mean  
 293 biomass observed within individual silver concentrations from day 0 to day 14. In both Ag NP  
 294 and AgNO<sub>3</sub> treatments, there was a significant increase ( $p < 0.05$ ) in mean earthworm biomass  
 295 at 0 mg kg<sup>-1</sup> but a significant decrease in biomass at 50 and 100 mg kg<sup>-1</sup> ( $p = 0.04$  and  $0.001$   
 296 for Ag NP and  $p = 0.001$  and  $0.001$  for AgNO<sub>3</sub> respectively).

297 Total silver concentration assessed after 14 days in earthworms exposed to Ag NP and AgNO<sub>3</sub>  
 298 suggested a positive association between concentration of silver in the soil and uptake in  
 299 earthworms (Table 3). Mean silver tissue concentrations were higher in AgNO<sub>3</sub> spiked soils  
 300 than Ag NP spiked soils at all concentration levels, however, recorded differences were not  
 301 significant ( $p > 0.05$ ).

## 302 Discussion

303 Results from the current work are compared with relevant literature and particular focus is  
 304 given to studies that employed epigeic earthworm species. However, it is important to  
 305 highlight that the validity of direct comparisons is often compromised by differences in  
 306 experimental design and in particular the use of coated NPs and standardised substrates.  
 307 Furthermore, it is recognised that stability, particle size and agglomeration can modify  
 308 biological interactions and toxicity of Ag NP (Reidy et al., 2013) and that dissolution rates can

309 be influenced by environmental conditions (e.g. soil type). It is also suggested that  
310 morphology and size of commercially available Ag NP may differ from reported manufacturer  
311 values (Foldbjerg et al., 2011). As Ag NP characterisation was not undertaken in this study a  
312 level of caution should be attributed to observed results.

313 *Effect of Ag NP and AgNO<sub>3</sub> on earthworm survival, biomass and uptake*

314 The biomass and survival results obtained in Experiment 2 are consistent with results of  
315 Experiment 1, with a significant decrease in *A. chlorotica* biomass recorded at Ag NP and  
316 AgNO<sub>3</sub> concentrations  $\geq 50 \text{ mg kg}^{-1}$  and mortality recorded at Ag NP / AgNO<sub>3</sub> concentration  
317 of  $100 \text{ mg kg}^{-1}$ . The concentrations eliciting a biomass decrease and mortality response are  
318 substantially lower than those recorded for *Eisenia spp.* in similar studies. Schlich et al. (2013)  
319 exposed *E. andrei* to Ag NP (containing a stabilising agent and with a particle size of 15 nm)  
320 and also to AgNO<sub>3</sub> and recorded no significant effect on mortality up to  $200 \text{ mg kg}^{-1}$ . Similarly,  
321 Shoults-Wilson et al. (2011a) found no influence of coated Ag NPs on *E. fetida* growth,  
322 mortality or reproduction at concentrations below  $773.3 - 801 \text{ mg kg}^{-1}$ . It is proposed that  
323 differences in behaviour were responsible for observed results. As an epigeic species, *E. fetida*  
324 is frequently found on the soil surface within organic matter and, unlike endogeic species, it  
325 is not geophagous and as a result is exposed to lower levels of soil contaminants. It is relevant  
326 at this point to refer further to Schlich et al. (2013) who interpreted the presence of *E. andrei*  
327 in organic matter (cow dung) on the soil surface as an avoidance response to Ag NP in the soil  
328 without also recognising that this is a location where this species might “naturally” occur  
329 (Sims and Gerard, 1999).

330 The enhanced negative effect of AgNO<sub>3</sub> on survival rates (33.3% at  $100 \text{ mg kg}^{-1}$ ) when  
331 compared with Ag NP (87.5%) is mirrored in other studies (e.g. Heckmann et al., 2011;  
332 Shoults-Wilson et al., 2011a; Schlich et al., 2013). Increased toxicity of AgNO<sub>3</sub> has been  
333 directly related to the presence of Ag<sup>+</sup> ions. Shoults-Wilson et al. (2011a) reported a 10-17%  
334 silver oxidation for Ag NP in OECD soil and suggested that a response for Ag NP at  $900 \text{ mg kg}^{-1}$   
335 should be similar to a response at  $100-200 \text{ mg kg}^{-1}$  for AgNO<sub>3</sub>. Furthermore, a study by  
336 Gomes et al. (2015), which investigated the effect of Ag NP and AgNO<sub>3</sub> on oxidative stress in  
337 *E. fetida*, suggested that response to AgNO<sub>3</sub> started earlier than Ag NP and was related to

338 oxidation time. This is supported by Diez-Ortiz et al. (2015) who found that toxicity of Ag NP  
339 in soils increased with time, as silver ion and AgNP effects merged to a common value.

340 *A. chlorotica* accumulated higher concentrations of Ag when exposed to AgNO<sub>3</sub> than Ag NP in  
341 accordance with results for *E. fetida* (Shoults-Wilson et al., 2011b).

#### 342 *Avoidance Behaviour*

343 Several studies have found that earthworm avoidance of contaminants can be more sensitive  
344 than traditional endpoints such as reproduction (e.g. van-Gestel, 2012). This is confirmed  
345 from results of the present study, where avoidance behaviour was observed at the lowest  
346 employed concentration of Ag NP / AgNO<sub>3</sub> (12.5 mg kg<sup>-1</sup>), whereas biomass was only effected  
347 at concentrations ≥ 50 mg kg<sup>-1</sup>. The increased sensitivity of avoidance behaviour to Ag NP is  
348 also reported by Shoults-Wilson et al. (2011c) who observed a significant avoidance response  
349 in *E. fetida* at a concentration of 6.97-7.42 mg kg<sup>-1</sup>, well below the 773.3-801 mg kg<sup>-1</sup> values  
350 reported to influence growth, mortality or reproduction in a related study under comparable  
351 conditions (Shoults-Wilson, et al., 2011a).

352 There was no significant difference in the distribution of earthworms in Ag NP and AgNO<sub>3</sub>  
353 gradients, which corroborates results of Shoults-Wilson et al. (2011c) and Velicogna et al.  
354 (2016). However, it is proposed that differences in avoidance response for *A. chlorotica* may  
355 become apparent at concentrations less than 12.5 mg kg<sup>-1</sup>. Establishing a no-observed-effect  
356 concentration (NOEC) and a lowest-observed-effect concentration (LOEC) for both chemicals  
357 should be a focus for future research and provide results that are directly related to PEC  
358 values in sewage sludge and soil. While it is clear that earthworms are able to detect the  
359 presence of Ag NP within the mineral soil, the mechanism triggering avoidance (sensory-  
360 based reaction or detrimental effect caused by uptake) is not known. It is also unclear as to  
361 whether the avoidance response is triggered solely by Ag<sup>+</sup> or if nano-particles also influence  
362 earthworm behaviour. The avoidance response observed in *E. fetida* by Shoults-Wilson et al.  
363 (2011c) was recorded over a short time period (48 h), but the study concluded that this was  
364 too short for a sufficient amount of Ag<sup>+</sup> to be oxidised from the Ag NPs. Because avoidance of  
365 Ag NP and AgNO<sub>3</sub> occurred at similar concentrations, silver ions may not have been entirely  
366 responsible for triggering avoidance behaviour with the authors questioning if nano-scale  
367 particles are able to interact with sensory structures.



368 The current study has shown that the use of soil dwelling earthworms in a linear gradient  
369 methodology developed by Lowe et al. (2016) is an effective and practical method of  
370 assessing avoidance behaviour to soil-based contaminants. Earthworm survival rates in  
371 experimental treatments also suggest that *A. chlorotica* is more sensitive than *E. fetida* to Ag  
372 NP and AgNO<sub>3</sub> pollution. However, further evidence is required to confirm if soil-dwelling  
373 earthworms are the most appropriate species to use as bioindicators of Ag NP soil pollution.  
374 This requires inclusion of a wider range of endogeic and anecic species to be studied alongside  
375 epigeic species under the same experimental conditions.

376

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