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

C. Brami, A.R. Glover, K.R. Butt, C.N. Lowe*

School of Forensic and Applied Sciences, University of Central Lancashire, Preston, PR1 2HE, United Kingdom

E-mail addresses:

Claire Brami: claire@brami.fr

Angus Glover: arglover@uclan.ac.uk

Kevin Richard Butt: krbutt@uclan.ac.uk

Christopher Nathan Lowe: cnlowe@uclan.ac.uk

* Corresponding Author

Abstract

Increasing commercial application of silver nanoparticles (Ag NP) and subsequent presence in wastewater and sewage sludge has raised concerns regarding their effects in the aquatic and terrestrial environment. Several studies have employed standardised acute and chronic earthworm-based tests to establish the toxicological effects of Ag NP within soil. These studies have relied heavily on the use of epigiec earthworm species which may have limited ecological relevance in mineral soil. This study assessed the influence of Ag NP (uncoated 80 nm powder) and $AgNO_3$ on survival, change in biomass and avoidance behaviour in a soil dwelling (endogiec) species, *Allolobophora chlorotica*. Earthworms were exposed for 14 days to soils spiked with Ag NP or $AgNO_3$ at 0, 12.5, 25, 50 and 100 mg kg^{-1} either separately for survival and biomass measurement, or combined within a linear gradient to assess avoidance. Avoidance behaviour was shown to provide the most sensitive endpoint with an observable effect at an Ag NP / $AgNO_3$ concentration of 12.5 mg kg^{-1} compared with 50 mg kg^{-1} for biomass change and 100 mg kg^{-1} for survival. Greater mortality was observed in $AgNO_3$ (66.7%) compared with Ag NP-spiked soils (12.5%) at 100 mg kg^{-1}, attributed to increased presence of silver ions. Although comparison of results with studies employing *Eisenia fetida* and *Eisenia andrei* suggest that the *A. chlorotica* response to Ag NP is more sensitive, further research employing both epigeic and endogeic earthworms under similar experimental conditions is required to confirm this observation.

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

Introduction

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

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

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

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

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

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

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

The main aim of this study was to assess the influence of Ag NP on a geophagous, soil dwelling earthworm, namely, *Allolobophora chlorotica*. The work also sought to provide further information on the mechanism of toxicity (the role of nanoparticles) by including comparative assessment with silver nitrate ($AgNO_3$). Earthworm survival, change in biomass and silver uptake were determined in soil spiked with either Ag NP or $AgNO_3$ concentrations, and avoidance behaviour was recorded in a linear concentration gradient.

Materials and Methods

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

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

In this initial, developmental experiment, the effect of ten Ag NP treatments (0, 1, 5, 10, 50, 125, 250, 500, 750 and 1000 mg kg^{-1}) was assessed on *A. chlorotica* survival and change in biomass over a 14-day period. The substrate selected for use in this study was Kettering loam (composition: Clay 24%, Silt 18%, Sand 58%, Organic content 6.72%, pH 6.8), obtained from Boughton Loam Ltd. Kettering loam has been widely used in earthworm research (e.g. Ellis *et al.,* 2010; Lowe et al., 2016) and is recommended for culture of soil dwelling species (Lowe and Butt, 2005). Uncoated, spherical 80 nm Ag NP powders (99.9 % purity) were purchased from GetNanoMaterials, Saint-Cannat, France. Particle size distribution was confirmed from manufacture certification. The soil was spiked with Ag NPs following the ISO11268-1:2012 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^{-1} 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 / $AgNO_3$ 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^{-1} – selected
161 from results of experiment 1) - of either Ag NP or $AgNO_3$ 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 $AgNO_3$ (n=5 replicates of
166 each). The 5 Ag NP/$AgNO_3$ 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_3 component was mixed with the remainder

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

In addition, three *A. chlorotica* were placed in circular (300 ml) clear plastic pots containing 250 g of soil, individually spiked with the same concentrations of either Ag NP or $AgNO_3$ used in the linear gradient: 0, 12.5, 25, 50 and 100 mg kg^{-1} (prepared as previously described). After earthworm introduction, the pots were maintained in the same environmental conditions as described for Experiment 1 with five replicates for each treatment (the 0 mg kg^{-1} treatment serving as a control for both types of silver). Individual earthworm biomass was recorded at day 0 and 14. Soil pH and concentration of total silver present in earthworms and in soil was recorded after 14 days as described for Experiment 1.

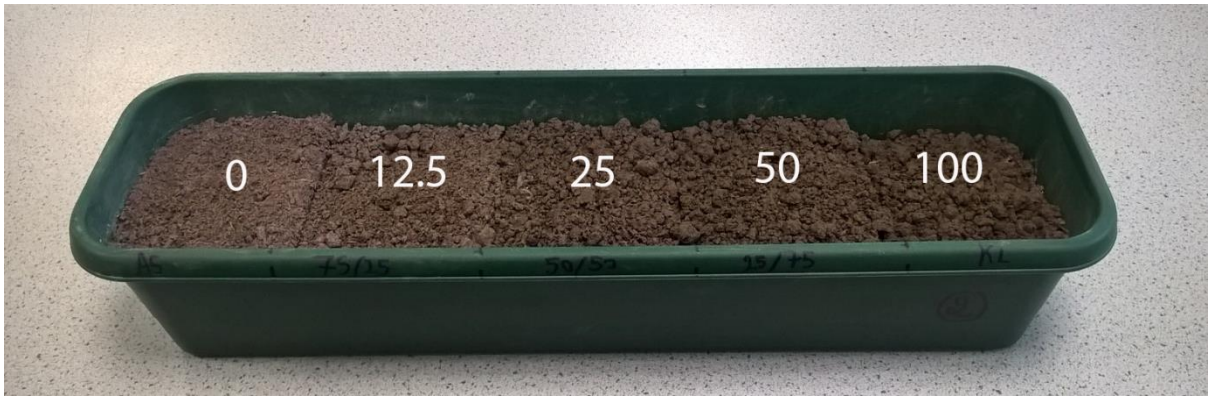


Figure 1. Linear gradient mescocosm (0.6 m x 0.13 m x 0.1 m) with nominal Ag NP / $AgNO_3$ concentrations (mg kg^{-1}) used in the Experiment 2 avoidance test. Each treatment occupied an equal soil volume (0.12 m x 0.13 m x 0.085 m).

Soil and Earthworm analysis

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

Statistical analysis

Statistical analyses were performed with Minitab software (Version 17). One-way ANOVA followed by a Tukey post-hoc test was used to analyse biomass results in the first experiment. In experiment 2, a Kruskal Wallis test, followed by a post hoc test executed using Mann Whitney with a Bonferroni correction, was used to analyse differences in earthworm location in the linear gradient. Welch's One-way ANOVA followed by a Games-Howell post hoc test was used to analyse earthworm biomass results (at day 0 and day 14) across the 5 separate Ag NP/$AgNO_3$ concentrations. A Welch's 2 sample t-test was used to analyse differences in

earthworm biomass between day 0 and day 14 in each Ag NP / $AgNO_3$ concentration and between each concentration level for Ag NP and $AgNO_3$ at day 14. A Student's 2 sample-t-test was also used to analyse differences in uptake of total Ag by earthworms in Ag NP and $AgNO_3$ treatments at 12.5, 25, 50 and 100 mg kg^{-1}. Where parametric tests have been applied, the Anderson-Darling Normality test and Levene's test for equality of variances were used to validate that test assumptions had been met. For all statistical tests a significance level of 5% ($p = 0.05$) was applied.

Results

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

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

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

Table 1. Total silver (Ag) concentration (mg kg^{-1}) in experimental soil and earthworm tissue (± Standard error), soil pH and earthworm survival after 14 days exposure to Ag NP.

[Ag] Nominal concentration (mg kg^{-1})	Mean [Ag] concentration in soil (mg kg^{-1})	Soil pH	Mean [Ag] concentration in *A. chlorotica* (mg kg^{-1})	% 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

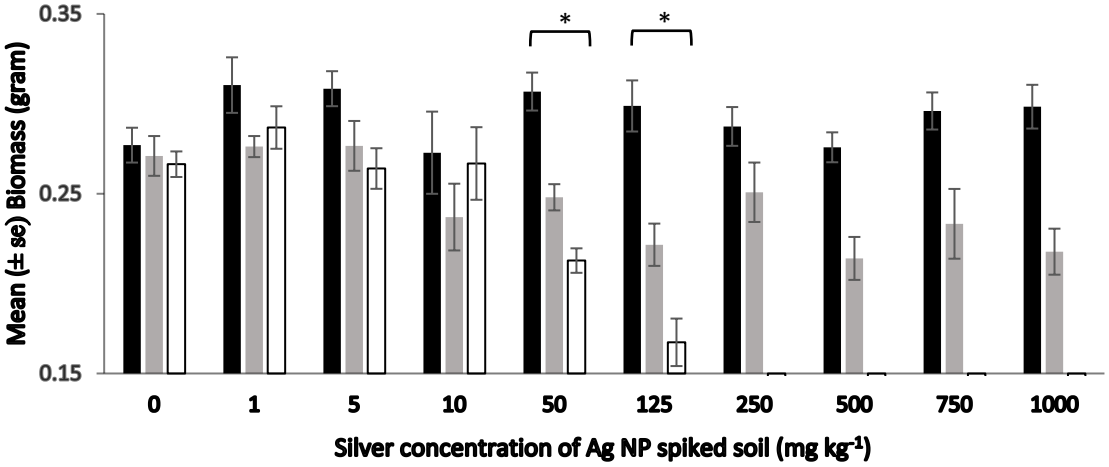


Figure 2. Mean (± standard error) biomass of A. chlorotica in Ag NP spiked soil after 0 (black bar), 7 (grey bar) and 14 (white bar) days. Asterisks indicate a significant difference in biomass between day 0, day 7 and day 14 for each Ag NP concentration ($p < 0.05$).

Experiment 2: Ag NP / $AgNO_3$ avoidance response and effects of exposure on survival and change in biomass in A. chlorotica

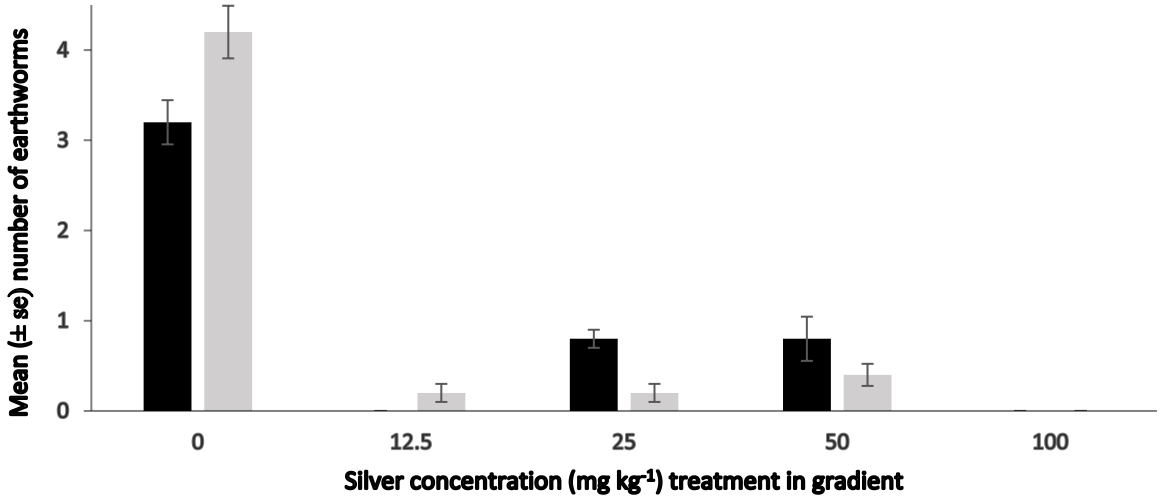
The pH of soils spiked with Ag NP and $AgNO_3$ was between 7.77 and 8.07 (Table 2). Recorded Ag concentrations in soils spiked with Ag NP and $AgNO_3$ were comparable with expected concentrations (based on experimental design) and suggest that both substances were homogenously distributed throughout the experimental substrate (Table 2).

Table 2. Total silver (Ag) concentration (mg kg^{-1}) (± Standard error) in soil spiked with Ag NP and $AgNO_3$ and associated pH.

[Ag] Nominal Concentration (mg kg^{-1})	Mean [Ag] concentration in soil spiked with AgNP (mg kg^{-1})	pH	Mean [Ag] concentration in soil spiked with $AgNO_3$ (mg kg^{-1})	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

After the avoidance test, one earthworm was not recovered. At 14 days the majority of earthworms in both Ag NP and $AgNO_3$ treatments were retrieved from the 0 mg kg^{-1} section of the gradient (64 and 84% respectively) and no individuals were located in the 100 mg kg^{-1} section (Figure 3). There was no significant difference in the distribution of earthworms in the Ag NP and $AgNO_3$ gradients.

There was a significant difference in earthworm numbers located in each section of both Ag NP ($p = 0.001$) and $AgNO_3$ ($p = 0.002$) gradients. However, a post hoc multiple comparison test showed no significant difference between each level of the gradient (results associated with the conservative approach of the statistical analysis).



266

267 *Figure 3. Mean (± standard error) number of A. chlorotica recorded in each section of Ag NP*
268 *(black bar) and $AgNO_3$ (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 $AgNO_3$ (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 $AgNO_3$ concentrations equal to or
275 below 50 mg kg^{-1} after 14 days (Table 3). However, at 100 mg kg^{-1} survival was reduced to
276 87.5 and 33.3% in AgNP and $AgNO_3$ spiked soils respectively.

277

278 *Table 3. Mean biomass of A. chlorotica in soil spiked with either Ag NP or $AgNO_3$ 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^{-1})	Mean Biomass (± s.e.) day 0	Mean Biomass (± s.e.) day 14	Mean [Ag] concentration (± s.e.) in *A. chlorotica* (mg kg^{-1})	% Survival
		Ag NP		
0	0.17 ± 0.01	0.21 ± 0.01*[a]	0.00	100
12.5	0.18 ± 0.01	0.19 ± 0.01[ab]	0.26 ± 0.03	100
25	0.17 ± 0.02	0.18 ± 0.02[abc]	0.60 ± 0.05	100
50	0.19 ± 0.01	0.15 ± 0.01*[bc]	0.92 ± 0.08	100
100	0.19 ± 0.01	0.13 ± 0.01*[c]	1.50 ± 0.12	87.5
		$AgNO_3$		

0	0.17 ± 0.01	0.21 ± 0.01*^{A}	0.00	100
12.5	0.17 ± 0.02	0.17 ± 0.02^{AB}	0.32 ± 0.03	100
25	0.17 ± 0.01	0.13 ± 0.01$^{B@}$	1.13 ± 0.10	100
50	0.16 ± 0.01	0.11 ± 0.01*$^{B@}$	1.50 ± 0.09	100
100	0.17 ± 0.01	0.12 ± 0.01*^{B}	1.67 ± 0.15	33.3

* denotes a significant difference in mean biomass day 0 to day 14. At day 14 mean biomass figures that do not share a letter (lower case for Ag NP and capitals for $AgNO_3$) are significantly different. $^{@}$ denotes a significant decrease in mean biomass in $AgNO_3$ compared with Ag NP at the same nominal Ag concentrations.

At day zero, there was no significant difference ($p = 0.757$) in mean earthworm biomass across all Ag NP and $AgNO_3$ concentrations ($n = 9$ manipulations). However, at day 14, significant differences in mean earthworm biomass were recorded in both Ag NP and $AgNO_3$ treatments (see table 3). In soil spiked with Ag NP, mean biomass at 50 and 100 mg kg^{-1} was significantly lower than at 0 mg kg^{-1}. In soil spiked with $AgNO_3$, mean biomass at 25, 50 and 100 mg kg^{-1} was significantly lower than at 0 mg kg^{-1}. Observed differences in biomass in the 5 Ag concentrations in the two treatments can be directly associated with changes in mean biomass observed within individual silver concentrations from day 0 to day 14. In both Ag NP and $AgNO_3$ treatments, there was a significant increase ($p<0.05$) in mean earthworm biomass at 0 mg kg^{-1} but a significant decrease in biomass at 50 and 100 mg kg^{-1} ($p = 0.04$ and 0.001 for Ag NP and $p = 0.001$ and 0.001 for $AgNO_3$ respectively).

Total silver concentration assessed after 14 days in earthworms exposed to Ag NP and $AgNO_3$ suggested a positive association between concentration of silver in the soil and uptake in earthworms (Table 3). Mean silver tissue concentrations were higher in $AgNO_3$ spiked soils than Ag NP spiked soils at all concentration levels, however, recorded differences were not significant ($p>0.05$).

Discussion

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

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

Effect of Ag NP and $AgNO_3$ on earthworm survival, biomass and uptake

The biomass and survival results obtained in Experiment 2 are consistent with results of Experiment 1, with a significant decrease in *A. chlorotica* biomass recorded at Ag NP and $AgNO_3$ concentrations ≥ 50 mg kg^{-1} and mortality recorded at Ag NP / $AgNO_3$ concentration of 100 mg kg^{-1}. The concentrations eliciting a biomass decrease and mortality response are substantially lower than those recorded for *Eisenia spp.* in similar studies. Schlich et al. (2013) exposed *E. andre*i to Ag NP (containing a stabilising agent and with a particle size of 15 nm) and also to $AgNO_3$ and recorded no significant effect on mortality up to 200 mg kg^{-1}. Similarly, Shoults-Wilson et al. (2011a) found no influence of coated Ag NPs on *E. fetida* growth, mortality or reproduction at concentrations below 773.3 – 801 mg kg^{-1}. It is proposed that differences in behaviour were responsible for observed results. As an epigeic species, *E. fetida* is frequently found on the soil surface within organic matter and, unlike endogeic species, it is not geophagous and as a result is exposed to lower levels of soil contaminants. It is relevant at this point to refer further to Schlich et al. (2013) who interpreted the presence of *E. andrei* in organic matter (cow dung) on the soil surface as an avoidance response to Ag NP in the soil without also recognising that this is a location where this species might "naturally" occur (Sims and Gerard, 1999).

The enhanced negative effect of $AgNO_3$ on survival rates (33.3% at 100 mg kg^{-1}) when compared with Ag NP (87.5%) is mirrored in other studies (e.g. Heckmann et al., 2011; Shoults-Wilson et al., 2011a; Schlich et al., 2013). Increased toxicity of $AgNO_3$ has been directly related to the presence of Ag^+ ions. Shoults-Wilson et al. (2011a) reported a 10-17% silver oxidation for Ag NP in OECD soil and suggested that a response for Ag NP at 900 mg kg^{-1} should be similar to a response at 100-200 mg kg^{-1} for $AgNO_3$. Furthermore, a study by Gomes et al. (2015), which investigated the effect of Ag NP and $AgNO_3$ on oxidative stress in *E. fetida,* suggested that response to $AgNO_3$ 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 $AgNO_3$ 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 / $AgNO_3$ (12.5 mg kg^{-1}), whereas biomass was only effected
347 at concentrations ≥ 50 mg kg^{-1}. 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^{-1}, well below the 773.3-801 mg kg^{-1} 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 $AgNO_3$
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^{-1}. 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^+ 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^+ to be oxidised from the Ag NPs. Because avoidance of
365 Ag NP and $AgNO_3$ 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 $AgNO_3$ 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

377 References:

378 Ahn, J-M., Eom. H-J., Yang, X., Meyer, J.N., Choi, J., 2014. Comparative toxicity of silver
379 nanoparticles on oxidative stress and DNA damage in the nematode *Caenorhabditis elegans.*
380 Chemosphere, 108: 343-352.

381 Akaighe, N., MacCuspie, R.I., Navarro, D.A., Aga, D.S., Banerjee, S., Sohn, M., Sharma, V.K.,
382 2011. Humic Acid-Induced Silver Nanoparticle Formation under Environmentally Relevant
383 Conditions. Environ. Sci. Technol., 45: 3895-3901.

384 Butt, K.R., Lowe, C.N., 2010. Controlled Cultivation of Endogeic and Anecic Earthworms. A.
385 Karaca (ed.), Biology of Earthworms*,* Soil Biology 24, Springer-Verlag, Berlin, Heidelberg, 107-
386 121.

387 Coutris, C., Hertel-Aas, T., Lapeid, E., Joner, E.J., Oughton, D.H., 2011. Bioavailability of cobalt
388 and silver nanoparticles to the earthworm *Eisenia fetida*. Nanotoxicology, 6 (2): 186-195.

389 Diez-Ortiz, M., Lahive, E., George, S., Schure, A.T., Van Gestel, C.A.M., Jurkschat, K., Svendsen,
390 C., Spurgeon, D.J., 2015. Short-term soil bioassays may not reveal the full toxicity potential
391 for nanomaterials; bioavailability and toxicity of silver ions ($AgNO_3$) and silver nanoparticles
392 to earthworm *Eisenia fetida* in long-term aged soils. Environ. Pollut., 203: 191-198.

393 Ellis, S.R., Hodson, M.E., Wege, P., 2010. The soil-dwelling earthworm *Allolobophora*
394 *chlorotica* modifies its burrowing behaviour in response to carbendazim applications. Ecotox.
395 Environ. Safe., 73 (6): 1424-1428.

396 European Commission, 2016. Sewage Sludge. Available at
397 [http://ec.europa.eu/environment/waste/sludge/]

398 Foldbjerg, R., Dang, D.A., Autrup, H., 2011. Cytotoxicity and genotoxicity of silver
399 nanoparticles in the human lung cancer cell line, A549. Arch. Toxicol., 85: 743-750.

400 Gomes, S.I.L., Hansen, D., Scott-Fordsmand, J.J., Amorim, M.J.B., 2015. Effects of silver
401 nanoparticles to soil invertebrates: Oxidative stress biomarkers in *Eisenia fetida*. Environ.
402 Pollut., 199: 49-55.

403 Hayashi, Y., Heckmann, L-H., Simonsen, V., Scott-Fordsmand, J.J., 2013. Time-course profiling
404 of molecular stress responses to silver nanoparticles in the earthworm *Eisenia fetida*. Ecotox.
405 Environ. Safe., 98: 219-226.

406 Heckmann, L-H., Hovgaard, M.B., Sutherland, D.S., Autrup, H., Besenbacher, F., Scott-
407 Fordsmand, J.J., 2011. Limit test toxicity screening of selected inorganic nanoparticles to the
408 earthworm *Eisenia fetida*. Ecotoxicology, 20: 226-233.

409 Homa, J., Klimek, M., Kruk, J., Cocquerelle, C., Vandenbulcke, F., Plytycz, B., 2010. Metal-
410 specific effects on metallothionein gene induction and riboflavin content in coelomocytes of
411 *Allolobophora chlorotica.* Ecotox. Environ. Safe., 73 (8): 1937-1943.

412 ISO 10390, 2005. Soil Quality - Determination of pH. ISO (International Organization for
413 Standardization), Geneva.
414 ISO 17512-1, 2008. Soil Quality - Avoidance test for determining the quality of soils and effects
415 of chemicals on behaviour - Part 1: Test with Earthworms (*Eisenia fetida* and *Eisenia andrei*).
416 ISO (International Organization for Standardization), Geneva.
417 ISO 11268-1, 2012. Soil Quality - Effects of pollutants on earthworms, Part 1: Determination
418 of Acute toxicity to *Eisenia fetida/Eisenia andrei*. ISO (International Organization for
419 Standardization), Geneva.
420 Kwak, J.I., An, Y-J., 2015. Ecotoxicological Effects of Nanomaterials on Earthworms: A review.
421 Hum. Ecol. Risk Assess, 26:1566-1575.

422 Lowe, C.N., Butt, K.R., 2005. Culture techniques for soil dwelling earthworms: a review.
423 Pedobiologia. 49 (5): 401-413.

Lowe, C.N., Butt, K.R., 2007. Earthworm culture, maintenance and species selection in chronic ecotoxicological studies: A critical review. Eur. J. Soil Biol., 43: 281-288.

Lowe, C.N., Butt, K.R., 2008. *Allolobophora chlorotica* (Savigny, 1826): Evidence for consideration as two separate species. Pedobiologia, 52, 81-84.

Lowe, C.N., Butt, K.R., Cheynier, K.Y-M., 2016. Assessment of avoidance behaviour by earthworms (*Lumbricus rubellus* and *Octolasion cyaneum*) in linear pollution gradients. Ecotox. Environ. Safe., 124: 324-328.

Makama, S., Piella, J., Undas, A., Dimmers, W.J., Peters, R., Puntes, V.F., van den Brink, N.C., 2016. Properties of silver nanoparticles influencing their uptake in and toxicity to the earthworm *Lumbricus rubellus* following exposure in soil. Environ.Pollut., 218: 870-878.

Novo, M., Lahive, E., Diez-Ortiz, M., Matzke, M., Morgan, A.J., Spurgeon, D.J., Svendsen, C., Kille, P., 2015. Different routes, same pathways: Molecular mechanisms under silver ion and nanoparticle exposures in the soil sentinel *Eisenia fetida*. Environ. Pollut., 205: 385-393.

OECD (Organisation for Economic Co-operation and Development), 1984. Earthworm Acute Toxicity Tests. OECD Guideline for testing of Chemicals, Test no. 207. OECD, Paris, France.

Ratte, H.T., 1999. Bioaccumulation and toxicity of silver compounds a review. Environ. Toxicol. Chem., 18 (1): 89-108.

Rayner, T.A., Mackevica, A., Wejdling, A., Zurera, J.G.O., Vaskio, S., Selck, H., 2010. Uptake and Toxicity of Silver Ions, Nanoparticles and Microparticles in *Nereis virens* in a Sediment Environment. Roskilde University, Unpublished Student Environmental Biology Project report, available at [http://rudar.ruc.dk/handle/1800/5119].

Reidy, B., Haase, A., Luch, A., Dawson, K.A., Lynch, I., 2013. Mechanisms of Silver Nanoparticle Release, Transformation and Toxicity: A Critical Review of Current Knowledge and Recommendations for Future Studies and Applications. Materials, 6: 2295-2350.

Sagee, O., Dror, I., Berkowitz, B., 2012. Transport of Silver nanoparticles (AgNPs) in soil. Chemosphere, 88: 670-675.

Schaefer, M., 2003. Behavioural Endpoints in Earthworm Ecotoxicology, Evaluation of Different Test Systems in Soil Toxicity Assessment. J Soils & Sediments, 3(2) 79-84.

452 Schlich, K., Klawonn, T., Terytze, K., Hund-Rinke, K., 2013. Effects of silver nanoparticles and
453 silver nitrate in the earthworm reproduction test. Environ. Toxicol. Chem., 32 (1): 181-188.

454 Shoults-Wilson, W.A., Reinsch, B.C., Tsyusko, O.V., Bertsch, P.M., Lowry, G.V., Unrine, J.M.,
455 2011a. Role or Particle Size and Soil Type in Toxicity of Silver Nanoparticles to Earthworms.
456 Soil Sci. Soc. Am. J., 75(2) 365-377.

457 Shoults-Wilson, W.A., Reinsch, B.C., Tsyusko, O.V., Bertsch, P.M., Lowry, G.V., Unrine, J.M.,
458 2011b. Effect of silver nanoparticle surface coating on bioaccumulation and reproductive
459 toxicity in earthworms (*Eisenia fetida*). Nanotoxicology 5(3) 432-444.

460 Shoults-Wilson, W.A., Zhurbich, O.I., McNear, D.H., Tsyusko, O.V., Bertsch, P.M., Unrine, J.M.,
461 2011c. Evidence for avoidance of Ag nanoparticles by earthworms (*Eisenia fetida*).
462 Ecotoxicology, 20: 385-396.

463 Sims, R.W., Gerard, B.M., 1999. Earthworms: Notes for the identification of British species.
464 Synposes of the British Fauna (New Series) No. 31 (Revised). Published for the Linnean Society
465 of London & the Estuarine and Coastal Sciences Association by Field Studies Council,
466 Montford Bridge, Shrewsbury.

467 Spurgeon, D.J., Weeks, J.M., Van Gestel, C.A.M., 2003. A summary of eleven years progress in
468 earthworm ecotoxicology. Pedobiologia, 47: 588-606.

469 Svendsen, T.S., Hansen, P.E., Sommer, C., Martinussen, T., Gronvold, J., Holter, P., 2005. Life
470 history characteristics of *Lumbricus terrestris* and effects of the veterinary antiparasitic
471 compounds ivermectin and fenbendazole. Soil Biol. Biochem., 37:927-936.

472 Sun, T.Y., Gottschalk, F., Hungerbühler, K., Nowack, B., 2014. Comprehensive probalistic
473 modelling of environmental emissions on engineered nanomaterials. Environ. Pollut., 185:
474 69-76.

475 Suthar, S., Singh, S., Dhawan, S., 2008. Earthworms as bioindicator of metals (Zn, Fe, Mn, Cu,
476 Pb and Cd) in soils: Is metal bioaccumulation affected by their ecological category? Ecol. Eng.,
477 32: 99-107.

478 Tsyusko, O.V., Hardas, S.S., Shoults-Wilson, W.A., Starnes, C.P., Joice, G., Butterfield, D.A.,
479 Unrine, J.M., 2012. Short-term molecular-level effects of silver nanoparticle exposure on the
480 earthworm, *Eisenia fetida*. Environ. Pollut. 171: 249-255.

481 USEPA (US Environment Protection Agency), 2012. OCSPP 850.3100: Earthworm Subchronic
482 Toxicity Test. Ecological Effect Test Guideline, EPA 712-C016. USEPA Office of Chemical Safety
483 and Pollution Prevention, Washington DC, US.

484 United States Environmental Protection Agency, 2016. Sewage Sludge/Biosolids Program.
485 https://www3.epa.gov/region9/water/npdes/sludge.html. Accessed 18 July 2016

486 Vance, M.E., Kuiken, T., Vejerano, E.P., McGinnis, S.P., Hochella, M.F., Rejeski, D., Hull, M.S.,
487 2015. Nanotechnology in the real world: Redeveloping the nanomaterial consumer products
488 inventory. Beilstein J. Nanotechnol., 6: 1769–1780.

489 van der Ploeg, M.J.C., Handy, R.D., Waalewujn-Kool, P.L., van der Berg, J.H.J., Herrera Rivera,
490 Z.E., Bovenschen, J., Molleman, B., Baveco, J.M., Tromp, P., Peters, R.J.B., Koopmans, G.F.,
491 Rietjens, I.M.C.M., van den Brink, N.W., 2014. Effects of silver nanoparticles (NM-300K) on
492 *Lumbricus rubellus* earthworms and particle characterization in relevant test matrices
493 including soil. Enivron. Toxicol. Chem., 33 (4): 743-752.

494 van Gestel, C.A.M., 2012. Soil ecotoxicology: state of the art and future directions. Zookeys,
495 176: 275-296.

496 Velicogna, J.R., Ritchie, E.E., Scroggins, R.P., Princz, J.I., 2016. A comparison of the effects of
497 silver nanoparticles and silver nitrate on a suite of soil dwelling organisms in two field soils.
498 Nanotoxicology, 10 (8): 1144-1151.

499