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

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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 epigeic earthworm species which may have limited ecological relevance in mineral soil. This study assessed the influence of Ag NP (uncoated 80 nm powder) and AgNO₃ 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 AgNO₃ 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 / AgNO₃ 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 AgNO₃ (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 (AgNO₃). Earthworm survival, change in biomass and silver uptake were determined in soil spiked with either Ag NP or AgNO₃ 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

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

Experiment 2: Ag NP / AgNO₃ avoidance response and effects of exposure on survival and change in biomass in A. chlorotica

A. chlorotica were exposed to 5 concentrations (0, 12.5, 25, 50 and 100 mg kg⁻¹ – selected from results of experiment 1) - of either Ag NP or AgNO₃ powder (99.9+% ultra-pure, Alfa Aesar) in sequential linear gradients (to assess avoidance) and separately in mesocosms (to assess survival and change in biomass).

The linear gradients (Figure 1) were created in opaque plastic containers (0.6 m x 0.13 m x 0.1 m), filled to a depth of 0.085 m with soil spiked with either Ag NP or AgNO₃ (n=5 replicates of each). The 5 Ag NP/AgNO₃ treatments were established sequentially in equal soil volumes (0.12 m x 0.13 m x 0.085 m) within the container. The spiked soil was prepared (a single batch of 9.5 kg dry weight for each dose) using the same protocol as Experiment 1 with the exception that the 50 g sand and Ag NP / Ag NO₃ 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 AgNO₃ 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 mesocosm (0.6 m x 0.13 m x 0.1 m) with nominal Ag NP / AgNO₃ 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/AgNO₃ 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 / AgNO₃ concentration and between each concentration level for Ag NP and AgNO₃ 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 AgNO₃ 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).

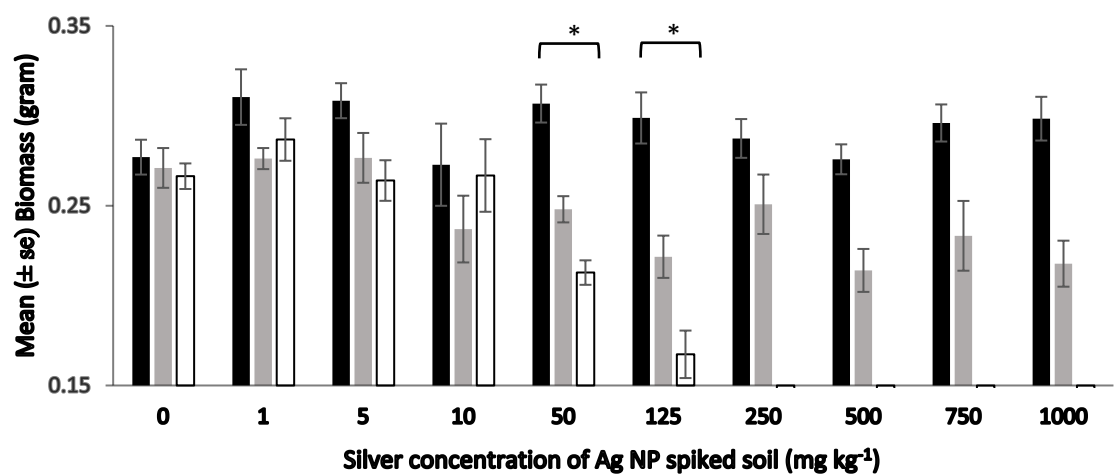
Total silver concentration in earthworms increased with silver concentration in the soil (Table 1).

Table 1. Total silver (Ag) concentration (mg kg⁻¹) in experimental soil and earthworm tissue (\pm 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 <i>A. chlorotica</i> (mg kg ⁻¹)	% Survival
0	0.38 \pm 0.24	8.11 \pm 0.1	0	100
1	2.24 \pm 0.92	8.03 \pm 0.07	0	100
5	6.57 \pm 0.33	8.13 \pm 0.03	0.04 \pm 0.01	100
10	7.68 \pm 0.62	7.88 \pm 0.01	0.06 \pm 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 / AgNO₃ avoidance response and effects of exposure on survival and
247 change in biomass in *A. chlorotica*

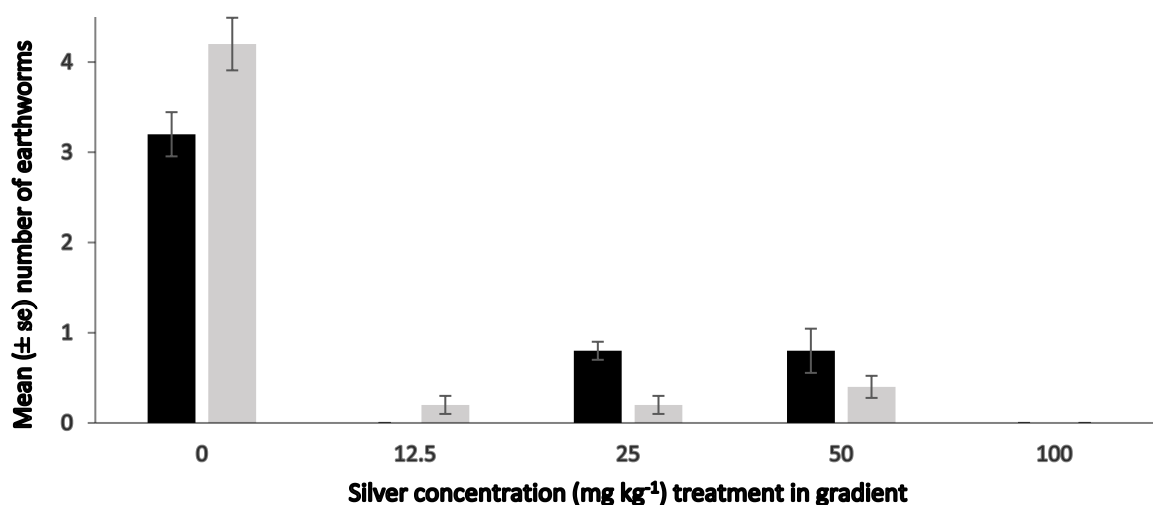
The pH of soils spiked with Ag NP and AgNO₃ was between 7.77 and 8.07 (Table 2). Recorded Ag concentrations in soils spiked with Ag NP and AgNO₃ were comparable with expected concentrations (based on experimental design) and suggest that both substances were homogenously distributed throughout the experimental substrate (Table 2).

Table 2. Total silver (Ag) concentration (mg kg⁻¹) (\pm Standard error) in soil spiked with Ag NP and AgNO₃ 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 \pm 0.05	0	7.77 \pm 0.05
12.5	8.36 \pm 1.02	7.89 \pm 0.02	7.19 \pm 0.83	8.02 \pm 0.03
25	15.55 \pm 0.72	7.77 \pm 0.03	18.26 \pm 1.78	8.07 \pm 0.01
50	37.99 \pm 1.94	7.89 \pm 0.01	35.27 \pm 1.84	7.97 \pm 0.01
100	85.39 \pm 5.84	7.99 \pm 0.02	81.94 \pm 11.33	7.93 \pm 0.05

After the avoidance test, one earthworm was not recovered. At 14 days the majority of earthworms in both Ag NP and AgNO₃ 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 AgNO₃ gradients.

There was a significant difference in earthworm numbers located in each section of both Ag NP ($p = 0.001$) and AgNO₃ ($p = 0.002$) 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 (\pm standard error) number of *A. chlorotica* recorded in each section of Ag NP
 268 (black bar) and AgNO₃ (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 AgNO₃ ($p=0.002$) 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 AgNO₃ 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 AgNO₃ spiked soils respectively.

277

278 Table 3. Mean biomass of *A. chlorotica* in soil spiked with either Ag NP or AgNO₃ 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 (\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 ⁻¹)	% Survival
Ag NP				
0	0.17 \pm 0.01	0.21 \pm 0.01 ^{*a}	0.00	100
12.5	0.18 \pm 0.01	0.19 \pm 0.01 ^{ab}	0.26 \pm 0.03	100
25	0.17 \pm 0.02	0.18 \pm 0.02 ^{abc}	0.60 \pm 0.05	100
50	0.19 \pm 0.01	0.15 \pm 0.01 ^{*bc}	0.92 \pm 0.08	100
100	0.19 \pm 0.01	0.13 \pm 0.01 ^{*c}	1.50 \pm 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 AgNO₃) are significantly different. @ denotes a significant decrease in mean biomass in AgNO₃ 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 AgNO₃ concentrations ($n = 9$ manipulations). However, at day 14, significant differences in mean earthworm biomass were recorded in both Ag NP and AgNO₃ 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 AgNO₃, 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 AgNO₃ 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 AgNO₃ respectively).

Total silver concentration assessed after 14 days in earthworms exposed to Ag NP and AgNO₃ suggested a positive association between concentration of silver in the soil and uptake in earthworms (Table 3). Mean silver tissue concentrations were higher in AgNO₃ 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 AgNO₃ 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 AgNO₃ concentrations $\geq 50 \text{ mg kg}^{-1}$ and mortality recorded at Ag NP / AgNO₃ 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. andrei* to Ag NP (containing a stabilising agent and with a particle size of 15 nm) and also to AgNO₃ 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 $773.3 - 801 \text{ mg kg}^{-1}$. 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 AgNO₃ 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 AgNO₃ 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 $100-200 \text{ mg kg}^{-1}$ for AgNO₃. Furthermore, a study by Gomes et al. (2015), which investigated the effect of Ag NP and AgNO₃ on oxidative stress in *E. fetida*, suggested that response to AgNO₃ started earlier than Ag NP and was related to

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

A. chlorotica accumulated higher concentrations of Ag when exposed to AgNO₃ than Ag NP in accordance with results for *E. fetida* (Shoults-Wilson et al., 2011b).

Avoidance Behaviour

Several studies have found that earthworm avoidance of contaminants can be more sensitive than traditional endpoints such as reproduction (e.g. van-Gestel, 2012). This is confirmed from results of the present study, where avoidance behaviour was observed at the lowest employed concentration of Ag NP / AgNO₃ (12.5 mg kg⁻¹), whereas biomass was only effected at concentrations ≥ 50 mg kg⁻¹. The increased sensitivity of avoidance behaviour to Ag NP is also reported by Shoults-Wilson et al. (2011c) who observed a significant avoidance response in *E. fetida* at a concentration of 6.97-7.42 mg kg⁻¹, well below the 773.3-801 mg kg⁻¹ values reported to influence growth, mortality or reproduction in a related study under comparable conditions (Shoults-Wilson, et al., 2011a).

There was no significant difference in the distribution of earthworms in Ag NP and AgNO₃ gradients, which corroborates results of Shoults-Wilson et al. (2011c) and Velicogna et al. (2016). However, it is proposed that differences in avoidance response for *A. chlorotica* may become apparent at concentrations less than 12.5 mg kg⁻¹. Establishing a no-observed-effect concentration (NOEC) and a lowest-observed-effect concentration (LOEC) for both chemicals should be a focus for future research and provide results that are directly related to PEC values in sewage sludge and soil. While it is clear that earthworms are able to detect the presence of Ag NP within the mineral soil, the mechanism triggering avoidance (sensory-based reaction or detrimental effect caused by uptake) is not known. It is also unclear as to whether the avoidance response is triggered solely by Ag⁺ or if nano-particles also influence earthworm behaviour. The avoidance response observed in *E. fetida* by Shoults-Wilson et al. (2011c) was recorded over a short time period (48 h), but the study concluded that this was too short for a sufficient amount of Ag⁺ to be oxidised from the Ag NPs. Because avoidance of Ag NP and AgNO₃ occurred at similar concentrations, silver ions may not have been entirely responsible for triggering avoidance behaviour with the authors questioning if nano-scale particles are able to interact with sensory structures.

The current study has shown that the use of soil dwelling earthworms in a linear gradient methodology developed by Lowe et al. (2016) is an effective and practical method of assessing avoidance behaviour to soil-based contaminants. Earthworm survival rates in experimental treatments also suggest that *A. chlorotica* is more sensitive than *E. fetida* to Ag NP and AgNO₃ pollution. However, further evidence is required to confirm if soil-dwelling earthworms are the most appropriate species to use as bioindicators of Ag NP soil pollution. This requires inclusion of a wider range of endogeic and anecic species to be studied alongside epigeic species under the same experimental conditions.

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