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Jessica R. Velicogna, Ellyn E. Ritchie, Richard P. Scroggins & Juliska I. Princz

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**Title:** A Comparison of the Effects of Silver Nanoparticles and Silver Nitrate on a Suite of Soil Dwelling Organisms in Two Field Soils

**Authors:** Jessica R. Velicogna*, Ellyn E. Ritchie, Richard P. Scroggins, and Juliska I. Princz

**Affiliation(s):** Biological Assessment and Standardization Section, Environment Canada.

*Corresponding Author: Jessica R. Velicogna, 613-998-8295, jessica.velicogna@canada.ca

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Abstract

Nanomaterials are increasingly used in a wide range of products, leading to growing concern of their environmental fate. In order to understand the fate and effects of silver nanoparticles in the soil environment, a suite of toxicity tests including: plant growth with *Elymus lanceolatus* (northern wheatgrass) and *Trifolium pratense* (red clover); collembolan survival and reproduction (*Folsomia candida*); and earthworm avoidance, survival and reproduction (*Eisenia andrei*) was conducted. The effect of silver nanoparticles (AgNP) was compared with the effect of ionic silver (as AgNO$_3$) in two agricultural field soils (a sandy loam and a silt loam). Lethal (LC50) or sub lethal (IC50) effect levels are presented for all endpoints and demonstrate that in most cases AgNO$_3$ (i.e., ionic silver) was found to be more toxic than the AgNP across test species. The difference in effects observed between the two forms of silver varied based on test species, endpoint and soil type. In tests that were conducted across different soil types, organisms in the sandier soil had a greater response to the Ag (ionic and nano) than those in soil with a high silt content. Earthworms (avoidance behaviour and reproduction) were the most sensitive to both AgNP and AgNO$_3$, while plant emergence was the least sensitive endpoint to both forms of Ag. The use of a test battery approach using natural field soils demonstrates the need to better quantify the dissolution and transformation products of nanomaterials in order to understand the fate and effects of these materials in the soil environment.
Introduction

Engineered nanomaterials are one of the most rapidly increasing new products entering the market around the world today, of which silver nanoparticles (AgNP) are most commonly used (Keller and Lazarava, 2014; Maurer-Jones et al. 2013; Weir et al. 2012). Many of these products are intended for and used in common household applications; therefore, it is likely that the nanomaterials will end up in either landfill or the waste water stream (Keller and Lazarava, 2014; Gottschalk et al. 2013; Kaegi et al. 2011; Sekine et al. 2015). There is increasing evidence that these nanomaterials will exist in biosolids as a result of waste water treatment processes (Kaegi et al. 2011; Hendren et al. 2013; Ma et al. 2014). In Canada, biosolids are commonly spread onto agricultural fields as a fertilizer, and as such, it is likely that nanomaterials will occur in the agricultural soil environment.

Due to their unique properties, varied forms, associations with stabilizing agents, and interactions with elements of the environment, the examination of the fate and effects of nanomaterials can be a complex challenge. In recent years, research on engineered nanomaterials in the environment, and the development of suitable analytical techniques, have been developing at a rapid pace. When considering nanoparticle toxicity in soil, challenges include: understanding how properties (physical and chemical) of the soil affect the fate of the nanoparticle; understanding how the properties of the nanoparticle (including stabilizing agent or surface coating) affect its interaction with the soil environment; tracking the fate of the nanoparticle in a soil toxicity test (soil, pore water, organism); and differentiating the effect of the nano-form from the ionized, bulk or transformed material. Some research has examined the effects of silver nanoparticles on soil dwelling organisms such as: plants (Dimkpa et al. 2013;
Mirzajani et al. 2013; Lee et al. 2012), earthworms (Hund-Rinke et al. 2012; Coutris et al. 2011; Shoults-Wilson et al. 2011(a-c); Schlich et al. 2013; Tsyusko et al. 2012; Coleman et al. 2013; van der Ploeg et al. 2014), nematodes (Kim et al. 2011(a); Ellegaard-Jensen et al. 2012), collembolan (Waalewijn-Kool et al. 2014) and soil micro-organisms (Schlich and Hund-Rinke, 2015; Mirzajani et al. 2013; Kim et al. 2011(b); Hansch and Emmerling, 2012; Langdon et al. 2014; Shin et al. 2012; De La Torre-Roche et al. 2013). The comparison of the effects of AgNP with the ionic form of Ag (as silver nitrate (AgNO$_3$)) is used in many of these studies to try to determine if observed effects are due to the release of Ag$^+$ from the nanoparticles. However, very few toxicity estimates (i.e., ECx) are provided, and most are rarely performed in a natural field soil. Some earthworm toxicity tests (survival, growth and, reproduction) indicate the release of Ag$^+$ as the cause of adverse effects when comparing between AgNP and AgNO$_3$ treatments (Shoults-Wilson et al. 2011(a-b); Schlich et al. 2013; Tsyusko et al. 2012). A study which compared the effects of AgNP and AgNO$_3$ on Folsomia candida found no effect on survival and reproduction in the highest test concentration of AgNP (673 mg kg$^{-1}$), but estimated an LC50 of 284 mg kg$^{-1}$ for survival and EC50 of 99.5 mg kg$^{-1}$ for reproduction in AgNO$_3$ exposed treatments, suggesting Ag$^+$ as the source of toxicity (Waalewijn-Kool et al. 2014). However, other studies and study designs suggest otherwise, for example: Coutris et al. (2011) determined that both AgNP and Ag$^+$ should be considered inert to Eisenia fetida based on the rapid excretion they observed of both forms of Ag. While Tsyusko et al. (2012) concludes that while Ag$^+$ was the source of toxicity based on gene expression in E. fetida; they suggested that AgNP may cause greater longer term effects due to dissolution that may occur after uptake. Similarly, a study which compared AgNP, Ag$^+$, and bulk Ag in wheat concluded that growth effects observed with AgNP were greater than those observed in treatments with equivalent Ag$^+$
concentration, and demonstrated that AgNP taken up into plants released more Ag$^+$ than in the growth environment (Dimkpa et al. 2013). A study using *Lumbriculus rubellus* showed greater effects on growth and reproduction in AgNP treatments compared to AgNO$_3$ treatments (van der Ploeg et al. 2014), and Shoults-Wilson et al. (2011c) concluded that avoidance behaviour observed by *E. fetida* in AgNP could not be explained by the release of Ag$^+$, nor by a change in the microbial community due to the Ag contamination.

The mixed results for individual tests suggest that the use of a test battery approach, using multiple species and biological endpoints, may better inform the risk assessment of substances in soil in order to capture both the structural and functional complexity of the system. The purpose of this research was to employ a test battery approach to assess the effects of AgNP in two natural field soils of varying physical and chemical properties. The tests were conducted using standardized soil toxicity test methods on a suite of plant and soil invertebrate species commonly found in agronomic systems. In order to help differentiate the effect of the ionic form and nano forms of silver, tests were completed with both AgNP and AgNO$_3$. 


Materials and Methods

Materials

Silver nitrate (AgNO₃) was used to assess the toxicity of the ionic form of silver (Ag⁺) and was purchased from Fisher Scientific (Canada). Silver nanoparticles were purchased in dry powder form as 20 nm PVP (0.3%) coated particles from NanoAmor (USA). Humic acid (CAS 1415-93-6) and PVP (40,000 mw.) were both purchased from Sigma Aldrich (USA).

Two agricultural soils were used to assess and compare between the effects of AgNP and AgNO₃ (Ag⁺): a sandy loam collected from Vulcan, Alberta, Canada (VSL); and a silt loam collected from Delacour, Alberta, Canada (DSL) (see Table 1). Due to poor performance of *E. andrei* in the DSL soil, earthworm tests were conducted in the VSL soil only. Plant and collembolan testing proceeded with both soils as control performance was acceptable. Formulated artificial soil (AS) was included as a negative control treatment for all tests to ensure that test cultures were healthy and, to confirm test validity (Environment Canada 2004, 2005a, 2007). The AS was formulated by mixing 10% *Sphagnum* sp. peat (air-dried and hand-sieved through a 2-mm screen), 20% kaolin clay (particle size < 40µm), and 70% silica sand (grade 70) using a mechanical stirrer. Calcium carbonate was then added in levels sufficient to produce a soil with a pH ranging between 6.5 and 7.5. After mixing, the soil was stored in an opaque container at room temperature and tested for conformance to optimal soil characteristics (e.g., soil pH, moisture content).

Plant tests were completed with *Elymus lanceolatus* (northern wheatgrass), and *Trifolium pratense* (red clover) using a standardized method for testing (Environment Canada, 2005a);
seeds were obtained from Pickseed Canada Inc. and William Dam Seeds Ltd., respectively.

Invertebrate tests were completed using standardized test methodologies with taxonomically verified springtails (*Folsomia candida*) (Environment Canada, 2007) and earthworms (*Eisenia andrei*) (Environment Canada, 2004), both derived from established in-house cultures. All test species selected for this study are found commonly in agricultural soils throughout Canada, and are well-established test species for soil ecotoxicity testing (Environment Canada 2004, 2005a, 2007).

**Spiking and Characterization**

The silver nanoparticles used were a dry powder with a 0.3% PVP coating. PVP is commonly used to coat or cap silver nanoparticles as it stabilizes the particles (prevents aggregation) and also acts as a surfactant to facilitate dispersion in water. However, the purchased particles did not disperse in water, even with repeated and extended sonication (using both bath and probe type sonication techniques). It has been previously noted that manufactured nanoparticles can be difficult to suspend in water homogenously and tend to aggregate quickly (Kool *et al.* 2011). In order to determine the best method for spiking test soils, a two part experiment was conducted, using the VSL soil, to (i) create an aqueous dispersion of purchased AgNP, and (ii) determine the best spiking method.

Discussion with the supplier indicated that a greater amount of PVP may be required to create a AgNP dispersion. Hund-Rinke *et al.* (2012) show that when using a wet application of nanoparticles to soils, the use of a dispersant is the preferred method compared to water. In order to confirm this, AgNP were added to 0.6% and 5.7% PVP solutions, representing about 2x
and 10x the amount of PVP in the purchased AgNP product. To find a more environmentally relevant surfactant to help disperse the AgNP in water, two additional options were investigated: the use of a soil extract (VSL mixed with water to saturation and then filtered through a P5 paper filter (Fisher Scientific, Canada)) (van der Ploeg et al. 2011); and a humic acid (HA) solution. Approximately 70% of the organic matter in soil is represented by humic acids (Adani and Spagnol, 2008), for the VSL soil, this equated to 1.8% humic acids. Humic acids are naturally found in soils, commonly found elevated in biosolids (Adani and Spagnol, 2008; Hernandez et al. 1988), and may help to stabilize particles in solution due to their negative charge at environmental pH (Tourinho et al. 2012). To determine the best spiking method, three options were evaluated: (i) dry mixing, in which dry AgNP were added to soil at 50% optimal moisture content (see Table 1) and mixed with a rotary mixer for 24 h; (ii) dry mixing using a soil carrier, in which dry AgNP were mixed by hand into a small amount of dried and ground soil, which was then added to the test soil (at 50% optimum moisture content) and mixed with a rotary mixer for 24 h; and (iii) wet mixing, in which a suspension of the AgNP was created by adding the dry AgNP to an aqueous-surfactant solution (i.e., PVP, soil extract, or humic acid), and then suspended by placing the solution in an ultrasonic bath for at least 1 h. The resultant suspension was then added to the test soil and mixed by hand with an electric mixer.

In all of the spiking methods described above, the test soil was brought to optimal moisture content using deionized (DI) water after the initial amendment and mixing was completed; the test soil was then mixed using a handheld electric mixer to ensure homogeneity. Small amounts of the VSL soil (100 g) were mixed with the AgNP at a low (4 mg kg⁻¹) and high (2000 mg kg⁻¹) nominal concentrations. Images of the spiked soils were taken using a scanning electron
microscope with energy dispersive x-ray spectroscopy (SEM-EDS) to confirm elemental components, to determine if the AgNP were evenly distributed throughout the soil, and to determine which method minimized aggregation. Additionally, total Ag analysis was completed via ICP-MS to confirm spiking concentrations.

Based on the results of the spiking experiment, the AgNP amendment method selected was wet mixing using 1.8% humic acid (HA) solution to disperse the AgNP. A stock solution of HA was made by adding 2 L of DI water to 1 g HA, adjusting the pH to 9.5 (± 0.5) with 0.02 M NaOH, and stirring on a hot plate (max. 50°C) until the pH was 7 (± 0.5). The solution was then filtered through a P5 paper filter (Fisher) to remove any undissolved HA particulates. The amount of AgNP powder required for each concentration was measured to $1 \times 10^{-5}$ g, and added to a flask, after which additional HA solution was added to make up 70% of the desired optimal moisture content (see Table 1) of the test soil. The resultant AgNP-HA solution was gently mixed by hand (i.e., swirled), covered and, ultra-sonicated in an ultrasonic bath for 1-2 h. The dispersion was then added to the air-dried test soil and mixed. Deionized water was used to rinse out the flask into the test soil and mixed thoroughly with the soil to achieve the optimal soil moisture content. For the AgNO$_3$ tests, test soils were amended using a stock solution of AgNO$_3$ in DI water, after which the soil moisture content was raised, and the test soil mixed thoroughly with an electric mixer. For plant growth and invertebrate reproduction tests, nominal treatments for both the AgNP and AgNO$_3$ spiked soils were: 0, 4, 10, 25, 60, 145, 347, 833, 2000 mg Ag kg$^{-1}$ dry soil; nominal concentrations for the *E. andrei* avoidance were: 0, 1, 10, 100, 1000 mg Ag kg$^{-1}$ dry soil.
Characterization of the AgNP-HA suspensions were completed by measuring particle hydrodynamic diameter and zeta potential through dynamic light scattering (DLS) (Malvern Zetasizer Nano), and particle size and agglomeration by transmission electron microscopy (TEM-EDS) (FEI, Technai, G2 F20). In addition to the negative control treatment using AS, additional controls included: unamended soil, soil amended with PVP equivalent to the PVP content of the highest concentration of AgNP, and soil amended with 1.8% HA. Soil samples from every concentration were taken at the beginning of toxicity tests to determine measured chemical concentrations in the soil. All soil samples required for chemical analysis were air-dried, frozen and stored at -20°C until analyzed by ICP-MS for total Ag content (analysis performed by Environmental Chemistry Laboratory, McGill University, Montreal, QC).

**Test Conditions**

For the plant tests, test vessels were incubated at a daily average of 24 ± 3°C for 16 h of light (300 ± 100 µmol m⁻² s⁻¹), and a nightly average of 15 ± 3°C for 8 h dark. The invertebrate test vessels were maintained at a daily average temperature of 20 ± 3°C at a diurnal cycle of 16 h light (>800 lux) and 8 h dark. Soil moisture content and pH were measured at the beginning and end of each test (soil moisture content was not measured at the end of plant tests).

**Effects Testing**

Plant definitive tests were performed in both test soils (DSL and VSL) according to standard methodology (Environment Canada, 2005a). Test measurements and observations included: general appearance of plants, mean (± standard deviation) emergence (EC50), and mean (± standard deviation) shoot and root length and dry mass (IC50) at the end of the test (day 14 for *T.*
pratense or day 21 for *E. lanceolatus*). Collembolan reproduction tests were also performed in both soils according to standard methodology (Environment Canada, 2007). Age-synchronized 10-12 day old adults were added to the test soils, and exposed to the test treatments for 28 d; granulated yeast (Fleischmann’s Quick-Rise Instant Yeast®) was added as a food source on days 0 and 14, and test vessels were aerated and monitored for moisture content weekly. Test endpoints included mean (± standard deviation) adult survival (LC50) and juvenile production (IC50). Earthworm reproduction and avoidance tests were performed according to standard methodology (Environment Canada, 2004) in the VSL soil only. The average wet weight of adult *E. andrei* added to the reproduction tests were 489 ± 114 mg; adults were exposed to test soil for 35 days, after which they were removed, and cocoons and juveniles incubated in the test soil for an additional 28 days. For the reproduction test, earthworm were fed cooked oatmeal (Quaker Oats™ ‘quick’ 3-5 min oatmeal) biweekly; test measurements included: mean (± standard deviation) adult survival (LC50) on day 35, and mean (± standard deviation) number of juveniles produced and mean individual juvenile dry mass (IC50) on day 63. According to Environment Canada (2004), a test option is provided to extend the adult incubation time from 28 to 35 days, depending on the cocoon production, which is checked on day 28. For the test conducted, the cocoon counts were relatively low, and as such, the adults were left within the test vessels for another 7 days. This resulted in the removal of adults on day 35 (rather than day 28), followed by an additional 28-day incubation period, resulting in the test concluding on day 63 (Environment Canada 2004). For the avoidance behaviour test, the number of earthworms in each soil compartment of the six-chambered test unit was counted following a 48 h exposure (Environment Canada, 2004).
Statistical Analysis and Data Reporting

Data was analyzed according to the Environment Canada guidance document on statistical methods for environmental toxicity tests (Environment Canada 2005b). Statistical calculations were completed with both nominal and measured values (where possible) and compared. Concentration values were transformed to log₁₀ for statistical analysis, after which results were back-transformed for reporting. Quantitative data (growth and juvenile production) were analyzed by nonlinear regression methods using SYSTAT (version 13) as a default, or ICPIN (Linear Interpolation; USEPA) in the event that data were heterogeneous, non-normal, or did not fit the available regression models. Quantal data (lethality and seedling emergence) was analyzed by Probit (USEPA) or Spearman-Kärber (USEPA) methods in the event that Probit could not be used (i.e., model assumptions were not met). Lethal (LC), effective (EC) and inhibitory (IC) concentrations were determined for 25 and 50% effect levels, with upper and lower confidence limits calculated at 95%. Toxicity estimates are reported as measured concentrations, unless indicated otherwise.
Results

Characterization of Silver Nanoparticles

Of the soil mixing methods assessed, the use of wet mixing using a dispersed solution was determined to be the best option (refer to Supplementary Information for a description of results for all methods). The best surfactant solution to disperse the AgNP for soil amendment included the 5.7% PVP and 1.8% humic acid (see Supplementary Information; Figure S1 and S2). The dispersion was characterized prior to addition to the test soils to confirm particle size and state of agglomeration. The mean diameter (n=3) measured by DLS and TEM were 65.0 nm and 30.1 nm respectively. Transmission electron microscopy images showed some agglomeration of particles, however, it was difficult to determine if this was an artifact of the sample preparation (i.e., drying of the solution on the TEM grid), rather than of the dispersion. In some cases, agglomerates formed a ring, suggesting that the particles had gathered at the edge of the droplet as it dried on the TEM grid; in other cases, agglomerates were scattered throughout the sample. It is likely that there were some agglomerates within the samples; however, in general, the particles were dispersed (Figure 1).

Soil Analysis

Soil samples were analyzed for total Ag by ICP-MS at the beginning of each toxicity test, with the exception of the collembolan test. The comparison between measured and nominal values yielded a consistent linear relationship ($r^2 \geq 0.98$) regardless of soil type and silver substance; the resultant comparison graphs (Figure S4) and linear equations (Table S2) are provided in the Supplementary Information. The mean recovery of total Ag from the soils was $87 \pm 6.8\%$. 

Toxicity Testing

The effect of AgNP was evaluated in two soil types (VSL and DSL), and compared to effects observed when exposed to AgNO₃ (i.e., Ag⁺); the earthworm tests were only conducted in the VSL soil due to poor control performance in the DSL soil (preliminary testing, results not presented). The resultant toxicity estimates are presented in Table 2; all tests met test validity criteria, and were not significantly influenced by the humic acid and PVP control treatments.

Plant growth was affected by both silver substances, with greater sensitivity demonstrated in the sandy loam (VSL) soil relative to the silt loam soil (DSL). With regards to toxicity, seedling emergence was the least sensitive endpoint; with varying degrees of sensitivity among the growth parameters (see Table 2). The growth of *E. lanceolatus* (northern wheatgrass) was more susceptible to the Ag (either form) in comparison to the *T. pratense* (red clover) and effects of the AgNO₃ were more pronounced than for the AgNP (Table 2). The plants that exhibited adverse effects on growth in the AgNP exposed soils had stunted roots (primary and lateral) with lighter coloured (green-yellow) leaves compared to control plants, whereas plants that exhibited adverse effects in AgNO₃ exposed treatments had both shoot and roots stunted.

*F. candida* were also more sensitive to the AgNO₃ in relation to the AgNP, with only slightly greater sensitivity observed in the VSL soil compared to the DSL soil (Figure 2). Adult survival was not compromised by the AgNP in either soil at the highest nominal test concentration (>2000 mg kg⁻¹), whereas there were no surviving adults in nominal test concentrations ≥476 mg AgNO₃ kg⁻¹ in the VSL soil. Juvenile production was significantly affected by the AgNO₃ treatments (Table 2) in both soils, with effects for AgNP visible in the VSL soil only (IC₅₀ =
694 mg kg\textsuperscript{-1}), a higher level than where juvenile production was inhibited in AgNO\textsubscript{3} contaminated soil (IC\textsubscript{50} = 124 mg kg\textsuperscript{-1}).

The concentrations at which the \textit{E. andrei} were able to detect and avoid the silver in soil were within the same order of magnitude as concentrations affecting their reproduction. Differences between the silver types were evident, such that \textit{E. andrei} avoided soil contaminated with 3.2 mg kg\textsuperscript{-1} AgNO\textsubscript{3} and 32 mg kg\textsuperscript{-1} AgNP (Figure 3). There was almost a 10-fold difference in sensitivity of \textit{E. andrei} to AgNO\textsubscript{3} impacted soil relative to AgNP-contaminated soil; these results are consistent with the observed plant and collembolan tests (Table 2). However, when results were evaluated for significance, no significant difference was detected for avoidance behaviour to the different silver forms (i.e., AgNO\textsubscript{3} and AgNP) in soil.

With regards to survival, live adult \textit{E. andrei} were found in (nominal) concentrations up to 2000 mg kg\textsuperscript{-1} in the AgNP treatments only, however, levels at which survival became compromised and inhibited by 50\% relative to the control was comparable to that determined in the AgNO\textsubscript{3} contaminated soil (Table 2; Figure 4). Similarly for juvenile production and dry mass, the difference in toxicity between the AgNO\textsubscript{3} and the AgNP was small relative to that observed for the plant and collembolan tests. Within the suite of toxicity endpoints presented here, earthworm reproduction and avoidance behaviour were the most sensitive to AgNP, whereby earthworm juvenile production and dry mass were significantly reduced at concentrations \(\geq 49\) mg AgNP kg\textsuperscript{-1}, and avoidance behaviour was significant at 32 mg kg\textsuperscript{-1}, with corresponding IC\textsubscript{50}s in the AgNO\textsubscript{3} contaminated soils of 29, 15 and 3.2 mg kg\textsuperscript{-1} for juvenile production, juvenile dry mass, and adult avoidance behaviour respectively (see Table 2).
Discussion

The greater sensitivity of both northern wheatgrass and red clover to Ag in the sandy loam soil relative to the silt loam soil are in agreement with Shoults-Wilson et al. (2011b), who also found greater toxicity in a sandy loam field soil of similar characteristics (76% sand, 16% silt, 7% clay, 1.8% organic matter and pH of 5.2) relative to a formulated artificial soil. Varying soil physical and chemical characteristics, such as pH, ionic strength, organic matter and clay content are known to affect the mobility and retention of silver, including engineered nanomaterials, which in turn affect the bioavailability and toxicity (Schlich and Hund-Rinke 2015; Sekine et al. 2015; Hansch and Emmerling, 2010; Tourinho et al. 2012; Cornelis et al. 2012; Cornelis et al. 2010). The literature suggests that a higher proportion of clay content increases substance binding (Cornelis et al. 2012; Cornelis et al. 2010, Schlich and Hund-Rinke, 2015) and possibly causes less bioavailability; however in the VSL soil, the clay content was greater (8.6%) than in the DSL (3.0%) soil. Schlich and Hund-Rinke (2015) demonstrate that soil grain size distribution plays the most significant role in changes in toxicity levels of AgNP to the soil microbial population in comparison to pH and organic carbon content. For both plant species, root length was the most sensitive endpoint to the AgNP in soil (Table 2); this agrees with other research that demonstrates altered morphology and significant root inhibition when exposed to AgNP (Dimkpa et al. 2013; Mirzajani et al. 2013; Lee et al. 2012). The mechanism of inhibition is unclear, although evidence suggests that the AgNP may aggregate on the root surface, and then proceed to translocate (in this case a greater effect was observed on shoot growth) (Dimkpa et al. 2013; Mirzajani et al. 2013), however it remains uncertain whether the resulting effect is due to the nanomaterial itself or ions released through dissolution of nanoparticles.
There are very few published studies on the toxicity of AgNP to springtails, despite their prevalence in soil systems, and the fact that *F. candida* is a model test organism for soil toxicity testing. Waalewijn-Kool *et al.* (2014) evaluated the effect of AgNO₃ and AgNP to *F. candida* in a field sand soil, and observed no toxicity with 3-8 nm AgNP coated in paraffin at a measured concentration of 673 mg Ag kg⁻¹ dry soil compared to a LC50 of 284 and an IC50 for juvenile production of 99.5 mg kg⁻¹ in AgNO₃ contaminated soil, which is comparable to the results reported here.

Earthworm results suggest no significant difference in avoidance behaviour between AgNO₃ and AgNP treatments, similar to findings reported by Shoults-Wilson *et al.* (2011c). The authors concluded that the earthworm avoidance response was sensitive to the nanoparticles, rather than to the dissolution of AgNP within the soil (i.e., Ag⁺) (Shoults-Wilson *et al.* 2011c). This contrasts with other research suggestive that effects of AgNP are attributed to Ag⁺ released from the AgNP (Cornelis *et al.* 2012; Cornelis *et al.* 2010; Bondarenko *et al.* 2013); and although some authors support this reasoning (Tourinho *et al.* 2012; Bondarenko *et al.* 2013; Allen *et al.* 2010), there is no general consensus (Dimkpa *et al.* 2013; Lee *et al.* 2012; Kim *et al.* 2011a; Ellegaard-Jensen *et al.* 2012; Cornelis *et al.* 2012). Juvenile production results were comparable to results reported by Schlich *et al.* (2013), who found *E. andrei* juvenile production affected by 15 nm AgNP at 74.3 - 146.0 mg kg⁻¹ and AgNO₃ at 42.0 - 46.9 mg kg⁻¹ in a field loam soil; since the amount of Ag⁺ did not differ between the silver substances, the authors concluded that the observed similarity in toxicity could be due to the released Ag⁺ measured in soil pore water. However, Shoults-Wilson *et al.* (2011a) found a marked difference in toxicity using artificial...
soil, such that *E. fetida* exposed to AgNO$_3$ experienced a significant decrease in cocoon production at 94.12 mg kg$^{-1}$ relative to those exposed to 30-50 nm PVP-coated AgNP, whereby cocoon production significantly decreased at 773.3 mg kg$^{-1}$. A study conducted by Diez-Ortiz *et al.* (2015) using a field sand soil demonstrated 50% inhibitory concentrations >4395 and 1420 mg kg$^{-1}$ for *E. fetida* adult survival and reproduction upon exposure to 50 nm AgNP; the soil had been aged for one week, and further aging yielded greater toxicity of the AgNP over time suggestive of slow dissolution over time. At nine weeks, the toxicity of the AgNP had increased by over two-fold and the EC50 for reproduction after 52 weeks reduced to 34 mg kg$^{-1}$ (Diez-Ortiz *et al.* 2015). As earthworms were the most sensitive of all species tested in this study, and involved the longest test duration (i.e., 63 days), it is possible that increased sensitivity was in part due to changes in dissolution and bioavailability over time. Alternately, the increased sensitivity could have been due to soil ingestion as a significant route of exposure. However, a dietary uptake study with *E. fetida* exposed to Ag$^+$ and AgNP demonstrated that 80 and 93% respectively were excreted within 48 h, and within two months, 97% of Ag$^+$ and 99% AgNP had been excreted (Lee *et al.* 2012).

**Conclusions**

The effect of nano and ionized forms of silver was compared in two agricultural field soils. In general, ionic silver was more toxic than the AgNP; however a clear relationship between these two forms of Ag is not obvious (i.e., the degree to which AgNO$_3$ is more toxic than AgNP). The difference in effect observed between the two forms of silver can vary based on test species, endpoint and soil type, regardless of form of silver. By examining two plant and invertebrate species in two different natural soils, this work demonstrates that exposure routes (e.g., soil pore...
water versus ingestion or interaction with soil particles), as well as soil physical and chemical properties all play an important role in the level of effect observed. In tests that were conducted across different soil types, organisms in the sandier soil exhibited a greater response to the Ag (ionic and nano) than those in soil with more silt. For single-species toxicity tests, the earthworms (avoidance and reproduction endpoints) were the most sensitive species to both AgNP and AgNO₃. To date, soil toxicity testing has tried to relate the effects of AgNP to Ag⁺ to understand the cause of any observed effects. However, it is now clear that this approach is not able to confirm this type of relationship as it does not take into consideration the ways in which nanoparticles and test organisms interact with the soil environment. In studies where the amount of Ag⁺ released from AgNP has been quantified, artificial or modelled systems are used. These study designs do not capture the complexity of a natural soil environment (Sekine et al. 2015; Lee et al. 2012; Ellegaard-Jensen et al. 2012; Leyard et al. 2012; Liang et al. 2013a-b). In order to better understand the fate and effect of nanoparticles in a soil environment, it will be critical to quantify the dissolution and transformation of these products in natural exposure scenarios.

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### Table 1: Selected physical and chemical characterization of the field soils.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>VSL</th>
<th>DSL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total cation exchange capacity (CEC) (meq/100g)</td>
<td>13</td>
<td>41</td>
</tr>
<tr>
<td>pH</td>
<td>5.8</td>
<td>7.2</td>
</tr>
<tr>
<td>Organic matter (%)</td>
<td>2.6</td>
<td>3.5</td>
</tr>
<tr>
<td>Clay (%)</td>
<td>8.6</td>
<td>3.0</td>
</tr>
<tr>
<td>Sand (%)</td>
<td>75</td>
<td>46</td>
</tr>
<tr>
<td>Silt (%)</td>
<td>16</td>
<td>51</td>
</tr>
<tr>
<td>Optimal moisture content (MC) (%)</td>
<td>25</td>
<td>21</td>
</tr>
<tr>
<td>Optimal MC as % WHC</td>
<td>50</td>
<td>37.5</td>
</tr>
<tr>
<td>Water holding capacity (WHC) (%)</td>
<td>50</td>
<td>55</td>
</tr>
</tbody>
</table>
Table 2: Estimated 50% effect levels (mg Ag kg\(^{-1}\) dry soil) of AgNO\(_3\) and 20 nm PVP-coated AgNP in sandy loam (VSL) and silt loam (DSL) soils; presented estimates are based on measured total silver in the soil at the beginning each test (unless otherwise noted). 95% upper and lower confidence levels are presented in parenthesis.

<table>
<thead>
<tr>
<th>Species/ Measurement</th>
<th>VSL Soil</th>
<th>DSL Soil</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AgNO(_3)</td>
<td>AgNP</td>
</tr>
<tr>
<td></td>
<td>EC/LC/IC50</td>
<td>EC/LC/IC50</td>
</tr>
<tr>
<td><strong>Elymus lanceolatus</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Emergence</td>
<td>298 (253-351)</td>
<td>&gt;1923</td>
</tr>
<tr>
<td>Shoot length</td>
<td>77 (65-91)</td>
<td>1104 (514-1496)</td>
</tr>
<tr>
<td>Root length</td>
<td>45 (34-55)</td>
<td>389 (305-470)</td>
</tr>
<tr>
<td>Shoot dry mass</td>
<td>40 (20-99)</td>
<td>805 (366-1361)</td>
</tr>
<tr>
<td>Root dry mass</td>
<td>99 (80-124)</td>
<td>732 (1167-3266)</td>
</tr>
<tr>
<td><strong>Trifolium pratense</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Emergence</td>
<td>188 (153-234)</td>
<td>&gt;1807</td>
</tr>
<tr>
<td>Shoot length</td>
<td>54 (45-64)</td>
<td>1167 (851-1601)</td>
</tr>
<tr>
<td>Root length</td>
<td>75 (69-84)</td>
<td>642 (579-707)</td>
</tr>
<tr>
<td>Shoot dry mass</td>
<td>85 (67-108)</td>
<td>&gt;1807</td>
</tr>
<tr>
<td>Root dry mass</td>
<td>106 (91-123)</td>
<td>&gt;1807</td>
</tr>
<tr>
<td><strong>Folsomia candida</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adult lethality</td>
<td>216 (172-279)(^a)</td>
<td>&gt;1792</td>
</tr>
<tr>
<td>Juvenile production</td>
<td>114 (71-185)(^a)</td>
<td>649 (204-2000)</td>
</tr>
<tr>
<td><strong>Eisenia andrei</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adult avoidance behaviour</td>
<td>3.2 (1.0-10)</td>
<td>32 (10-100)</td>
</tr>
<tr>
<td>Adult lethality</td>
<td>152 (132-179)</td>
<td>226 (192-260)</td>
</tr>
<tr>
<td>Juvenile production</td>
<td>29 (10-40)</td>
<td>&gt;49(^b)</td>
</tr>
<tr>
<td>Juvenile dry mass</td>
<td>15 (7.9-28)</td>
<td>&gt;49(^b)</td>
</tr>
</tbody>
</table>
a Estimated values based on measured concentrations in plant and earthworm toxicity tests (see table S2).

b A significant depression in juvenile production and mean dry mass was measured at test concentrations >49 mg kg\(^{-1}\) relative to the control response; however, a lack of reproduction in the control soil precluded statistical analysis of toxicity endpoints.

c Definitive testing not conducted, as earthworms did not meet test validity criteria for this soil type.

Figure 1

Figure 2
Figure 3

Figure 4
Figure Captions

**Figure 1:** Example of TEM image (A) showing 20 nm silver nanoparticles dispersed in 1.8% humic acid solution; (B) shows the 20 nm AgNP agglomerating in a ring, an artifact of sample preparation (drying of the droplet on the TEM grid).

**Figure 2:** The effect of AgNO$_3$ (solid line) and 20 nm PVP-coated AgNP (dashed line) on mean (± standard deviation) *F. candida* adult survival (left) and juvenile production (right) in VSL (top) and DSL (bottom).

**Figure 3:** Percent avoidance of *E. andrei* AgNO$_3$ and 20 nm PVP-coated AgNP in VSL soil.

**Figure 4:** The effect of AgNO$_3$ (solid line) and AgNP (dashed line) on mean (± standard deviation) *E. andrei* adult survival (top), juvenile production (mid) and individual juvenile dry mass (bottom) in VSL soil.