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Terrestrial Fate and Effects of Nanometer-Sized Silver

David R. Johnson, Robert E. Boyd, Anthony J. Bednar,
Cynthia J. Banks, Charles A. Weiss, Jr., Jessica G. Coleman,
Burton C. Suedel, and Jeffery A. Stevens

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Abstract

Although engineered nanomaterials are active components in a wide variety of commercial products, there is still limited information related to the effects of these nanomaterials once released into the terrestrial environment. A high number of commercial applications use silver nanoparticles (nAg) due to its anti-microbial activity. This may be of concern for waste management since nAg could be applied to soil (e.g., biosolids) or disposed of in traditional landfills, which could lead to possible leaching into surrounding soil. This report aims to provide additional insight into the fate and effects of nAg in terrestrial systems. The studies in this report examine the leachability of nAg in field soil and compares the soil migration to bulk (i.e., micron-sized) silver; examine the ecotoxicity of nAg to earthworms in four field soils spanning several different soil orders; and examine the behavioral effects of earthworms when exposed to engineered nanoparticles in field soil. These data provide additional insight into engineered nanoparticle fate and effects to terrestrial receptors in field soils, an important distinction from laboratory-generated soils. These data will also assist ecological risk assessors to better determine the acute environmental risks of nAg in terrestrial ecosystems with different soil compositions.

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Preface

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1 Introduction

1.1 Background

A variety of commercial products contain engineered nanomaterials as active components, but information regarding their fate in a product's end-of-life management and their effects on the environment are limited. Due to its anti-microbial activity, many commercial applications use silver nanoparticles (nAg), which could be concerning for waste management since nAg could be applied to soil (e.g., biosolids) or disposed of in traditional landfills. This has the possibility of leaching into the surrounding soil and impacting the terrestrial environment. A few studies have been conducted examining the leachability of nAg in field soil, which provides more insight into these nanoparticles.

1.2 Objective

The aim of this technical report (TR) is to have a better understanding of the effect of nAg in different soils. Studies within this TR examined the leachability of nAg in field soil and compared the soil migration to bulk (i.e., micron-sized) silver; examined the ecotoxicity of nAg to earthworms, compared to bulk silver, in four field soils that spanned several soil orders; and examined changes in earthworm behavior when exposed to engineered nanoparticles in field soil.

1.3 Approach

This report compiles information from three independent studies describing different aspects of the environmental fate and ecotoxicological effects of nAg in terrestrial systems. These data will also assist ecological risk assessors to better determine the acute environmental risks of nAg in terrestrial ecosystems with different soil compositions.

2 Soil Leachability of Engineered Silver Nanoparticles using a Column Method

Summary: Silver nanoparticles (nAg) are engineered nanomaterials used in several commercial applications due to its anti-microbial activity. This fact is of concern because guidance is scarce concerning engineered nanomaterial waste management. Many nAg products may be directly applied to soil (e.g., pesticide applications) or disposed in traditional landfills, resulting in possible leaching into surrounding soil. The aim of this soil column study was to determine the leaching potential of soil spiked with nAg (35 nm), micron (μm)-sized Ag (20 nm, 1.5-2.5 μm), and polyvinylpyrrolidone (PVP)-coated nAg (PVP-nAg; 20 nm) as a function of particle size, coatings, and/or leachate solution pH. Uncontaminated field soil was spiked with nano- and micron-Ag at concentrations of 0.01 and 100 mg/kg. Leachate samples were collected every hour for 48 hr and were subjected to total Ag analysis by inductively coupled plasma-mass spectrometry (ICP-MS). The analyses revealed that for 100 mg/kg spiked soils, nAg, micron-sized Ag, and PVP-nAg were not mobilized through the soil when leached with ultrapure water or synthetic acid rain solutions; however, silver mobility did increase when leached with a 1% nitric acid solution. In 0.01 mg/kg spiked soils, less Ag leached from the PVP-nAg soil when compared to nAg and micron-sized Ag soils. The present experiments indicate that it is unlikely Ag from engineered nAg will leach from a soil system except under extremely acidic conditions, resulting in an increased potential for exposure to terrestrial organisms.

2.1 Introduction

Nanosized silver (nAg) is a metal-based engineered material with strong anti-microbial properties. Silver has long been used in wound and burn dressings (Chen and Schluesener 2008; Burrell 2009) but recently, it has become one of the most utilized nanoparticles in commercial applications. It has gained popularity among consumer products such as anti-microbial fabrics (e.g., military force protection), surface coatings, disinfectants, and health food supplements (Luoma 2008). With the rapid pace of nAg developments, the usage of nAg and its presence in multiple waste streams should be expected to increase. According to the National Nanotechnology Initiative, potential uses of nAg for large-scale applications include clean-up of oil spills and other hazardous chemicals

(<http://www.nano.gov/html/facts/nanoapplicationsandproducts.html>). Environmental

regulations for Ag have undergone several iterations (Purcell and Peters 1999; Luoma 2008). Although nAg is a relevant material in commercial and military applications, these materials are under limited regulation. nAg will likely be released into the environment through leaching from landfills, rinsing of personal products and discharge in wastewater treatment plant effluent (Adams and Kramer 1999a and 1999b), and intentional or accidental spills (Burrell 2009); therefore, it is critical to gain more knowledge regarding nanomaterials waste management (Bystrzejska-Piotrowska et al. 2009).

Although recent studies have investigated soil mobility of nano-aluminum and other metals (Bednar et al. 2008, 2009; Doshi et al. 2008; Darlington et al. 2009), limited information is available on the effects of nAg in the soil environment. To assess the effects of nAg in aquatic systems, toxicity and bioaccumulation studies have been conducted with Japanese medaka (*Oryzias latipes*) (Chae et al. 2009), particle feeding invertebrates (*Ceriodaphnia dubia*) (Gao et al. 2009), fathead minnow (*Pimephales promelas*) embryos, (Laban et al. 2010), freshwater algae (*Chlamydomonas reinhardtii*) (Navarro et al. 2008), and rainbow trout (*Oncorhynchus mykiss*) (Scown et al. 2010). There have been several nAg studies investigating the antibacterial effects on common microorganisms, *Streptococcus mutans* (Espinosa-Cristobal et al. 2009), *Escherichia coli*, *Staphylococcus aureus*, *Aspergillus niger*, and *Penicillium phoeniceum* (Khaydarov et al. 2009). Luoma (2008) determined that nAg can enter cells through active transport called endocytosis. In addition to the environmental impact of nAg, media outlets have publicized that the dermal penetration, ingestion, or inhalation of colloidal Ag can cause considerable side effects (<http://today.msnbc.msn.com/id/22536241>). Two recent studies revealed that fabrics impregnated with nAg release the nanomaterials in wastewater after repeated agitation (Benn and Westerhoff 2008; Duran et al. 2010). To our knowledge, there is currently no published data on leaching of nAg into a terrestrial system. However, the US Environmental Protection Agency (USEPA) is considering conditional registration for pesticides containing nAg (http://www.epa.gov/oppfead1/cb/csb_page/updates/2010/nanosilver.html), so the fate and effects of nAg in soil ecosystems is a subject area that needs close attention.

Leaching and soil migration has been traditionally studied with the use of column methods for soil contamination including polycyclic aromatic hydrocarbons (Enell et al. 2004), fly ash (Jackson et al. 1984), halogenated

organic compounds (Kartal et al. 2009), metals, and oxyanions. The purpose of this research was to perform a series of soil column studies to determine the leachability of engineered nAg (35 nm), micron (μm)-sized Ag (1.5-2.5 μm), and polyvinylpyrrolidone (PVP)-coated nAg (20 nm) through a field soil as a function of size and/or coating related effects. Further studies examined the effects of synthetic acid rain leachate on the nAg migration in soil, thus providing more realistic Ag leachate information to further assist the regional environmental risk assessments of nAg in the environment. This research will help elucidate the potential risks posed by the management of waste nanomaterials and provide fundamental information on various nAg soil migration.

2.2 Materials and methods

2.2.1 Test soil

A silty loam soil of the Grenada-Loring (GL) series (Alfisols order) collected from Learned, MS was used in all experiments. After shipment, the soil was sieved (<1 cm) and characterized as follows: texture (3% sand, 72% silt, and 26% clay), total organic carbon (0.7%), percent organic matter (1% loss on ignition), pH (6.7), and cation and anion exchange capacity (0.075 and 0.025 meq g^{-1} , respectively) (Bednar et al. 2008; Inouye et al. 2006).

2.2.2 Chemical analysis

Elemental analysis of soil and water samples was performed by inductively coupled plasma-mass spectrometry (ICP-MS; Elan DRC II, Perkin Elmer, Waltham, MA) following USEPA Method 6020. The soil samples were digested following USEPA Method 3050B. All samples were diluted in 1% nitric acid prior to ICP-MS analysis. Scandium, yttrium, rhodium, terbium, and holmium were added on-line prior to the nebulizer using a mixing T as internal standards to correct for instrumental drift. Calibration and second source verification standards were NIST-traceable and purchased from SPEX Certiprep (Metuchen, NJ) and CPI International (Santa Rosa, CA). All calibration verification and matrix spike results were within 10% of the nominal values. Additionally, a commercially available reference solution from Environmental Resource Associates (Golden, CO; Lot number P136-500) was also analyzed in each analytical batch with analyte recoveries within 20% of the certified value.

Less than 0.100 mg/kg of Ag was detected in the unamended field soil (data not shown).

2.2.3 Reagents and eluent solutions

All experiments used ultrapure water collected from a Millipore Solution 2000 Water Purification (Billerica, MA) system and had a pH of 6.0. Nitric and sulfuric acids were both at >90.0% purity and obtained from Fisher Scientific (Pittsburg, PA). The 1% HNO₃ solution was prepared by adding 145 ml of nitric acid to 10 L of ultrapure water and mixed for 20 min. The pH of the resulting solution was 1.0. The synthetic acid rain solution was adapted from USEPA Method 1312 in which two pH solutions were prepared to be representatives of acid rain conditions east and west of the Mississippi River, US. Eastern US conditions were created by dilution of a 60/40 weight percent mixture of sulfuric and nitric acids in ultrapure water until a pH of 4.2 ± 0.05 was achieved. To represent Western US conditions, the same acid mixture was diluted in ultrapure water to yield a pH of 5.0 ± 0.05 .

2.2.4 Material characterizations

Engineered nAg, micron-sized Ag, and PVP-nAg powders of sizes 35 nm, 1.5-2.5 μm , and 20 nm, respectively, (NanoAmor, Houston, TX) were used for all soil spiking experiments. All engineered materials were received from the manufacturer in the form of dry powders. Once received from the manufacturer, the Ag powders were kept in an “as is” condition. Dynamic light scattering (DLS) was utilized to determine particle size and stability (90 Plus/BI-MAS, Brookhaven Instruments, Holtsville, NY). To characterize nAg particles in solutions comparative to test media, DLS measurements were conducted in a 1% nitric acid solution. To measure primary particle size, all nAg particles were sonicated (Branson Sonifier 450, Branson Ultrasonic) for 30 min at a 20-Watt, 40% duty cycle. Measurements for micron-sized Ag were not possible due to large particle size, which is outside of the upper diameter limit for the DLS instrumentation. To determine particle morphology, imaging analysis of the Ag powders was obtained through use of low vacuum, field emission scanning electron microscopy (SEM) (Nova 600 NanoSEM, FEI, Hillsboro, OR). Images were simultaneously collected of both secondary and back scattered electrons providing topographical and chemical information. Energy dispersive x-ray (EDX) spectroscopy (Quantax

System, Ewing, NJ) verified chemical composition of the soils spiked with 100 mg/kg Ag.

2.2.5 Soil leaching tests

The mobility of nAg through soil was tested in a manner similar to previously described procedures (Bednar et al. 2008). The GL soil was spiked with nAg, micron-sized Ag, and PVP-nAg dry powders at concentrations of 0.01 and 100 mg/kg. Ag powders were added to the soil in a 20 L glass jar, rapidly shaken, and then placed on a rolling apparatus at four rotations per minute for 24 hr. Three clear polycarbonate columns (10.2 cm diameter by 5.1 cm tall) were slurry packed by mixing 410 g dry spiked field soil and 171 ml ultrapure water. The columns had a porous polyethylene frit at the bottom and a piece of filter paper (Whatman #4) was placed over the frit to retain soil fines. The slurry was poured into the appropriate column, and a piece of filter paper was placed on top of the slurry. Each column was tapped lightly to remove any air trapped in the slurry.

Fresh columns were prepared for each leaching solution, which included ultrapure water, 1% nitric acid, and pH 4.2 and 5.0 synthetic acid rain solutions. The eluent was pumped through the columns in parallel with a diaphragm pump (CHEM-TECH, Lowell, MA) at a constant flow rate of approximately 35 ml per column per hour. An automated fraction collector (Waters, Milford, MA, USA) collected samples hourly over the course of 48 hr. The leachate samples were analyzed for total Ag as described above.

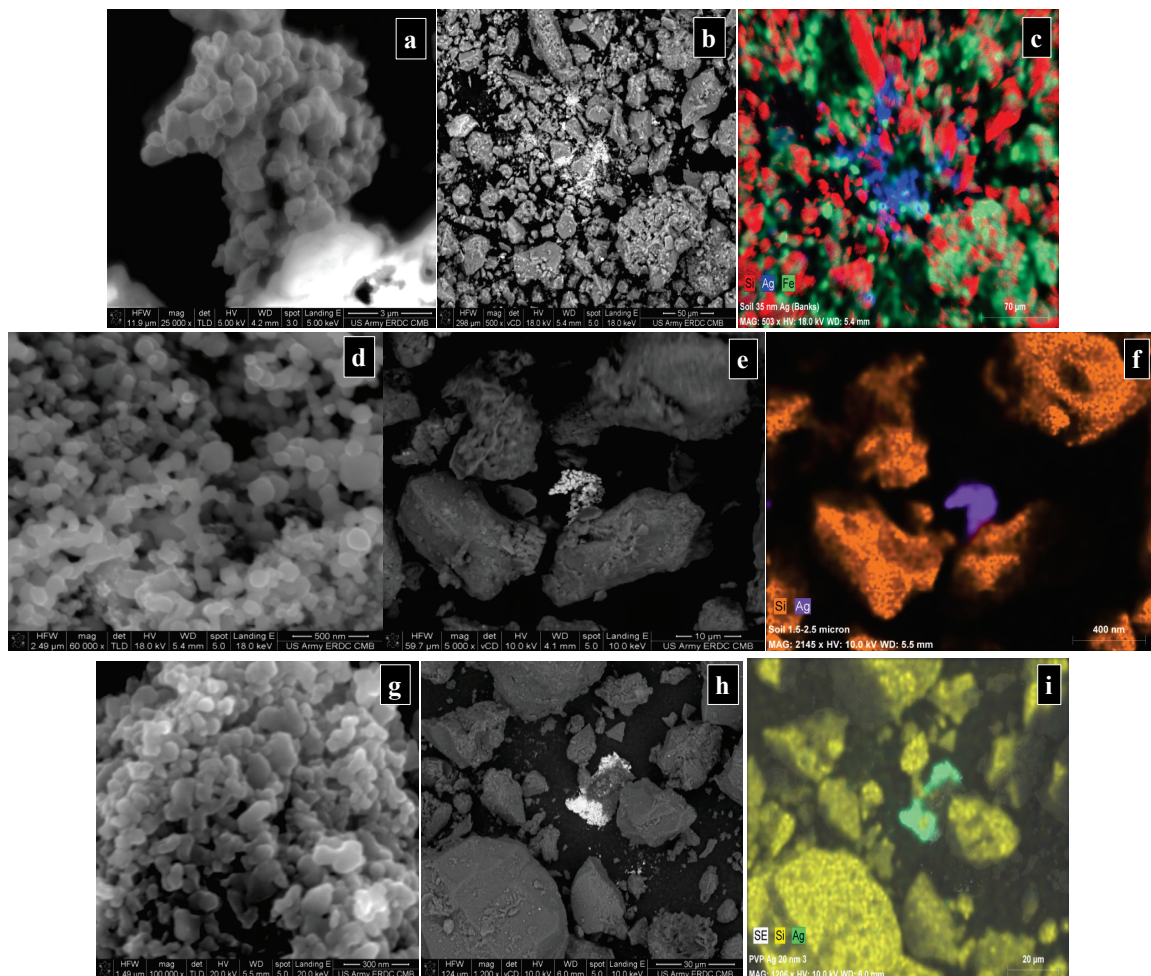
2.3 Results

2.3.1 Material characterization

Nanoparticle characterization can prove challenging even with the most sophisticated analytical equipment (Domingos et al. 2009); however, characterization is essential. Thus, particle characterization was conducted to verify the manufacturer's reported information. DLS analysis of nAg in 1% HNO₃ resulted in bimodal size groups of 180-259 and 926-1427 nm. The PVP-nAg contained trimodal size groups in the range of 80-132, 247-432, and 846-1324 nm. Although DLS results provide an overview of particle size within solutions, it also assumes samples consist of all spherical particles (Chappell et al. 2008); therefore, results should be interpreted carefully in correlation with additional particle size measurements. For all three Ag powders, most particles appeared

spherical, though they varied in size and agglomeration status. These data illustrate that nAg was highly agglomerated, mostly below one micrometer, as corroborated by SEM analysis (Figure 1). The nAg particle agglomerates did not disperse once mixed into the soil but were simply randomized nanoparticle hotspots distributed throughout the soil sample.

Figure 1. SEM/EDX analyses of Ag nanopowder. A) Ag micron-sized powder (manufacturer size: 1.5-2.5 μm ; size bar: 3 μm); B) Ag powder in field soil (size bar: 50 μm); C) EDX analysis of metallic elements in Ag-spiked field soil (EDX elements: blue, silver; red, silicon; green, iron) (size bar: 70 μm); D) nAg nanopowder (manufacturer size: 35 nm; size bar, 500 nm); E) nAg powder in field soil (size bar: 10 μm); F) EDX analysis of metallic elements in nAg-spiked field soil (EDX elements: purple, silver; orange, silicon) (size bar: 400 nm); G) PVP-nAg nanopowder (manufacturer size: 20 nm; size bar: 300 nm); H) PVP-nAg nanopowder in field soil (size bar: 30 μm); I) EDX analysis of metallic elements in PVP-nAg-spiked field soil (EDX elements: green, silver; yellow, silicon) (size bar: 20 μm).



2.3.2 Soil leaching tests

Leaching of the 100 mg/kg Ag-spiked field soil with all three Ag particle types resulted in similar leachate profiles. As shown in Figure 2, no Ag was detected in the column leachate when the spiked field soils were leached with ultrapure water. Silver leaching through the field soil column was observed over the 48-hr study period when leached with a 1% HNO₃ solution (Figures 3 and 4). In 100 mg/kg spiked soils (Figure 3), it took approximately 15 hr for Ag to increase in field soil leachates above baseline levels for both nano and micron-sized Ag columns. Micron-sized Ag peaked at 20 hr (3.5 mg Ag leached), then decreased to near non-detectable levels by 30 hr. Nano-sized Ag-spiked field soil also demonstrated a maximum mass occurring at approximately 20 hr (2.0 mg Ag leached); although a smaller amount of Ag was leached from the system compared to micron-sized Ag. The PVP-nAg-spiked field soil behaved differently with the elution peak of Ag starting after only 7 hr. Silver peaked in the PVP-nAg field soil at 10 hr (7.5 mg Ag leached). Overall, the nAg-spiked field soil leached less total Ag when compared to micron-sized Ag and PVP-nAg-spiked field soils; however, the PVP-nAg-spiked field soil leached Ag more rapidly.

Figure 2. Mass of Ag leached using ultrapure water from a 100 mg/kg Ag spiked soil.

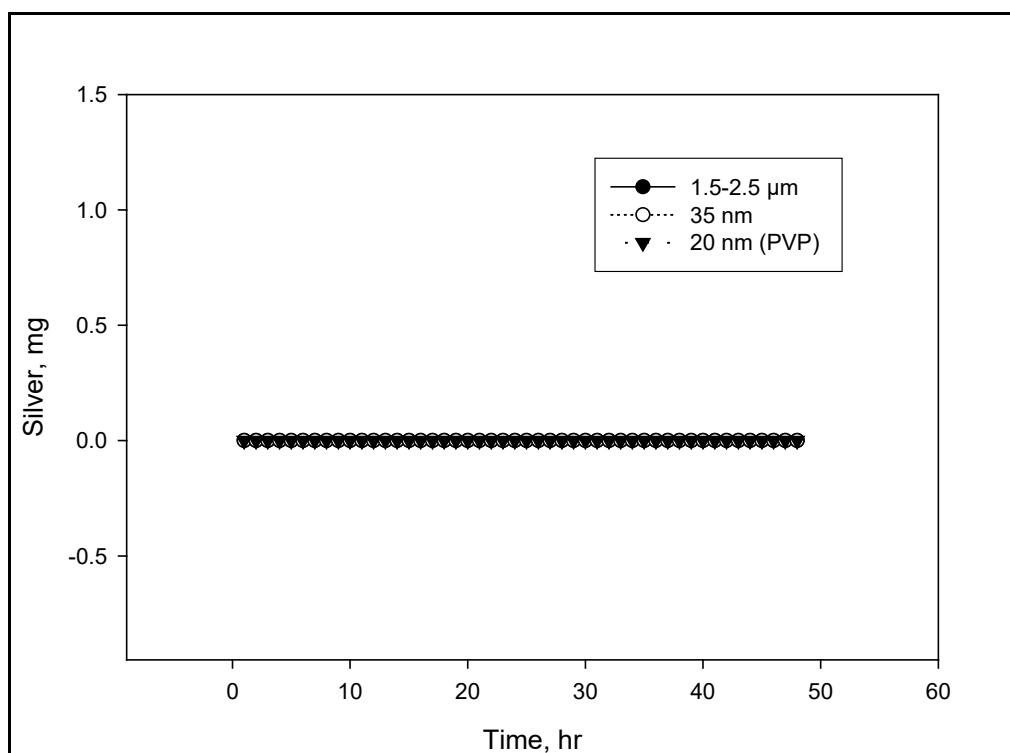


Figure 3. Mass of Ag leached using 1% HNO₃ from a 100 mg/kg Ag-spiked soil.

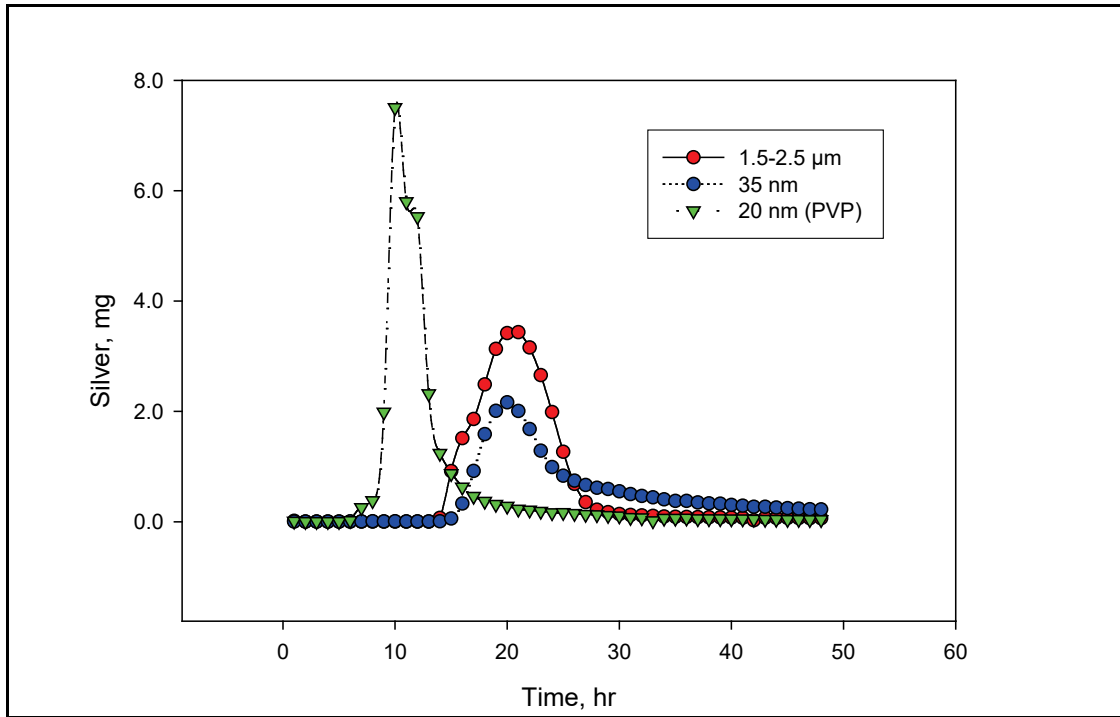
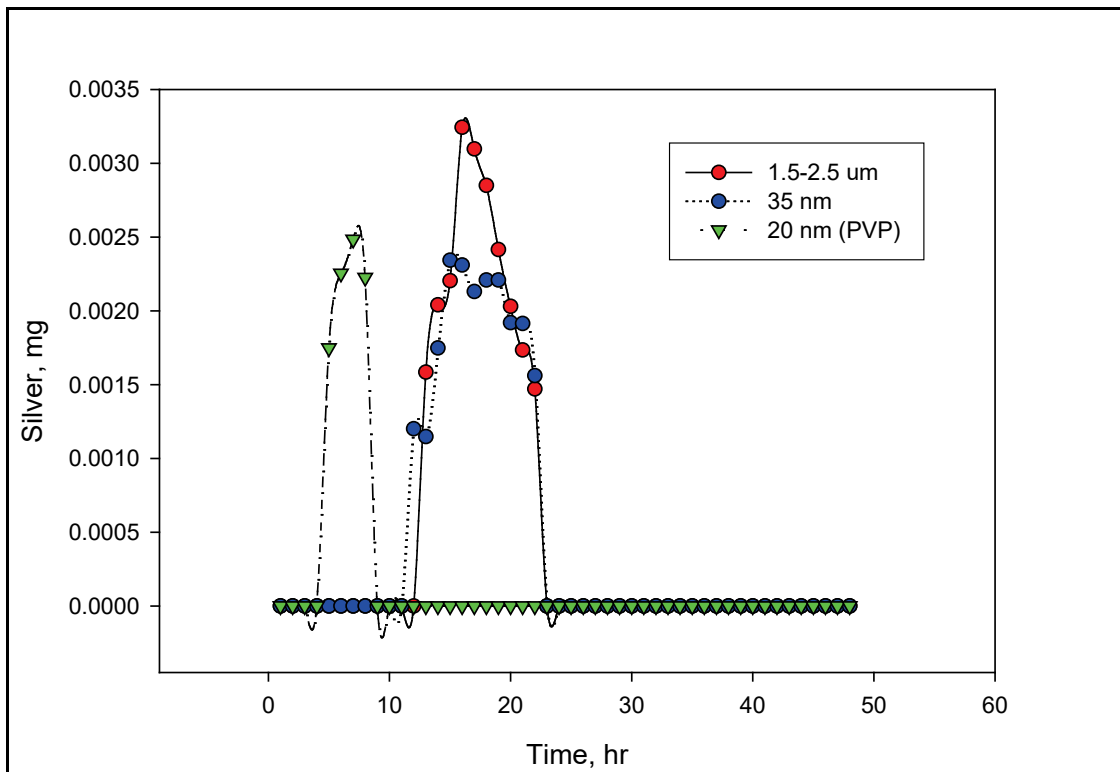


Figure 4. Mass of Ag leached using 1% HNO₃ from a 0.01 mg/kg Ag-spiked soil.



In 0.01 mg/kg spiked field soils (Figure 4), Ag mass increased above baseline after 12 hr in both nAg and micron-sized Ag-spiked field soils. The highest Ag mass for both nAg (0.0024 mg Ag leached) and micron-sized Ag (0.0033 mg Ag leached) -spiked field soil was at 15-16 hr. PVP-nAg mass peaked at 7 hr (0.0025 mg Ag leached). When compared to the nAg and micron-sized Ag-spiked field soil, less Ag was leached from the PVP-nAg-spiked field soil. Similar to the high concentration soil, the 0.01 mg/kg PVP-nAg-spiked field soil leached Ag more rapidly than nAg and micron-sized Ag.

Synthetic acid rain leaching of the Ag-spiked field soil resulted in very low Ag elution from soil under the Eastern or Western US acid rain solutions regardless of the high Ag concentration (Figures 5 and 6, respectively). The present data indicated that it is unlikely Ag will leach from a field soil system unless under extremely acidic scenarios (e.g., 1% nitric acid).

Figure 5. Mass of Ag leached from field soil in an Eastern US acid rain scenario (pH 4.2); field soils were spiked with 100 mg/kg Ag. Top, silver leachate scale from 0-1.5 mg; bottom, silver leachate scale reduced approximately 1000x (0-0.0012 mg).

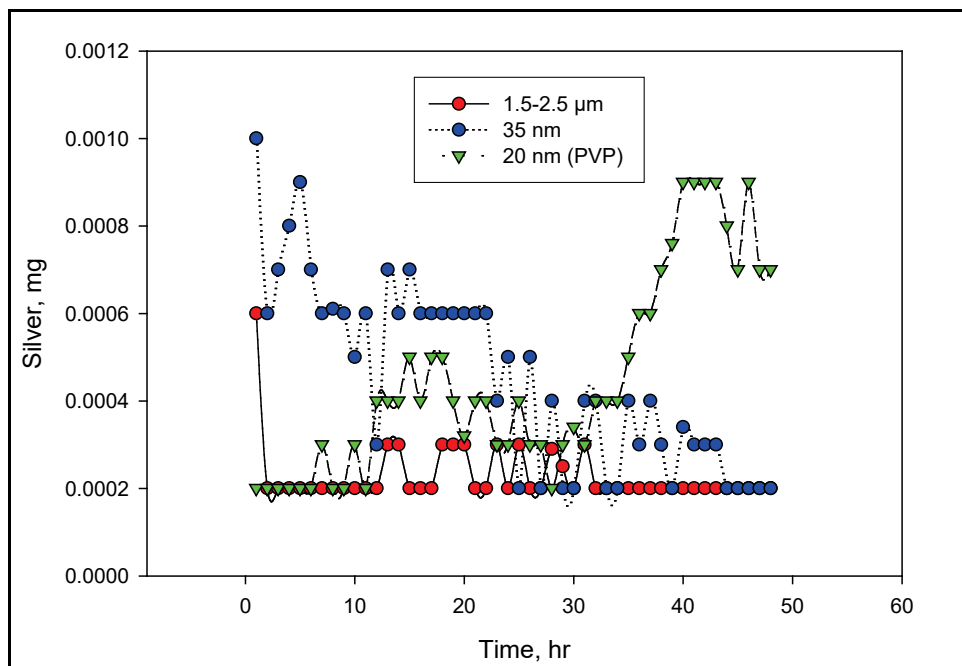
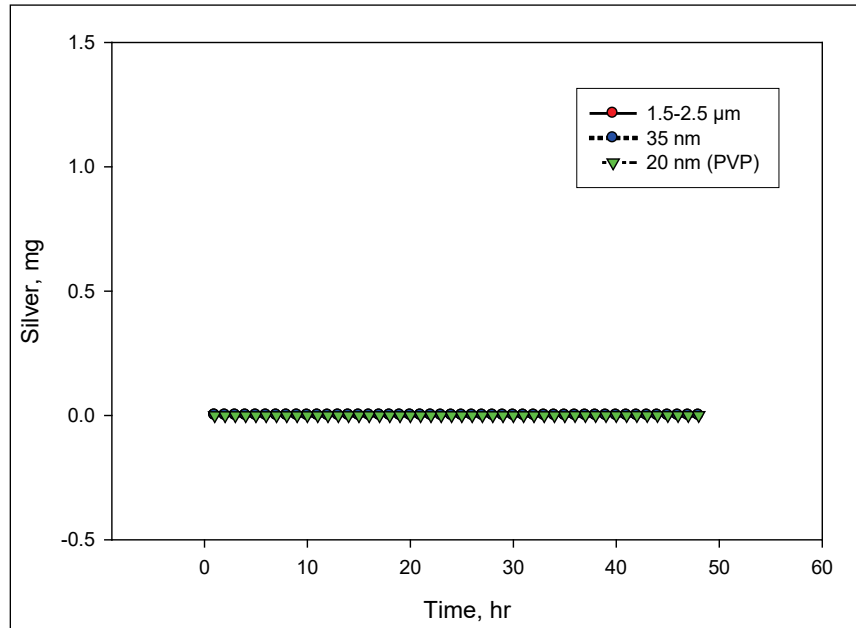
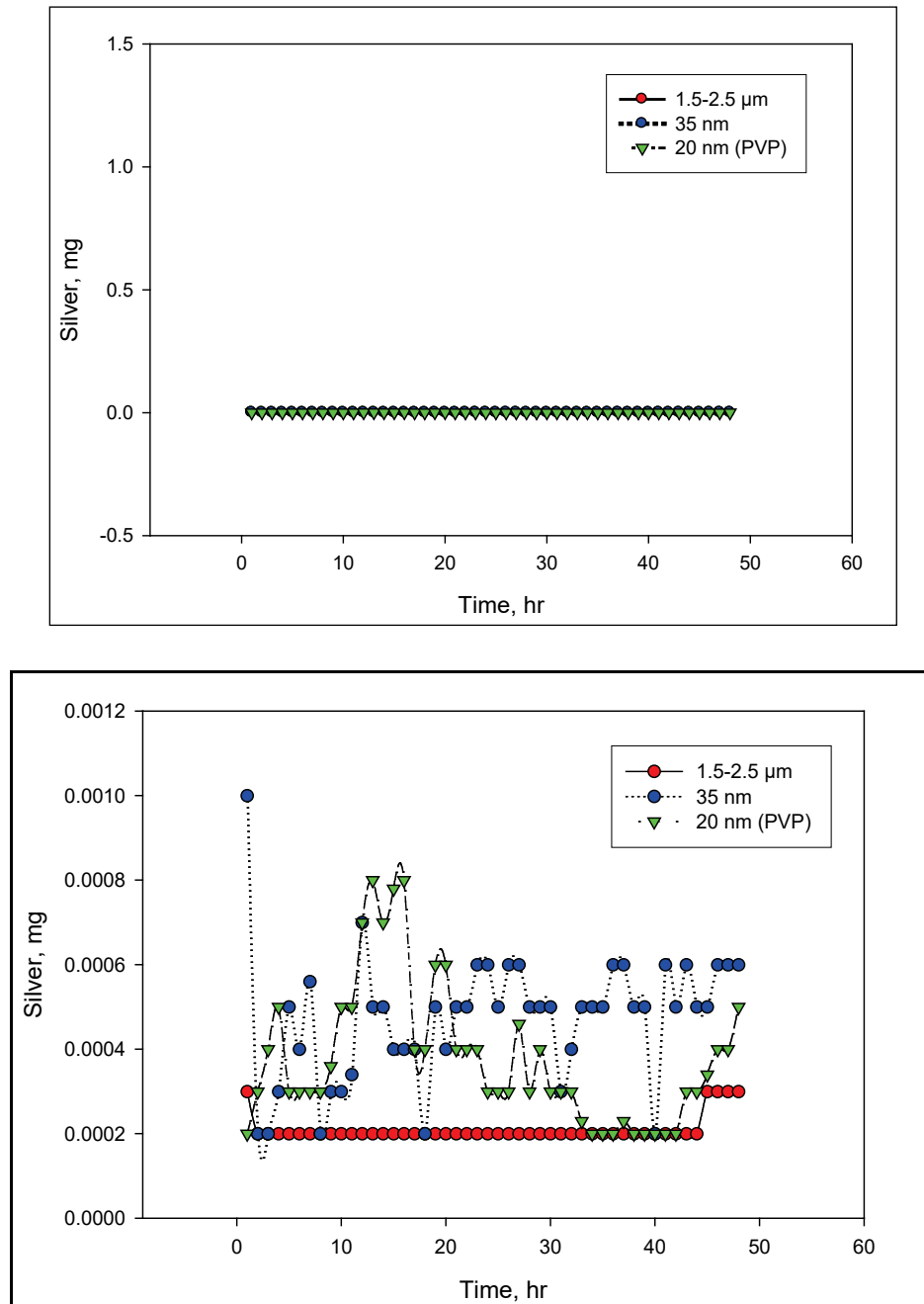


Figure 6. Mass of Ag leached from field soil in a Western US acid rain scenario (pH 5.0); field soils were spiked with 100 mg/kg Ag. Top, silver leachate scale from 0-1.5 mg; bottom, silver leachate scale reduced approximately 1000x (0-0.0012 mg).



2.4 Discussion and conclusion

Silver is a naturally occurring element in the earth's crust, usually present in low soil concentrations except at major deposits (Purcell and Peters 1998; Luoma 2008). While some Ag is found naturally as nanosized particles, advances in materials sciences are dramatically increasing the

use of nAg in consumer products and, thus, the potential for release of these engineered nanoparticles into the environment. The purpose of this study was to investigate Ag leaching through a field soil system by column tests, with emphasis put on the importance of particle size, coatings, and leaching solution pH. Initial leaching tests did not result in Ag leaching in ultrapure water, regardless of Ag particle size, coating, or agglomeration. This result seems intuitive given the low solubility of Ag oxide which will likely coat the surface of a metallic Ag particle as it oxidizes and binds to soil particles. Furthermore, nAg coated with PVP will be stabilized by the organic coating, resulting in limited Ag ion dissolution until the coating degrades.

Little or no Ag leaching was observed with weakly acidic solutions that mimic acid rain conditions. However, Ag mobilization was observed with highly acidic solutions, likely as a result of the release of Ag ions. In 100 mg/kg spiked field soils, the nAg soil leached less total Ag while less Ag was leached out of the PVP-nAg soil in 0.01 mg/kg spiked soils. This suggests that at higher concentrations, nAg is less likely to leach from soil. The data also suggested the PVP coating of nAg may play a role in rapid leaching, affecting the release of Ag⁺ ions or the mobility of nAg particles in soil. These results illustrate that Ag migrated through soil only at very low pH levels, indicating that the soil leachability of nAg was leachate pH dependent. Silver particles are therefore likely to remain on the soil surface where they can be contacted by organisms living in the upper soil horizons (top 10 in.) and terrestrial plants.

In a related research effort (chapter 3 of this report), we found that earthworms (*Eisenia fetida*) displayed avoidance behavior of field soil containing nAg at >100 mg/kg yet was not affected in field soil containing micron-sized Ag at the same concentration. These data suggest that if more than 100 mg/kg nAg accumulates in the soil (mimicking chronic application or spills), this could cause terrestrial organisms to leave field soil containing nAg.

Engineered nanoparticles of aluminum oxide (Al₂O₃) (Coleman et al. 2010), titanium dioxide (TiO₂), and zinc oxide (ZnO) (Hu et al. 2010) in soil could pose risks to terrestrial organisms if these materials accumulate sufficiently in the soil environment. Bioaccumulation and soil avoidance data indicated only concentrations >2,500 mg/kg of nano Al₂O₃ would result in reduced reproduction and trigger avoidance behavior in

earthworms (Coleman et al. 2010). Research also indicated that TiO_2 and ZnO concentration exceeding >1000 mg/kg in soil yielded bioaccumulation in earthworms sufficient to produce harmful effects as manifested by oxidative stress and DNA damage (Hu et al. 2010).

It is estimated that 1,814 products were added to the Nanotechnology Consumer Product Inventory in 2014, with nanosilver being the most frequently used nanomaterial (435 products, or 24% of all products on the inventory) (Vance et al. 2015). As production and usage of these materials increase, so will the amount of waste that is produced (Bystrzejewska-Piotrowska et al. 2009). Currently, regulations are limited regarding the management of nanomaterials. For example, only 10% of manufactured fullerenes are usable while the remaining 90% are considered waste and therefore sent to landfills (RCEP 2008). Since nAg is currently the most commercially utilized nanomaterial, it is critical to gain knowledge of the fate of Ag particles in soil, including leaching and migration potential, to better understand the risk posed to the environment.

The leaching data in this study may provide useful information to assist in developing future regulations and remediation strategies for nAg; however, additional research is warranted. Future experiments describing kinetics of dissolution and elution of particles vs. dissolved Ag, as well as the role of soil matrix components (e.g., organic matter content) would build on the current study. Overall, the data presented here provide fundamental insights into the release and removal of nAg in soil protecting environmental and human health.

3 Silver Nanoparticle-Induced Toxicity to Earthworms in Different Soil Orders

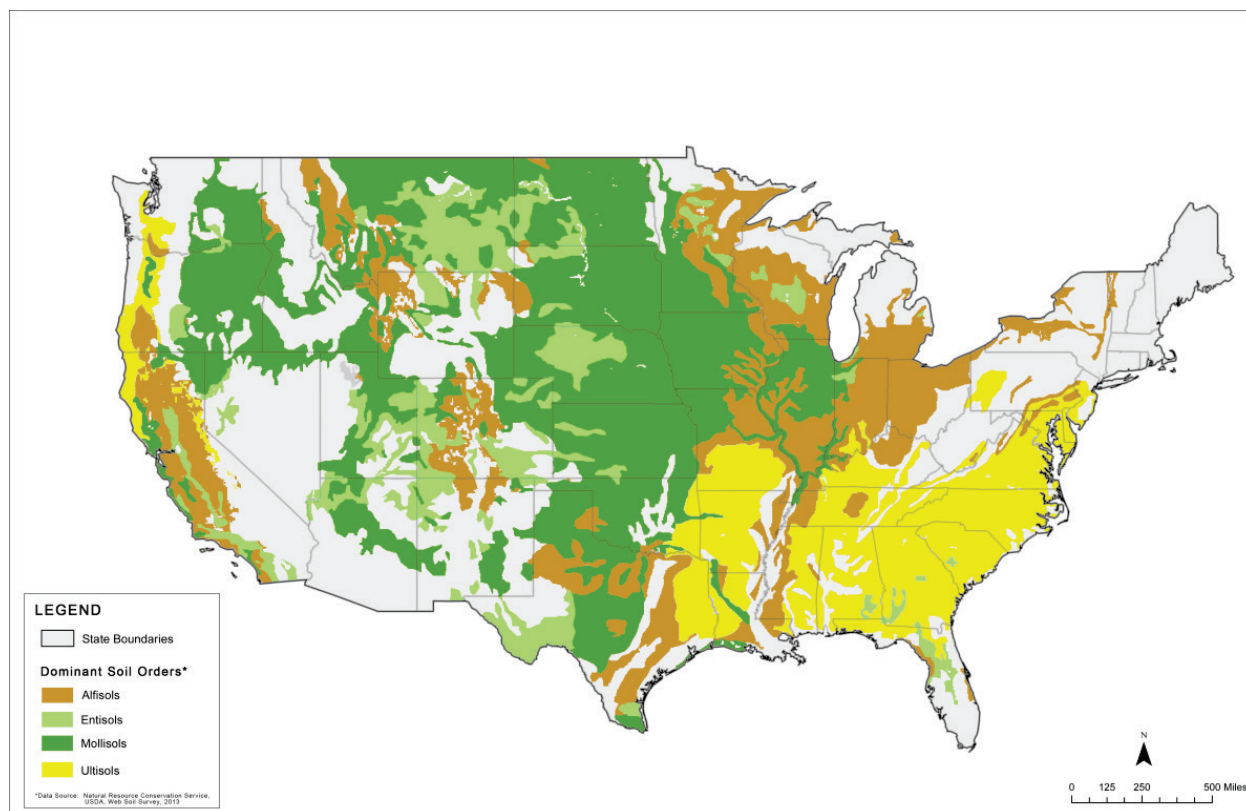
Summary: This study examines the acute toxicity of polyvinylpyrrolidone (PVP)-coated silver nanoparticles (nAg; 38.6 nm diameter) to terrestrial invertebrates (earthworms, *Eisenia fetida*) in four field soils spanning several soil orders. The 14-day median lethal concentrations for 50% of the population (LC₅₀) for PVP-nAg in an Alfisol soil was 2,828 mg/kg; in contrast, AgNO₃ was more toxic than PVP-nAg in the same soil (LC₅₀=223 mg/kg). PVP coating does not contribute to PVP-nAg soil toxicity (LC₅₀ > 40,000 mg/kg). PVP-nAg LC₅₀ in Mollisol, Ultisol, and Entisol soils were >1,000 mg/kg. These data demonstrate that PVP-nAg is not acutely toxic to earthworms in natural soils used in these experiments except at concentrations that may result from high or accidental releases. These data will assist ecological risk assessors to better determine the acute environmental risks of silver nanoparticles in terrestrial ecosystems with different soil compositions.

3.1 Introduction

The unique properties of engineered nano-sized silver particles (nAg; <100 nm in size) have led to its ever-increasing presence in consumer products, such as clothing, washing detergents, soaps, bandages, and various coating applications (Benn and Westerhoff 2008). Studies have modeled nAg entering the environment through end of product life disposal through waste streams, such as landfills and wastewater treatment facilities (Gottschalk et al. 2010; Voelker et al. 2015). As a result, wildlife receptors are potentially exposed to nAg in the environment. The effects of nAg and other engineered nanoparticles on terrestrial invertebrates (i.e., earthworms) have been characterized. Most nAg studies to date demonstrate limited toxicity; however, these studies are either performed with artificial soil or use a limited dose range (Heckmann et al. 2011; Shoults-Wilson et al. 2011; Schlich et al. 2013). Artificial soil provides data that may be useful for preliminary toxicity screening tests, but it does not reflect the true complexity of natural soils. For example, natural soil characteristics (e.g., clay content, organic matter content, cationic exchange capacity, etc.) can dramatically alter a chemical or particle's bioavailability and toxicity. Thus, the objective of this study was to examine the acute toxicity of nAg on earthworms in natural soils.

Soils are categorized into orders that reflect similar physical and chemical characteristics that affect a response to the experimental manipulation and management. Out of the 12 soil orders that exist in the United States, four were utilized for this study (alfisol, entisol, mollisol, ultisol). These soils comprise approximately 57% of the soil in the contiguous US (Figure 7). The soil orders used represent soils that range from fairly poorly developed soils to highly weathered soils. These acute lethality data will aid scientists and risk assessors in evaluating the effects of nAg on wildlife if released into the terrestrial environment.

Figure 7. Distribution of Alfisol, Entisol, Mollisol, and Ultisol soil orders in the United States. Data source: Natural Resource Conservation Service, USDA, Web Soil Survey, 2013.



3.2 Materials and methods

3.2.1 Chemicals

All chemicals were reagent or assay grade or higher. Polyvinylpyrrolidone (PVP) was purchased from Sigma-Aldrich (St. Louis, MO), silver nitrate (AgNO_3) was purchased from Fisher Scientific (Pittsburgh, PA), and the Econix version of PVP-coated Ag (PVP-nAg) nanopowder (Lot #DAG1157-MGM1712) was purchased from nanoComposix (Ronson, CT). The PVP-

nAg was sold as 50 nm diameter (TEM), but lot characterization showed a 38.6 nm diameter (TEM).

3.2.2 Material characterization

Dry engineered nanoparticles, both as received from the manufacturer and in amended field soil, were characterized by low vacuum field emission scanning electron microscopy (SEM) (Nova630 NanoSEM, FEI, Hillsboro, OR) with energy dispersive x-ray (EDS) spectroscopic capability (Quantax System, Bruker AXS, Madison, WI).

3.2.3 Field Soils

Memphis silt field soil (Order Alfisol) came from Vicksburg, MS; Sunev field soil (Order Mollisol) came from Cameron, TX; Camp Shelby field soil (Order Ultisol) came from Camp Shelby, Hattiesburg, MS; and Big Black field soil (Order Entisol) came from ERDC's Big Black River test site (Warren County, MS). Bulk soil samples were collected, air dried, gently ground to break apart any aggregates, and stored at room temperature until used in experiments. Physical and chemical properties of these field soils are listed in Table 1. All soils were hydrated with tap water that was dechlorinated with an activated carbon purification system.

Table 1. Physical and chemical characteristics of natural field soils.

Soil	Order	US Land Coverage, % ^a	Soil Texture	pH	Sand, %	Silt, %	Clay, %	Carbon, %	Organic Matter	Electrical Conductivity, mmhos/cm
Memphis Silt	Alfisol	13.9	Silt Loam	7.88	5.8	82	12.2	1.6	<0.2	0.27
Big Black	Entisol	12.3	Silt Loam	5.08	39.8	52	8.2	1.22	1.7	0.06
Sunev	Mollisol	21.5	Sandy Clay Loam	7.48	57.8	14	28.2	3.43	4.3	0.13
Camp Shelby	Ultisol	9.2	Loam	4.27	39.8	48.1	12.1	0.85	1.4	0.05

^a Data provided by the University of Idaho, College of Agriculture and Life Sciences (<http://www.cals.uidaho.edu/soilorders/>)

3.2.4 Test organisms

Individuals of the earthworm species *Eisenia fetida* that were sexually mature (i.e., displayed a prominent clitellum) were used in all exposure

experiments. Earthworms were originally obtained from the Worm Farm (Durham, CA); the identity of *E. fetida* was confirmed by the transverse banding on its segments. Earthworms were cultured in-house in a mixture of peat moss and horse manure and maintained in large fiberglass bins at a temperature of 20-23°C and light-controlled environment (continuous light exposure [24 hr]). The earthworms were fed a grain-based dry food (Magic Worm Food, Amherst Junction, WI) and allowed to acclimate to laboratory conditions for a minimum of seven days before testing. The health of the earthworm cultures was monitored by routine potassium chloride reference toxicity tests (Environment Canada 2004).

3.2.5 Acute earthworm toxicity studies

All soils were rehydrated to 15% moisture by weight with either dechlorinated tap water or dechlorinated tap water containing resuspended PVP-nAg, AgNO₃, or PVP. Soils were spiked with PVP-nAg by placing the amount needed to reach the desired nominal concentration of Ag in the soil (0, 10, 100, 1,000, 2,000, and 4,000 mg/kg) into the water used to rehydrate the soil, then bath sonicated for ten minutes until all agglomerates were suspended. The PVP-nAg suspensions were then added to the soils in a small stainless-steel bucket and mixed until uniformly hydrated. Soils were spiked with AgNO₃ by dissolving the amount needed to reach the desired nominal concentrations of Ag in soil (0, 100, 500, 750, and 1,000 mg/kg). The Ag solutions were poured onto the soils in a small stainless-steel bucket and mixed until uniformly hydrated. Soils were spiked with PVP by dissolving the amount needed to reach the desired nominal concentrations in soil (0, 100, 500, 750, 1000, 10,000, and 40,000 mg/kg), then added to the soil in a small stainless-steel bucket and mixed until uniformly hydrated. All soils were sieved through a #5 screen to break up any soil clumps, then thoroughly homogenized and placed in experimental jars. Each jar received 294 g of hydrated soil.

Acute (14-d) earthworm toxicity studies were conducted according to Environment Canada (2004). Briefly, mature (i.e., clitellate) earthworms (0.3-0.6 g, fresh weight) were sorted from on-site culture bins, rinsed, blotted, and weighed (n = 10) before being placed into an experimental jar. Worms (n = 10, 4 replicates per dose) were added to experimental jars with Ag concentrations ranging from 0-4,000 mg Ag/kg soil or experimental jars with PVP concentrations ranging from 0-40,000 mg/kg. Earthworms were exposed to spiked soils for 14 days in a growth chamber with 24 hr light to maximize soil exposure and constant 80% humidity to

control evaporation. At the end of 14 days, surviving worms were removed, counted, rinsed, blotted, and weighed in groups without being depurated.

3.2.6 Statistics

Survival response curves were plotted using SigmaPlot (SPSS) on a log concentration scale. Statistical significance was determined using a Student t-test ($p < 0.05$) (SigmaStat, version 3.11, Systat Software, Inc., San Jose, CA). Fifty percent lethal concentration values (LC50s) were determined by the trimmed Spearman-Kärber method (ToxCalc 5.0, Tidepool Scientific Software, McKinleyville, CA).

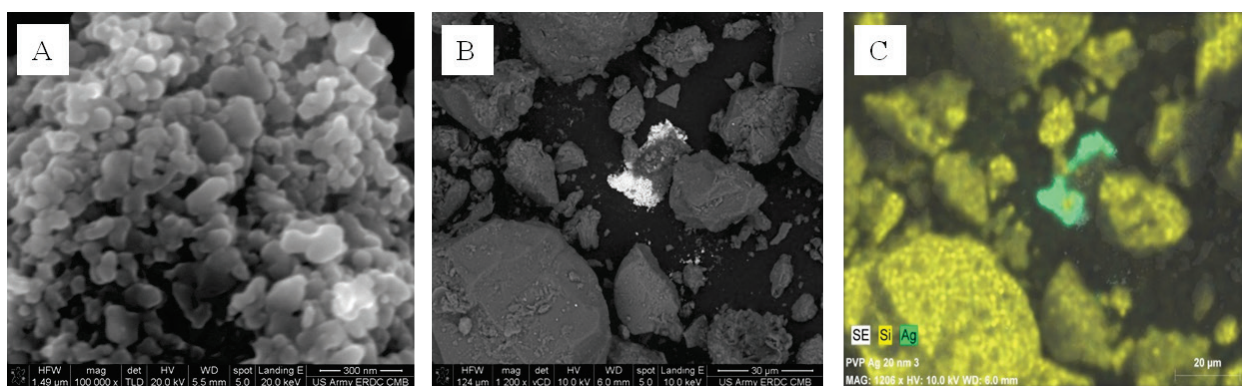
3.3 Results and discussion

The objective of this study was to identify sub-acute toxicity levels of coated-nAg to terrestrial invertebrates in field-collected soils that more realistically simulate the natural environment. Artificial soils, while useful for preliminary toxicity screening, do not simulate other important characteristics found in natural soils, such as clay and organic matter, and in some instances result in increased toxicity compared to field soils (Spurgeon 1997). Thus, field soils provide a more realistic environmental media in which to evaluate chemical toxicity. Hoppe et al. (2014) demonstrated that nAg is generally retained in soils with higher clay content and either low pH (<5.1) or high pH (>7.0). This indicates that any nAg present in soils (intentionally or unintentionally applied) is likely to remain present in the topsoil and lead to potential exposure to terrestrial invertebrates. By using several soils collected around the US, we attempted to identify potential changes in nAg soil toxicity due to soil types across the US.

SEM-EDX analysis of PVP-nAg used in this study demonstrated that the nanoparticle sizes ranged from approximately 50 to 200 nm in diameter, with particle shapes ranging from spherical, ovals, and irregular polyhedra (Figure 8A). However, the PVP-nAg particles were agglomerated, making the effective particle size much larger (Figure 8A). Supplemental analysis of the PVP-nAg particles by dynamic light scatter (DLS) analysis showed that hydrodynamic particle diameter (\pm standard deviation) of PVP-nAg after suspension and sonication for 30 min was 463 ± 24 nm (data not shown). This was further confirmed after sonication and mixing into the experimental field soils. In Figure 8B, PVP-nAg agglomerates (bright objects) were apparent among the darker soil particles. Elemental analysis

of this same micrograph identified the bright objects as silver (Figure 8C). This demonstrates that nanoparticles are often agglomerated during terrestrial toxicity studies, despite the coating which is supposed to reduce agglomeration.

Figure 8. Scanning electron microscopy (SEM) analysis of coated-nanosilver in an Alfisol field soil. A) SEM image of PVP-nAg particles showing spherical and oval Ag particles about 100-200 nm in diameter. Scale bar, 300 nm. B) SEM images of the PVP-nAg agglomerates in the natural field soil at 100 mg/kg. The bright objects in the center of the backscatter image are agglomerated PVP-nAg nanoparticles. Scale bar, 30 μm . C) energy dispersive x-ray (EDS) spectroscopic analysis of image (B) confirms the bright objects as PVP-nAg particles (green). Naturally occurring silicon (Si) particles are shown in yellow. Scale bar, 20 μm .



Sub-acute (i.e., 14-d) toxicity tests for PVP-nAg were conducted in four different soils that had different physical and chemical characteristics (Table 2). In general, dissolved silver (in the form of AgNO_3) was more toxic than PVP-coated silver nanoparticles. The LC_{50} value for PVP-nAg in Memphis silt soil (order Alfisol; silt loam) was 2,828.43 mg/kg, whereas the LC_{50} value for AgNO_3 in Memphis silt soil was 223.61 mg/kg (Table 8 and Figure 9). A similar differential in toxicity between PVP-nAg and AgNO_3 was described for *Enchytraeus albidus*, another terrestrial invertebrate (Gomes et al. 2013). The LC_{50} value of PVP in Memphis silt soil was >40,000 mg/kg (data not shown), so PVP appears to contribute to the low toxicity of PVP-nAg.

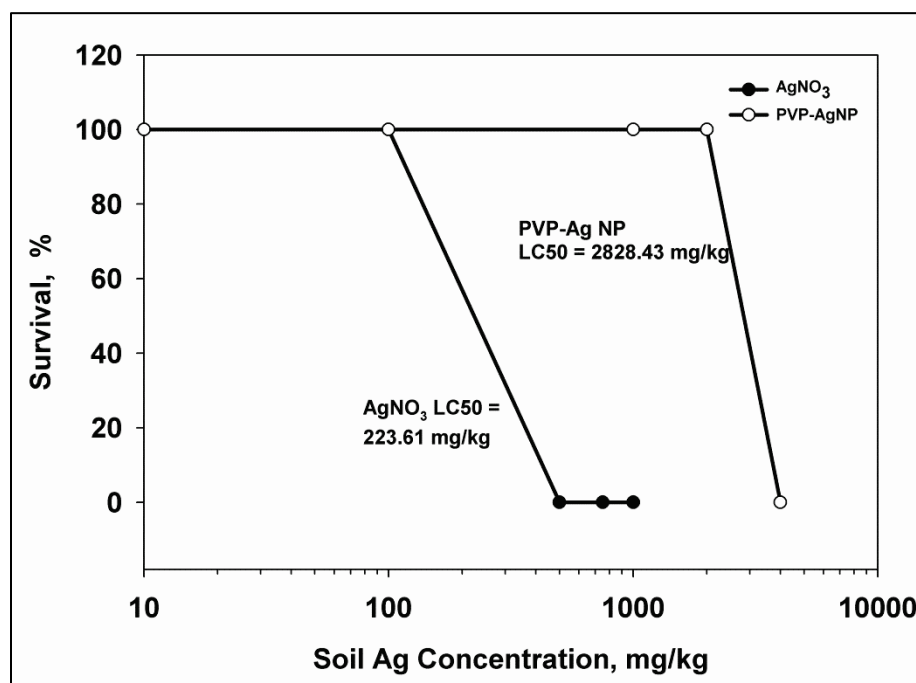
Table 2. Sub-acute terrestrial toxicity of PVP-coated silver nanoparticles and silver nitrate in natural field soils.

Soil	Soil Order	Particle/Chemical	LC50, mg/kg
Memphis Silt	Alfisol	PVP-nAg	2828.43
		AgNO ₃	223.61*
		PVP	>40,000
Big Black	Entisol	PVP-nAg	> 1,000 ^b
Sunev	Mollisol	PVP-nAg	> 1,000 ^b
Camp Shelby	Ultisol	PVP-nAg	> 1,000 ^b

* Statistically significant ($p < 0.05$) between PVP-nAg and AgNO₃

^b LC50 determination was limited by the availability of soil and PVP-nAg

Figure 9. Fourteen-day sub-acute lethality test for earthworms (*E. fetida*) in Memphis Silt soil (Order Alfisol) spiked with PVP-coated nanosilver.



Sub-acute toxicity tests for PVP-nAg were also conducted in Sunev soil (order Mollisol; sandy clay loam), Camp Shelby soil (order Ultisol; loam), and Big Black soil (order Entisol; silt loam). Due to limited availability of soils from remote locations and limited availability of PVP-nAg from the manufacturer, the experimental design was scaled down to provide meaningful data. Sub-acute toxicity tests were conducted at 0, 100, and 1,000 mg/kg in the Sunev and Camp Shelby soils, while sub-acute toxicity tests were conducted at 0 and 1000 mg/kg in the Big Black soil. PVP-nAg was not toxic to earthworms in all three soil types at concentrations up to

1,000 mg/kg (100% survival) (Table 2). Thus, the LC₅₀ values for Sunev, Camp Shelby, and Big Black soils were >1,000 mg/kg.

3.4 Conclusion

These data provide a more refined LC₅₀ value for PVP-nAg in field soil, supporting inconclusive results of previous studies that used PVP-nAg in field soils (Heckmann et al. 2011; Shoults-Wilson et al. 2011; Schlich et al. 2013). These data demonstrate that PVP-nAg is not acutely toxic to earthworms in natural soils except at concentrations that may result from an accidental release. These data will assist ecological risk assessors to better determine the acute environmental risks of silver nanoparticles in the terrestrial ecosystems with different soil orders.

Although toxicity to PVP-nAg may be at higher concentrations than likely to be encountered under normal deposition, sub-lethal effects may occur at lower concentrations, especially during chronic exposures. Silver nanoparticles have been demonstrated to cause earthworm reproductive toxicity by increasing juvenile mortality (Schlich et al. 2013; Diez-Ortiz et al. 2015). Furthermore, nAg may affect oxidative stress biomarkers and metabolic biomolecules that have long term implications on earthworm populations (Li et al. 2014; Gomes et al. 2015; Novo et al. 2015; Hayashi et al. 2016). However, the chronic fate and effects of nAg will be most critical for determining the long-term risk of nAg in the environment. A 52-week study by Diez-Ortiz et al. (2015) showed that nAg toxicity increased with time, likely related to the dissolution of Ag ions. Thus, ecological risk assessments for nAg, such as Voelker et al. (2015), albeit highly conservative by design, will likely need to incorporate these chronic data studies to better estimate the risk of nAg in the terrestrial environment.

4 Behavioral Response of Earthworms (*Eisenia fetida*) to Agglomerated Engineered Nanomaterials in a Natural Field Soil

Summary: Environmental conditions often cause nanomaterials to agglomerate which may cause the nanomaterials to elicit ecotoxicological responses more akin to micron-sized particles. Thus, 48-hr behavioral tests were conducted using the earthworm *Eisenia fetida* to assess the avoidance behavior of earthworms to an extensively characterized field soil amended with a series of engineered micron- and agglomerated nano-sized particles of silver (Ag), cerium oxide (CeO₂), zinc oxide (ZnO), and titanium dioxide (TiO₂) at nominal soil concentrations of 0.1, 1.0, 10 and 100 mg/kg. Earthworms did not avoid field soils amended with micron- or agglomerated nano-sized CeO₂, ZnO, or TiO₂ after 48-hr (no observable adverse effect levels [NOAELs] > 100 mg/kg). In contrast, earthworms significantly avoided field soil containing raw Ag nanoparticle agglomerates in a dose-dependent manner, yielding a NOAEL and lowest observed adverse effect level (LOAEL) of 10 mg/kg and 100 mg/kg, respectively. Interestingly, polyvinylpyrrolidone-coated Ag (PVP-Ag) nanoparticle agglomerates did not elicit the same soil avoidance behavior as raw (i.e., uncoated) Ag nanoparticle agglomerates, which suggests that Ag surface characteristics (i.e., Ag ion dissociation) may cause the soil avoidance behavior. These data demonstrate the importance of using field soils to conduct experiments that more accurately characterize organism responses to engineered nanomaterials in the terrestrial environment.

4.1 Introduction

Engineered nanomaterials are increasingly being used in a wide variety of applications, with thousands of consumer products in the areas of material sciences, medicine, energy, environment, communications, and electronics among others (<http://www.nanotechproject.org/inventories/consumer/>). Therefore, there is potential for widespread dispersal of these materials in the environment. Much emphasis has been placed on aquatic system studies; however, the effects of these materials on organisms living in the terrestrial environment are less characterized yet likely to be encountered due to soil remediation (Cundy et al. 2008; Pan and Xing 2012), land application of biosolids (Mueller and Nowack 2008; Gottschalk et al. 2009; Coleman et al. 2013),

and atmospheric deposition (Murr 2008; Cassee et al. 2011; Batley et al. 2013). Earthworms are used as a research animal model because they are vital to the terrestrial environment. Through bioturbation, earthworms affect the physical properties of soil (e.g., porosity, bulk density) and redistribute nutrients in the soil (Hooper et al. 2011). The earthworm *Eisenia fetida* (Oligochaeta, Lumbricidae), a commonly used species in ecotoxicological testing programs, is sensitive to many contaminants in soil, including organic compounds and metals, resulting in decreased growth, reproduction, and survival (Malecki et al. 1982; Venter and Reinecke 1985; Van Gestel et al. 1992; Spurgeon and Hopkin 1996; Bustos-Obregon and Goicochea 2002) that, as a result, affects soil quality (Shoults-Wilson et al. 2011b). Recent studies have focused on the effects of engineered nanomaterials on earthworms and whether they exhibit different toxicity profiles than their corresponding dissolved or bulk metals. These studies demonstrated that some metal and metal oxide nanoparticles, such as silver (Ag), copper (Cu), and zinc oxide (ZnO), produced mild to severe toxicity to earthworms, while others, such as titanium dioxide (TiO₂) and aluminum oxide (Al₂O₃), were much less toxic (Coleman et al. 2010; Unrine et al. 2010; Bigorgne et al. 2011; Canas et al. 2011; Hooper et al. 2011; Shoults-Wilson et al. 2011a; McShane et al. 2012). Furthermore, a common feature seen with dissolvable NPs (e.g., Ag, Cu, and ZnO) is that the NPs are less toxic than their respective ionic metals (Unrine et al. 2010; Hooper et al. 2011; Shoults-Wilson et al. 2011a).

Earthworms utilize their sensory systems to detect chemicals in the environment, often at sublethal concentrations, resulting in either positive or negative responses. As a result, soil avoidance by earthworms can be used as a rapid, low-cost screening tool for ecological risk assessments (Environment Canada 2004). The soil avoidance endpoint has been found to be equally or more sensitive than lethality, reproductive, or growth endpoints during exposures of several terrestrial invertebrate species to various pesticides, explosives, and NPs (Achazi 2002; Hund-Rinke et al. 2003; Environment Canada 2004; Natal-da-Luz et al. 2004; Schaefer 2004; Loureiro et al. 2005; Kuperman et al. 2006; Garcia et al. 2008; Natal-da-Luz et al. 2008; Shoults-Wilson et al. 2011b). Thus, it was of interest to examine the effects of several metal-based engineered nanomaterials on the behavior (i.e., soil avoidance) of a common earthworm species, *E. fetida*, in a silt-loam field soil. Behavioral effects were compared between nano-sized and traditional micron-sized particles used in numerous consumer products (e.g., Ag in textiles and antibacterial

products, TiO₂ in sunscreen and paints, ZnO in sunscreen, and CeO₂ in coatings and fuel additives). Furthermore, the nanoparticles used in this experiment were agglomerated to simulate anthropogenic activities and/or natural environmental conditions that cause monodispersed nanomaterials to agglomerate in the environment (Murr et al. 2004; Donaldson et al. 2005; Ghosh et al. 2008; Cornelis et al. 2011; Batley et al. 2013). Our null hypothesis (H₀) was that earthworm soil avoidance will not differ between micron- and nano-sized particle agglomerates at increasing concentrations in a field soil; our alternative hypothesis (H_a) was that earthworms will avoid the field soil amended with nano-sized particle agglomerates more than field soil with micro-sized particles. To test this hypothesis, 48-hr soil avoidance tests were conducted in the Kaushik avoidance wheel (Environment Canada 2004). These tests were conducted in natural soil, an important distinction from previously reported studies that used artificial media and soils. The use of natural soils is important because of the presence of naturally occurring nanoparticles that organisms are exposed to daily (Shoults-Wilson et al. 2011; Wiesner et al. 2011; Tourinho et al. 2012) and thus do not generate confounding results due to artificial exposure conditions.

4.2 Materials and methods

4.2.1 Engineered nanomaterials

Micron-sized metal and metal oxide particles (Sigma-Aldrich Chemical Co, St. Louis, MO), engineered nano-sized particles (NanoAmor, Houston, TX) of silver (nAg; 35 nm primary particle size), polyvinylpyrrolidone (PVP)-coated nAg (PVP-nAg; 20 nm primary particle size), cerium oxide (nCeO₂; 15-30 nm primary particle size), zinc oxide (nZnO; 20 nm primary particle size), and titanium dioxide (nTiO₂; 10-30 nm primary particle size) were used based on their current and projected use in a variety of commercial and military applications. Average nanoparticle diameters as reported by the manufacturer are presented in Table 1. Average micron-sized particle diameters (as reported by the manufacturer) were 1.5-2.5, <5, <5, and 45 μm for Ag, CeO₂, ZnO, and TiO₂, respectively.

4.2.2 Material characterizations

To measure hydrodynamic particle size, all nanoparticles were suspended in deionized water and sonicated (Branson Sonifier 450, Branson Ultrasonic) for 30 min at a 20 W, 40% duty cycle. Nanoparticle suspensions were then

characterized by dynamic light scattering analysis (DLS; 90Plus /BI-MAS, Brookhaven Instruments, Holtsville, NY). Dry engineered nanoparticles—raw (i.e., as-received) and in amended soil—were characterized by low vacuum field emission scanning electron microscopy (SEM) (Nova600 NanoSEM, FEI, Hillsboro, OR) with energy dispersive x-ray (EDS) spectroscopic capability (Quantax System, Ewing, NJ).

4.2.3 Test soil

A silt loam soil of the Grenada-Loring series (Alfisol order), obtained from the Brown Loam Branch Experiment Station (Learned, MS), was used in all experiments. This soil contains 3% sand, 72% silt, and 26% clay; 0.7% total organic carbon, 1% organic matter, pH 6.7, and cation and anion exchange capacities of 0.075 and 0.025 mEq/g, respectively (Inouye et al. 2006; Bednar et al. 2008). Chemical analyses indicated no anthropogenic chemical contamination in the field soil (data not shown).

4.2.4 Test organisms

Individuals of the earthworm species *E. fetida* (0.3-0.6 g fresh weight; n=10), that were sexually mature (i.e., displayed a prominent clitellum), were used in all exposures. Earthworms were originally obtained from the Worm Farm (Durham, CA), and the identity of *E. fetida* was confirmed by the transverse banding on its segments. Earthworms were cultured in-house in a mixture of peat moss and cow manure and maintained in large fiberglass bins at a constant temperature range (20-23°C). The earthworms were fed a grain-based dry food (Magic Worm Food, Amherst Junction, WI). The earthworms acclimated to laboratory conditions for a minimum of seven days before testing. Mature earthworms culled from cultures were selected, rinsed with dechlorinated tap water, and blotted dry before use.

4.2.5 Earthworm behavioral testing

Dry field soil placed in 20-L glass jars was dry-amended with 100, 10, 1, or 0.1 mg/kg of either nano- or micron-sized particles. Jars were placed on a roller apparatus (Wheaton Science Products, Millville, NJ) at four rpm for 24 hr to homogenize the mixtures. The resultant soil/material mixtures were weighed and distributed into separate polypropylene containers for each replicate, hydrated with dechlorinated tap water to achieve 15%

moisture content (44.7% water holding capacity), and allowed to equilibrate for 2 hr at $20 \pm 3^\circ\text{C}$ before experimentation.

The avoidance experiments were conducted utilizing the Kaushik avoidance wheel (5.4 cm inner diameter, 23 cm outer diameter, 1 cm holes between the inner chamber and test chamber, and multiple 1 cm holes in the removable partitions to allow free movement of earthworms throughout the chamber's pie-shaped compartments) (Stephenson et al. 1998; Environment Canada 2004; ISO 2008; Coleman et al. 2010). The avoidance wheel chamber compartments were alternately filled with either control or particle-spiked soil. To begin the experiment, earthworms were added individually into the central chamber of each avoidance wheel. Once an earthworm completely entered a soil compartment, the behavior was recorded (entry into either a control or spiked soil chamber) and another earthworm placed in the center chamber (devoid of soil) until every individual avoidance wheel contained ten earthworms. A clear Plexiglas lid was placed on top of each avoidance wheel to prevent escape while still permitting airflow between chambers. The avoidance wheels were placed in a temperature-controlled room at $20 \pm 3^\circ\text{C}$. Each wheel was covered with aluminum foil to block light and encourage earthworm movement across chamber compartments. The earthworms were not fed during exposures. After 48 hr of exposure, the soils were systematically removed and the earthworm location (i.e., control or spiked soil) and other observations (e.g., presence of lesions, dead worms) were recorded. A minimum of three replicates of ten earthworms was performed for each bulk or nano-sized particle. A potassium chloride-spiked experiment was also conducted as a positive control for soil avoidance.

4.2.6 Chemical analysis

Elemental analysis of soil samples was performed by inductively coupled plasma-mass spectrometry (ICP-MS; Elan DRC II, Perkin Elmer, Waltham, MA) following USEPA Method 6020 (USEPA 2003a). Prior to analysis, soil samples were dried, ground with a mortar and pestle, and sieved through an ASTM #40 sieve to improve sample homogeneity (Felt et al. 2008). Soil aliquots (0.5 g) were subsequently digested following USEPA Method 3050B (USEPA 2003b). However, due to the low solubility of titanium dioxide in nitric and hydrochloric acid digestions, the acid cocktail was modified by addition of hydrofluoric acid, modified from methods described by Taggart (2002). Soil samples were digested in

triplicate and included predigestion matrix spike quality assurance samples, as well as NIST standard reference material soils.

All digestates were filtered and diluted in 1% nitric acid prior to ICP-MS analysis. To correct for instrumental drift, scandium, yttrium, rhodium, terbium, and holmium were added as internal standards on-line prior to the nebulizer using a mixing T. Internal standard drift was less than 10% of the intensity measured in the calibration blank over the course of the analytical batch. Calibration and second source verification standards were NIST-traceable and purchased from SPEX Certiprep (Metuchen, NJ) and CPI International (Santa Rosa, CA). Recoveries were within 10% of the nominal values for all calibration verification and matrix spike values. In addition, a reference solution from Environmental Resource Associates (Golden, CO; Lot number P136-500) was analyzed in each analytical batch. Analyte recoveries were within 20% of the certified values.

4.2.7 Statistical analysis

Statistical analyses were performed using chi-square analysis to determine whether the distribution of earthworms at time 0 and 48 hr was random (SigmaStat, Version 3.1). Treatments were considered statistically different at $p < 0.05$.

4.3 Results and discussion

4.3.1 Nanoparticle and soil characterizations

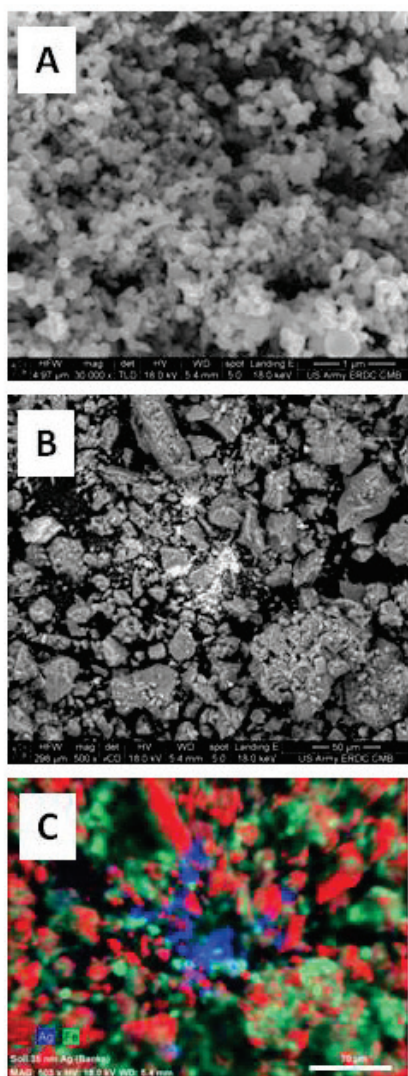
Raw, agglomerated forms of the engineered nanoparticles were used in this study to simulate anthropogenic uses and environmental factors that cause changes in nanoparticle dispersion in the terrestrial environment. Natural field collected soil and extensive characterization techniques were employed to assess particles/agglomerates in an environmentally relevant scenario. DLS analysis of nanoparticle suspensions in water indicated substantial agglomeration before testing (Table 3). Average hydrodynamic diameters of nanoparticle agglomerates, even after sonication, were 426 nm for nZnO, 463 nm for PVP-coated nAg, 628 nm for raw nAg, 767 nm for nTiO₂, and 1,575 nm for nCeO₂. These nanoparticle agglomerates were between one or two orders of magnitude greater than the primary particle diameters reported by the manufacturers, which ranged from 10-35 nm; however, the DLS does provide measurements which are skewed toward the larger particles in a suspension. Measured micron-sized Ag particles were similar

to, although slightly less than, the reported 1,500 to 2,500 nm range reported by the manufacturer. SEM characterization of raw nAg particles (35 nm) showed original spherical nano-sized particles that agglomerated in the 100-200 nm range (Figure 10A). These results were indicative of the other metal and metal oxide nanoparticles tested as well (data not shown). The characterization of agglomerated nAg nanoparticles in amended test soils were similar to raw nAg nanoparticles alone (Figure 10B and 10C). These results were consistent across the other nanomaterials tested (data not shown). Nanoparticle agglomeration and/or aggregation is often the outcome with anthropogenic uses (e.g., combustion, suspension by-products) and dispersion in the environment (Greiger et al. 2010; Bigorgne et al. 2011; Poda et al. 2013; Batley et al. 2013), so these data appear to reflect the state of nanoparticles that will occur in environmental matrices.

Table 3. Size characterization of engineered nanoparticles used in the earthworm avoidance tests. The primary particle diameters were determined by TEM image analysis by the manufacturer. Hydrodynamic particle diameter was determined by dynamic light scatter analysis with nanoparticles suspended in deionized water and sonicated for 30 min.

Material	Primary Particle Diameter (nm)	Hydrodynamic Particle Diameter (nm) (Mean \pm SD)
TiO ₂	10-30	767 \pm 114
ZnO	20	426 \pm 59
CeO ₂	15-30	1,575 \pm 134
Uncoated Ag	35	628 \pm 1
PVP-Coated Ag	20	463 \pm 24

Figure 10. Scanning electron microscopy analysis of engineered nanoparticles. A) SEM image of nano-sized Ag particles (35 nm primary particle size) showing spherical Ag particles about 100-200 nm in diameter. Scale bar, 1 μ m. B) SEM images of the Ag nanoparticle agglomerate in the natural silt-loam field soil at 100 mg/kg. The bright objects in the center of the backscatter image are agglomerated Ag nanoparticles. Scale bar, 50 μ m. C) energy dispersive x-ray (EDS) spectroscopic analysis of image (A) confirms the bright objects as Ag nanoparticles (blue). Naturally occurring silicon (Si) and iron (Fe) particles are depicted in red and green, respectively. Scale bar, 70 μ m.



Total concentrations of Ag, Ce, Zn, and Ti (mean \pm SD, n=3) in control and 0.1-100 mg/kg nanoparticle-amended field soils were measured by ICP-MS (Table 4). The natural Ag concentration in the field soil was low, averaging less than the detection limit (< 0.1 mg/kg). The addition of nAg and micron-sized Ag particles resulted in detectable increases in soil

concentrations, though at lower concentrations than the nominal values. In contrast to the low natural levels of Ag, natural concentrations of Ce, Ti, and Zn averaged 41.0, 59.7, and 29.1 mg/kg, respectively. The high concentration of these metals naturally occurring in the field soil masked the engineered nanoparticles that were amended to this soil (Table 4) except at the 100 mg/kg spike level. This was particularly true with Ti where measured concentrations in amended soil did not differ significantly from the untreated soil concentration (59.7 mg/kg in untreated soil vs. 65.9 mg/kg in 100 mg/kg TiO₂-treated soil). Acid digestion of metal-spiked soils with hydrofluoric acid did not result in substantially increased metal concentrations compared to the results in Table 4 (data not shown), likely indicating the variability in the acid extractable and refractory phases is larger than the amount of engineered materials added. Despite the discrepancy between nominal and measured concentrations, the presence of nanoparticle agglomerates in the soil was confirmed by SEM techniques (Figure 10).

Table 4. Nominal and measured concentrations of nano- and micron-sized particles amended to field soil used in 48-hr earthworm avoidance tests. Values are presented as the mean \pm SD (n=3). All units in dry weight.

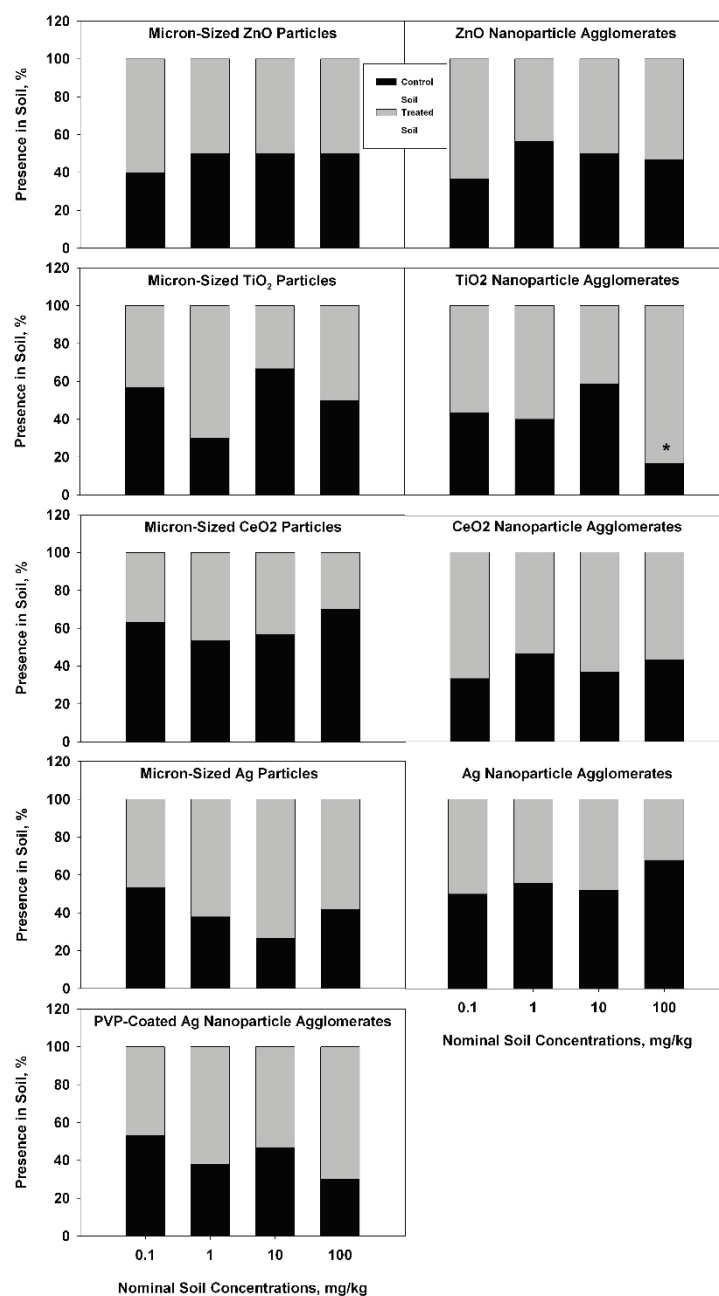
Nominal Soil Concentration (mg/kg)	Nano-Spiked Soil (mg/kg)	Micron-Spiked Soil (mg/kg)
Ag		
Control	<0.10	NA
0.1	<0.10	0.114 \pm 0.014
1.0	0.206 \pm 0.156	0.371 \pm 0.014
10	2.43 \pm 2.45	3.86 \pm 0.22
100	58.8 \pm 13.69	88.5 \pm 2.62
CeO₂		
Control	41.0 \pm 0.29	NA
0.1	42.0 \pm 1.10	41.4 \pm 3.30
1.0	43.1 \pm 1.17	41.9 \pm 1.62
10	44.8 \pm 1.60	45.9 \pm 1.45
100	112 \pm 8.5	87.0 \pm 3.09
ZnO		
Control	29.1 \pm 1.38	NA
0.1	30.4 \pm 0.75	30.7 \pm 1.88
1.0	28.0 \pm 4.3	29.9 \pm 3.69
10	32.8 \pm 0.9	34.0 \pm 2.14
100	101 \pm 10.9	104 \pm 0.6
TiO₂		
Control	59.7 \pm 20.4	NA
0.1	58.8 \pm 1.4	58.7 \pm 1.2
1.0	62.5 \pm 2.7	62.5 \pm 2.5
10	61.9 \pm 3.0	59.2 \pm 4.4
100	65.9 \pm 1.9	58.7 \pm 0.92

NA=Not applicable

4.3.2 Behavioral tests

Behavioral tests were conducted to determine whether earthworms will exhibit positive or negative responses to the presence of agglomerated nanoparticles in soil. Upon test initiation, *E. fetida* did not exhibit a significant avoidance when burrowing into the soils amended with micron- or nano-sized particles of raw Ag, PVP-coated Ag, CeO₂, ZnO, or TiO₂ (Figure 11). These data indicate the micron- and nanoparticle concentrations tested do not elicit an immediate avoidance response by earthworms upon initially encountering these particles in the field soil tested.

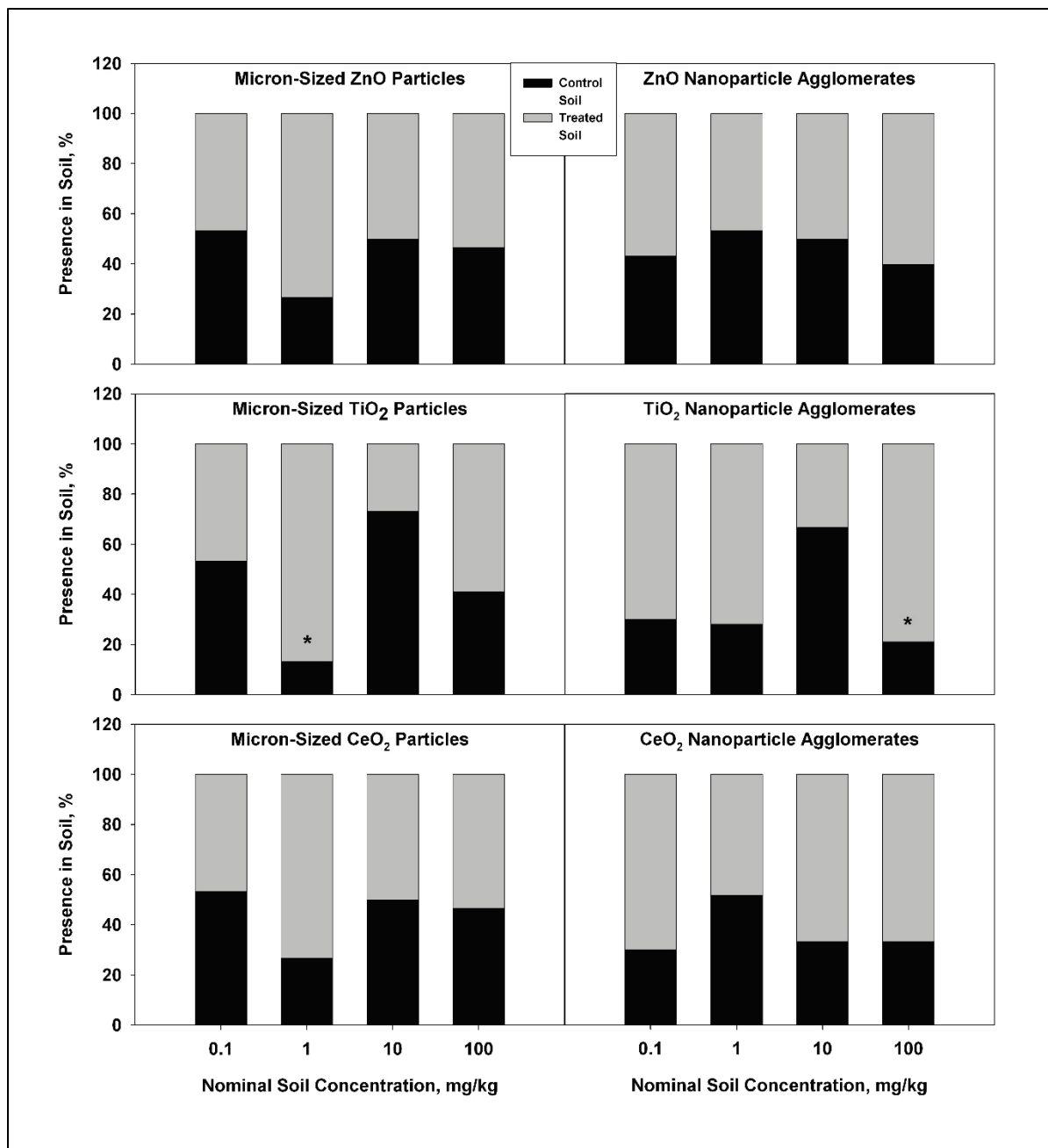
Figure 11. Behavioral response of the earthworm *E. fetida* at the initiation of the 48-hr exposure to field soil amended with micron- and nano-sized ZnO, CeO₂, TiO₂, raw Ag, and PVP-coated Ag. Asterisk indicates statistical significance between the control and amended soil ($p < 0.05$). Black, worms present in control soil; grey, worms present in treated soil.



No mortality or other outward toxicity effects on earthworms were observed during the avoidance experiments conducted with any of the micron- or nano-sized particles. In general, after 48 hr exposure, *E. fetida* did not

significantly avoid soils spiked with micron- or nano-sized particles of ZnO, CeO₂, and TiO₂ at concentrations ranging from 0.1 to 100 mg/kg (Figure 12). In fact, earthworms were attracted to field soils containing 100 mg/kg nano-sized TiO₂ and 1 mg/kg micron-sized TiO₂ (Figure 12). These data confirm results described by McShane et al. (2012) where earthworms only avoided TiO₂-spiked soil at high concentrations (1,000-5,000 mg/kg) and that earthworms were attracted to soil at 100 mg/kg nTiO₂, albeit not at statistically significant levels. Similar behavioral results were seen with earthworms exposed to nano-aluminum oxide at 1,250-2,500 mg/kg in soil (Coleman et al. 2010). The no observable adverse effect levels (NOAEL) for *E. fetida* exposed to micron- and nano-sized particles of ZnO, CeO₂, and TiO₂ were >100 mg/kg. The lack of avoidance behavior noted for both micron-sized particles and nanoparticle agglomerates of ZnO, CeO₂, and TiO₂ at 100 mg/kg suggests high concentrations of these nanoparticle agglomerates that may occur in field soils due to anthropogenic activity (e.g., manufacturing site release/spill, biosolid application, site remediation) may not be perceived by earthworms as a threat and thus does not elicit a negative behavioral response. However, nanoparticles at these concentrations may elicit unique ecological effects on earthworms that warrant further investigation, such as altered earthworm reproduction by nTiO₂ (Schlich et al. 2012).

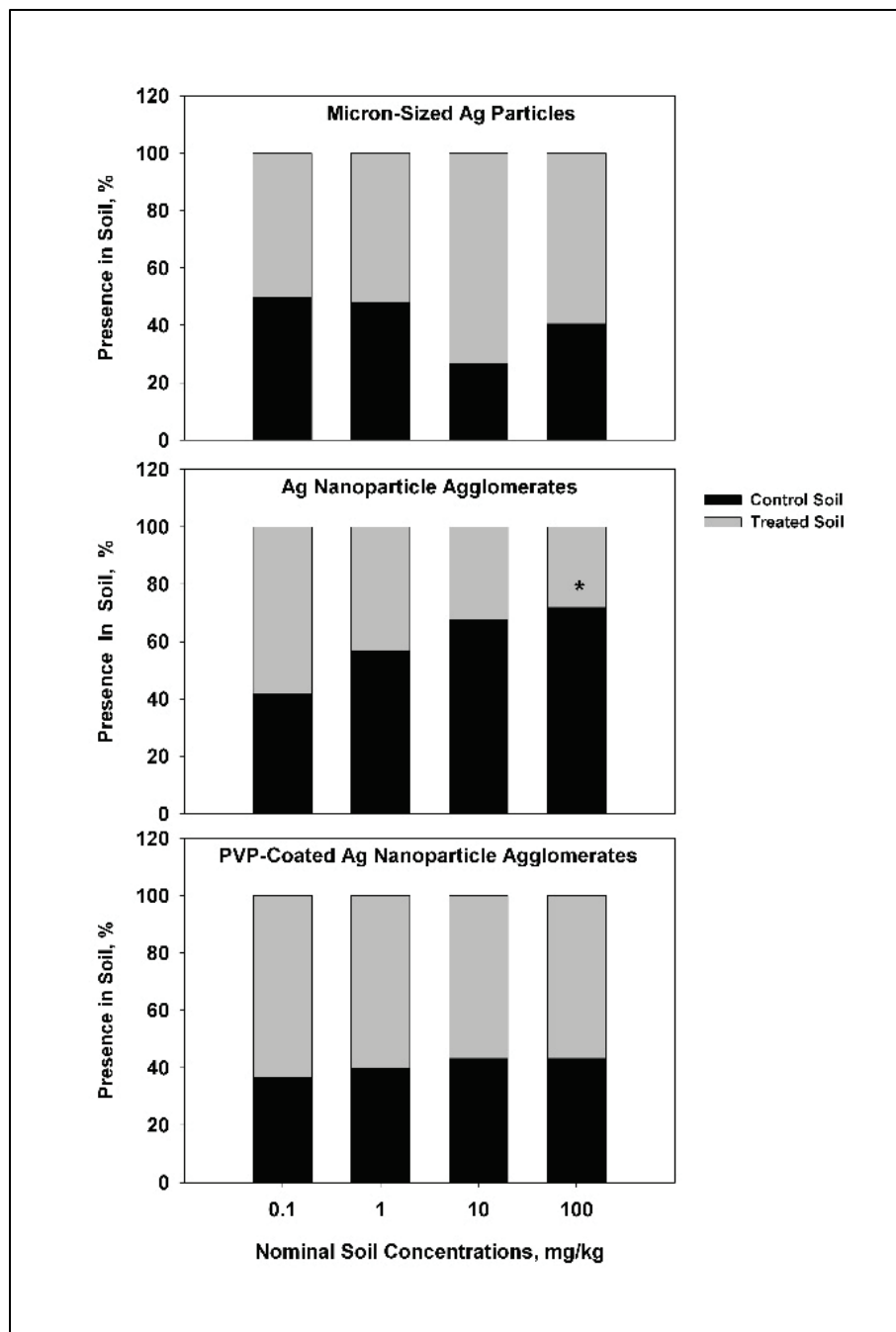
Figure 12. Behavioral response of the earthworm *E. fetida* after 48-hr of exposure to field soil amended with nano- and micron-sized ZnO, CeO₂, and TiO₂. Asterisk indicates statistical significance between the control and amended soil ($p < 0.05$). Black, worms present in control soil; grey, worms present in treated soil.



No avoidance response was observed at test initiation and termination when *E. fetida* was exposed to soil containing micron-sized Ag at concentrations from 0.1 to 100 mg/kg. In contrast, *E. fetida* significantly avoided soil containing raw nAg agglomerates after 48-hr exposure. Earthworm avoidance increased with raw nAg in a dose-dependent

manner, with 72% avoidance reached at 100 mg/kg ($p = 0.048$) (Figure 13). These data are confirmed by Shoults-Wilson et al. (2011b). The observed NOAEL and lowest observable adverse effect level (LOAEL) for raw nAg agglomerates were 10 mg/kg and 100 mg/kg, respectively. These data suggest that if raw nAg agglomerates deposit or accumulate at concentrations ≥ 100 mg/kg in soil, this may result in harm to terrestrial organisms, such as earthworms, contacting these soils (Schlich et al. 2013; Diez-Ortiz et al. 2015). However, the fact that earthworms actively avoid soils spiked with nanoparticle agglomerates at sublethal concentrations suggests the potential for reduced adverse effects on organisms in the terrestrial food web.

Figure 13. Behavioral response of the earthworm *E. fetida* after 48-hr of exposure to field soil amended with micron-sized Ag, raw nAg, and PVP-coated nAg. Asterisk indicates statistical significance between the field and amended soil ($p < 0.05$). Black, worms present in control soil; grey, worms present in treated soil.



Interestingly, PVP-coated nAg agglomerates did not elicit an avoidance behavior (Figure 13). This response is different than what was reported by Shoults-Wilson et al. (2011b) where earthworm soil avoidance (EC₅₀ values for 10 nm and 30-50 nm PVP-nAg were 8.39 mg/kg and 4.8 mg/kg,

respectively) was comparable to avoidance seen with silver nitrate ($EC_{50} = 7.76$ mg/kg). The authors speculated that the increased avoidance with PVP-nAg might be due to dissolved Ag^+ ion release from the nanoparticles. The differences between our results and Shoults-Wilson et al. (2011b) may also be due to the soil types used in the different studies (current study: 3% sand / 72% silt / 26% clay vs. Shoults-Wilson et al. (2011) study: 76.34% sand / 16.53% silt / 7.13% clay), where earthworms are more likely to be affected by chemicals in soil with higher sand content.

4.4 Conclusion

Data presented in this study demonstrate that only raw nAg agglomerates elicit a negative behavioral (soil avoidance) response in earthworms in a spiked silt loam field soil, whereas nano-sized agglomerates of TiO_2 , ZnO , and CeO_2 and micro-sized particles – including Ag – did not affect earthworm avoidance behavior. Furthermore, PVP-coated nAg agglomerates also eliminated negative behavioral responses, demonstrating the importance of NP coatings on biological endpoints. These data confirm previous reports of nAg and $nTiO_2$ eliciting behavioral responses in soil at high environmental concentrations (Shoults-Wilson et al. 2011b; McShane et al. 2012). These data also demonstrate that ZnO NPs, a component of sunscreen, and CeO_2 NPs, a component in fuel additives, also do not elicit a behavioral response in earthworms. These data are the first to detail the earthworm response to $nCeO_2$ in soil and will assist with future $nCeO_2$ hazard assessments, especially with the potential for increased uses as fuel additives (Batley et al. 2013). Furthermore, these studies demonstrate the importance of using field-collected soils to conduct experiments that more accurately reflect how organisms will respond to chemical stressors than artificial soils. These data may be useful for future risk assessments of terrestrially applied nanotechnologies to fulfill regulatory guidelines (e.g., USEPA's Toxic Substance Control Act [TSCA] or Federal Insecticide, Fungicide, and Rodenticide Act [FIFRA], and the European Union's Registration, Evaluation, Authorization and Restriction of Chemicals [REACH] regulations).

While this field soil study demonstrates the usefulness of behavioral studies for ecological risk assessments for nanoparticles, additional studies are necessary to differentiate the effects of soil matrix components (e.g., grain size, organic matter content) on nanoparticle effects on earthworms (Li et al. 2011). In addition, despite not eliciting behavioral responses, lower nanoparticle agglomerate concentrations may still elicit adverse

effects to the organism, such as oxidative stress and apoptosis (Hu et al. 2010; Bigorgne et al. 2011; Lapied et al. 2011; Novo et al. 2015; Hayashi et al. 2016). Thus, additional research is needed to examine potential chronic, sublethal biological effects of these engineered nanomaterials (Coleman et al. 2013).

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Acronyms and Abbreviations

Ag, silver

AgNO₃, silver nitrate

ASTM, American Society for Testing and Materials

C, Celsius

CeO₂, cerium oxide

cm, centimeters

DLS, dynamic light scattering

EDS, energy dispersive x-ray spectroscopy

Fe, iron

g, grams

GL, Grenada-Loring soil

Ha, alternative hypothesis

HNO₃, nitric acid

Ho, null hypothesis

ICP-MS, inductively-coupled plasma-mass spectrometry

L, liter

LC₅₀, median lethal concentration

LOAEL, lowest observed adverse effect level

μm, micrometers

mEq, milliequivalent

mg/kg, milligram per kilogram

n, sample size

nAg, nanometer-sized silver

nCeO₂, nanometer-sized cerium oxide

NIST, National Institute of Standards and Technology

nm, nanometer

NOAEL, no observable adverse effect levels

NP, nanoparticle

nTiO₂, nanometer-sized titanium dioxide

nZnO, nanometer-sized zinc oxide

PVP, polyvinylpyrrolidone

RPM, revolutions per minute

SD, standard deviation

SEM, scanning electron microscopy

Si, silicon

TEM, transmission electron microscope

TiO₂, titanium dioxide

USEPA, United States Environmental Protection Agency

ZnO, zinc oxide

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14. ABSTRACT Although engineered nanomaterials are active components in a wide variety of commercial products, there is still limited information related to the effects of these nanomaterials once released into the terrestrial environment. A high number of commercial applications use silver nanoparticles (nAg) due to its anti-microbial activity. This may be of concern for waste management since nAg could be applied to soil (e.g., biosolids) or disposed of in traditional landfills, which could lead to possible leaching into surrounding soil. This report aims to provide additional insight into the fate and effects of nAg in terrestrial systems. The studies in this report examine the leachability of nAg in field soil and compares the soil migration to bulk (i.e., micron-sized) silver; examine the ecotoxicity of nAg to earthworms in four field soils spanning several different soil orders; and examine the behavioral effects of earthworms when exposed to engineered nanoparticles in field soil. These data provide additional insight into engineered nanoparticle fate and effects to terrestrial receptors in field soils, an important distinction from laboratory-generated soils. These data will also assist ecological risk assessors to better determine the acute environmental risks of nAg in terrestrial ecosystems with different soil compositions.					
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