Environmental Toxicology and Chemistry—Volume 39, Number 6—pp. 1257–1266, 2020
Received: 6 April 2019 | Revised: 30 April 2019 | Accepted: 12 March 2020

Environmental Toxicology

Antioxidant Enzyme Activity and Lipid Peroxidation in *Aporrectodea caliginosa* Earthworms Exposed to Silver Nanoparticles and Silver Nitrate in Spiked Soil

Nadir Saleeb,[a] Brett Robinson,[a] Jo Cavanagh,[b] James Ross,[a] Kiran Munir,[a] and Ravi Gooneratne[a,*]

[a]Faculty of Agriculture and Life Sciences, Lincoln University, Lincoln, Canterbury, New Zealand
[b]Landcare Research, Lincoln, Canterbury, New Zealand

Abstract: Silver nanoparticles (AgNPs) from industrial use, discharged via the land application of sewage sludge, are interacting with soil biota, including earthworms. In affected organisms, excessive production of reactive oxygen species can result in lipid peroxidation, shifting the balance between oxidants and antioxidants to cause oxidative stress. We determined selected lower-tier biomarkers such as antioxidant responses and lipid peroxidation in *Aporrectodea caliginosa* earthworms exposed to soils spiked with AgNPs or silver nitrate ($AgNO_3$). *Aporrectodea caliginosa* were exposed to AgNPs at 0 (control), 0.3, 3, 30, and 300 mg/kg or Ag^+ (as $AgNO_3$) at 0, 0.03, 0.3, 3, and 10 mg/kg in soil for 4 wk. At 1, 2, 3, and 4 wk, the activity of the antioxidant enzymes superoxide dismutase, catalase, glutathione peroxidase, glutathione *S*-transferase, as well as lipid peroxidation (malondialdehyde content), increased as a function of concentration, with a much larger response for Ag^+ than AgNPs. Given the likelihood of ever-increasing AgNP concentrations in soil, where AgNPs can transform to ionic Ag (Ag^+), our findings of antioxidant response to oxidative stress in a common indicator organism even at an environmentally realistic exposure concentration of 0.03 mg/kg demonstrate that AgNPs may affect soil fertility and, thus, agricultural production. Evaluating selected lower-tier biomarkers offers a meaningful assessment of AgNPs and Ag^+ effects on terrestrial earthworms. *Environ Toxicol Chem* 2020;39:1257–1266. © 2020 SETAC

Keywords: Biomarkers; Ecotoxicology; Emerging pollutants; Nanotoxicology; Silver nanoparticles; Silver nitrate

INTRODUCTION

Silver nanoparticles (AgNPs) are increasingly used in a variety of industries (Xiong et al. 2013) and reach the soil from sewage sludge, waterways, air, volcanic eruption, mining, and from vehicle exhaust (Tripathi et al. 2017). This implies their increasing presence in the environment, making exposure of ecologically relevant organisms and humans possible (Kühnel and Nickei 2014). In the face of rapid growth in nanotechnology and environmental policy instruments that have not been designed to regulate the novel physical or chemical properties of substances like NPs, there is now an urgent need to understand their environmental impact, including that of AgNPs (Judy et al. 2011). Silver NPs may be released into the environment, including into soil, during the production and disposal of AgNP-containing materials (Stensberg et al. 2011; Dobias and Bernier-Latmani 2013). Many industries release wastes into sewerage systems that end in sludge, which is sometimes spread on the land as fertilizer (Meier et al. 2016). Silver NPs in sewage sludge when applied on soil may inhibit plant growth (Lee et al. 2012) and impact on terrestrial organisms and on aquatic organisms via leaching of land contaminants into waterways. Thus, AgNPs may accumulate in soil and be transmitted via the food chain to humans.

Earthworms contribute significantly to ecosystem health by modifying the physical, chemical, and biological properties of soil; recycling organic material; increasing nutrient availability; providing food for other soil organisms; and improving the soil structure profile, all of which improve soil fertility (Lemtiri et al. 2014). Thus, earthworms are of importance to agriculture by increasing pastoral productivity (Blouin et al. 2013). *Eisenia fetida* is the most common earthworm species used to assess chemical toxicity (Organisation of Economic Co-operation and Development 1984). In New Zealand, however, the most abundant earthworm species in agricultural land is *Aporrectodea caliginosa*, and this species has been shown to be equally suitable for monitoring soil toxicity (Gooneratne et al. 2011) and genotoxicity (Cataldo et al. 2011).

This article includes online-only Supplemental Data.
* Address correspondence to Ravi.Gooneratne@lincoln.ac.nz
Published online 18 March 2020 in Wiley Online Library (wileyonlinelibrary.com).
DOI: 10.1002/etc.4713

The size and surface coating of AgNPs have impacts on the toxic response (Makama et al. 2016). Soil organic matter and the thiol compounds therein can reduce the toxicity of AgNPs to soil microorganisms but nevertheless still affect soil, plant, and animal enzymes (Levard et al. 2012). Exposure of earthworms to AgNPs results in increased lipid peroxidation (LPO) and elevated antioxidant defence enzyme systems such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione *S*-transferase (GST; Zhang et al. 2013). However, AgNPs in soil tend to react with ligands such as sulfide but can also react with fluvic and humic acids in organic matter, resulting in reduced toxicity (Levard et al. 2012).

Biomarkers such as SOD, CAT, GPx, and GST enzymes as well as LPO at suborganismal levels have been considered to be measures of responses to stressors (Huggett et al. 2017). Several biomarkers have been used at the cellular level as efficient tools because of their sensitivity, quickness, and accurate relationship between toxicant exposure and respective biological responses (Connon et al. 2012). Gordon et al. (2010) reported that Ag ions inactivate enzymes by binding to sulfhydryl (thiol) groups in amino acids and promote release of iron with subsequent free radical formation by a variety of mechanisms, mostly mediated by reactive oxygen species (ROS). Sources of O_2^- and H_2O_2 are functionally and spatially related to the production and cellular localization of natural antioxidant enzymes including SOD and CAT (Gobe and Crane 2010). Superoxide dismutases detoxify O_2^- into H_2O_2, which is then converted to water and molecular O_2 by CAT. Glutathione *S*-transferase is a phase II enzyme that catalyzes glutathionylation of xenobiotics. Glutathione peroxidase enzymes mediate the reduction of peroxides to alcohols. The balance between ROS production and the antioxidant system leads to regulated intracellular steady-state levels of ROS in aerobic organisms (Sies 1997).

Oxidative stress is a deleterious process caused by an imbalance between oxidants and antioxidants in steady state, which plays a role in the pathogenesis of many diseases including some caused by environmental agents including metal ions (Samet and Wages 2018). It can result in damage to proteins and DNA and cause LPO (Wible and Bratten 2018). It is suggested that the major toxicity pathway for AgNPs in environmental organisms is via the impairment of oxidative phosphorylation, generation of ROS, and LPO (Terevinto et al. 2010; Cataldo et al. 2011). The aim of the present study was to compare time- and dose-related responses of 4 antioxidant enzymes and LPO in *A. caliginosa* earthworms chronically exposed to AgNPs and Ag^+ (as $AgNO_3$) for 4 wk.

MATERIALS AND METHODS

Chemicals and reagents

Trisodium citrate (99% purity), sucrose, and dipotassium hydrogen phosphate (98% purity) were purchased from BDH. Analytical-grade $AgNO_3$ (99% purity) and all other chemicals (e.g., ferrous sulfate heptahydrate, 98% purity) were from Sigma-Aldrich.

AgNP synthesis and quantification

On the day of the experiments AgNPs were freshly prepared. We used citrate-coated AgNPs because these are easy to synthesize, stable, and one of the less toxic AgNPs (Nguyen et al. 2013). Citrate-coated AgNPs were synthesized from $AgNO_3$, reduced by ferrous sulfate heptahydrate, and coated with trisodium citrate dehydrate, as reported by Lea (1889). The dissolved Ag (in the supernatant ~0.1 mg/mL) was removed from AgNP pellets by centrifugation at 1865 *g* for 10 min. The pellet was suspended in 10 mL of deionized water (MilliQ), and the synthesized AgNPs were kept at 4 °C. This method produced spherical particles, which did not aggregate because of the electrostatic repulsion of the citrate capping agent. Silver NPs were diluted from stock colloidal solution by diluting 0.35 (Ag) µL in 50 mL deionized water. The Ag concentration in AgNPs (diluted 1400×) was determined by flame atomic absorption spectroscopy (FAAS; model AA.6200; Shimadzu), calibrated with the Merck ICP certiPUR multielement standard (Sigma-Aldrich).

AgNP charge measurement and particle size

The average mean particle size and zeta potential (a measure of the magnitude of the electrostatic or charge repulsion or attraction between particles) of the synthesized AgNPs were determined using a Zetasizer Nano ZS (Malvern Instruments) at 25 °C. The conditions employed were He/Ne laser (wavelength = 633 nm), scattering angle 90°, refractive index 1.33, and viscosity 0.887 mPa. Prior to the measurements, the concentration of the AgNPs was diluted 200-fold with deionized water.

The morphology and size of AgNPs were determined using a Philips CM200 transmission electron microscope (TEM). The AgNPs were diluted with deionized water to obtain a more uniform distribution and directly transferred onto a TEM gold-carbon grid for size characterization, using the high-resolution TEM fitted with a Gatan digital camera.

Experimental design (soil studies)

Aporrectodea caliginosa earthworms were used in the study from a colony typed to genus in our laboratory by Bate (2015). Adult earthworms ($n = 200$; average weight = 0.8 + 0.08 g, range = 0.66–1.02 g), 3 to 4 mo old with a well-developed clitellum, were kept in plastic containers ($n = 40$; with small holes drilled in caps for air circulation) containing 200 g of test soil with a 5-g total mixture of ground cow manure and grass clippings added weekly. Approximately 50 kg of Templeton Silt Loam soil (see Supplemental Data, Table S1, for soil properties) were sieved, and the moisture content (22%) was adjusted to field capacity (41%), as described by Grewal et al. (1990). The concentration of Ag in the control soil was 0.06 µg/L. Because this is negligible compared to the exposure concentrations, throughout present study it is stated as 0 mg/kg.

Earthworms were exposed to 0, 0.3, 3, 30, and 300 mg/kg (as AgNPs) or 0, 0.03, 0.3, 3, and 10 mg/kg Ag^+ (as $AgNO_3$)

soil, with 20 earthworms (4 containers of 5 replicates) per exposure concentration (2 control and 8 Ag treatment groups). The exposure concentrations were selected based on a preliminary experiment conducted to determine the median lethal concentrations (LC50s) of *A. caliginosa* exposed to AgNPs and Ag^+, calculated using probit analysis as 2649.2 and 305 mg/kg, respectively (Supplemental Data, Figure S1). Because these LC50 results showed that Ag^+ was approximately 10-fold more lethal than AgNPs, relatively much lower concentrations of $AgNO_3$ were used in the present study. The highest concentrations for each Ag form used in the present study were approximately 10% of the LC50 doses. At each of 1, 2, 3, and 4 wk of exposure to AgNPs or $AgNO_3$, 5 worms from each exposure concentration were removed, rinsed, dried with filter paper, labeled, and frozen at −20 °C for subsequent analysis of LPO and antioxidant enzyme activities (SOD, CAT, GPx, and GST). Earthworm Ag concentration was determined in the week-4 samples.

Because of the negligible soil Ag concentration and analysis of the spiking solution, we assumed the soil concentration of Ag to be approximately nominal. We did not analyze the soils directly.

Earthworm homogenization and measurements

Earthworms were homogenized (FJ 200, Gao Su Fen San Jun2HI JI Shanghai Specimen and Model Factory homogenizer) at a ratio of 1:3 (w/v) in 0.15 M phosphate saline buffer (PSB; pH 7.4) at high speed for 25 s in ice. Each 3 mL of PSB contained 175 µL of a mixture of protease inhibitors (aprotinin, leupeptin, and pepstatin, 5 µg/mL; antipain, 1 µg/mL; trypsin inhibitor, 1 mg/mL; Concetti et al. 1984; Laguerre et al. 2009). The homogenate was then centrifuged (Beckman J2-MI; GMI Laboratory Solutions) at 30 390 *g* relative centrifugal field for 20 min at 4 °C to remove cell debris and most of the cell organelles. Aliquots of the supernatant for enzyme analysis and Ag and protein concentrations were transferred to 15-mL capped plastic tubes and kept on ice. All antioxidant enzymes and LPO were measured using spectrophotometric (Shimadzu 1200) methods.

Total protein in earthworm homogenate

Total protein was determined by the method of Bradford (1976) with bovine serum albumin as the standard. All enzyme activities are expressed as a function of the protein concentration in the earthworm homogenate supernatant.

Ag concentration in earthworms

Earthworm homogenates were digested using concentrated nitric acid in a microwave (CEM MARS Xpress operating in selectable output of 0–1600 watts ± 15%). The Ag concentration was determined using an inductively coupled plasma optical emission spectrophotometer (Varian 720 ICP-OES). The calibration internal standards were prepared by serially diluting the Merck ICP standard. The recovery of certified reference material for Ag concentrations ranging from 0.1 to 1000 µg/L ranged from 86 to 103% (Supplemental Data, Table S2).

SOD

Measurement of SOD activity in earthworm homogenate was based on the inhibition of pyrogallol autoxidation with the extinction coefficient of pyrogallol as 2640 M/cm (Terevinto et al. 2010). Briefly, 2.85 mL of phosphate buffer (pH 8), 75 µL of 10 mM pyrogallol, and 75 µL of homogenate were mixed; and the kinetics was followed by measuring the increase in absorbance at 340 nm, every minute for 4 min. The enzyme activity is expressed as µmol/min/mg protein. The assay buffer was used as the blank.

CAT

Measurement of CAT activity was based on determination of the kinetics of the degradation of H_2O_2 with the extinction coefficient of H_2O_2 as 43.6 M/cm (Cataldo et al. 2011). Briefly, 72.5 µL of 50 mM phosphate buffer (pH 6.5), 2.5 mL of 300 mM H_2O_2 (30% w/w), and 0.5 mL of earthworm homogenate were mixed; and the kinetics was followed by measuring the decrease in absorbance at 240 nm, every minute for 4 min. The blank contained sodium phosphate buffer and H_2O_2. The enzyme activity is expressed as µmol/min/mg protein. The assay buffer was used as the blank.

GPx

The assay mixture consisted of 1.97 mL of assay buffer, made up of 1 mM reduced glutathione (GSH), 50 mM phosphate buffer, 0.15 mM nicotinamide adenine dinucleotide phosphate hydrogen (NADPH), 1 mM sodium azide, 1.5 U glutathione reductase (GR), 0.15 mM H_2O_2, and 0.0073 g ethylenediaminetetraacetic acid (EDTA). To this, 30 µL of earthworm homogenate was added and the GPx activity measured for 4 min at 22 °C based on the oxidation of NADPH monitored by the decrease in absorbance of the incubation mixture at 340 nm, measured every minute for 4 min. The extinction coefficient of NADPH was 6220 M/cm (Terevinto et al. 2010). The GPx activity is expressed as µmol oxidized NADPH per minute per mg protein. The assay buffer was used as the blank.

GST

Measurement of GST in earthworm homogenates was based on the increase in absorbance at 340 nm because of the conjugation of GST to 1-chloro-2,4-dinitro-benzene (CDNB) and the extinction coefficient of GSH as 9.6 M/cm (Cataldo et al. 2011). Briefly, the reaction mixture contained 100 µL of freshly prepared GSH (40 mM) in phosphate buffer (0.01 M, pH 7.6), 100 µL of CDNB (1 mM), and 500 µL of potassium phosphate buffer (0.1 M, pH 6.5), which were mixed and incubated for 5 min at 25 °C. The reaction was initiated by adding 30 µL of earthworm homogenate and the increase in absorbance

at 340 nm followed every minute for 4 min. The enzyme activity is expressed as mmol/min/mg protein. The assay buffer was used as the blank.

LPO

The assay of thiobarbituric acid (TBA) reactive substances measures malondialdehyde (MDA), which is formed by LPO as described by Cataldo et al. (2011). Initially, the proteins in the earthworm homogenates were removed by precipitation with trichloroacetic acid (30%) and centrifuged at 2000 *g* for 20 min. To 1 mL of the supernatant, 1 mM EDTA and 1% TBA were added, and the mixture was heated in a bath of boiling water (deionized) for 15 min. The sample was cooled on ice and the absorbance of MDA measured at 532 nm. The concentration of MDA was calculated using the molar extinction coefficient of MDA (156 000 M/cm), and the results are expressed as milligrams of MDA equivalents normalized to total earthworm homogenate protein.

Statistical analysis

Throughout, the results are reported as the mean ± standard error of the mean (SEM). The relationship between mean soil Ag concentration and mean earthworm Ag concentration after 4 wk was analyzed using linear regression. Differences between the means ($n = 5$) at each concentration were then analyzed using a one-way analysis of variance (ANOVA). Repeated-measures ANOVA was used to analyze differences for the 5 different soil concentrations at 1-wk intervals. The LPO data ($n = 5$ worms per treatment; Table 1) are expressed on a weekly basis and comparisons made between the 5 soil concentrations at each time point. All comparisons of means used Fisher's least significant difference post hoc multiple comparison test ($\alpha = 0.05$), with different letters indicating statistical significance. The data were analyzed using either Minitab® 17 Statistical Software for the repeated-measures ANOVA. The data were also analyzed using Genstat (2018, 17th ed.; VSN International) to derive regression (either linear or nonlinear) fitting curves for the earthworm antioxidant enzyme responses on exposure to the different concentrations of AgNPs and Ag^+ ions. The regression (either linear or nonlinear) fitting curves are shown in Supplemental Data, Figures S4 to S11. From the enzyme profiles generated, in general, for each biomarker enzyme, there was an obvious increasing trend over time (the detailed estimate parameters with week in regression functions are shown in Supplemental Data, Figures S4–S11), except some at concentration 0. This is what we expected. So we concluded that the enzyme activities at week 4 > week 3 > week 2 > week 1.

RESULTS

AgNPs

The concentration of the synthesized AgNPs used in these earthworm experiments as measured by FAAS ranged from 12 to 13 g/L. The morphology of AgNPs examined by TEM varied from circular to sometimes oval. The size distribution of the samples synthesized varied between 10 and 40 nm, but most ranged from 10 to 30 nm, with an average size of approximately 25 ± 8 nm (Supplemental Data, Figure S2). The AgNP size measured by the zetasizer was 30 ± 4 nm. The zeta potential of the synthesized AgNPs was −41 ± 6 mV, indicating good stability of Ag.

Ag accumulation in earthworms

No mortality was observed during the study. Following a 4-wk exposure, the Ag concentration in the earthworms increased in a dose-responsive fashion as a function of exposure concentration (Figure 1), to a maximum of 0.84 mg/kg in the 300-mg/kg AgNPs soil treatment ($R^2 = 0.94$, $y = 0.0023x + 0.1418$, $p = 0.0311$) and 0.26 mg/kg in those exposed to the highest Ag^+ concentration of 10 mg/kg soil ($R^2 = 0.96$, $y = 0.023x + 0.0211$, $p = 0.0186$).

SOD

A concentration-dependent significant increase ($p < 0.05$) in SOD enzyme activity in earthworms exposed to both AgNPs

TABLE 1: Lipid peroxidation measured as malondialdehyde equivalents normalized to earthworm *Aporrectodea caliginosa* homogenate total protein (mean ± standard error of the mean)[a] following exposure to different concentrations of Ag nanoparticles (AgNPs) or Ag^+ ($AgNO_3$) over 4 wk

Ag (mg/kg soil)	Week 1	Week 2	Week 3	Week 4
AgNPs				
0	25.9 ± 4.97A	51.21 ± 7.06A	41.84 ± 11.94A	52.25 ± 10.23A
0.3	111.37 ± 8.91AB	155.4 ± 12.2A	222.52 ± 39.44B	285.63 ± 29.21B
3	179.73 ± 14.39B	212.2 ± 25.49B	258.4 ± 36.67BC	407.46 ± 28.83C
30	238.73 ± .33.03C	281.5 ± 41.34B	325.22 ± 50.52C	438.6 ± 26.12C
300	587.58 ± 38.82D	608.4 ± 22.5B	629.23 ± 7.15D	738.31 ± 20.46D
Ag^+				
0	46.51 ± 11.26A	36.61 ± 4.06A	34.09 ± 11.94A	52.25 ± 10.23A
0.03	119.42 ± 25.38A	234.88 ± 21.78AB	366.54 ± 62.84AB	485.84 ± 128.42B
0.3	174.32 ± 54.6A	303.08 ± 28.21BC	563.08 ± 83.76AB	575.88 ± 154.88C
3	382.24 ± .74.19B	454.45 ± 29.2CD	600.32 ± 77.74BC	951 ± 302.8C
10	546 ± 123.86B	710.23 ± 57.7CD	808.11 ± 7.15C	1277 ± 358.34D

[a]Means with different letters denote significant differences ($p < 0.05$) between treatments ($n = 5$ worms per treatment) at each time point for each Ag form separately.

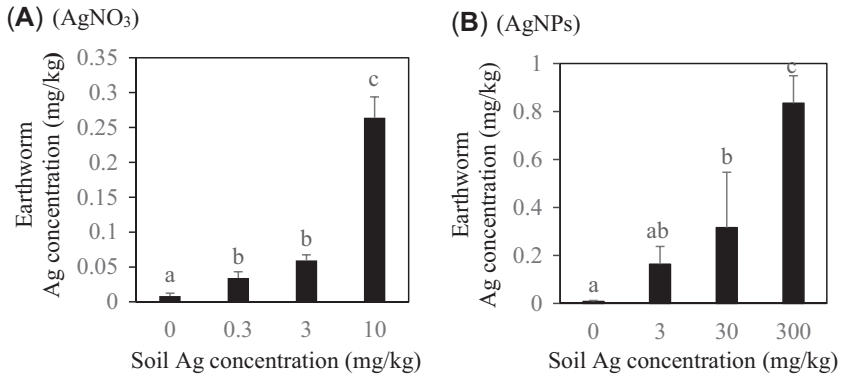

FIGURE 1: Silver concentration (mean ± standard error of the mean) in *Aporrectodea caliginosa* ($n = 5$) homogenates exposed to (**A**) Ag^+ as $AgNO_3$ (0, 0.3, 3, and 10 mg/kg), and (**B**) Ag nanoparticles (NPs; 0, 3, 30, and 300 mg/kg) at week 4. Means with different letters on bars are significantly different ($p < 0.05$) from the respective controls (0 exposure) at each time point.

and $AgNO_3$ was evident at most time points (Figure 2). The increase in SOD activity in earthworms exposed to $AgNO_3$ (Ag^+) was approximately twice that observed in the worms exposed to AgNPs.

CAT

The CAT enzyme activity in *A. caliginosa* exposed to AgNPs or Ag^+ over a 4-wk period is shown in Figure 3. A concentration-dependent significant increase ($p < 0.05$) at most time points was observed, with the elevated CAT enzyme activities most marked in the earthworms exposed to the highest concentration of Ag^+.

GPx

The GPx enzyme activity in *A. caliginosa* exposed to AgNPs or Ag^+ over a 4-wk period is shown in Figure 4. The GPx activity increased significantly ($p < 0.05$) in a concentration-dependent manner, with the values observed on exposure to Ag^+ not significantly different from those observed in earthworms exposed to AgNPs.

GST

The GST enzyme activity was markedly elevated in earthworms exposed to both AgNPs and Ag^+ compared to other enzyme activities. There was a significant ($p < 0.05$) concentration-dependent GST increase in earthworms exposed to AgNPs or Ag^+, and this was most marked in those exposed to Ag^+ (Figure 5).

Antioxidant enzymes (linear or nonlinear fitting curves)

The regression (either linear or nonlinear) fitting curves derived from either linear or nonlinear fitting curves for the earthworm antioxidant enzyme responses (SOD, CAT, GPx, GST) on exposure to the different concentrations of AgNPs and Ag^+ ions as analyzed by using Genstat are shown in Supplemental Data, Figures S4 to S11. From the enzyme profiles generated, in general, for each biomarker enzyme, there was an obvious increasing trend over time (the detailed estimate parameters with week in regression functions are shown in Supplemental Data, Figures S4 to S11), except some at concentration 0. This is what we expected. Based on this, we

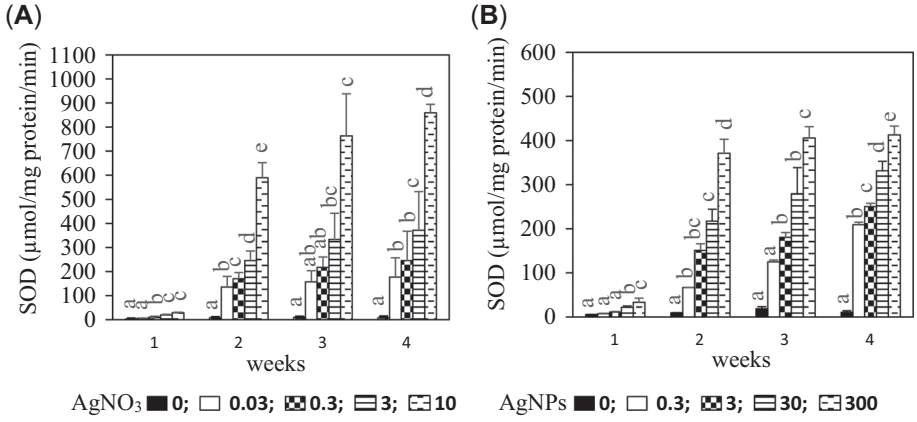

FIGURE 2: Superoxide dismutase enzyme activity (mean ± standard error of the mean) in *Aporrectodea caliginosa* ($n = 5$) exposed to (**A**) Ag^+ as $AgNO_3$ (0, 0.03, 0.3, 3, and 10 mg/kg), and (**B**) Ag nanoparticles (NPs; 0, 0.3, 3, 30, and 300 mg/kg) for 1, 2, 3, and 4 wk. Means with different letters on bars are significantly different ($p < 0.05$) from the respective controls (0 exposure) at each time point. SOD = superoxide dismutase.

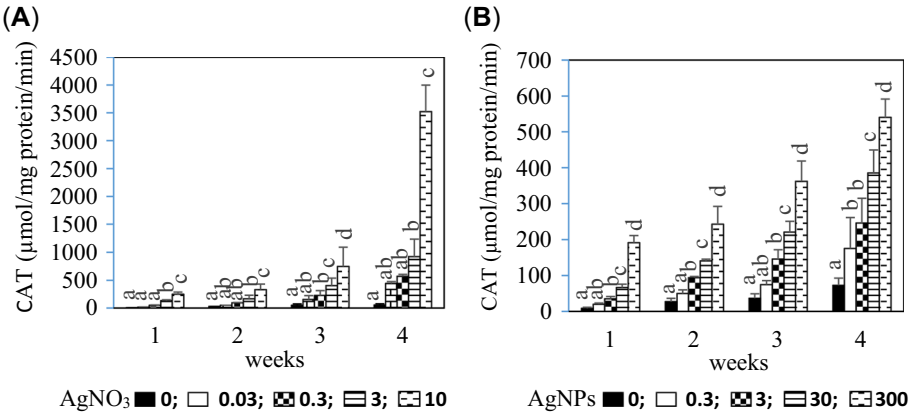

FIGURE 3: Catalase enzyme activity (mean ± standard error of the mean) in *Aporrectodea caliginosa* ($n = 5$) exposed to (**A**) Ag^+ as $AgNO_3$ (0, 0.03, 0.3, 3, and 10 mg/kg), and (**B**) Ag nanoparticles (NPs; 0, 0.3, 3, 30, and 300 mg/kg) for 1, 2, 3, and 4 wk. Means with different letters on bars are significantly different ($p < 0.05$) from the respective controls (0 exposure) at each time point. CAT = catalase.

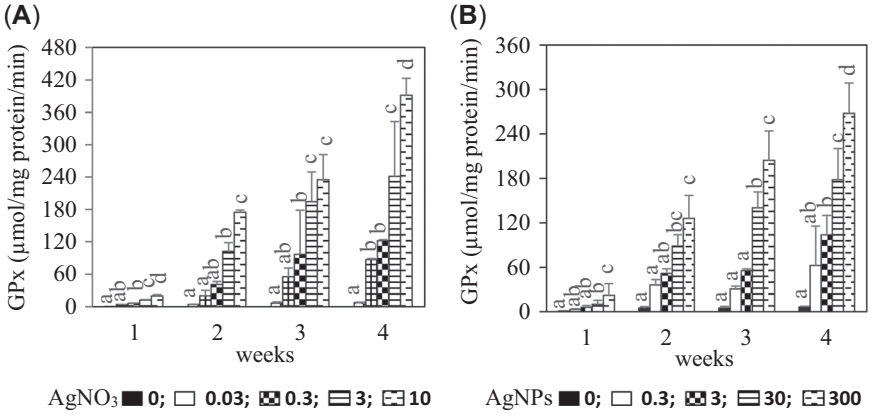

FIGURE 4: Glutathione peroxidase enzyme activity (mean ± standard error of the mean) in *Aporrectodea caliginosa* ($n = 5$) exposed to (**A**) Ag^+ as $AgNO_3$ (0, 0.03, 0.3, 3, and 10 mg/kg), and (**B**) Ag nanoparticles (NPs; 0, 0.3, 3, 30, and 300 mg/kg) for 1, 2, 3, and 4 wk. Means with different letters on bars are significantly different ($p < 0.05$) from the respective controls (0 exposure) at each time point. GPx = glutathione peroxidase.

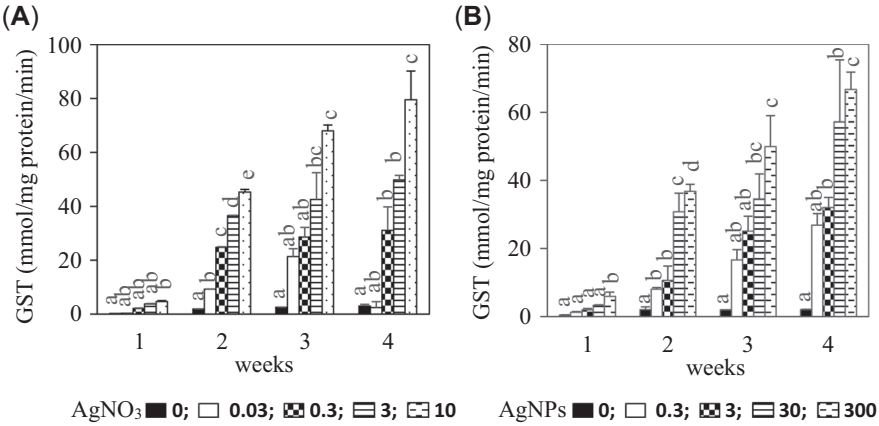

FIGURE 5: Glutathione *S*-transferase enzyme activity (mean ± standard error of the mean) in *Aporrectodea caliginosa* ($n = 5$) exposed to Ag^+ (**A**) $AgNO_3$ (0, 0.03, 0.3, 3, and 10 mg/kg), and (**B**) Ag nanoparticles (NPs; 0, 0.3, 3, 30, and 300 mg/kg) for 1, 2, 3, and 4 wk. Means with different letters on bars are significantly different ($p < 0.05$) from the respective controls (0 exposure) at each time point. GST = glutathione *S*-transferase.

concluded that the enzyme activities at week 4 > week 3 > week 2 > week 1.

LPO

The LPO activity as measured by MDA adduct formation is shown in Table 1. There was a concentration- and time-responsive increase in LPO following exposure to both AgNPs and Ag^+. The LPO activity was higher in earthworms exposed to Ag^+ > AgNPs over 4 wk but not significantly different ($p > 0.05$; Table 1).

DISCUSSION

With many industries now using AgNPs, potential exists for increased environmental and human exposure to occur with deleterious toxicological implications (Martirosyan et al. 2014). In the present study, both AgNPs and Ag^+ increased antioxidant enzyme activities and increased LPO. In soil AgNPs dissociate to Ag^+ with time, resulting in an increase in toxicity, but over an extended period will bind with soil ligands to reduce Ag bioavailability and toxicity (Diez-Ortiz et al. 2015). Short-term exposure of the earthworm *Eisenia fetida* to AgNPs/Ag^+ induced oxidative damage, with a significant increase in the protein carbonyl, and by day 3 upregulated 9 stress response genes including CAT and heat shock protein 70 (Tsyusko et al. 2012). *Eisenia fetida* earthworms altered their behavior to avoid soil contaminated with AgNPs/Ag^+ within 48 h when exposed to AgNPs and immediately on exposure to $AgNO_3$ (Shoults-Wilson et al. 2010).

The earthworm *A. caliginosa* was selected for the present study because it is widespread throughout New Zealand, which makes it a good animal model to study the effects of AgNPs in this country's terrestrial environment. One of the common mechanisms of toxicity mediated by NPs is the induction of oxidative stress (Oberdörster et al. 2005). This has been demonstrated with AgNPs, which induce high levels of ROS within cells (Kawata et al. 2009). The present study has clearly shown that the antioxidant enzymes (Figures 2–5) that counteract oxidative stress and LPO (Table 1) were increased in *A. caliginosa* exposed to either Ag^+ or AgNPs in a concentration-dependent manner.

Two modes of action of AgNPs have been proposed: 1) the release of Ag^+ ions on long-term incubation in soil and induction of ROS (McShan et al. 2014), and 2) direct induction of ROS (Wijnhoven et al. 2009). Both modes of action can initiate oxidative stress, which can irreversibly damage proteins, lipids, and nucleic acids and stimulate the production of antioxidant enzymes such as SOD, CAT, and GPx (McShan et al. 2014). Both mechanisms can also lead to the harmful genotoxic effects associated with AgNP exposure (Durán et al. 2016; Wang et al. 2017). Silver NPs can also cause toxicity through surface oxidation, leading to the release of Ag^+, which can interact with nucleic acids, lipid molecules, and proteins in biological systems (McShan et al. 2014). One of the most important differences between our earthworm AgNP study and those reported by others is that we used a mixture of protease inhibitors—aprotinin, leupeptin, pepstatin, and antipain—in the homogenizing buffer (Concetti et al. 1984; Laguerre et al. 2009). In a preliminary study, the protein yield of the earthworm homogenate supernatant prepared with the PSB containing protease inhibitors was approximately 35% higher than with PSB alone (Supplemental Data, Figure S11). We believe the addition of antiproteases may have prevented enzyme degradation and hence contributed to higher enzyme activities even when expressed as a fraction of the protein concentration. This is an important finding and may explain the differences in antioxidant enzyme activities and LPO between the present study and those reported by others (Pan et al. 2010; Aldarraji et al. 2013; Muthu and Durairaj 2015).

The biomarkers of oxidative stress used in the present study comprised the antioxidant enzymes SOD, CAT, GPx, and GST as well as LPO, all of which were in general significantly increased in a concentration-dependent manner on exposure to both Ag^+ and AgNPs; and there was a trend for values in week 4 > week 3 > week 2 > week 1 in general but not always (Figures 2–5). Superoxide dismutase enzymes play a significant role in protecting cells from oxidative stress by catalyzing the dismutation of O_2^- anions to H_2O_2 and O_2, and CAT converts H_2O_2 to water (Roubalová et al. 2015). The higher antioxidant enzyme activities in earthworms exposed to Ag^+ even at concentrations 10 times lower than those of AgNPs are probably due to the high solubility and ready absorption of Ag^+ by *A. caliginosa* (Zhan 2012), which is similar to what is observed in rats (Qin et al. 2017). Shoults-Wilson et al. (2011), in their study on the effect of AgNP surface coating on bioaccumulation and reproductive toxicity in earthworms, reported that surface coating with polyvinylpyrrolidone (PVP) and oleic acid prevented excessive oxidation (<15%) and dissolution of NPs. Based on a study of a 28-d exposure of earthworms to both PVP- and oleic acid–coated AgNPs in artificial soil, Shoults-Wilson et al. (2011) suggested that the marked toxicity of AgNPs was primarily due to the release of Ag^+. Also, AgNPs can react with ligands present in soil, to form sulfides, thus reducing toxicity (Levard et al. 2012; Doolette et al. 2015).

On a molar basis, Ag^+ ions are some 5.6×10^{14} times more concentrated than an equal mass of AgNPs (Supplemental Data, Table S3). Given this large difference in molarity, it is likely that the toxicity of AgNPs is due in part to conversion of the AgNPs into Ag^+ ions because such a dissociation can occur in the long term. Thus, the Ag^+ released from AgNPs can lead to LPO in earthworms, either *A. caliginosa* or *E. fetida* (Li et al. 2014). Exposure of the soil invertebrate *Enchytraeus crypticus* to AgNPs showed that the oxidative stress mechanism caused by AgNPs may be somewhat different from that of Ag^+, with AgNPs taking a longer time than Ag^+ to produce toxic effects (Ribeiro et al. 2015).

Among the antioxidant enzymes, CAT appears to be the most induced (Ribeiro et al. 2015). This agrees with our present study, which found a >2-fold increase in CAT activity by Ag^+. Gomes et al. (2015) showed that exposure of *E. fetida* to Ag^+ mixed in Organisation of Economic Co-operation and Development soil spiked with 25 to 200 mg/kg for 28 d resulted in

increases in GPx, SOD, and GR by 300, 100, and 200%, respectively, but reduced the activities of CAT and GST. These findings in earthworms exposed to very high concentrations are somewhat dissimilar to our much lower dose 4-wk study, where we observed a continued increase of all antioxidant enzymes up to 4 wk. Gomes et al. (2015) used *E. fetida* (whereas we used *A. caliginosa* earthworms) and high doses of Ag^+ up to 200 mg/kg and uncapped AgNPs up to 1500 mg/kg, which could be considered unrealistic environmental concentrations. Such high concentrations can lead to marked irreversible enzymatic and metabolic changes. Mendes et al. (2015), in a similar study but with *Folsomia candida* (a member of the order Collembola, a common and widespread arthropod that occurs in soils throughout the world), found that the increases in metallothionein and GST > CAT, GR, GSH, and LPO were more marked in those exposed to Ag^+ than in those exposed to AgNPs.

A comparison of the antioxidant enzyme profiles in earthworms exposed to the same concentrations (0.3 and 3 mg/kg soil) of Ag^+ and AgNPs showed that the activities of antioxidant enzymes were markedly higher in earthworms exposed to Ag^+. There are several reasons Ag^+ may be more toxic than AgNPs: 1) the solubility of Ag^+ is much higher than that of AgNPs because Ag^+ (from $AgNO_3$) is a salt, although AgNPs are considered to be a base that forms a colloidal solution in water; 2) on a molar basis, Ag^+ ions are some 5.6×10^{14} times more concentrated than an equal mass of AgNPs; 3) Ag from AgNPs accumulates in the cell membranes, whereas Ag^+ is localized in the cytosolic fraction (Li et al. 2014); and 4) the elimination rate of Ag when exposed to AgNPs is greater than that of Ag^+ (Ribeiro et al. 2015). Schlich et al. (2013) observed higher accumulation of Ag in the AgNP (sized 15 nm) group than in *Eisenia andrei* earthworms exposed to Ag^+. However, the induction of antioxidant enzymes and LPO was 2.5- to 3-fold higher in earthworms exposed to 0.3 and 3 mg/kg of Ag^+ concentration compared to AgNPs (Table 1). When *E. fetida* earthworms were exposed to AgNPs of 2 particle sizes at doses up to 500 mg/kg for 14 d, the smaller 10-nm particles were more toxic than the 80-nm ones (Li et al. 2012). Thus, it appears that AgNP size influences uptake and degree of toxicity. In addition to a greater accumulation rate of Ag^+ (Qin et al. 2017), certain toxicokinetic parameters, such as total body clearance, mean residence, half-life, bioaccumulation factor, and elimination rate constant (Ramsey et al. 2009; Waalewijn-Kool et al. 2014; Bednarska et al. 2016; Świątek et al. 2017), may have also contributed to the greater toxicity of Ag^+ than AgNPs to *A. caliginosa*, which is in agreement with Qin et al. (2017).

According to Zhan (2012), the LC50 of Ag^+ to *A. caliginosa* is 418 mg/kg, which is higher than the LC50 of 305 mg/kg measured in $AgNO_3$ (Ag^+) by us (Supplemental Data, Figure S1). In the present study, AgNPs (LC50 2649 mg/kg; Supplemental Data, Figure S1) were acutely less toxic than Ag^+, and earthworms exposed to AgNPs took a longer time than with Ag^+ to show toxic effects, which is in agreement with Ribeiro et al. (2015). Choi and Park (2015) observed no mortality in *E. fetida* exposed to up to 100 mg/kg of AgNPs (citrate-coated, particle size 11 nm) mixed with artificial soil and concluded that it was not acutely toxic. In contrast, Brami et al. (2017) observed that exposure of *Allolobophora chlorotica* earthworms to Ag^+ or AgNPs mixed in Kettering loam soil (composed of 24% clay, 18% silt, 58% sand, 6.7% organic matter, pH 6.8) at 100 mg/kg soil caused 12.5% mortality with AgNPs (uncoated, spherical, 80 nm particle size) compared to 66.7% with Ag^+. The highest concentrations of Ag^+ and AgNPs used in the present study were 10 and 300 mg/kg, respectively, mixed in Templeton Silt Loam soil in a 4-wk exposure period. No deaths occurred. This shows different degrees of toxicity to different earthworm species exposed to Ag in different soil types. Shoults-Wilson et al. (2010) mixed artificial soil with 10, 100, and 1000 mg/kg AgNPs and with 10 or 100 mg/kg Ag^+ and observed that earthworms exposed to Ag^+ accumulated higher Ag concentrations than those exposed to AgNPs, which is in agreement with our results. This is also in agreement with our pharmacokinetic studies of a higher bioaccumulation in *A. caliginosa* with Ag^+ when exposed to either AgNPs or Ag^+ at a concentration of 20 mg/kg (N. Saleeb, Lincoln University, Lincoln, Canterbury, New Zealand, unpublished observation).

Pollution by heavy metals of anthropogenic origin has long been recognized as a serious threat to terrestrial ecosystems. And heavy metals, including Ag, accumulate in earthworms by soil ingestion, via ion exchange of dissolved heavy metals across the lipophilic outer membrane and/or adsorption on membrane surfaces. For example, divalent metals such as cadmium (Cd^{2+}), copper (Cu^{2+}), chromium (Cr^{2+}), lead (P^{2+}), and zinc (Zn^{2+}) accumulated in tissues of the earthworm *Eudrilus euginiae* exposed to 0.1 and 1.5 g of waste (vermicomposting municipal solid waste; Kumar et al. 2008). These divalent heavy metals may also be present in brownfields and public parks (Jennings et al. 2002). When the earthworm *Lampito mauritii* was exposed to different concentrations of Pb and Zn separately for 28 d (Maity et al. 2008), the activities of GST, GPx, GSH, and GR were linearly increased after exposure to Pb; but on exposure to Zn, only GPx and GR activities were increased, and that occurred only after 14 d and at >300 mg/kg soil. It appears that different metals accumulate in earthworms at different rates, and such differences may be attributable to differences in earthworm species and potentially the exposure concentration and length.

CONCLUSION

Exposure to AgNPs or Ag^+ resulted in an accumulation of Ag in *A. caliginosa* earthworms. Dose-dependent significant increases in antioxidant enzyme activities (CAT, GPx, SOD, and GST) and increased LPO were observed in earthworms exposed to Ag^+ > AgNPs. The increases in antioxidant enzyme activity tended to be time-dependent, and this trend was more marked in LPO activity. In general, Ag^+ was 2.5- to 4-fold more toxic than AgNPs to *A. caliginosa*.

Supplemental Data—The Supplemental Data are available on the Wiley Online Library at https://doi.org/10.1002/etc.4713.

Acknowledgment—The present study was supported by Lincoln University and Landcare Research, Lincoln, New Zealand. We thank C. Bunt for carrying out the zeta-potential measurement. The technical assistance of M. Wellby is gratefully acknowledged. The present study was partly funded by the Centre for Integrated Biowaste Research.

Data Availability Statement—Data are available on request to the corresponding author (Ravi.Gooneratne@lincoln.ac.nz).

REFERENCES

Aldarraji QM, Halimmon N, Majid NM. 2013. Antioxidant activity and total phenolic content of earthworm paste *Lumbricus rubellus* (red worm) and *Edurilus eugenia* (African night crawler). *J Entomol Nematol* 5:33–37.

Bate A. 2015. Speciation of and the effects of silver nanoparticles on the earthworm *Aporrectodea caliginosa*. Master's thesis. Lincoln University, Lincoln, Canterbury, New Zealand.

Bednarska AJ, Laskowski R, Pyza E, Semik D, Świątek Z, Woźnicka O. 2016. Metal toxicokinetics and metal-driven damage to the gut of the ground beetle *Pterostichus oblongopunctatus*. *Environ Sci Pollut Res* 23:22047–22058.

Blouin M, Hodson ME, Delgado EA, Baker G, Brussaard L, Butt KR, Dai J, Dendooven L, Peres G, Tondoh JE, Cluzeau D, Brun JJ. 2013. A review of earthworm impact on soil function and ecosystem services. *Eur J Soil Sci* 64:161–182.

Bradford MM. 1976. A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72:248–254.

Brami C, Glover AR, Butt KR, Lowe CN. 2017. Effects of silver nanoparticles on survival, biomass change and avoidance behaviour of the endogeic earthworm *Allolobophora chlorotica*. *Ecotoxicol Environ Saf* 141(Suppl. C): 64–69.

Cataldo J, Hidalgo ME, Neaman A, Gaete H. 2011. Use of molecular biomarkers in *Eisenia foetida* to assess copper toxicity in agricultural soils affected by mining activities. *J Soil Sci Plant Nutr* 11:57–70.

Choi JS, Park JW. 2015. Molecular characterization and toxicological effects of citrate-coated silver nanoparticles in a terrestrial invertebrate, the earthworm (*Eisenia fetida*). *Mol Cell Toxicol* 11:423–431.

Concetti A, Fioretti E, Barra D, Ascoli F. 1984. Protease inhibitors from the parasitic worm *Parascaris equorum*. *Eur J Biochem* 145:417–421.

Connon RE, Geist J, Werner I. 2012. Effect-based tools for monitoring and predicting the ecotoxicological effects of chemicals in the aquatic environment. *Sensors (Basel)* 12:12741–12771.

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

Dobias J, Bernier-Latmani R. 2013. Silver release from silver nanoparticles in natural waters. *Environ Sci Technol* 47:4140–4146.

Doolette CL, Mcaughlin MJ, Kirby JK, Navarro DA. 2015. Bioavailability of silver and silver sulfide nanoparticles to lettuce (*Lactuca sativa*): Effect of agricultural amendments on plant uptake. *J Hazard Mater* 300:788–795.

Durán N, Durán M, de Jesus MB, Seabra AB, Fávaro WJ, Nakazato G. 2016. Silver nanoparticles: A new view on mechanistic aspects on antimicrobial activity. *Nanomedicine* 12:789–799.

Gobe G, Crane D. 2010. Mitochondria, reactive oxygen species and cadmium toxicity in the kidney. *Toxicol Lett* 15:49–55.

Gomes SI, Hansen D, Scott-Fordsmand JJ, Amorim MJ. 2015. Effects of silver nanoparticles to soil invertebrates: Oxidative stress biomarkers in *Eisenia fetida*. *Environ Pollut* 199C:49–55.

Gooneratne R, Buser A, Lindsay P, Wellby M. 2011. Ecotoxicological assessment of acid mine drainage: Electrophysiological changes in earthworm (*Aporrectodea caliginosa*) and aquatic oligochaete (*Lumbriculus variegatus*). *J Environ Monit* 13:1360–1365.

Gordon O, Slenters TV, Brunetto PS, Villaruz AE, Sturdevant DE, Otto M, Landmann R, Fromm KM. 2010. Silver coordination polymers for prevention of implant infection: Thiol interaction, impact on respiratory chain enzymes, and hydroxyl radical induction. *Antimicrob Agents Chemother* 54:4208–4218.

Grewal KS, Buchan GD, Tonkin PJ. 1990. Estimation of field capacity and wilting point of some New Zealand soils from their saturation percentages. *N Z J Crop Hortic Sci* 18:241–246.

Huggett RJ, Klmerle RA, Mehrle PM Jr, Bergman HL, eds. 2017. *Biomarkers: Biochemical, Physiological and Histological Markers of Anthropogenic Stress*. CRC, Boca Raton, FL, USA.

Jennings AA, Cox AN, Hise SJ, Petersen EJ. 2002. Heavy metal contamination in the Brownfield soils of Cleveland. *Soil Sediment Contam* 11:719–750.

Judy JD, Unrine JM, Bertsch PM. 2011. Evidence for biomagnification of gold nanoparticles within a terrestrial food chain. *Environ Sci Technol* 45:776–781.

Kawata K, Osawa M, Okabe S. 2009. In vitro toxicity of silver nanoparticles at non-cytotoxic doses to HepG2 human hepatoma cells. *Environ Sci Technol* 43:6046–6051.

Kühnel D, Nickei C. 2014. The OECD meeting on ecotoxicology and environmental fate—Towards the development of improved OECD guidelines for the testing of nanomaterials. *Sci Total Environ* 472:347–353.

Kumar S, Sharma V, Bhoyar RV, Bhattacharyya JK, Chakrabarti T. 2008. Effect of heavy metals on earthworm activities during vermicomposting of municipal solid waste. *Water Environ Res* 80:154–161.

Laguerre C, Sanchez-Hernandez JC, Köhler HR, Triebskorn R, Capowiez Y, Rault M, Mazzia C. 2009. B-type esterases in the snail *Xeropicta derbentina*: An enzymological analysis to evaluate their use as biomarkers of pesticide exposure. *Environ Pollut* 157:199–207.

Lea MC. 1889. On allotropic forms of silver. *Am J Sci* 37:476–491.

Lee WM, Kwak JI, An YJ. 2012. Effect of silver nanoparticles in crop plants *Phaseolus radiatus* and *Sorghum bicolor*: Media effect on phytotoxicity. *Chemosphere* 86:491–499.

Lemtiri A, Colinet G, Alabi T, Cluzeau D, Zirbes L, Haubruge É, Francis F. 2014. Impacts of earthworms on soil components and dynamics. A review. *Biotechnol Agron Soc Environ* 18:121–133.

Levard C, Hotz EM, Lowry GV, Brown GE Jr. 2012. Environmental transformations of silver nanoparticles: Impact on stability and toxicity. *Environ Sci Technol* 46:6900–6914.

Li CHM, Wang W, Cui Y, Chen J, Yang L. 2012. Ecotoxicity of silver nanoparticles on earthworm *Eisenia fetida*: Responses of the antioxidant system, acid phosphatase and ATPase. *Toxicol Environ Chem* 94: 732–741.

Li L, Wu H, Peijnenburg WJGM, van Gestel CAM. 2014. Both released silver ions and particulate Ag contribute to the toxicity of AgNPs to earthworm *Eisenia fetida*. *Nanotoxicology* 9:792–801.

Maity S, Roy S, Chaudhury S, Battacharya S. 2008. Antioxidant responses of the earthworm *Lampito mauritii* exposed to Pb and Zn contaminated soil. *Environ Pollut* 151:1–7.

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

Martirosyan A, Bazes A, Schneider YJ. 2014. In vitro toxicity assessment of silver nanoparticles in the presence of phenolic compounds—Preventive agents against the harmful effect? *Nanotoxicology* 8:573–582.

McShan D, Ray PC, Yu H. 2014. Molecular toxicity mechanism of nanosilver. *J Food Drug Anal* 22:116–127.

Meier C, Voegelin A, Pradas del Real A, Sarret G, Mueller CR, Kaegi R. 2016. Transformation of silver nanoparticles in sewage sludge during incineration. *Environ Sci Technol* 50:3503–3510.

Mendes LA, Maria VL, Scott-Fordsmand JJ, Amorim MJB. 2015. Ag nanoparticles (Ag NM300K) in the terrestrial environment: Effects at population and cellular level in *Folsomia candida* (Collembola). *Int J Environ Res Public Health* 12:12530–12542.

Muthu S, Durairaj B. 2015. Evaluation of antioxidant and free radical scavenging activity of *Annona muricate*. *Eur J Exp Biol* 5:39–45.

Nguyen KC, Seligu VL, Massarsky A, Moon TW, Rippstein P, Tan J, Tayabali AF. 2013. Comparison of toxicity of uncoated and coated silver nanoparticles. *J Phys Conf Ser* 429:012025.

Oberdörster G, Oberdörster E, Oberdörster J. 2005. Nanotoxicology: An emerging discipline evolving from studies of ultrafine particles. *Environ Health Perspect* 113:823–839.

Organisation of Economic Co-operation and Development. 1984. Test No. 207: Earthworm: Acute toxicity tests. *OECD Guidelines for the Testing of Chemicals*. Paris, France.

Pan R, Zhang ZJ, He RQ. 2010. Earthworm protease. *Appl Environ Soil Sci* 2010:294258.

Qin G, Tang S, Li S, Lu H, Wang Y, Zhao P, Li B, Zhang J, Peng L. 2017. Toxicological evaluation of silver nanoparticles and silver nitrate in rats following 28 days of repeated oral exposure. *Environ Toxicol* 32:609–618.

Ramsey H, Levine H, Kennedy J. 2009. Development of a dynamic pharmacokinetic model to estimate bioconcentration of xenobiotics in earthworms. *Environ Model Assess* 14:411–418.

Ribeiro MJ, Maria VL, Scott-Fordsmann JJ, Amorim MJB. 2015. Oxidative stress mechanisms caused by Ag nanoparticles (NM300K) are different from those of $AgNO_3$: Effects in the soil invertebrate *Enchytraeus crypticus*. *Int J Environ Res Public Health* 12:9589–9602.

Roubalová R, Procházková P, Dvořák J, Škanta F, Bilej F. 2015. The role of earthworm defense mechanisms in ecotoxicity studies. *Invertebrate Surviv J* 12:203–213.

Samet JM, Wages PA. 2018. Oxidative stress from environmental exposures. *Curr Opin Toxicol* 7:60–66.

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

Shoults-Wilson A, Reinsch BC, Tsyusko OV, Bertsch PM, Lowry G, Unrine JM. 2010. Role of particle size and soil type in toxicity of silver nanoparticles to earthworms. *Soil Sci Soc Am J* 75:365–377.

Shoults-Wilson W, Zhurbich OI, McNear DH, Tsyusko OV, Bertsch PM, Unrine JM. 2011. Evidence for avoidance of Ag nanoparticles by earthworms (*Eisenia fetida*). *Ecotoxicology* 20:385–396.

Sies H. 1997. Oxidative stress: Oxidants and antioxidants. *Exp Physiol* 82:291–295.

Stensberg MC, Wei Q, McLamore ES, Porterfield DM, Wei A, Sepúlveda MS. 2011. Toxicological studies on silver nanoparticles: Challenges and opportunities in assessment, monitoring and imaging. *Nanomedicine* 6:879–898.

Świątek ZM, Van Gestel CAM, Bednarska AJ. 2017. Toxicokinetics of zinc-oxide nanoparticles and zinc ions in the earthworm *Eisenia andrei*. *Ecotoxicol Environ Saf* 143:151–158.

Terevinto ARA, Ramos A, Castroman G, Cabrera MC, Saadoun A. 2010. Oxidative status, in vitro iron-induced lipid oxidation and superoxide dismutase, catalase and glutathione peroxidase activities in rhea meat. *Meat Sci* 84:706–710.

Tripathi DK, Tripathi A, Schweta, Singh S, Vishwakarma K, Yadav G, Sharma S, Singh VK, Mishra RK, Upadhyay RG, Dubey NK, Lee Y, Chauhan DK. 2017. Uptake, accumulation, and toxicity of silver nanoparticles in autotrophic plants, and heterotrophic microbes: A concentric review. *Front Microbiol* 8:7.

Tsyusko OV, Hardas SS, Shoults-Wilson WA, Starnes CP, Joice G, Butterfield DA, Unrine JM. 2012. Short-term molecular-level effects of silver nanoparticle exposure on the earthworm, *Eisenia fetida*. *Environ Pollut* 171:249–255.

Waalewijn-Kool PL, Klein K, Forniés RM, van Gestel CAM. 2014. Bioaccumulation and toxicity of silver nanoparticles and silver nitrate to the soil arthropod *Folsomia candida*. *Ecotoxicology* 23:1629–1637.

Wang J, Che B, Zhang LW, Dong G, Luo Q, Xin L. 2017. Comparative genotoxicity of silver nanoparticles in human liver HepG2 and lung epithelial A549 cells. *J Appl Toxicol* 37:495–501.

Wible DJ, Bratten SB. 2018. Reciprocity in ROS and autophagic signaling. *Curr Opin Toxicol* 7:28–36.

Wijnhoven SWP, Peijnenburg WJGM, Herberts CA, Hagens WI, Oomen AG, Heugens EHW, Roszek B, Bisschops J, Gosens I, Van De Meent D, Dekkers D, De Jong WH, van Zijverden M, Sips AJAM, Geertsma R. 2009. Nano-silver—A review of available data and knowledge gaps in human and environmental risk assessment. *Nanotoxicology* 3:109–138.

Xiong J, Xue Q, Wu X. 2013. Synthesis of stable dispersions of water-soluble and SERS-active silver nanoparticles using PAM. *Nano* 8:1350046.

Zhan Y. 2012. Effect of silver nanoparticles on bacteria and earthworms. Master's thesis. Lincoln University, Lincoln, Canterbury, New Zealand.

Zhang Q, Zhu L, Wang J, Xie H, Wang J, Han Y, Yang J. 2013. Oxidative stress and lipid peroxidation in the earthworm *Eisenia fetida* induced by low doses of fomesafen. *Environ Sci Pollut Res* 20:201–208.

