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EFFECTS OF SILVER NANOPARTICLES (NM-300K) ON *LUMBRICUS RUBELLUS* EARTHWORMS AND PARTICLE CHARACTERIZATION IN RELEVANT TEST MATRICES INCLUDING SOIL

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Abstract: The impact of silver nanoparticles (AgNP; at 0 mg Ag/kg, 1.5 mg Ag/kg, 15.4 mg Ag/kg, and 154 mg Ag/kg soil) and silver nitrate ($AgNO_3$; 15.4 mg Ag/kg soil) on earthworms, *Lumbricus rubellus*, was assessed. A 4-wk exposure to the highest AgNP treatment reduced growth and reproduction compared with the control. Silver nitrate ($AgNO_3$) exposure also impaired reproduction, but not as much as the highest AgNP treatment. Long-term exposure to the highest AgNP treatment caused complete juvenile mortality. All AgNP treatments induced tissue pathology. Population modeling demonstrated reduced population growth rates for the AgNP and $AgNO_3$ treatments, and no population growth at the highest AgNP treatment because of juvenile mortality. Analysis of AgNP treated soil samples revealed that single AgNP and AgNP clusters were present in the soil, and that the total Ag in soil porewater remained high throughout the long-term experiment. In addition, immune cells (coelomocytes) of earthworms showed sensitivity to both AgNP and $AgNO_3$ in vitro. Overall, the present study indicates that AgNP exposure may affect earthworm populations and that the exposure may be prolonged because of the release of a dissolved Ag fraction to soil porewater. *Environ Toxicol Chem* 2014;33:743–752. © 2013 SETAC

Keywords: Soil organisms Population model Histopathology Coelomocytes Exposure characterization

INTRODUCTION

Silver nanoparticles (AgNP) are among the most widely used nanoparticles in consumer products and there are concerns about unintended exposure of humans and the environment [1,2]. Consequently, effort has focused on collecting data relating to the hazards and behavior of AgNP in the environment [1–3]. Kahru and Dubourguier [4] evaluated the toxicity of nanoparticles to a range of aquatic species (from bacteria to invertebrates and fishes) from 77 studies, and classified AgNP as extremely toxic. However, the risks of AgNP exposure to terrestrial ecosystems, and especially soil organisms, is still poorly understood [5].

Only a limited number of studies have investigated the impact of AgNP exposure to earthworms (i.e., *Eisenia fetida* and *Lumbricus terrestris* earthworms), showing effects on survival, growth, avoidance, and inhibition of the Na^+,K^+-ATPase and apoptotic activity in tissues [5–10]. The toxicity of metal nanoparticles to soil organisms has been tentatively associated with ionic metal fractions appearing in the soil porewater [9,11]. The behavior of AgNP in soil may depend on interactions of these nanoparticles with soil components, such as organic matter, metal oxides and clay, and on soil characteristics, including ionic strength, pH, and dissolved organic matter [8,9,12–15]. Therefore, for a good interpretation of soil toxicity experiments, characterization of the nanoparticles in different soil compartments (i.e., soil solid phase and soil porewater) is required [3,12,16,17].

Our previous work on Buckminsterfullerene (C_{60}) exposure to earthworms highlighted some important timescale issues in nanoparticle toxicity [18–20]. In vivo experiments demonstrated lower reproduction after 4-wk C_{60} exposure, and reduced survival and growth after long-term C_{60} exposure, with all these parameters being critical endpoints to population dynamics [18]. For these C_{60} exposed earthworms, tissue pathology and genomic responses were observed as well [19]. Additional in vitro experiments using earthworm immune cells (coelomocytes) demonstrated effects of exposure to C_{60} and polymer nanoparticles, which implicated the immunotoxic potential of nanoparticles [20].

In the present study, a similar approach was employed to investigate the effects of AgNP exposure on *Lumbricus rubellus* earthworms. Earthworms were exposed to AgNP in vivo for 4 wk with a subsequent long-term exposure of the offspring and a range of endpoints was assessed, including survival, growth, reproduction, and histopathology. A population model was used to quantify the potential impact of AgNP induced changes of these individual endpoints on *L. rubellus* populations. In an attempt to link exposure to effects, Ag was characterized in different test matrices, using several independent analytical techniques. In addition, given our previous observations of immunotoxicity of nanoparticles [20] and the importance of immune health in natural populations, an in vitro experiment

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with coelomocytes was performed to aid interpretation of the data as well as providing insight into possible modes of action of AgNP exposure.

MATERIAL AND METHODS

Three experiments were conducted, 2 in vivo experiments and 1 in vitro experiment. The first in vivo experiment encompassed a 4-wk exposure of adult earthworms to different levels of AgNP and $AgNO_3$, and assessment of the effects of exposure on survival, growth, and reproduction of the earthworms. In the second in vivo experiment, offspring from the first experiment were exposed and monitored long-term for survival and growth under experimental conditions similar to their parents. In addition, an in vitro experiment was conducted to assess the possible immunotoxic effects of AgNP. In this experiment, earthworm coelomocytes were isolated and exposed overnight to different levels of AgNP and cell survival was determined (compared with Van der Ploeg et al. [20]).

Test compounds

Commercially available NM-300K silver nanoparticles (AgNP) were obtained from Mercator GmbH (Germany), with a mean reported particle diameter of 15 nm (90% < 20 nm). These nanoparticles have been selected as a representative nanomaterial by the Organisation for Economic Co-operation and Development Working Party on Manufactured Nanomaterials international testing program [21]. The stock suspension of AgNP contained 10.16% Ag (w/w) dispersed in a stabilizing vehicle material, consisting of polyoxyethylene glycerol trioleate (4%; w/w) and polyoxyethylene (20) sorbitan mono-laurate (Tween 20; 4%; w/w; manufacturer's information). The vehicle material without AgNP was also purchased from Mercator as NM-300K DIS Ag-dispersant, and used as a control. Silver nitrate ($AgNO_3$) was obtained from Merck KGaA as a powder with a purity of 99.8% (w/w), and used to benchmark the AgNP effects against Ag salt.

The AgNP stock suspension and the $AgNO_3$ powder were dissolved in 0.14 M HNO_3 at a ratio of 1/1000 (w/v) to determine the presence of metal impurities. Metal concentrations in the digests were measured by a high-resolution inductively coupled plasma-mass spectrometer (Element 2; Thermo Scientific). The levels of metal impurities were very low (Supplemental Data, Table S1); therefore, no additional adverse effects were expected from these impurities.

In vivo experiments

Experimental design. The in vivo experiments were conducted following International Organization for Standardization guidelines 11268-2:1998 [22], with minor adjustments in exposure scenarios and using the earthworm species *L. rubellus*. For the 4-wk exposure experiment, adult (clitellated) earthworms were acquired (Nijkerkerveen). The average weight of the earthworms was 1373 ± 33 mg (mean $\pm$ standard error of the mean [SEM]; $n = 180$), and the weights did not differ significantly between those assigned to the exposure groups (analysis of variance [ANOVA], $p > 0.05$). Prior to the experiment, the earthworms were acclimatized for 2 wk under constant conditions (24-h light, 15 °C, and 61% relative humidity) in uncontaminated sandy test soil containing 1.6% clay and 4.3% organic matter with a pH of 6.0.

For the in vivo experiments, the soil was prepared as described by van der Ploeg et al. [18], using a wet-spiking procedure with a soil extract. For each treatment, 6 glass containers (650 g soil) were used ($n = 6$), each housing 5 earthworms. Different soil exposure concentrations of Ag in the form of AgNP and $AgNO_3$ were used. Three nominal exposure concentrations of AgNP were tested: 1.5 (low) mg Ag/kg, 15.4 (medium) mg Ag/kg, and 154 (high) mg Ag/kg soil. Because of limitations in the experimental logistics of the in vivo experiments, only 1 concentration of $AgNO_3$ was tested at 15.4 mg Ag/kg soil. This $AgNO_3$ concentration was chosen to compare the Ag salt with the highest AgNP treatment, assuming approximately 10% dissolution within the time frame of (maximum) 4 wk [9,14]. In addition, soil without Ag or vehicle material and soil with just vehicle material were used as controls. The soil extract used to spike the soil was obtained by the addition of soil to ultrapure water (400 g/L), after which the suspension was shaken and filtered. Physicochemical characteristics of the soil extract are presented in Supplemental Data, Table S2. The obtained soil extract was spiked with AgNP (2.0 g Ag/L), $AgNO_3$ (0.2 g Ag/L), vehicle material (20 g/L; as a control for AgNP) or without any addition (control for $AgNO_3$), and added to the soil in specific amounts to obtain the desired nominal concentrations. To the reference soil and low- and medium-exposure levels, nonspiked extracts were added so all treatments received the same amount of extract. The soil was mixed thoroughly and added to the container, after which the earthworms were placed in the container immediately. In the Supplemental Data, further details about the soil and the spiking procedure are provided. For the 4-wk experiment, endpoints included survival, growth (weight gain compared between beginning and end of experiment), and reproduction (the cocoon production per container at the end of the experiment, converted to the average number of cocoons produced per surviving earthworm per day). Furthermore, at the end of the experiment, histological observations of different tissues were made and total Ag concentrations were analyzed in tissues. In addition, imaging of Ag particles taken up by the earthworms (only for the high AgNP treatment) was conducted at the end of the experiment, to underpin the analytical results of the total Ag concentrations in the earthworms. In the long-term experiment, earthworms were checked monthly for survival and growth. Furthermore, histological examinations were made at the end of the long-term experiment (after 10 mo).

Exposure characterization. An overview of the analytical techniques used to characterize the AgNP prior to spiking of the soil and during exposure are presented here. Because of the large number of analytical techniques, details on all these techniques are provided in the Supplemental Data.

Characterization of the AgNP and total Ag concentrations in the soil extract (used for addition of AgNP, $AgNO_3$ and vehicle material to the soil), the soil solid phase, the soil porewater, and the earthworms was performed where technically and practically possible. An overview of the in vivo exposure characterization is presented in Table 1 (discussed in the *Results and Discussion* section on *In vivo experiments*). The particle diameter of the AgNP suspended in the soil extract, which was used to spike the soils in the in vivo experiments, was determined using asymmetric flow field flow fractionation (AF4), For this purpose, a fresh suspension of AgNP in the soil extract was prepared at a Ag concentration of 2 g Ag/L. In an accompanying experiment, colloidal stability of NM-300K AgNP in the soil extract was monitored while the ionic strength of the soil extract was increased using calcium nitrate ($Ca(NO_3)_2$). This experiment was performed because as a result of the high soil to solution ratio employed during the soil extraction procedure (of 400 g/L), the ionic strength of 2.3 mM in the soil extract (as shown in Supplemental Data, Table S2)

Table 1. Overview of the different analytical techniques used to characterize in vivo exposure

Matrix	SE and UPW	Soil	Soil pore water	Earthworm	SE, soil, earthworms
Endpoint	Size	(Total Ag)	(Total Ag)	(Total Ag)	Imaging of Ag particles
Method[a]	AF4	ICP-MS	F-AAS	ICP-MS	SEM/EDX
Time point[b]	t = 0	t = 1 and t = 10	t = 1 and t = 10	t = 1	t = 0 (SE), t = 1 (S, E)
Pretreatment	None	Acid digestion	Water saturation and filtration	Acid digestion	Drying and coating
Treatment unit analyzed	NA	All AgNP and $AgNO_3$ treatments	All AgNP and $AgNO_3$ treatments	All AgNP and $AgNO_3$ treatments	High AgNP and $AgNO_3$ (S), high AgNP (E)

[a]The exposure was assessed in soil extract (SE) and ultrapure water (UPW) by asymmetric flow field flow fractionation system (AF4) to determine the size of silver nanoparticles (AgNP) before spiking the soil, and inductively coupled plasma mass spectrometry (ICP-MS) was used to quantify total Ag concentrations in soil and earthworms. Furthermore, soil porewater was analyzed by flame atomic absorption spectrometry (F-AAS) to measure total Ag concentrations present in soil porewater samples.
[b]Samples for these analyses were freshly made (t = 0), taken at the end of the 4 wk (t = 1 mo) or at the end of the long-term experiment (t = 10 mo). In addition, freshly made samples of soil extract (SE), as well as samples from soil (S) and earthworms (E) taken at the end of the 4-wk experiment were analyzed by field emission gun scanning electron microscopy in combination with energy dispersive analysis of X-rays (SEM/EDX).
NA = not applicable.

was much lower than what was expected for soil porewater during the in vivo experiments. In many soil porewaters, the ionic strength amounts to an average of 30 mM [23]. As colloidal stability decreases with increasing ionic strength, AgNP aggregation is expected to occur during the in vivo experiments when adding the soil extract to the soil [12,15]. For analysis of total Ag concentrations in the soil solid phase, soil samples were taken at the end of the 4-wk and long-term experiments (after 10 mo). After acid digestion of the soil, inductively coupled plasma mass spectrometry (ICP-MS) was used to measure the total Ag concentrations. For analysis of total Ag in the soil porewater, samples were obtained from the soil samples taken at the end of the 4-wk and long-term experiments (after 10 mo). Soil porewater was generated by saturation of the soils with water and subsequent centrifugation and filtration over 0.45-μm filters. Total Ag concentrations in the soil porewater filtrates were directly measured with flame atomic absorption spectrometry (F-AAS). For the measurement of the total Ag concentrations in the earthworms, whole earthworm samples were collected at the end of the 4-wk experiment. The total Ag concentrations in acid digests of the earthworms were analyzed by ICP-MS. Furthermore, in almost all matrices of the in vivo experiment (soil extract, soil, and earthworm) the presence of Ag particles was qualitatively assessed by field emission gun scanning electron microscopy in combination with energy dispersive X-ray analysis (SEM/EDX). The SEM/EDX provides information about the Ag particle size and composition and the elements associated with these particles.

Effect assessment: Individual and population endpoints. At the start of the 4-wk experiment, earthworms were weighed and added to the containers. Earthworms were fed alder leaves (*Alnus glutinosa*) from an uncontaminated location (Vossemeerdijk, Dronten, The Netherlands) ad libitum. After 4 wk, the experiment was terminated and the earthworms were counted and weighed again. The number of cocoons produced per container was determined by wet sieving and hand sorting. One earthworm per container was stored in 4% buffered formal saline for histological examination [24] after segments were carefully cut with a sharp scalpel blade, covering the region approximately 1 centimeter anterior and posterior to the clitellum. The other earthworms were put in liquid nitrogen and stored at –80 °C until further analysis.

For the consecutive long-term experiment, cocoons from the first experiment were incubated in Petri dishes with soil of the corresponding treatment of their parent earthworms. After hatching, juveniles were transferred to containers with soil with the same concentrations as their parents, again with 6 containers per treatment ($n = 6$) and 5 earthworms per container. Earthworms were checked monthly over a period of 10 mo, to determine weight and life stage: juvenile, subadult (showing a *tubercula pubertatis*, but before the clitellum has fully developed), and adult (with a fully developed clitellum) [25]. At the end of the experiment, earthworms were counted, weighted, and then prepared for histology or frozen in liquid nitrogen as described above.

The individual effect markers on adult growth, mortality, and reproduction and offspring survival and growth, were integrated to assess consequences at the population level, using a continuous-time life-history model as described by van der Ploeg et al. [18]. Within this model, several parameters were taken from the literature [26], including the duration of the cocoon stage (τ_0; 42 d) and the mortality chance per day (with 1 = 100% mortality at day 1) for the cocoon stage (μ_c; 0.001), the subadult stage (μ_s; 0.0043), and the adult life stage (μ_{ad}; 0.0027).

Effect assessment: Histological observations. For the histological examinations, the fixed segments were processed into wax blocks and transverse sections of 7 μm were cut from each segment. The staining of these sections was performed using Mallory's trichrome. All sections were prepared simultaneously in batches containing both samples from earthworms of the control, AgNP, and $AgNO_3$ treatments to eliminate differences in fixation or staining artifacts between treatments. Sections were examined with an Olympus Vanox-T microscope and photographs were obtained using an Olympus digital camera (C-2020 Z). Eventually, not all earthworms were examined because of some preservation artifacts, which made the histological observations semiquantitative.

In vitro experiment

Exposure characterization. Dynamic light scattering (DLS) and Zetasizer were applied on freshly made stocks of AgNP (10 μg/mL) in ultrapure water and cell culture medium containing 10% fetal calf serum, to measure particle diameter and zeta potential for AgNP (see *Results and Discussion* section). No DLS and Zetasizer measurements were discussed for stocks of $AgNO_3$, as AgNO3 will dissolve and the measurements would show results from protein (aggregates) mostly. Furthermore, the presence, morphology, and composition of Ag particles were qualitatively established for both AgNP and $AgNO_3$ stocks using SEM/EDX.

Effect assessment: Coelomocyte viability. Primary immune cells from the coelomic fluid (coelomocytes) were exposed to

AgNP and $AgNO_3$ in vitro. Coelomocytes were extruded from unexposed adult *L. rubellus* earthworms as described by van der Ploeg et al. [20].

Serial dilutions of AgNP and $AgNO_3$ in cell culture medium containing 10% fetal calf serum were made to obtain a concentration range of exposure medium from 0 μg Ag/mL to 2000 μg Ag/mL cell culture medium, for AgNP as well as $AgNO_3$. In addition, serial dilutions of vehicle material without AgNP were prepared and used as negative control for AgNP exposure. Then 50 μL of exposure medium was added to 50 μL of coelomocytes suspension (with $\sim 5 \times 10^6$ coelomocytes/mL) in a 96 well-plate, to obtain the acquired final concentrations of AgNP and $AgNO_3$ from 0 μg Ag/mL to 1000 μg Ag/mL cell culture medium. The coelomocytes were incubated overnight (18–20 h), at 15 °C. Hereafter, cell viability was assessed using 0.05% trypan blue (final concentration) as described by van der Ploeg et al. [20].

Statistical analyses

Differences between treatments were analyzed using ANOVA (with $\alpha = 0.05$), with least significant differences as the posthoc test [27]. The ANOVA tests were carried out using GENSTAT (14th ed., VSN International Ltd.). If data were not normally distributed, a prerequisite for the use of parametric methods like ANOVA [27], they were transformed into their natural logarithm prior to statistical analysis. Results are presented relative to the corresponding control as mean ± SEM per treatment, unless specified otherwise.

RESULTS AND DISCUSSION

In vivo experiments

Exposure characterization. In the present study, several analytical techniques were used to characterize AgNP prior to spiking of the soil and during the in vivo exposure of earthworms. With these different techniques, a thorough characterization of AgNP was established at different stages of the experiment; before addition of AgNP to the soils with the soil extract, during exposure of the earthworms in the soil, in the soil porewater, and finally the uptake of AgNP by the earthworms was characterized.

Exposure characterization: Soil extract. The particle diameter of AgNP in the soil extract, which was used for spiking the soil, was determined by AF4 using ultraviolet absorbance at a wavelength of 413 nm for AgNP detection. The results demonstrated a clear peak with a maximum absorbance at a particle diameter of 16 nm (Supplemental Data, Figure S1), although the cross flow employed during AF4 analysis was not high enough to fully separate the peak with AgNP from the void peak. The theoretical calculation of the AgNP particle diameter was not validated with independent measurements of AgNP size in the eluent fractions using DLS or electron microscopy. Nevertheless, the estimated AgNP particle diameter of 16 nm is in good agreement with Klein et al. [28] for the same NM-300K AgNP, who reported a particle diameter varying from 14 nm to 17 nm using transmission electron microscopy. Based on integration of the fractograms with total Ag measurements in the AF4 eluent fractions, more than 90% of the total Ag amount in the soil extract had a particle diameter < 32 nm (Supplemental Data, Figure S2). The SEM/EDX analysis of the soil extract containing AgNP demonstrated the presence of single Ag particles, with a particle diameter of approximately 20 nm (data not shown). However, some larger structures (50–250 nm) of Ag were present as well, consisting of Ag only or Ag in combination with chloride and sulfide. In the soil extract with $AgNO_3$, large Ag flake-like structures (≥ 1 μm) were present, which also contained chloride.

Addition of calcium to the soil extract with AgNP, increasing the calcium levels from its initial value of 0.33 mM (Supplemental Data, Table S2) to 5 mM and higher, caused complete destabilization of the colloidal AgNP suspension (Supplemental Data Figure S3), suggesting clustering and settling of the AgNP. The ionic strength of the soil extract was rather low (2.3 mM; Supplemental Data, Table S2), while the ionic strength in the soil porewater during the in vivo experiment may have been (much) higher [23]. Therefore, although AgNP were monodisperse when added to the soil, higher ionic strength of the soil porewater during the in vivo experiments may have led to aggregation of AgNP after addition of the soil extract to the soil [14,29].

Exposure characterization: Soil. Aggregation of AgNPs because of porewater ionic strength may explain the presence of larger Ag structures within the solid phase of the soil, as indicated by SEM/EDX analysis taken at the end of the 4-wk experiment. These analyses indicated the presence of AgNP in the soil in 3 different forms: 1) single AgNP of approximately 20 nm in size, which were most abundant; 2) larger spherical structures with a size of 50 nm to 250 nm, containing only Ag (Supplemental Data, Figure S4A), which were likely aggregated AgNP; and 3) structures of approximately 500 nm in the shape of a star (Figure S4B), which consisted of Ag particles of 50 nm to 100 nm in combination with carbon and chloride. These star-shaped structures were probably formed after oxidation and dissolution of Ag from AgNP, followed by precipitation of Ag with chloride or binding of Ag to soil particles [30,31]. In the SEM/EDX imaging of soil with $AgNO_3$, no Ag particles and structures were detected. The Ag from $AgNO_3$ may have undergone rapid fixation to the soil via binding with soil organic matter or reactive surfaces of other soil constituents (metal oxides and clay), without forming large and detectable amounts of poorly soluble Ag precipitates such as AgCl and AgS particles [13,32].

Soil samples were taken at the end of the 4-wk and the long-term (10 mo) in vivo experiments. The total Ag concentrations in the 4-wk experiment were 1.2 ± 0.03, 10.5 ± 0.2, and 118 ± 4 mg Ag/kg for the low, medium, and high AgNP treatments, respectively (Table 2). Hence, 68% to 80% of the nominal AgNP soil exposure concentration was recovered from the soil samples with the acid digestion method. In the soil samples taken after 10 mo, the total Ag concentrations of the low, medium, and high AgNP treatments were slightly lower than after 4 wk, with concentrations of 1.3 ± 0.3 mg Ag/kg, 8.3 ± 0.3 mg Ag/kg, and 104 ± 4 mg Ag/kg soil, respectively. The $AgNO_3$ treatment (nominal concentration of 15.4 mg Ag/kg soil) showed a measured total Ag concentration of 11.5 ± 0.1 mg Ag/kg after 4 wk (Table 2) and 6.3 ± 0.01 mg Ag/kg soil after 10 mo.

Exposure characterization: Soil porewater. Analyses of the soil porewater samples were carried out at the end of the 4-wk and the long-term in vivo experiments to test whether any changes of the total Ag levels in the soil porewater occurred over time. The total Ag concentrations were measured after filtration of the samples to separate dissolved Ag from the rest of the sample. As 0.45-μm membrane filters were used, the dissolved total Ag concentrations measured may have consisted of Ag ions as well as some Ag particles greater than 0.45 μm. However, measurements by Kool et al. [11] indicate that in case of zinc nanoparticles, all zinc is present in the porewater in the ionic form, and this may account for AgNP as well. Total Ag

Table 2. Exposure characterization of the 4-wk experiment presented with the nominal concentrations (mg/Ag/kg soil) for the silver nanoparticle (AgNP) and silver nitrate ($AgNO_3$) treatments, as well as the total Ag concentrations measured in soil (mg/Ag/kg soil), soil porewater (μg Ag/L soil porewater), and the earthworms (μg Ag/g earthworm) are demonstrated

Treatment	Nominal [Ag]	Total Ag in soil	Total Ag in soil porewater	Total Ag in earthworms	Relative weight gain[a]	Relative # cocoons[a]
Vehicle control	0	<1	<3	<1	100 ± 12	100 ± 4
AgNP	1.5	1.2 ± 0.03	<3	<1	121 ± 17	103 ± 5
	15.4	10.5 ± 0.2	<3	4.5 ± 0.5	136 ± 4	91 ± 11
	154	118 ± 4	96 ± 6	2.7 ± 0.3	44 ± 12*[c]	18 ± 3*[c]
Control[b]	0	<1	<3	<1	100 ± 17	100 ± 8
$AgNO_3$	15.4	11.5 ± 0.1	16 ± 7	2.3 ± 0.7	118 ± 14	60 ± 10*[c]

[a]In addition, effects of 4-wk exposure to AgNP and $AgNO_3$ on relative weight gain and cocoon production of the *Lumbricus rubellus* earthworms (n = 6 containers) are shown.
[b]Values are compared with the corresponding control (=100%) and are presented as mean ± standard error of the mean.
[c]For the effects, significant differences were determined between vehicle material control and AgNP treatments or control and $AgNO_3$ treatments, and for these data an asterisk sign (*) shows an assessed significant difference.

concentrations in the soil porewater samples from the low and medium AgNP treatments were below the detection limit of the F-AAS (<3 μg Ag/L). Soil porewater samples from the high AgNP treatment showed an Ag concentration of 96 ± 6 μg Ag/L after 4 wk (Table 2), and this concentration had increased after 10 mo to 260 ± 21 μg Ag/L. These results from the high AgNP treatment suggest that Ag particles and structures within the soil solid phase act as a continuous source of Ag to the soil porewater. This suggestion is in line with Coutris et al. [13], who also observed a long-term release of (dissolved) Ag in their study using uncoated AgNP of 20 nm in size. However, on a pro rata basis, this also suggests that the medium AgNP treatment should release a total Ag concentration of approximately 25 Ag μg/L, but Ag concentrations were below the detection limit. The most likely explanation for this discrepancy is that any Ag releases in the medium and low AgNP treatments were rapidly complexed with the porewater chloride unlike the high AgNP treatment that may have exhausted the anion supply in the porewater or saturated the absorption sites. For the $AgNO_3$ treatment, Ag could only be detected in the soil porewater samples after 4 wk, with an Ag concentration of 16 ± 7 μg Ag/L (Table 2). Later, Ag appears to be bound to soil particles, as was also indicated by the SEM/EDX analysis.

Exposure characterization: Earthworms. Total Ag concentrations in earthworms exposed in vivo for 4 wk were measured after acid digestion of the tissues. Total Ag concentrations of the earthworms sampled from the low AgNP treatment remained below the detection limit for the ICP-MS (<1 μg Ag/g earthworm). Earthworms from the medium AgNP treatment had a total Ag concentration of 4.5 ± 0.5 μg Ag/g earthworm (Table 2). Strikingly, earthworms from the high AgNP treatment showed a lower total Ag concentration of 2.7 ± 0.3 μg Ag/g earthworm (Table 2). These results indicate that Ag bioavailability is not a simple function of the measured total Ag in the soil or soil porewater. Shoults-Wilson et al. [5] made similar observations for *E. fetida* earthworms exposed to polyvinylpyrrolidone (PVP)-coated or oleic acid (OA)-coated AgNP through the soil. Unfortunately, the details of Ag chemical speciation could not be measured in the present study. Nevertheless, results from other studies on AgNP [9] and zinc nanoparticles [11] suggest that the bioavailability and uptake of metals by soil organisms may be associated with the ionic fraction present in the soil porewater. For $AgNO_3$ exposed earthworms, the total Ag concentration was 2.3 ± 0.7 μg Ag/g earthworm (Table 2) and similar to the earthworms exposed to the equivalent dose of AgNPs, despite the fact that total Ag levels in the porewater were very dissimilar. This implies that the bioavailability from the porewater is also dependent on the form of the Ag addition to the soil.

The SEM/EDX analysis of the earthworm slices from the high AgNP treatment of the 4-wk experiment demonstrated the presence of Ag particles in the intestine, consisting of Ag only (Supplemental Data, Figure S5) or along with sulfide and chloride. The AgCl and Ag_2S heteroaggregate formation has been observed during in vivo oral exposure in mammals to AgNP and Ag metal salts as well [31,33]. In the present study, Ag particles were also found within epithelial cells of the dermis. These were single AgNP with a size of approximately 20 nm (Figure 1), but also larger particles (≤100 nm) consisting mainly of Ag. These results on both the gut and dermal epithelia provide evidence that Ag particles were in close contact with the earthworm tissues, especially the external barriers. However, the SEM/EDX technique does not penetrate far into the cells, and especially for the gut it was not able to distinguish between adsorption onto the external epithelium and true uptake (particles internalized in the cells). It is therefore also not clear what fraction of the total Ag measured in the earthworms is surface adsorption or true internal accumulation.

Effect assessment: Individual and population endpoints. All adult earthworms survived the 4-wk exposure. Table 2 shows that the adult weight gain in the high AgNP treatment group was significantly reduced (down to 44%), compared with the vehicle material control group, as was reproduction (down to 18%). Exposure to $AgNO_3$ did not reduce weight gain, but a significant reduction in cocoon production down to 60% was observed, as compared with the unexposed control treatment (Table 2).

Other 4-wk reproduction studies exposing earthworms to AgNP also demonstrated effects of $AgNO_3$ at lower Ag concentrations than AgNP [5,6]. This indicates that $AgNO_3$ is more toxic than AgNPs on short term, and that the presence of dissolved Ag may be at least causing a significant part of the toxicity.

Effects on growth and reproduction after 4-wk exposure to AgNP in the present study were observed at lower concentrations than in other studies. Shoults-Wilson et al. [5] noted a decrease in reproduction for *E. fetida* earthworms of 40% at 1000 mg Ag/kg soil after 4-wk exposure to PVP-coated or OA-coated AgNP, with a size of 30 nm to 50 nm. Heckmann et al. [6] tested the effect of PVP-coated AgNP (30–50 nm) on *E. fetida* earthworms only at 1000 mg Ag/kg soil for 4 wk, and the exposed earthworms responded with a reduced growth (down to 73% compared with the control) and did not reproduce. The earthworms of the present study demonstrated a reduction in growth down to 44% and a very low reproduction (down to 18%)

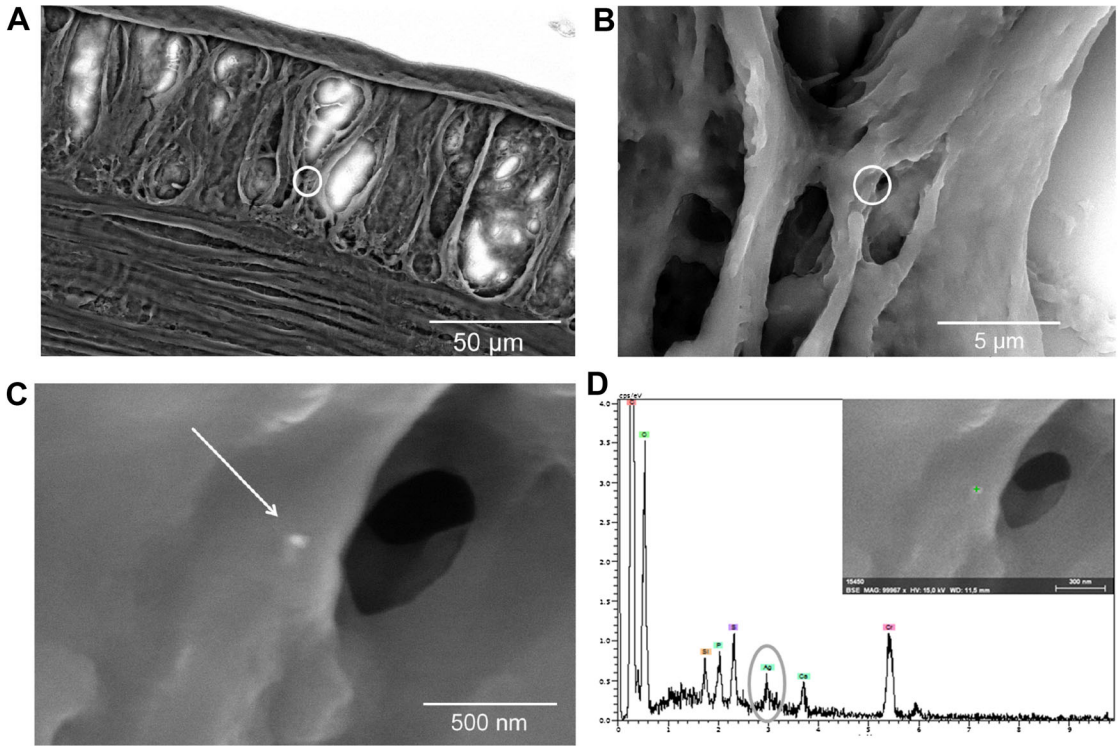

Figure 1. Scanning electron microscopy in combination with energy dispersive X-ray analysis (SEM/EDX>) images and EDX spectrum analysis obtained from a *Lumbricus rubellus* earthworm exposed to the high silver nanoparticles (AgNP) treatment (154 mg AgNP/kg soil) for 4 wk, indicating the presence of a single AgNP in the dermis. **(A–C)** Zooming into the single AgNP in the dermis of the earthworm is demonstrated. **(D)** The EDX spectrum of this AgNP is shown, with the grey oval line encircling the Ag element peak. [Color figure can be viewed in the online issue which is available at wileyonlinelibrary.com]

after only 4-wk exposure to 154 mg Ag/kg (Table 2). These differences in sensitivity may be explained by the surface coating and functionalization of the AgNPs. For example, toxicity of 10-nm citrate-coated AgNP and 20 nm PVP-coated AgNPs to the bacteria *Nitrosomonas europaea*, depended on coating and/or size of the material [2]. Other factors explaining differences between the toxicity studies may be the characteristics of the soil [9] and differences in species sensitivity to AgNP [34].

In the long-term experiment, the hatchability of the offspring was only significantly affected in the high AgNP treatment (ANOVA, $p = 0.001$), where only 2 cocoons hatched and the hatched juveniles died shortly afterwards. Furthermore, survival was significantly lower for juveniles in the medium AgNP treatment than for the vehicle material control treatment (ANOVA, $p = 0.036$). These results demonstrate that reproductive effects are more striking for AgNP than for $AgNO_3$ exposure, and also indicate that juveniles may be more sensitive to (chronic) AgNP exposure than adults, as was also observed for C_{60} [18].

Table 3 shows the parameters used in the continuous-time life-history model for the different treatments, which were estimated from the individual endpoints, for example, adult reproduction, and survival and growth of the offspring. When these parameters were introduced into the population model, the low and medium AgNP treatments demonstrated significantly reduced population growth rates (ANOVA, $p < 0.001$), decreasing down to 94.1% and 91.8%, respectively, compared with the corresponding vehicle material control. For the high AgNP, no population growth calculation was possible because of the 100% mortality shortly after hatching. The population growth rate of the $AgNO_3$ treatment was significantly reduced compared with the corresponding unexposed control (93.1%; ANOVA, $p < 0.001$). These

Table 3. Input parameters for the population model, estimated from the 4-wk and long-term in vivo experiments, are presented per treatment group[a]

Treatment	Input parameters						
	Y	R_m	L_b	L_s	L_{ad}	L_m	μ_j
Vehicle control	0.022 ± 0.005	0.0033 ± 0.0005	2.49 ± 0.3	10.5 ± 0.7	11.2 ± 0.7	13.3 ± 0.8	0.0003
Low AgNP	0.023 ± 0.005	0.0039 ± 0.0003	2.43 ± 0.1	9.9 ± 0.4	11.2 ± 0.5	12.5 ± 0.6	0.0004
Medium AgNP	0.021 ± 0.006	$0.0027 \pm 0.0004^*$	2.46 ± 0.2	10.4 ± 0.7	11.8 ± 0.8	14.0 ± 1.0	0.0015*
Control	0.021 ± 0.004	0.0032 ± 0.0005	2.68 ± 0.1	9.9 ± 0.8	11.4 ± 0.9	13.4 ± 1.0	0
$AgNO_3$	0.021 ± 0.005	$0.0018 \pm 0.0002^*$	2.69 ± 0.2	10.2 ± 0.6	11.5 ± 0.7	13.9 ± 0.9	0.0003

[a]The growth rate constant for the individual earthworms (γ) is given in $mg^{1/3}/mg^{1/3}/d$ and the maximum reproduction rate (R_m) per treatment group is presented as cocoons/$mg^{1/3}$/d. Earthworm length at birth (L_b), the lengths at reaching subadulthood (L_s) and adulthood (L_{ad}), and the estimated maximum length of the earthworms (L_m) are displayed as $mg^{1/3}$. In addition, the mortality chance for the juveniles (μ_j) is given per day (with 1 = 100% mortality at day 1). For all data, the mean values are presented and the standard deviation (SD) is displayed when this was required for the model. Significant differences between vehicle material control and silver nanoparticle (AgNP) treatments or control and silver nitrate ($AgNO_3$) treatments are displayed with an asterisk sign (*).

population growth rate data demonstrate that earthworm populations may be affected by AgNP exposure even at the lowest concentration tested.

Effect assessment: Histological observations. For the 4-wk experiment, observations of the tissue segments anterior to the clitellum of the control and the vehicle material control earthworms showed a normal histology, with an epithelium consisting of columnar epithelial cells, some mucous and basal cells, and normal-looking underlying circular and longitudinal muscles (Figure 2A and B). One of the 4 vehicle material control earthworms, however, showed some slight eosinophilic granular material in the epithelium. The AgNP treatments caused mild or moderate erosion in parts of the epithelium (Figure 2C, D, and E), for 3 of the 4 low AgNP treated earthworms and 2 of the 4 medium AgNP and high AgNP earthworms examined, respectively. Mild fibrosis was noted in the circular muscles of these earthworms as well. Earthworms from the $AgNO_3$ treatment showed some hyperplasia in the epidermis and some granular lipofuscin-like deposits in the circular muscle (2 of 3 earthworms; Figure 2F).

The tissues of the clitellum region were also affected by 4-wk exposure to AgNP and $AgNO_3$. Control treatments showed normal histology, with a pseudostratified epithelium with soft parenchyma underneath and normal circular as well as longitudinal muscle layers. Exposure to the low and medium AgNP treatment caused granular lipofuscin-like deposits in the clitellum tissue and epidermis, and mild fibrosis of the circular muscle, in 2 of 6 (low) and 2 of 3 (medium) examined earthworms. For the high AgNP treatment, only tissue from 1 earthworm, with normal histology, was examined. One of the 3 $AgNO_3$ exposed earthworms showed hyperplasia of the pseudostratified epithelium.

For the long-term experiment, examination of the segments anterior to the clitellum of the control and the vehicle material control earthworms showed normal histology. The tissue of earthworms from the low and medium AgNP treatments showed some erosion of the epithelium. In addition, 1 earthworm from the medium AgNP treatment showed excessive hyperplasia of mucus cells in the epidermis, which suggests increased mucus production (a defense mechanism). For the $AgNO_3$ treatment,

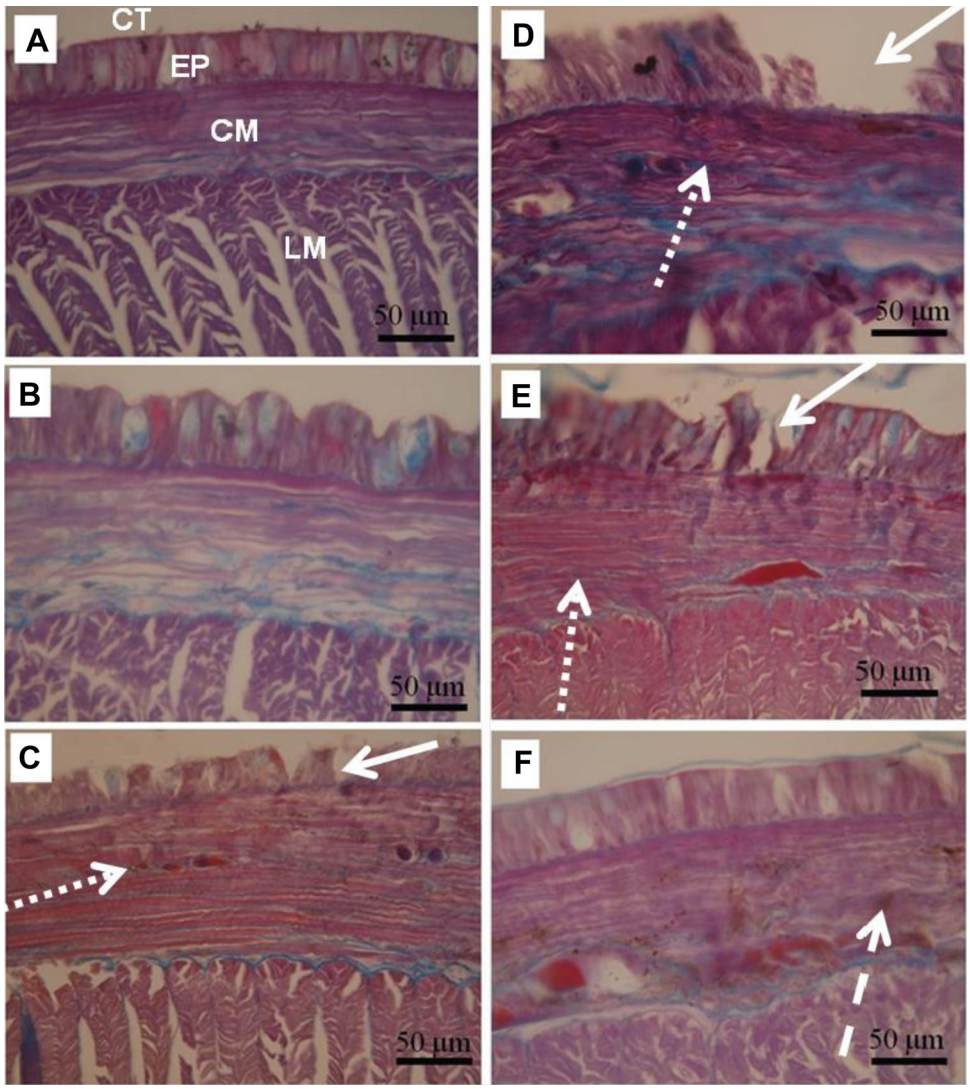

Figure 2. Transverse sections of segments from the anterior region of *Lumbricus rubellus* earthworms exposed for 4 wk to control **(A)**; vehicle material control **(B)**; and low **(C)**, medium **(D)**, high **(E)** silver nanoparticle- (AgNP-) or silver nitrate- ($AgNO_3$-) treated **(F)** soil. Controls and vehicle material controls showed normal morphology of the cuticle (CT), epidermis (EP), and underlying circular (CM) and longitudinal (LM) muscles. Note the erosion of the epithelium (solid arrow) and fibrosis of the circular muscle (dotted arrow) in panels **C**, **D**, and **E** because of AgNP exposure, with the medium treatment usually worse than the others. The $AgNO_3$ treatment (**F**) had only minor impact with some granular lipofuscin-like deposits (dashed arrow), mainly in the circular muscle. Magnification ×400. Sections cut 7 µm stained with Mallorys trichrome. [Color figure can be viewed in the online issue which is available at wileyonlinelibrary.com]

slight erosion of epithelial cells (with otherwise intact epithelium) and some thickening of the circular muscle were noted in 2 of the 3 earthworms.

The clitellum of the control and vehicle material control earthworms from the long-term experiment showed normal histology. Low AgNP treatment resulted in hyperplasia of mucus cells in the epidermis and some loss of the clitellum tissue thickness in 1 of the 5 earthworms. All 4 earthworms from the medium AgNP treatment demonstrated slight or moderate loss of architecture for the epithelial cells, and 1 earthworm showed additional fibrosis and lipofuscin-like deposits in the circular muscle, as well as some damage to the longitudinal muscle. The $AgNO_3$ treatment had less effect than the AgNP treatments, causing some erosion of the epithelium, and some thickening of the circular muscle in 2 of the 3 earthworms.

These histological examinations, together with the SEM/EDX analysis of AgNP-exposed earthworms, indicate that AgNP may affect the external barriers but are not expected to penetrate far into the earthworm body. Lapied et al. [8] also demonstrated that the external barriers of the earthworm *L. terrestris* were affected most by AgNP exposure. Damage to the outer skin and the intestine may seriously affect the health of earthworms because this damage interferes with the correct functioning of these tissues and potentially the homeostasis of the earthworms [8]. Furthermore, when looking at the long-term experiment, tissue injuries were more severe for the medium AgNP treatment compared with the $AgNO_3$ treatment. This complements the idea indicated by the juvenile mortality data that chronic AgNP treatment was more harmful for the earthworms than chronic $AgNO_3$ treatment. This difference may be explained by the exposure characterization data, which suggests that AgNP may prolong the presence of a bioavailable fraction of Ag. In addition, the lipofuscin-like deposits and fibrotic change observed in both in vivo experiments suggest some inflammation. This indicates that the immune system was trying to respond to the exposure, but failing to prevent tissue injury. This raises concern about the toxicity of AgNPs to the immune cells.

In vitro experiment

Exposure characterization. For the in vitro experiment, Ag exposure media were analyzed using SEM/EDX, DLS, and zeta potential. The SEM/EDX analysis of cell culture medium with AgNP demonstrated single Ag particles of approximately 20 nm (most abundant) and some larger Ag structures (50–250 nm), which appeared to include chloride and sometimes sulfide. The DLS analysis of AgNP in the ultrapure water demonstrated particles with a hydrodynamic diameter of 50 nm (Supplemental Data, Figure S6) and a zeta potential of -25 ± 1. When AgNP was analyzed in the cell culture medium, the particle hydrodynamic diameter became slightly larger (58 nm; Supplemental Data, Figure S6) and the zeta potential became less negative (-11 ± 1 mV). This is in good agreement with DLS results discussed by Klein et al. [28] for the same NM-300K AgNP. This result suggested that the coelomocytes were exposed in vitro mainly to single- or 2-clustered AgNP (Supplemental Data, Figure S6). The AgNP was presumably kept stable in this state because of a protein corona [35,36], which caused the zeta potential to become less negative in cell culture medium when compared with ultrapure water.

The SEM/EDX imaging of $AgNO_3$ in the cell culture medium showed some large structures of Ag (≥ 1 μm), which were associated with chloride.

Effect assessment: Coelomocyte viability. Figure 3 shows that the viability of the coelomocytes decreased with increasing AgNP concentration, resulting in a median effective concentration (EC50) of 290 μg Ag/mL cell culture medium. The viability of the coelomocytes was affected more by $AgNO_3$ exposure with an EC50 of 21 μg Ag/mL cell culture medium (Figure 3).

When comparing the AgNP EC50 with the EC50 for $AgNO_3$, a 14-fold difference is observed. The soluble Ag fraction could not be measured in the present study, but Hayashi et al. [35] demonstrated an ionic Ag fraction between 2% and 8% for the AgNP in cell culture medium after incubation for 24 h. If this is similar for the AgNP used in the present study, this would result in ionic concentrations that are 12.5 times to 50 times lower for AgNP as compared with $AgNO_3$. This indicates that a large portion of the effects of AgNP exposure may be explained by the ionic Ag fraction. Overall, this in vitro experiment indicates that AgNP exposure may impair the immune functioning of the coelomocytes, and these in vitro observations are consistent with histological injury (insufficient tissue repair) observed in vivo. Effects on immune functioning and tissue repair may have consequences for the bioenergetics of the exposed earthworms, increasing the body maintenance and leaving less energy for other processes like growth and reproduction [37], as is observed for the AgNP and $AgNO_3$ in vivo exposed earthworms (Table 2). However, as these factors are one of many mechanisms that may affect reproduction and growth, more research is required to fully elucidate the mode of action.

CONCLUSIONS

The present study demonstrates that AgNP exposure may impact earthworm populations by affecting growth, reproduction, juvenile survival, tissue integrity, and immune cell viability

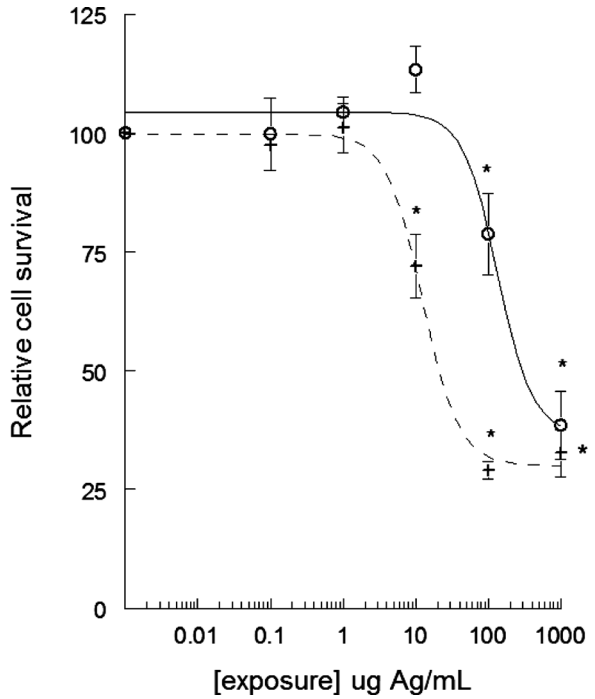

Figure 3. Viability of the coelomocytes exposed to increasing concentrations of silver nanoparticles (AgNP; black open circle) or silver nitrate ($AgNO_3$; grey cross). Values are expressed compared with the control (= 100%) and are shown in mean ± standard error of the mean ($N = 3$). An asterisk sign (*) indicates a statistically significant difference compared with the control. The regression curves for AgNP (continuous black line) and $AgNO_3$ (dashed grey line) exposure are displayed.

of the earthworms. Chronic effects of AgNP exposure are readily observed at 1.5 mg Ag/kg soil; hence, the no observed adverse effect level is below that concentration. It is also shown that dermal uptake of AgNP occurs and that uptake of AgNP through the gut is likely. Expected environmental concentrations of AgNP in soil are modeled at the ng/Ag/kg level [38], but deposited AgNP may accumulate in the top layer of the soil, as is also demonstrated for more conventional contaminants, such as zinc, lead, and polycyclic aromatic hydrocarbons [39,40]. In this way, organisms living in the upper soil layer, including *L. rubellus* earthworms, may be at risk of chronic exposure to Ag.

SUPPLEMENTAL DATA

Figures S1–S6. (675 KB DOCX).
Table S1. (15 KB XLS).
Table S2. (16 KB XLS).

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