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**Author(s):** Rajala, Juho; Vehniäinen, Eeva-Riikka; Väisänen, Ari; Kukkonen, Jussi

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Dissolved Silver and Promoted by Low Sediment pH

Juho E. Rajala, Eeva-Riikka Vehniäinen, Ari Väisänen, and Jussi V.K. Kukkonen

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Low sediment pH promotes toxicity of AgNP

Toxicity of Silver Nanoparticles to *Lumbriculus variegatus* is a Function of Dissolved Silver and

Promoted by Low Sediment pH

Juho E. Rajala,<sup>a,\*</sup> Eeva-Riikka Vehniäinen,<sup>a</sup> Ari Väisänen,<sup>b</sup> and Jussi V.K. Kukkonen<sup>a</sup>

<sup>a</sup>Department of Biological and Environmental Science, University of Jyväskylä, Jyväskylä,

Finland

<sup>b</sup>Department of Chemistry, University of Jyväskylä, Jyväskylä, Finland

\* Address correspondence to jurajala1987@gmail.com

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**Abstract:** Toxicity of silver nanoparticles (AgNP) to benthic organisms is a major concern. The use of AgNP in industry and consumer products leads to increasing release of AgNP into the aquatic environment, sediments being the major sink. Effects of sediment pH on the toxicity of AgNP to benthic oligochaeta *Lumbriculus variegatus* was studied in a 23 day toxicity test. Artificially prepared sediments (pH 5 and pH 7) were spiked with varying concentrations of uncoated AgNP, polyvinylpyrrolidone-coated AgNP, and silver nitrate as dissolved Ag reference. Number of individuals and biomass change were used as endpoints for the toxicity. The toxic effects were related to the bioaccessible concentration of dissolved Ag in the sediments, assessed with a two-step extraction procedure. The toxicity of two AgNP was similar and greatly enhanced in the acidic sediment. As the toxic effects were well related to the bioaccessible concentration of dissolved Ag in the sediments, the toxicity of sediment-associated AgNP to *L. variegatus* is suggested to be a function of dissolved Ag rather than result from nanoparticle specific modes of toxicity. This article is protected by copyright. All rights reserved

**Keywords:** Sediment toxicity, Benthic worms, Nanoecotoxicology, Nanoparticles, Silver nanoparticles, Sequential extraction

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## INTRODUCTION

The use of nanoparticles in the industry and consumer products is annually increasing, silver nanoparticles (AgNP) having the widest variety of applications within a number of products (Vance et al. 2015). In the European Union, one estimate of the annual production of AgNP is 32 t, of which 2.09 t is predicted to enter the aquatic environment (Sun et al. 2014). Heteroaggregation of AgNP with suspended solids is the primary mechanisms leading to precipitation of AgNP into sediment that is believed to be the final sink for AgNP (Velzeboer et al. 2014).

AgNP are extremely toxic to many benthic organisms (Khan et al. 2015; Park et al. 2015). Most of the toxicity studies have been conducted using waterborne exposure, not considering the natural environment of the benthic species. The capacity of sediment to mitigate the AgNP-derived toxicity is reported to be high, thus the risk to the benthic ecosystem may be overestimated if only waterborne exposures are used (Park et al. 2015; Lee et al. 2016; Rajala et al. 2016). With current knowledge, the environmental risk of AgNP in sediments cannot be modelled due to the lack of sufficient toxicity data (Coll et al. 2016). This emphasizes the need of sediment studies where the organisms are exposed to AgNP in an environmentally realistic way.

The toxicity of AgNP is often linked to the Ag ions ( $\text{Ag}^+$ ), which are highly toxic to aquatic organisms (Notter et al. 2014). The oxidation of AgNP leads to the release of  $\text{Ag}^+$  (Loza et al. 2014). The process is referred to as dissolution and is affected by environmental factors, such as temperature, pH, ionic strength and the concentration of complexing ligands, e.g. chloride and sulfide anions, divalent cations and dissolved or suspended solids (Liu and Hurt 2010; Levard et al. 2011, 2013; Peretyazhko et al. 2014; Metreveli et al. 2016; Zhang et al.

2016). Also aggregation status, size, morphology and surface chemistry of AgNP affect the dissolution rate, which altogether is very complex to predict (Liu et al. 2010; Li et al. 2011; Li and Lenhart 2012; Zhang et al. 2016). AgNP can also pose nanoparticle-specific toxicity in addition to or over the effects of dissolved Ag species (Fabrega et al. 2009; Chan and Chiu 2015; Cozzari et al. 2015; Park et al. 2015). One suggested mechanism is endocytic uptake of AgNP leading to internal release of Ag<sup>+</sup>, so called “Trojan horse” -type of behavior (Garcia-Alonso et al. 2011). Also activation of oxidative stress response by AgNP has been found to differ from that caused by Ag<sup>+</sup>, indicating a different toxic pathway (Cozzari et al. 2015).

The effect of sediment pH on the toxicity of AgNP has not been thoroughly studied. In our earlier experiment, AgNP were observed to be toxic to benthic oligochaeta *Lumbriculus variegatus* only in the acidic sediment (pH 5.1) (Rajala et al. 2016). The low pH is known to increase the dissolution of AgNP in studies done in water and this can potentially be true also in sediment (Liu and Hurt 2010; Peretyazhko et al. 2014). A higher concentration of Ag<sup>+</sup> together with increased mobility of Ag<sup>+</sup> in the acidic environmental media predicts also increased toxicity (Cornelis et al. 2014; Wang et al. 2015). However, in complex environmental media the dissolution of AgNP is suggested to be independent of the pH, possibly due to the increased coagulation, leading to decreased surface area of the particles (Fabrega et al. 2009; Cornelis et al. 2012). In contrast to Ag<sup>+</sup>, the mobility of AgNP is expected to decrease in low pH due to the negative surface charge of AgNP (Cornelis et al. 2014; Wang et al. 2015). If AgNP pose nanoparticle-specific toxicity, the toxic effects are expected to be more pronounced in more alkaline sediments, where the mobility of AgNP is higher compared to acidic conditions.

In the present study, the effect of sediment pH on the toxicity of sediment-associated AgNP to *L. variegatus* was examined. The hypothesis was that the low pH of the sediment would

increase the dissolution of AgNP, leading to a higher bioaccessible concentration of dissolved Ag, and thus increase toxicity to *L. variegatus*. To test the hypothesis, varying concentrations of uncoated AgNP (uc-AgNP) and polyvinylpyrrolidone-coated AgNP (PVP-AgNP) were spiked to artificial sediments, with pH adjusted to 5 and 7. Silver nitrate (AgNO<sub>3</sub>) was used as a reference compound for the toxicity of Ag<sup>+</sup>. The toxicity was observed as a decrease of the biomass and the number of individuals compared to the control, and further related to the bioaccessible concentration of dissolved Ag in the sediment evaluated by a two-step extraction procedure. In addition, the critical body burden (CBB) of Ag in *L. variegatus* was estimated for each Ag form.

## **MATERIAL & METHODS**

### *Silver nanoparticles*

Two types of AgNP were obtained from Nanostructured and Amorphous materials Inc. (Nanoamor) as dry powder. The PVP-AgNP were reported to measure from 30 to 50 nm in diameter, to have 99.9 % purity and to contain 0.2 % of polyvinylpyrrolidone. The size distribution of 205 particles was measured by transmission electron microscopy and the diameter varied from 7.5 to 74.8 nm resulting in a mean of 28.6 nm (standard deviation, SD, ± 5.8) (Rajala et al. 2017). Reported diameter of uc-AgNP was 35 nm and the purity 99.5 %. The size distribution of 344 uc-AgNP was measured and the diameter varied from 11.9 to 48.8 nm, resulting in a mean of 39.4 nm (SD ± 12.6) (Rajala et al. 2017). Both AgNP formed large agglomerates when introduced to water but further characterization of AgNP in water suspension was not considered to be relevant, as AgNP were spiked directly to the sediment (Rajala et al. 2017).

### *Test organisms*

*Lumbriculus variegatus* oligochaetes were cultured at the University of Jyväskylä in 1.0 mM L<sup>-1</sup> (as Ca + Mg) artificial fresh water (AFW). Culture aquaria were constantly aerated. The temperature of the culture room was 20 °C (± 1 °C) and the light regime adjusted to 16 h light and 8 h dark. Cellulose paper tissues were used as a substrate for the worms and ground tetra fish food was added once a week (Tetrawerke). Worms were synchronized by cutting them in half 13 days before the start of the experiment, and only the posterior parts of the worms were selected for use to ensure the similar reproductive states of the test organisms.

### *Sediments*

The test sediments were prepared as recommended in the OECD guideline 225 (OECD 2007). Final constituents were 75 % of combusted quartz sand (58 % grain size from 100 to 200 µm, 42 % grain size from 700 to 1200 µm), 5 % of finely ground (< 500 µm) sphagnum peat (Kekkilä) and 20 % of kaolin (Sigma-Aldrich). The prepared sediment was divided into two containers and the pH was adjusted to 5 and 7 by adding 0.02 % (of total dry weight, dw) calcium carbonate to pH 5 sediment (S5) and 0.17 % to pH 7 sediment (S7) and let to equilibrate in dark at 20 °C temperature for 14 days. The pH -values were selected based on our previous studies with natural sediments collected from boreal lakes and are thus considered to be environmentally relevant (Rajala et al. 2016, 2017). *Urtica dioica* powder (0.4 % of total dw) was added as a food source prior to the experiment. For S7, the final characteristics were; pH = 6.56 (SD ± 0.28), dw = 63.2 % (SD ± 0.5, n = 4), loss of ignition = 4.84 % (SD ± 0.02, n = 4) and estimated organic carbon content = 1.24 % (SD ± 0.02, n = 4), and for S5; pH = 4.63 (SD ± 0.08), dw = 63.5 % (SD ± 0.7, n = 4), loss of ignition = 4.74 % (SD ± 0.03, n = 4) and estimated organic carbon content = 1.18 % (SD ± 0.02, n = 4).



The test sediments S5 and S7 were spiked with uc-AgNP, PVP-AgNP (S5: 10 to 1000 mg kg<sup>-1</sup> dw, S7: 50 to 1400 mg kg<sup>-1</sup> dw) and AgNO<sub>3</sub> (5 to 300 mg kg<sup>-1</sup> dw). Dry AgNP powder was weighed and transferred in ultrapure water (conductivity 0.056 μS cm<sup>-1</sup>, resistivity 18.2 MΩ cm) in a volume of 1 mL, sonicated for 10 min (120 W, 50 kHz, Bransonic B-220) to break down the largest agglomerates and then mixed into the sediment with a metal spoon. Stock solution of AgNO<sub>3</sub> (analytical grade, supplied by J.T. Baker) was prepared in ultrapure water (50 mg mL<sup>-1</sup>), and suitable volume of stock solution was mixed into the sediments with a metal spoon to obtain the desired concentrations of Ag. The spiked sediments were rotated for 48 h in an overhead mixer (12 rpm) to ensure homogenous distribution of Ag. The exact concentrations (Supporting info, Table S1) and the details of the preliminary test used in the selection of the concentration range can be found in the supporting information.

#### *Toxicity test*

The toxicity test was conducted according to the OECD guideline 225 in low form 250 mL glass beakers, using 4 replicates per treatment (OEC 2007). Separate control groups were used for each Ag compound in both sediments ( $n = 3$ ). The amount of sediment in each unit was 60 g (wet weight), topped with 230 mL of AFW. The test units were constantly aerated and left to settle for 24 h before introducing 10 synchronized *L. variegatus* worms. The temperature of the test room was 20 °C ( $\pm 1$  °C) and the light regime adjusted to 16 h light and 8 h dark. The duration of the test was shortened from 28 to 23 days according to the preliminary test (supporting info, Figure S1). The pH and the ammonium concentration were measured weekly from the overlying water. At the end of exposure period, worms were sieved out of the sediment, counted and transferred to clean AFW with tetramin food to empty their gut for 6 h. The dw was measured after drying the worms overnight at 105 °C. The dried worms were stored in dark at 4

°C temperature prior to the Ag analysis. The decrease of total biomass and the number of worms compared to the control groups were used as endpoints for the toxicity. The effect concentrations where the response was 50 % of the control (EC50) were calculated using log-logistic 3 parameter model and compared with z-test ( $p = 0.05$ ) in R version 3.0.1 (R foundation for Statistical Computing), using the *drc* package (Supporting info, Table S5, Table S6).<sup>29</sup> The no observed effect concentrations (NOEC) and lowest observed effect concentrations (LOEC) were estimated by stepwise step-down Jonckheere-Terpstra test using IBM SPSS Statistics for Windows, version 24 (IBM Corp).

#### *Sediment extractions*

The bioaccessibility of dissolved Ag in the test sediments was evaluated by a two-step extraction procedure. The amount of AgNP in the extracted samples was experimentally and theoretically proven to be low and Ag in the samples is considered to be in form of dissolved Ag complexes (Supporting info). At the first step, a 2.5 g wet sediment sample was extracted with 1.5 mL of ultrapure water. The sample was shaken and centrifuged for 15 min at  $6450 \times g$  (Sanyo MSE, Harrier 18/80R). The supernatant was removed and the residue washed again with 1.5 mL of ultrapure water. Both supernatants were combined as one sample, filtered (Whatman No. 42) and acidified with 0.1 mL of 65 %  $\text{HNO}_3$  (Merck kGaA) and diluted to a volume of 5 mL (fraction 1). The residue was further extracted with 2 mL of 1 M ammonium nitrate ( $\text{NH}_4\text{NO}_3$ , Merck kGaA) for  $2 \times 2.5$  min under sonication (120 W, 50 kHz, Bransonic B-220). The sample was shaken between the sonication steps and centrifuged for 15 min at  $6450 \times g$  after the sonication. Supernatant was removed and the residue washed with 1.5 mL of ultrapure water. Both supernatants were again combined, filtered, acidified and diluted to a volume of 5 mL (fraction 2). The extractions were done in 3 replicates, and all the samples were analyzed for Ag

by inductively coupled plasma optical emission spectrometer (ICP-OES, Perkin Elmer model Optima 8300). Level of detection and level of quantification values were calculated from the parameters of the calibration line ( $y = bx + a$ , where  $b$  is the slope of the line and  $a$  is the intercept) by substituting  $a + 3sa$  or  $a + 10 sa$  ( $sa =$  standard deviation of  $a$ ) respectively into the calibration line. Accepted relative SD of 3 replicate measurements was less than 10 % and the level of detection was  $1.9 \mu\text{g L}^{-1}$ . Samples that did not meet these criteria, were removed from the data set. Reagent blank samples were analyzed between the actual samples. Fraction 1 corresponds to the readily water-soluble Ag and fraction 2 to the ion-exchangeable concentration of dissolved Ag. The sum of fraction 1 and 2 is later referred as total bioaccessible Ag.

#### *Modelling the bioaccessibility of Ag*

The water-soluble, ion-exchangeable and the total bioaccessible concentration of dissolved Ag were modelled using the Freundlich equation:  $C_e = (C_s / K_f)^n$ , where  $C_e =$  concentration in the extractant,  $C_s =$  concentration in the sediment,  $K_f =$  Freundlich capacity factor and  $n =$  Freundlich slope. For the comparisons among the treatments, the concentration of extracted Ag was modelled in 10, 20, 40, 80  $\text{mg kg}^{-1}$  dw (Supporting info, Table S2, Table S3).

#### *Total Ag concentration in the sediment*

For the analysis of the total Ag concentrations, a 250 mg (dw) sediment sample was digested in 5 mL of 1:3 (v:v)  $\text{HNO}_3$ :HCl solution in three replicates prior the experiment. The samples were sonicated (650 W, 35 kHz, ELMA Transsonic T820/H) at  $60^\circ\text{C}$  temperature for 3 x 3 min and shaken between every 3 min step. The filtered samples (Whatman No. 42) were diluted into a volume of 25 mL and analyzed for Ag by ICP-OES. All the reagents used were analytical grade and supplied by Merck kGaA. Measured total Ag concentrations are given in the supporting info (Table S1).

### *Silver concentration in the overlying water*

Overlying water from the test units was analyzed for Ag by ICP-OES after the 23 days toxicity test ( $n = 4$ ). Unfiltered water samples (10 mL) were acidified with 100  $\mu\text{l}$  of  $\text{HNO}_3$ , and stored in the dark at 4  $^\circ\text{C}$  temperature prior the analysis.

### *The body burden of Ag in L. variegatus*

The total body burdens (TBB) of Ag were obtained by digesting the worms in a similar way as the sediment samples. Due to the small biomass, only 1 mL of acid was used and the dilution was done in 5 mL. All the worms in each test unit were digested together. If the dry biomass in one unit was under 1 mg, two replicates were pooled together for more precision. The concentration of Ag in the unfiltered samples was analyzed by ICP-OES. The obtained TBB ( $\mu\text{g g}^{-1}$  of dry tissue) for each concentration was plotted against the biomass of the worms, and log-logistic three parameter model was used to model the critical body burdens of Ag that led to the biomass of the worms being 50 % smaller compared to the control treatments (CBB50,  $\mu\text{g g}^{-1}$  dry tissue) (Supporting info, Figure S3). The CBB50 were calculated and compared with z-test ( $p = 0.05$ ) in R version 3.0.1 (R foundation for Statistical Computing), using the *drc* package (Ritz et al. 2015) (Supporting info, Table S7).

## **RESULTS**

### *The bioaccessibility of Ag*

The proportion of water-soluble Ag in S5 was highest when spiked as  $\text{AgNO}_3$ , followed by uc-AgNP, PVP-AgNP and  $\text{AgNO}_3$  in S7 (Figure 1A). The water-soluble concentration of Ag was estimated to be 8.2 ( $\text{SD} \pm 0.5$ ) times higher for  $\text{AgNO}_3$  in S5 than in S7 and 1.6 ( $\text{SD} \pm 0.02$ ) times higher for uc-AgNP than PVP-AgNP (Supporting info, Table S3). In AgNP spiked S7 the

concentration of water-soluble Ag was low and only few samples contained measurable concentrations of Ag (Table 1).

The proportion of ion-exchangeable Ag was overlapping in the low total Ag concentration among AgNO<sub>3</sub>, uc-AgNP and PVP-AgNP in the S5 sediment but a lower concentration of dissolved Ag was extracted in the higher total Ag concentrations with AgNP compared to AgNO<sub>3</sub> (Figure 1B). In contrast to water-soluble Ag, on average 1.6 (SD ± 0.2) times more Ag was extracted from ion-exchangeable fraction with PVP-AgNP than uc-AgNP (Supporting info, Table S3). The concentration of ion-exchangeable Ag in the S7 was low in every treatment, especially with AgNP, as only few samples contained measurable concentrations of Ag (Table 1).

The proportion of bioaccessible concentration of Ag was highest in AgNO<sub>3</sub>-spiked S5, followed by PVP-AgNP and uc-AgNP in S5 (Figure 1C). Total bioaccessibility of uc-AgNP and PVP-AgNP followed a similar trend, PVP-AgNP resulting in on average 1.3 (SD ± 0.1) times more bioaccessible Ag than uc-AgNP (Supporting info, Table S3). The model predicts that at low concentrations, the bioaccessibility of AgNO<sub>3</sub>, uc-AgNP and PVP-AgNP in S5 overlap, but in higher doses AgNO<sub>3</sub> generates more bioaccessible Ag (Figure 1C). In the S7 sediment the total bioaccessibility of Ag was highest with AgNO<sub>3</sub> and only low concentrations of bioaccessible Ag were observed in AgNP spiked S7 sediment (Table 1).

#### *Concentration of Ag in the overlying water*

The Ag concentration in the overlying water was higher with S5 sediment than S7 sediment, when spiked as AgNO<sub>3</sub> and uc-AgNP (Figure 2AB). Contradicting results were obtained with the PVP-AgNP-spiked sediments, where more Ag was measured from the overlying water with S7 sediment than with S5 sediment (Figure 2C). The sample preparation

did not include any filtration or centrifugation, and the results are given as total Ag concentration in the water, including also AgNP.

#### *Toxicity test*

The total dry biomass of the worms in the control units increased from 4.6 mg ( $\pm 0.4$ ,  $n = 12$ ) to 14.8 mg ( $\pm 2.3$ ,  $n = 8$ ) in the S7 and from 4.1 mg ( $\pm 0.6$ ,  $n = 12$ ) to 10.8 mg ( $\pm 1.0$ ,  $n = 9$ ) (Figure 3A) in the S5 and the number of individuals from 10 to 16.6 ( $\pm 4.8$ ,  $n = 8$ ) in the S7 and to 14.8 ( $\pm 3.5$ ,  $n = 9$ ) (Figure 3B) in the S5. The concentration of ammonia stayed below toxic levels in both sediments (Supporting info, Table S4). The pH of the S5 was lower than the recommended 6 (OECD 2007) but the increasing biomass and reproduction illustrate the suitability of S5 sediment for *L. variegatus* (Supporting info, Table S4).

*Toxicity related to the total Ag concentration.* In the S5 sediment, the EC50 for the number of individuals were lower for AgNP than AgNO<sub>3</sub> but the differences were not significant ( $p = 0.085$  and  $0.25$ ) (Table 2). Also the EC50 for AgNO<sub>3</sub> was similar between the S5 and S7 sediment ( $p = 0.99$ ) and between uc-AgNP and PVP-AgNP in S5 ( $p = 0.25$ ) (Table 2). In S7, exposure to AgNP did not affect the number of individuals (Table 3). The comparisons of EC50 values were affected by the unexpected behaviour of the test organisms in the AgNO<sub>3</sub> spiked S5. Worms curled into tight balls on the sediment surface in the high Ag concentrations and some of the individuals survived through the 23-day test period. In the other treatments similar behaviour was not observed and small individuals were not found or present. The biomass of the survived individuals in S5 was very small, and they would have not been expected to survive if a longer exposure time was used (Figure 3A). Thus the decrease of the biomass was considered to be a more reliable endpoint for toxicity.

Toxicity observed as loss of biomass was decreasing among the treatments in the following order:  $\text{AgNO}_3$  (S5) >  $\text{AgNP}$  (S5) >  $\text{AgNO}_3$  (S7) >  $\text{AgNP}$  (S7). In the S5 sediment, the EC50 for the biomass decrease were approximately 2 times higher for  $\text{AgNP}$  than for  $\text{AgNO}_3$  ( $p < 0.001$  and  $0.004$ ) (Table 2). Low sediment pH significantly promoted the toxicity of  $\text{AgNO}_3$  and EC50 was approximately 6 times higher in S5 than in S7 ( $p < 0.001$ ) (Table 2). No difference in EC50 was observed between uc- $\text{AgNP}$  and PVP- $\text{AgNP}$  in S5 ( $p = 0.44$ ) (Table 2). In the S7, only the highest concentration of uc- $\text{AgNP}$  ( $1360 \text{ mg kg}^{-1} \text{ dw}$ ) affected the biomass of the test organisms and no effects were seen with PVP- $\text{AgNP}$  (Table 3).

*Toxicity related to the water-soluble and bioaccessible Ag concentration.* To evaluate if the toxicity of  $\text{AgNP}$  can be explained by the dissolved Ag concentration in the sediment, the toxic responses were also plotted against the water-soluble and bioaccessible Ag (Supporting information, Figure S2). The unexpected behavior of the test organisms in the  $\text{AgNO}_3$  spiked S5 resulted in higher EC50 values for the number of individuals than other treatments ( $p = 0.002$ – $0.004$ ). The EC50 for  $\text{AgNO}_3$  in S7 was estimated to be similar to that for uc- $\text{AgNP}$  in S5 (water-soluble) and similar to that for PVP- $\text{AgNP}$  in S5 (bioaccessible). No differences in EC50 between uc- $\text{AgNP}$  and PVP- $\text{AgNP}$  in S5 were observed ( $p = 0.29$  and  $0.84$ ).

The EC50 for the biomass decrease calculated using the concentration of water-soluble Ag were similar among Ag compounds in S5 ( $p = 0.38$  to  $0.92$ ) (Table 2). For  $\text{AgNO}_3$  in S7, the EC50 was approximately 2 times higher than with  $\text{AgNO}_3$  or  $\text{AgNP}$  in S5 (Table 2). The bioaccessible concentration of dissolved Ag seemed to explain the toxicity of Ag compounds, as no difference in the EC50 values among  $\text{AgNO}_3$  (S5, S7) and  $\text{AgNP}$  (S5) was observed ( $p = 0.053$ – $0.90$ ) (Table 2). Also the LOEC for uc- $\text{AgNP}$  in S7 compares well with other treatments

(Table 3). The concentration of water-soluble and bioaccessible Ag in PVP-AgNP spiked S7 was too low to pose any toxicity to *L. variegatus* (Table 3).

#### *The total body burden of Ag*

The TBB of Ag was steeply increasing as a function of increasing exposure concentration, intensified with the decreasing biomass of the worms (Figure 3AC). The log-logistic modelled CBB50 (95 % confidence intervals) were  $83.7 \mu\text{g g}^{-1}$  (58.2 to  $109 \mu\text{g g}^{-1}$ ) for AgNO<sub>3</sub> in S7 and  $133.2 \mu\text{g g}^{-1}$  (69.0 to  $197 \mu\text{g g}^{-1}$ ),  $98.9 \mu\text{g g}^{-1}$  (38.3 to  $160 \mu\text{g g}^{-1}$ ) and  $94.7 \mu\text{g g}^{-1}$  (2.28 to  $187 \mu\text{g g}^{-1}$ ) for AgNO<sub>3</sub>, uc-AgNP and PVP-AgNP, respectively, in S5 (Supporting info, Figure S3A). No differences among the CBB50 were observed ( $p = 0.15$  to  $0.94$ ). In S7 sediment, the TBB of Ag spiked as AgNP did not correlate with the total biomass of the worms, although the TBB were higher than the modelled CBB50 in other treatments (Supporting info, Figure S3B).

## **DISCUSSION**

In the present study the toxicity of uc-AgNP and PVP-AgNP was similar and greatly increased by the low sediment pH. Toxicity was well related to the total bioaccessible concentration of dissolved Ag, estimated by the extraction procedure. Therefore, we accept our study hypothesis and suggest that the toxicity of sediment-associated AgNP to *L. variegatus* is a function of dissolved Ag and promoted by the low pH of the sediment.

#### *The bioaccessibility of Ag*

The bioaccessibility of Ag in the acidic S5 was higher than in S7 with all tested Ag forms. In low pH, the sorption sites for Ag<sup>+</sup> are more limited as there is excess of H<sup>+</sup> ions competing on the sorption making Ag<sup>+</sup> more bioaccessible (Jacobson et al. 2005; Oromieh 2011). This was seen as 8.2 times increased water-soluble Ag concentration in S5 compared to



S7 when spiked as AgNO<sub>3</sub>. With AgNP the difference was even more pronounced, as the modelled concentrations suggest 39 to 51 times more water-soluble Ag with uc-AgNP and 10 to 25 times more with PVP-AgNP in S5 than in S7 (supporting data, Table S3). Comparisons are rough estimates, as the water-soluble Ag concentration in S7 was low with AgNP. However, the estimates suggest slower overall concentration of dissolved Ag, and thus a lower dissolution rate of AgNP in S7 compared to more acidic S5. In our earlier study using natural sediment, the dissolution rate of uc-AgNP and PVP-AgNP was independent of sediment pH (Rajala et al. 2017). Also the dissolution of AgNP in soil porewater was reported to be independent of pH, possibly due to the higher coagulation of AgNP (Fabrega et al. 2009; Cornelis et al. 2012). Increased dissolution of AgNP in low pH observed in the present study is supported by the research done in water media (Liu and Hurt 2010; Peretyazhko et al. 2014) and by the soil study conducted by Hedbeg et al. 2015. Due to the varying results reported in the literature, it seems evident that the effect of pH on the dissolution rate of AgNP can be suppressed by other media properties, and site-specific effects should be always considered when evaluating the environmental behaviour of AgNP.

The difference in the concentration of ion-exchangeable and bioaccessible dissolved Ag between the sediments was larger than in the water-soluble fraction. Interestingly in the low total Ag concentrations, the bioaccessible and ion-exchangeable concentrations among Ag compounds were overlapping in S5. In the higher concentrations, relatively less Ag was extracted from the ion-exchangeable fraction with AgNP, possibly due to the decreased dissolution rate of AgNP in higher total sediment Ag concentration (Loza et al. 2014). If the released Ag<sup>+</sup> from AgNP is assumed to behave in a similar way as AgNO<sub>3</sub> originated Ag<sup>+</sup>, overlapping concentration in low total Ag concentrations suggests that all AgNP had dissolved in S5. This is unlikely, as we

observed difference in the water-soluble fraction and in the toxicity between AgNO<sub>3</sub> and AgNP.

Thus the partitioning of dissolved Ag originating from AgNP is considered to be different from AgNO<sub>3</sub>. Similar observation has also been made in the soil studies (Coutris et al 2012b, Benoit et al. 2013). One possible explanation for the relatively higher concentrations of Ag in the ion-exchangeable fraction is binding of Ag<sup>+</sup> on the surface of AgNP (Liu and Hurt 2010; Liu et al. 2010; Klitzje et al. 2014). Sediment properties and AgNP surface chemistry is suggested to influence the affinity of Ag<sup>+</sup> to AgNP surface (Rajala et al. 2017). In the present study, the ion-exchangeable concentration of dissolved Ag was very similar between uc-AgNP and PVP-AgNP. Thus the PVP-coating did not seem to have an influence on the proposed surface binding of Ag<sup>+</sup>. It is possible, that after the 23-day exposure in the sediment, the surface chemistry of the two AgNP was quite similar, due to the tendency of NOM to interact with AgNP, coating the uc-AgNP and replacing the coating on PVP-AgNP (Yang et al. 2014; Seitz et al 2015).

#### *Concentration of Ag in the overlying water*

Despite the lower bioaccessibility of AgNP in S7 compared to S5, overlying water of the test units contained relatively high concentrations of Ag. This can be explained by the sample treatment. The water samples were not centrifuged before the analysis, and also nanoparticulate Ag, which was removed by the centrifugal force from the sediment samples, was analyzed. The presence of AgNP in the samples was not experimentally verified but because the bioaccessibility of Ag<sup>+</sup> was low in S7, majority of Ag measured from the overlying water is expected to be in the form of AgNP. This indicates that the mobility of PVP-AgNP was higher than the mobility of uc-AgNP. Results are in line with the finding that the sterically stabilized PVP-coated particles are mobile and resistant to agglomeration under environmental conditions (Thio et al. 2011; Topuz et al. 2015).

## Toxicity of $\text{AgNO}_3$ and AgNP

As predicted by the higher bioaccessibility of dissolved Ag, the toxicity of all Ag compounds was promoted by the low sediment pH,  $\text{AgNO}_3$  was more toxic than AgNP, and the PVP-coating did not affect the toxicity of AgNP. The effect of pH was more pronounced with AgNP than  $\text{AgNO}_3$ . This can be explained by a two-step process. Firstly, the dissolution of AgNP was promoted by the low pH and secondly, the bioaccessibility of dissolved  $\text{Ag}^+$  was increased. The suggested higher dissolution of AgNP in S5 based on the comparisons of water-soluble concentrations of Ag in sediments is also supported by the toxicity test. When the LOEC of AgNP, calculated by using the total Ag concentrations in the sediment, are compared to LOEC of  $\text{AgNO}_3$ , the ratio in S7 is multiple times higher than in S5.

When the toxic responses were related to the bioaccessible concentrations of dissolved Ag, no differences in the EC50 (biomass decrease) were observed among Ag compounds in S5 and  $\text{AgNO}_3$  in S7. This indicates that the toxicity of AgNP to *L. variegatus* is a function of dissolved Ag. In S7, EC50 values could not be modelled for AgNP due to the low toxicity. However, LOEC values for uc-AgNP (S7) indicate, that the toxic response is somewhat related to the dissolved Ag also in S7. Higher total Ag concentrations for AgNP in S7 should have been used to confirm this result, but as the concentration is already unrealistically high on the environmental point of view, this was not considered relevant. We cannot out rule the possibility of nanoparticle-specific toxicity observed in earlier studies, if more sensitive endpoints were used (Chan and Chiu 2015; Cozzari et al. 2015; Park et al. 2015). However, the environmental relevance of the possible nanoparticle-specific toxicity is considered low, as *L. variegatus* tolerated extreme concentrations of AgNP in S7. According to the present study, the acute

toxicity of AgNP to *L. variegatus* in sediment exposures is not expected to be higher than the toxicity of Ag<sup>+</sup>.

#### *The critical body burdens of Ag*

The hypothesis of Ag<sup>+</sup> driven toxicity and similar dissolution rate of uc-AgNP and PVP-AgNP were also supported by the residual concentration of Ag in the test organisms. In the S5, calculated CBB50 for the decrease of the biomass were similar among the Ag compounds. This indicates that Ag was mostly taken up as dissolved Ag in all treatments. In the S7, the TBB-values for AgNP were higher than modelled CBB50 values for AgNO<sub>3</sub>, which suggests that organisms contained Ag that is not as toxic as dissolved Ag. The extraction procedure for the TBB did not separate between nanoparticulate and dissolved Ag, and it is likely that most of the measured Ag was in the form of AgNP. In soil studies the bioaccumulation factors for AgNP in earthworms (*E. fetida*) has been reported low, and the observed accumulation of Ag has been explained by incomplete gut clearance before the Ag analysis (Shoults-Wilson et al. 2011; Coutris et al. 2012a). This may explain the unexpectedly high TBB in S7, as already a small amount of retained sediment may lead to significantly increased TBB in high total concentration of Ag. In S5, the bioaccumulation or incomplete gut clearance of AgNP may also overestimate the CBB50, but the effect is considered to be insignificant as the toxicity was already seen at relatively low AgNP concentrations. In conclusion, dissolved Ag seems to mediate the toxicity of sediment-associated AgNP to *L. variegatus*. However, it is possible that also AgNP are taken up by the organisms, and the possibility of chronic toxicity due to the uptake of AgNP should be considered in further studies.

*Supplemental Data*—The Supplemental Data are available on the Wiley Online Library at DOI: 10.1002/etc.xxxx.

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*Data Availability*—All data can be accessed by contacting the corresponding author, Juho Rajala (jurajala1987@gmail.com)

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Graphical abstract. Toxicity of silver nanoparticles to benthic oligochaete *Lumbriculus variegatus* was found to be higher in the more acidic sediment. Toxicity was explained by increased dissolution of silver nanoparticles in the acidic test sediment.

Figure 1. Concentration ( $\text{mg kg}^{-1} \text{ dw}$ ) of A) water-soluble Ag, B) ion-exchangeable Ag and C) the total bioaccessible Ag plotted against the total concentration of Ag in artificial sediment pH 7 (S7) and artificial sediment pH 5 spiked with silver nitrate ( $\text{AgNO}_3$ ), uncoated silver nanoparticles (uc-AgNP) and polyvinylpyrrolidone-coated silver nanoparticles (PVP-AgNP). The lines are modelled using Freundlich equation.

Figure 2. The concentration of Ag ( $\mu\text{g L}^{-1}$ ) in the overlying water of the test set-up at different Ag concentrations ( $\text{mg kg}^{-1} \text{ dw}$ ) spiked as A) silver nitrate ( $\text{AgNO}_3$ ), B) uncoated silver nanoparticle (uc-AgNP) and C) polyvinylpyrrolidone-coated silver nanoparticles (PVP-AgNP) into the artificial sediment pH 7 (S7) and artificial sediment pH 5 (S5).

Figure 3. A) Total dry biomass of the *L. variegatus*, B) number of individuals and C) the total body burdens of Ag in the varying exposure concentration ( $\text{mg kg}^{-1} \text{ dw}$ ) of silver nitrate ( $\text{AgNO}_3$ ), uncoated silver nanoparticle (uc-AgNP) and polyvinylpyrrolidone-coated silver nanoparticles (PVP-AgNP) in the artificial sediment pH 7 (S7) and artificial sediment pH 5 (S5) after the 23 days toxicity test as a mean of 4 replicates ( $\pm \text{SD}$ ). The solid symbols and lines represent S5 sediment and open symbols with dashed lines S7 sediment.

**Table 1** Concentration of water soluble Ag, ion-exchangeable Ag, and total bioaccessible Ag in artificial sediment pH 7

| Treatment<br>(mg kg <sup>-1</sup> dw) | Ag concentration<br>(mg kg <sup>-1</sup> dw) |                    |        |
|---------------------------------------|--|--------------------|--------|
|                                       | F1   | F2                 | F1+2   |
| Uc-AgNP 760                           | 0.0467 <sup>a</sup>                          | 0.145 <sup>b</sup> | 0.191  |
| S7 1360 <sup>c</sup>                  | 0.0603 <sup>b</sup>                          | 0.372 <sup>b</sup> | 0.433  |
| PVP-AgNP 529                          | 0.0894 <sup>b</sup>                          | ND                 | 0.0894 |
| S7 1130 <sup>c</sup>                  | 0.0713 <sup>d</sup>                          | 0.219 <sup>b</sup> | 0.290  |

<sup>a</sup> SD = 0.0065, *n* = 3

<sup>b</sup> 2/3 replicates under the detection limit

<sup>c</sup> Data are from reference 27

<sup>d</sup> SD = 0.0006, *n* = 2

AgNO<sub>3</sub> = silver nitrate; uc-AgNP = uncoated silver nanoparticles; PVP-AgNP = polyvinylpyrrolidone-coated silver nanoparticles; S7 = artificial sediment pH 7; F1 = water-soluble; F2 = ion-exchangeable; F1+2 = bioaccessible

**Table 2.** Effective concentrations (with 95 % confidence intervals) where the response is 50 % smaller than in the control group for the number of individuals for the decrease of biomass

| Treatment         | EC50 for the number of individuals<br>(mg kg <sup>-1</sup> ) |                            |                            | EC50 for the biomass decrease<br>(mg kg <sup>-1</sup> ) |                            |                            |
|-------------------|--|----------------------------|----------------------------|---|----------------------------|----------------------------|
|                   | Total Ag <sup>a</sup>  | Bioaccessible <sup>b</sup> | Water-soluble <sup>c</sup> | Total Ag <sup>a</sup>                                   | Bioaccessible <sup>b</sup> | Water-soluble <sup>c</sup> |
| AgNO <sub>3</sub> | 44.1   | 2.17                       | 0.637                      | 9.58  | 0.136                      | 0.057                      |
| S5                | (20.9-67.4)  | (1.34-3.00)                | (0.267-1.01)               | (7.17-12.0)   | (0.029-0.240)              | (0.025-0.089)              |
| AgNO <sub>3</sub> | 44.4   | 0.100                      | 0.079                      | 56.7  | 0.128                      | 0.096                      |
| S7                | (34.7-54.0)  | (0.075-0.126)              | (0.057-0.100)              | (49.3-64.1)   | (0.110-0.145)              | (0.084-0.108)              |
| uc-AgNP           | 29.7   | 0.343                      | 0.064                      | 18.5  | 0.195                      | 0.043                      |
| S5                | (20.3-39.0)  | (0.191-0.495)              | (0.025-0.103)              | (13.7-23.4)   | (0.128-0.262)              | (0.041-0.045)              |
| PVP- AgNP         | 22.9   | 0.378                      | 0.043                      | 21.4  | 0.363                      | 0.043                      |
| S5                | (16.2-29.7)  | (0.055-0.701)              | (0.033-0.053)              | (16.6-26.2)   | (0.125-0.603)              | (0.035-0.052)              |

<sup>a</sup> Modelled using the total Ag concentration (mg kg<sup>-1</sup> dw).

<sup>b</sup> Modelled using the concentration of bioaccessible Ag (mg kg<sup>-1</sup> dw).

<sup>c</sup> Modelled using the concentration of water soluble Ag (mg kg<sup>-1</sup> dw).

EC50 = effective concentration, where the response is 50 % smaller than in the control group, S5

= artificial sediment pH 5, AgNO<sub>3</sub>

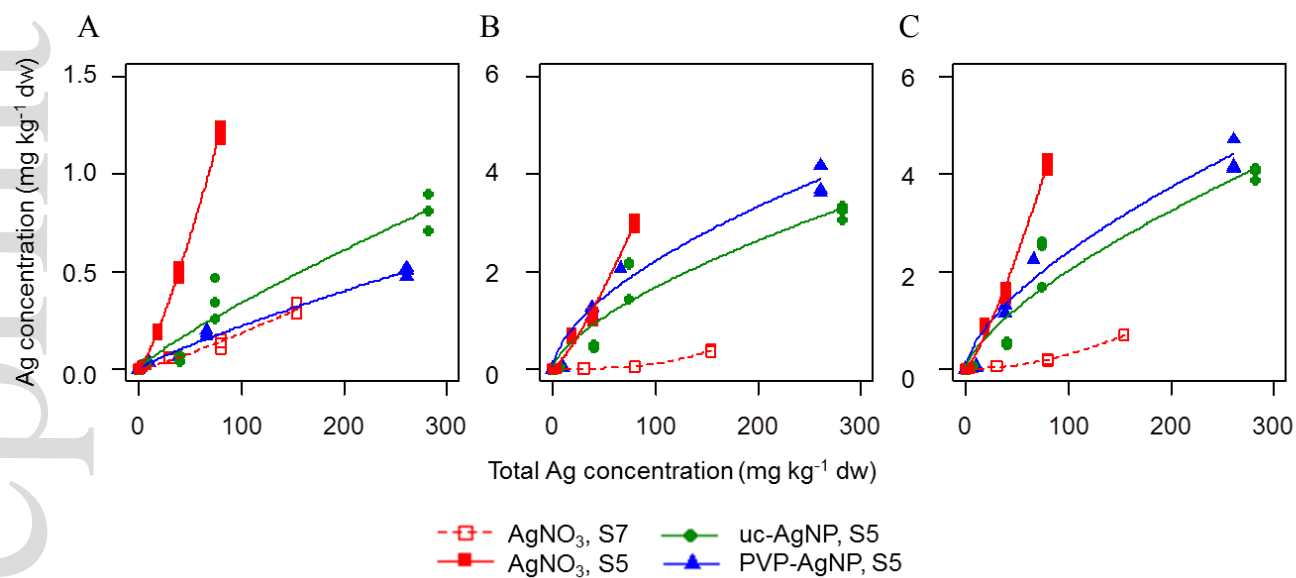
**Table 3.** No observed effect concentrations and lowest observed effect concentrations for the number of individuals and for the decrease of biomass

| Treatment         | Sediment | Total Ag <sup>a</sup> |                   | Bioaccessible <sup>b</sup> |                    | Water-soluble <sup>c</sup> |                    |
|-------------------|----------|-----------------------|-------------------|----------------------------|--------------------|----------------------------|--------------------|
|                   |          | NOEC                  | LOEC              | NOEC                       | LOEC               | NOEC                       | LOEC               |
| AgNO <sub>3</sub> | S5       | 3.67                  | 18.1              | 0.011                      | 0.856              | 0.009                      | 0.195              |
|                   | S7       | 29.8                  | 80.2              | 0.067                      | 0.182              | 0.056                      | 0.129              |
| uc-AgNP           | S5       | 6.99                  | 40.4              | 0.062                      | 0.509              | 0.037                      | 0.085              |
|                   | S7       | 760 <sup>d</sup>      | 1360 <sup>d</sup> | 0.191 <sup>d</sup>         | 0.433 <sup>d</sup> | 0.047 <sup>d</sup>         | 0.060 <sup>d</sup> |
|                   |          | 1360 <sup>e</sup>     | -                 | 0.433 <sup>e</sup>         | -                  | 0.060 <sup>e</sup>         | -                  |
| PVP-AgNP          | S5       | 9.48                  | 37.8              | 0.053                      | 1.27               | 0.028                      | 0.058              |
|                   | S7       | 1130                  | -                 | 0.290                      | -                  | 0.089                      | -                  |

<sup>a</sup> Modelled using the total Ag concentration (mg kg<sup>-1</sup> dw), <sup>b</sup> modelled using the concentration of bioaccessible Ag (mg kg<sup>-1</sup> dw), <sup>c</sup> modelled using the concentration of water soluble Ag (mg kg<sup>-1</sup> dw), <sup>d</sup> for the biomass decrease, <sup>e</sup> for the number of individuals

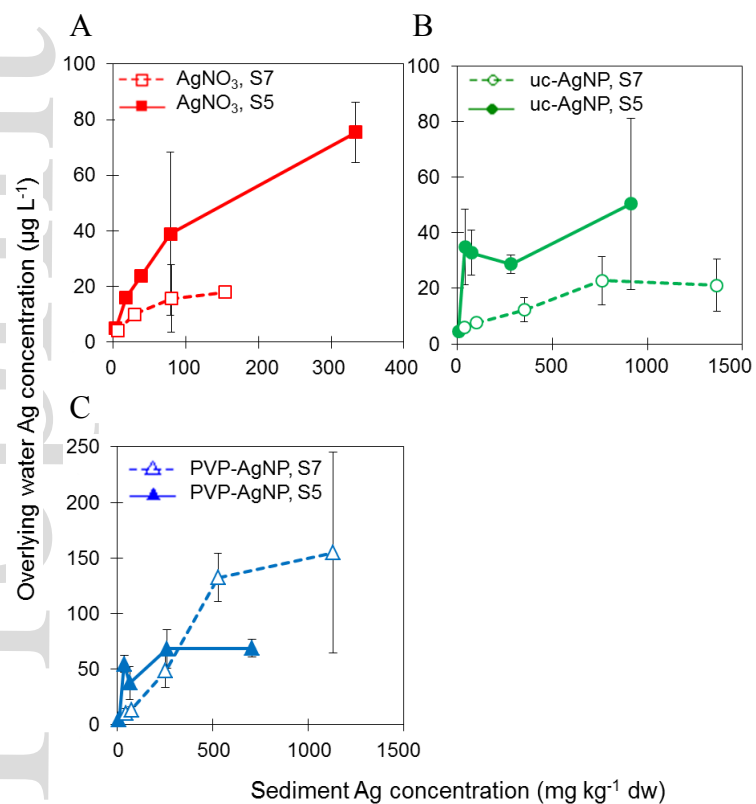
NOEC = no observed effect concentration, LOEC = lowest observed effect concentration.

AgNO<sub>3</sub>, uc-AgNP, PVP-AgNP, S5, S7 = Table 1 and 2

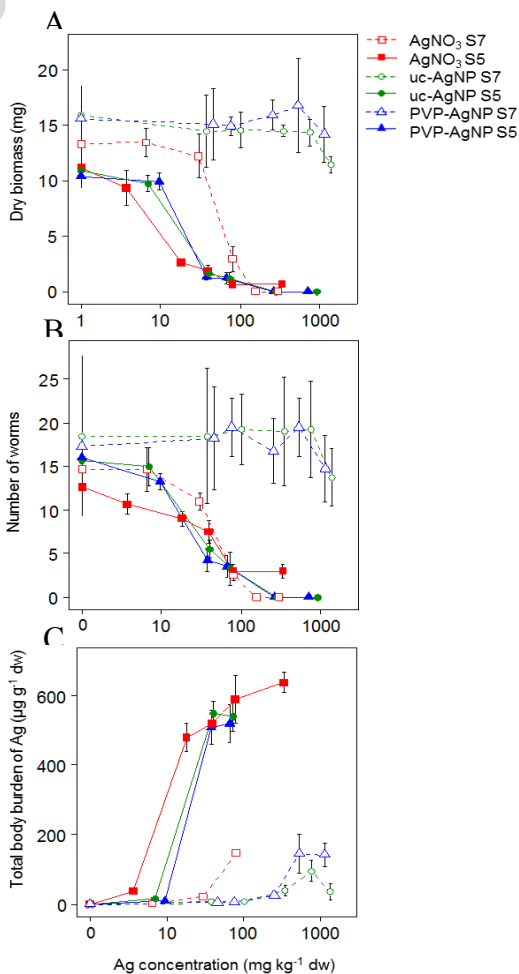


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**Figure 2.** The concentration of Ag ( $\mu\text{g L}^{-1}$ ) in the overlying water of the test set-up at different Ag concentrations ( $\text{mg kg}^{-1} \text{ dw}$ ) spiked as A) silver nitrate ( $\text{AgNO}_3$ ), B) uncoated silver nanoparticle (uc-AgNP) and C) polyvinylpyrrolidone-coated silver nanoparticles (PVP-AgNP) into the artificial sediment pH 7 (S7) and artificial sediment pH 5 (S5).



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