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Title:

Toxicity testing of silver nanoparticles in artificial and natural sediments using the benthic organism *Lumbriculus variegatus*

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ABSTRACT

The increased use of silver nanoparticles (AgNP) in industrial and consumer products worldwide has resulted in their release to aquatic environments. Previous studies have mainly focused on the effects of AgNP on pelagic species, while few studies have assessed the risks to benthic invertebrates despite the sediments acts a large potential sink for nanoparticles. In this study, the toxicity of sediment-associated AgNP was evaluated with the standard sediment toxicity test for chemicals provided by the Organization of Economic Cooperation and Development. The freshwater benthic oligochaete worm *Lumbriculus variegatus* was exposed to sediment-associated AgNP in artificial and natural sediments at concentrations ranging from 91 to 1098 mg Ag/kg sediment dry weight. Silver nitrate ($AgNO_3$) was used as a reference compound for Ag toxicity. The measured endpoints of toxicity were mortality, reproduction and total biomass. In addition, the impact of sediment-associated AgNP on the feeding rate of *L. variegatus* was studied in a similar test set-up as mentioned above. The addition of AgNP into the sediment significantly affected the feeding rate and reproduction of the test species only in the highest concentration (1098 mg/kg) of Ag in the natural sediment with the lowest pH. In comparison, the addition of $AgNO_3$ resulted in reproductive toxicity in every tested sediment, and Ag was more toxic when spiked as $AgNO_3$ than AgNP. In general, sediments were observed to have a high capacity to eliminate the AgNP derived toxicity. However, the capacity of sediments to eliminate the toxicity of Ag follows a different pattern when spiked as AgNP than $AgNO_3$. This study emphasizes the importance of sediment toxicity testing and the role of sediment properties when evaluating the environmental effects and behavior of AgNP in sediments.

Keywords; Silver nanoparticles, environmental toxicology, nanoecotoxicology, sediment toxicity

1. INTRODUCTION

Silver nanoparticles (AgNP) used, e.g., in healthcare, textiles, paints, cosmetics and cleaning agents, have the highest degree of commercialization (as a number of products) of all nanoscale material due to the unique optical and antibacterial properties (Vance et al. 2015). In surface waters, AgNP are mainly released through wastewater treatment plants and untreated wastewater (Gottschalk et al. 2009). Sediment is the final sink for the AgNP, and modeled annual increase of sediment concentrations varies between 0.15 and 10.18 µg/kg/y, resulting in a possible hazard for aquatic organisms (Gottschalk et al. 2009).

In environmental media AgNP may be oxidized, which leads to dissolution and release of Ag ions (Ag^+) (Loza et al. 2014). Ionic Ag is highly toxic to aquatic organisms, and thus the toxicity of AgNP may be related to the concentration of dissolved Ag^+ (Navarro et al. 2008; van Aerle et al. 2013). However, the concentration of freely dissolved Ag^+ in environmental media is typically low due to strong complexation with chloride, sulfide and natural organic matter (Levard et al. 2013; Loza et al. 2014). Silver nanoparticles also pose nanoparticle-specific toxicity (Chan and Chiu 2015; Cozzari et al. 2015; García-Alonso et al. 2014). One of the primary identified toxic mechanisms at the molecular level is the generation of reactive oxygen species resulting in oxidative stress (Cozzari et al. 2015; Roh et al. 2009).

The behavior and toxicity of AgNP in sediment is still poorly understood and there is an urgent need for studies and standardized test methods. The biggest challenge in studies with nanomaterials in sediment and other complex environmental media is the lack of proper characterization methods. As most of the nanomaterials are not stable in water, sediment studies are still considered to be relevant and sometimes even more representative of environmental

exposure than aqueous tests (Petersen et al. 2015). In water-only exposure AgNP with varying coatings results in LC-50 (lethal concentration to kill 50 % of the test organisms in 96 h) of 0.07-0.33 mg/L to the benthic organism *Lumbriculus variegatus* (Khan et al. 2015). When the same species is exposed via sediment, AgNP shows no mortality upon exposure at 367 mg/kg (Coleman et al. 2013). However in sediment exposure AgNP induces oxidative stress in *Nereis diversicolor* already at concentrations lower than 10 mg/kg (Cozzari et al. 2015). Results indicate that the AgNP induced toxicity is reduced when particles are introduced into sediment, but the role of sediment properties has not yet been studied.

The aims of this study were: 1) to examine how the sediment properties influence the toxicity of AgNP, 2) to compare the toxicity of Ag spiked as AgNP to dissolved Ag spiked as silver nitrate ($AgNO_3$), and 3) to evaluate the suitability of the OECD standard test method guideline 225 for use with nanomaterials. Artificial and two natural sediments that differed in their characteristics were selected and spiked with polyvinylpyrrolidone-coated AgNP and $AgNO_3$. The OECD standard test guideline 225 “Sediment-Water Lumbriculus Toxicity Test Using Spiked Sediment” (OECD 2007) was followed, and mortality, reproduction and changes in biomass were used as indicators of toxicity to the endobenthic aquatic Oligochaeta *Lumbriculus variegatus*. In addition, the feeding rate of *L. variegatus* was used as an endpoint of toxicity for AgNP.

2. MATERIAL AND METHODS

2.1 Silver nanoparticles

Silver nanoparticles (polyvinylpyrrolidone coating 0.2 %, NanoAmor) had a nominal reported surface area of 5–10 m^2/g and a diameter of 30–50 nm with a purity of 99.9 %. The characterization of the particles was published in the same year as the experimental part of this study was done (Heckmann et al. 2011). Particles were stored as dry powder and kept away from the direct sunlight, as recommended by the manufacturer, to minimize the possible changes in particle properties during the storage. The characterization of AgNP included: transmission electron microscopy (Phillips CM20, Phillips/FEI), dynamic light scattering and zeta potential measurements (Malvern Zetasizer Nano, Malvern Instruments Ltd). The characterization of AgNP was done in deionized water suspension due to the lack of methods to characterize the particles in complex environmental media. Characterization in the test water was not considered to be relevant, as particles were never introduced into the test water. The mean diameter of AgNP has been reported to be 82 ± 2 nm (n=294) measured from the transmission electron microscope images and 235 ± 4 nm (n=4) with a zeta potential of -28.6 ± 0.6 mV (n=8) by the dynamic light scattering (Heckmann et al. 2011). Agglomeration of the AgNP in water suspension explains the larger diameter of the particles measured by the dynamic light scattering. For further details of the characterization, see Heckmann et al. (2011).

2.2 Test Organisms

Endobenthic oligochaeta *Lumbriculus variegatus* originated from the laboratory culture maintained at the Department of Biology, University of Eastern Finland, Joensuu, Finland. Worms were cultured in 5 L tanks, containing artificial fresh water (AFW, pH 7, hardness 1.0

mM/L as [Ca] + [Mg]) with a constant aeration. The light regime was adjusted to 16 hours light and 8 hours dark, and temperature was held constant at 20 ± 2 °C**.** A layer of paper towels was used as a substrate. Worms were fed twice a week with a Tetramin fish food (Tetrawerke) and water was renewed once a week. Acclimation phase of 24 h was used to adapt the worms to test water.

2.3 Sediments

One artificial sediment (AS) and two natural sediments collected from Lake Höytiäinen (HS) and Lake Kuorinka (KS) were used in this study. Both natural sediments have been used as clean reference sediments in similar experiments, and possible trace amounts of organic chemicals are low and not believed to have an influence on the outcome of current experiments (Mäenpää et al. 2008; Ristola et al. 1996). The sediment AS was prepared using the OECD guideline 225 (OECD 2007). The exact constituent composition was 5 % peat, 74 % quartz sand (60 % < 0.2 mm, 40 % 0.2–1.0 mm), 20 % kaolin and 51 % water (of total dw). *Urtica dioica* powder (0.5 %) was added as a food source to AS and pH was adjusted to 6.7 with $CaCO_3$.

For analyses, natural sediments were sieved through a 1 mm sieve to remove large particles and debris. Subsamples of the sediments were dried at 105 °C over-night to measure dry weight. The determination of organic carbon, inorganic carbon and black carbon were done with Analytik Jena TOC analyzer with a solid sample module (Analytik Jena N/C 2100). Furthermore, subsamples of the sediments were heated for 2 h at 550 °C in a muffle furnace oven (Naber 2804 L47) to obtain the loss of ignition percent. All analyses were done in three replicates.

The heavy metal concentrations of sediments were measured from two different test vessels for each treatment, and the total Ag concentrations were determined in triplicate for each treatment.

133 The sediment samples were stored frozen at -20 °C prior the extraction. The extraction was as
134 follows: A subsample of approximately 200 mg (500 mg for total Ag) was taken from dry
135 sediment, and digested in 1:3 nitric acid:hydrochloric acid (v:v) solution for 9 minutes in three
136 minute intervals in ultrasound water bath (650 W, 35 kHz, ELMA Transsonic T820/H) at 60 °C.
137 The sample tubes were shaken between each 3 minute step. The digested sediment samples were
138 filtered (Whatman No. 41) and diluted to a volume of 20 ml (50 ml for total Ag) with ultrapure
139 water prior to the analysis. The samples were analyzed with Perkin-Elmer model Optima 8300
140 inductively coupled plasma optical emission spectrometry. The cyclonic spray chamber equipped
141 with the GemCone Low-Flow nebulizer was used throughout. The plasma power of 1500 W and
142 nebulizer flow of 0.6 l/min was used in order to get robust plasma conditions for the accurate
143 analysis of the elements. Reagent blank samples were used in between of the samples to ensure
144 the analytical procedure. The accepted relative standard deviation of three replicate
145 measurements was less than 10 %, and the detection limit was 1.9 µg/L. All the used reagents
146 were of analytical grade and supplied by Merck.

147 **2.4 Spiking of the sediments**

148 Direct addition of dry AgNP powder to the sediment was chosen as the spiking method due to the
149 unstable behavior of the particles in the water suspension. The final Ag concentrations were
150 selected based on the preliminary test (Table 1). The sediments were spiked with AgNP by first
151 mixing the nanoparticle powder to a small subsample of the sediment with a metal spoon. The
152 subsample was then mixed to the rest of the sediment. To ensure the homogenous distribution of
153 the compounds, the sediment was mixed with a rotating metal blade for one hour. Silver nitrate
154 (high grade: 99.5% purity, supplied by J.T. Baker) was used as a source of dissolved Ag, and

added to the sediment in a stock solution dissolved in water (400 g/L). The sediment was treated in a similar way as the AgNP-spiked sediment.

2.5 Toxicity test

The toxicity of AgNP was tested according to the OECD guideline 225, using $AgNO_3$ as a reference for Ag^+ toxicity (OECD 2007). The test was conducted in 250 ml beakers (diameter 6 cm) with 4 replicates for each treatment, and 6 replicates for the control treatment. The amount of the sediment was adjusted to the ratio of 1:50 (dry biomass of worms:total organic carbon of the sediment, w:w). The sediment-overlying water ratio was adjusted to approximately 1:3 (v:v). The water hardness of AFW was 2.5 mM/L ([Ca] + [Mg] concentration) and pH was 7.5 (OECD 2007). The sediments were allowed to settle for 7 days with gentle aeration before adding 10 similar-sized *L. variegatus* into the test vessels. The worms were not synchronized for the toxicity test based on the consistent results with only low variation in reproduction and biomass of the worms in the preliminary test (data not shown). During the incubation, the temperature was kept constant at 20 ± 2 °C, and the light regime was 16 h light to 8 h dark. Oxygen and pH were measured once a week during the test. After the 28-day exposure time, the worms were removed from the sediment, counted and placed on a petri dish with a small amount of AFW. A depuration time of 4 h was used to let the worms empty their gut before placing the worms in an oven at 105 °C for overnight. The dry weight was measured with a microbalance (Sartorius 4503). Missing worms were interpreted as mortality and extra worms as reproduction in the test vessels after the exposure period.

2.6 Feeding rate test

176 The feeding rate test was done according to Leppänen and Kukkonen (1998) in similar
177 conditions as described above for the toxicity test. Three replicates were used for each treatment,
178 and two control treatments were done for each sediment (total n=6). A portion of 23 g of wet test
179 sediment was added on the bottom of the 50 ml beakers, which were then filled with 2.5 mM/L
180 ([Ca] + [Mg] concentration) AFW (OECD 2007). The oxygen level and pH in the overlying
181 water were measured during the test, and the water was renewed using aerated AFW every two
182 days. Before adding the organisms into the beakers, the sediments were allowed to settle for 2
183 days. Each beaker received five worms of a similar size. Immediately after the worms buried
184 themselves into the sediment, a layer of a few millimeters of combusted quartz sand (grain size
185 1–2 mm) was added on the top of the sediment. The egestion rate of the worms was followed by
186 collecting fecal pellets every second day for 14 days. The fecal pellets were dried overnight at
187 105 °C, and the dry weight was measured with a microbalance. On the last day of the
188 experiment, the worms were removed from the sediment. After a 4 h depuration time in clean
189 AFW, the worms were counted and dried at 105 °C overnight to measure their dry weight.

190 **2.7 Statistical testing**

191 The effective (EC) and inhibition (IC) concentrations were estimated using a three parameter
192 log-logistic model. The normality of data was tested with Shapiro-Wilk normality test and the
193 homogeneity of variances with Bartlett's test. Normally distributed data with equal variances
194 between groups was studied with one-way analysis of variance (ANOVA) followed by pairwise
195 t-test. When the data was not normally distributed, Kruskal-Wallis rank sum test was used and
196 multiple comparisons between groups were done according to Siegel & Castellan (1988).

One-way ANOVA with Tukey's HSD (honestly significant difference) post-hoc test ($p < 0.05$) was used to compare the amount of fecal pellets in the feeding rate test. The normality of the data was tested with Shapiro-Wilk normality test, and the homogeneity of variances with Levene's test. Due to the small sample size (n=3) the normality of the treatment groups was assumed from the normally distributed control groups (n=6) in all sediments. Statistical analyses and graphical illustrations were done with R version 3.0.1.

3. RESULTS

3.1 Sediment characteristics

The HS sediment had the highest pH (7.10) and organic carbon percent (OC% = 3.12 %) of the tested sediments (Table 2). In the KS sediment the pH was low (5.10) and OC% (2.22) lower than in HS but higher than in the AS sediment (0.59 %), which had also higher pH (6.70) than the KS sediment (Table 2). The visual detection and smaller dw% indicated that the natural sediments KS and HS consisted of finer material compared to AS (Table 2). The artificial sediment contained only low levels of heavy metals. The natural sediments had higher concentrations, the HS sediment containing approximately 2 to 3 fold higher concentrations in comparison to the KS sediment (Table 2). The determined Ag concentrations were in good agreement with the nominal concentrations, and standard deviation among the replicates was relatively small, which indicates homogenous distribution of Ag in the sediments (Table 1.).

3.2 Toxicity test

The pH of the overlying water was at acceptable levels (6–9) for *L. variegatus* in AS and HS, but in the KS sediment the pH was lower than recommended in the guideline (OECD 2007). The oxygen saturation was over 90 % throughout the experiment in all sediments, but the validity criteria of an 1.8-fold increase in the number of individuals was only fulfilled in the AS sediment (OECD 2007).

Exposure to AgNP-spiked sediments caused no mortality in any sediment type or exposure concentration, but reproduction was significantly decreased compared to the control in the highest concentration in the KS sediment (pairwise *t*-test, $p < 0.001$) (Fig 1a). In this treatment the worms were also avoiding the sediment. The $AgNO_3$-spiked AS sediment was the most toxic

to *L. variegatus* and the only sediment where mortality was observed (Fig 1b). Reproduction was decreasing with increasing $AgNO_3$ concentration in all of the tested sediments (Fig 1b). The calculated IC50 values for the reproduction and EC50 values for the number of worms (compared to control) indicate that the Ag spiked as AgNP was only toxic in KS, and Ag spiked as $AgNO_3$ showed highest toxicity in AS, followed by HS, and the lowest toxicity was observed in KS (Table 3).

No statistically significant differences in the total dry biomass of the worms were observed among the exposure concentrations in any of the test sediments spiked with AgNP (Fig 1c). The total dry biomass decreased with increasing $AgNO_3$ concentration in AS, but stayed constant in KS and HS (Fig 1d). Furthermore, the sediment properties affected the total biomass of the test species among the test sediments. In the beginning of the experiment the total dry biomass of the worms was 15.69 mg (SD 0.17) in AS and HS, and 19.04 mg (SD 1.78) in KS. After the 28-day test period the total dry biomass of worms increased in the control groups by on average 49 % (23.31 mg SD 1.89) in AS but decreased by on average 7 % (14.56 mg SD 1.03) in HS and 16 % (16.01 mg SD 1.60) in KS.

3.3 Feeding rate

Silver nanoparticle exposure had no effect on the fecal pellet production of the worms in HS and AS (Fig 2a). In the KS sediment, however, the fecal pellet production increased with increasing exposure concentration of AgNP with an exception that at the highest exposure concentration (1098 mg/kg) the worms were avoiding the sediment and the pellet production thus decreased (Fig 2a).

In the natural sediments HS and KS the worms reproduced during the 14-day exposure period (Fig 2b). In the HS sediment the worms reproduced in each concentration somewhat evenly, but in the KS sediment reproduction was observed only in the controls and in the two lowest exposure concentrations (Fig 2b). In the AS sediment only few extra worms were found in occasional test vessels. No significant differences were found in the total dry biomass of the worms between the different Ag concentrations. The biomass gain was different among the sediments, as also observed in the toxicity test. The biomass of the worms increased during the 14-day exposure period in AS (71 %), stayed constant in KS, and decreased in HS (17 %) (Fig 2c).

4. DISCUSSION

Silver nitrate and AgNP are known to be extremely toxic to the benthic organisms (Khan et al. 2015; Nair et al. 2013). However, the majority of the toxicity studies have been done using waterborne exposures, not considering the natural environment of the benthic organisms. In waterborne exposures the uptake of Ag occurs primarily over the respiratory body surface. Sediment exposures are more environmentally realistic, as organisms feed on the sediment, and Ag is also internalized into the organisms through the gut epithelium. Dietary uptake is especially important when Ag is spiked as AgNP, as particles can be internalized directly via endocytosis (García-Alonso et al. 2011). Endocytic uptake can lead to nanoparticle-specific modes of toxicity, which cannot be considered in water-only exposures.

In this study, the toxicity of Ag spiked as AgNP and $AgNO_3$ to *L. variegatus* in sediment exposures was remarkably lower compared to waterborne exposures in the literature. Khan et al. (2015) reported the LC50 concentrations in the 96 h acute toxicity test to be 64.6 µg/L for PVP-coated AgNP and 4.4 µg/L for $AgNO_3$ in the OECD 225 standard AFW. In the present study no mortality was observed in any of the tested sediments even in the highest 1098 mg/kg (dw) concentration of sediment-associated AgNP. The EC50-value for $AgNO_3$ was 38 mg/kg (dw) in AS sediment, but no mortality was observed in other test sediments. The decrease of toxicity of Ag in sediments compared to the waterborne exposures is dramatic, especially when spiked as AgNP, despite the possible direct uptake of AgNP by endocytosis. The capacity of sediment to decrease the toxicity of Ag emphasizes the need of sediment toxicity tests when evaluating the environmental effects of AgNP. Our results indicate that the toxicity to benthic fauna may be highly overestimated if only waterborne exposures are used.

278 Dissolved Ag spiked as $AgNO_3$ was more toxic to *L. variegatus* in the artificial sediment than in
279 the natural sediments. This suggests that the Ag^+ binding capacity is greater in the natural
280 sediments compared to the AS sediment. The higher OC content of the HS and KS sediments
281 compared to the AS sediment partly explains the lower toxicity of $AgNO_3$, as Ag is known to
282 form complexes with OC (Erickson et al. 1998). Also the grain size of the natural sediments is
283 small; 79.0 % (HS) and 77.9 % (KS) of the particles are under 63 µm in diameter (Mäenpää et al.
284 2003). The high dw% in the AS sediment indicates that the sediment was mainly reconstructed
285 from coarse quartz sand resulting in a smaller surface area in the AS sediment components to
286 bind Ag. The concentration of acid volatile sulfides (AVS) in the sediment is often considered to
287 be the most important individual factor in anoxic sediments, since Ag has a strong affinity
288 towards organic and inorganic sulfur groups (Bell and Kramer 1999; Berry et al. 1999). In this
289 study the tested sediments were treated under oxidized conditions, where the concentration of
290 AVS can be considered negligible (Di Toro et al. 1990). Silver has also high affinity towards Cl^-
291 anions (Wingert-Runge and Andren 1993). In our test set-up the amount of Cl anions in the
292 overlying AFW was theoretically high enough to complex all Ag^+, but as the Ag compounds
293 were spiked directly to the sediment, the effect of Cl and other anions is considered small. This is
294 proved by the toxicity of $AgNO_3$ in the test sediments despite the complexing anions in overlying
295 water.

296 The toxicity of Ag increases when pH decreases, due to the increased free Ag^+ concentration in
297 the media (Erickson et al. 1998). Low pH also increases the dissolution of AgNP, which leads to
298 a higher free Ag^+ concentration and increased toxicity (Navarro et al. 2008; Peretyazhko et al.
299 2014; van Aerle et al. 2013). The natural sediment KS had the lowest pH of the tested sediments.
300 Whereas the toxicity of Ag spiked as $AgNO_3$ was lowest in KS, it was the only sediment in

301 which the addition of AgNP resulted in reproductive toxicity. This indicates that low pH may
302 increase the toxicity of AgNP more than that of $AgNO_3$. The IC50 values for reproduction were
303 approximately 2 times higher for AgNP than for $AgNO_3$ in KS sediment. If the toxicity is
304 proposed to be solely a function of Ag^+, around 50 % of the particles would be dissolved. The
305 partitioning studies done in sediment however show that the bioavailable concentration of Ag^+ in
306 sediment is higher when added as AgNP than when added as $AgNO_3$ (Coutris et al. 2012). Direct
307 comparisons between the toxicity data and the dissolution of AgNP cannot thus be made. AgNP
308 can also pose nanoparticle-specific toxicity over Ag^+ (Chan and Chiu 2015; Cozzari et al. 2015;
309 García-Alonso et al. 2014) or "Trojan horse" -type of behavior, leading to the intracellular
310 release of Ag^+ (Moore 2006; Park et al. 2010; Wang et al. 2013). If these nanoparticle-specific
311 modes of toxicity would explain the toxicity of AgNP in the KS sediment, the bioavailability of
312 AgNP should be higher in the KS sediment compared to the other tested sediments, as no toxicity
313 was observed in the HS or AS sediments. This is unlikely as the relatively low pH in KS actually
314 suggests lower bioavailability of AgNP compared to the other more alkaline sediments due to a
315 stronger electrostatic attraction between the negatively charged particles and positively charged
316 matrix (Cornelis et al. 2014). Considering these facts, we suggest that the AgNP toxicity in KS
317 was mainly caused by dissolved Ag^+ released from the particles and that the dissolution is
318 promoted by the low pH of the sediment.

319 The nutritional value of sediment to *L. variegatus* varied between the tested sediments. The AS
320 sediment was the only sediment where the worms were gaining weight. The total biomass of the
321 worms was decreasing in the HS and KS sediments despite that the worms ingested both natural
322 sediments. This indicates the poor nutritional value of the natural sediments compared to the AS
323 sediment. Especially the KS sediment seems to have a poor nutrient content, since the biomass-

normalized ingestion rate was highest among the test sediments but the biomass loss was the largest. No significant difference in the total biomass was found between the treatments in toxicity or feeding rate test, despite the significant decrease in the ingested amount of sediment in the highest concentration of AgNP in KS. The biomass change seems not to be an applicable endpoint for the acute toxicity tests in the natural sediments with poor nutritional value, as the worms were losing weight also in the control groups of the HS and KS sediments.

The feeding behavior of *L. variegatus* has been shown to give an immediate response to the exposure, and it is considered to be a more sensitive endpoint than mortality, biomass gain or reproduction (Leppänen and Kukkonen 1998). Generally the ingestion rate tends to decrease with increasing concentration of contaminant, but in the KS sediment *L. variegatus* ingested more sediment with increasing AgNP concentration. We suggest that the antibacterial properties of AgNP disturbed the microbial growth in the sediment, which impeded adequate nutrition of *L. variegatus*, and thus worms had to compensate for the nutrient-poor food by ingesting more sediment. In the highest exposure concentration, however, the AgNP-induced stress seemed to become too high for the *L. variegatus*, as the worms avoided the sediment throughout the test period and thus the feeding rate was minimal. The increase in the feeding rate was only observed in the KS sediment. The microbes can be a more important food source in KS compared to the other test sediments due to the poor nutritional value. Also the low pH of KS is believed to be an intensifying factor for AgNP toxicity as discussed before.

The natural sediments HS and KS used in this study were selected to represent typical unpolluted Finnish lake sediments from a watershed without industrial influence, and have been used in studies as clean reference sediments (Mäenpää et al. 2003; Ristola et al. 1996). The geochemical background level of metals is slightly elevated if compared to the consensus-based threshold

effect concentrations (TEC), meaning that these metals possibly cause toxic effects in a freshwater ecosystem (MacDonald et al. 2000). In HS the Cd, Cr, Cu, Ni, Pb, and Zn concentrations are above the TEC. In KS the metal concentrations are also elevated but somewhat lower compared to HS, and only Cd, Cu and Ni are above the TEC values. The background metal concentrations are however typical for the sediments in this area (Ristola et al. 1996). When a test sediment is amended with Ag, it is possible that Ag^+ and AgNP displace sediment-bound metals and release them into the sediment pore water. Especially Zn and Ni are known to be displaced by Ag (Call et al. 1999). The measured toxicity in the natural sediments may therefore be a mixture effect of metals, Ag being the predominant active substance. Higher concentration of background metals may therefore explain the higher toxicity of $AgNO_3$ in HS sediment compared to the KS sediment. In the AgNP treatments this effect is not pronounced, as the dissolution of nanoparticles is believed to be more promoted by the lower pH of KS compared to HS, leading to the higher toxicity in KS. The environmental relevance is often a key factor when considering the behavior of nanoparticles in the aquatic environment. As the properties of the natural sediments differ greatly from the artificially prepared standard sediment, we consider testing in the natural sediments highly important, despite the fact that the environmental factors apart from the nanoparticle exposure may complicate interpreting the results.

The OECD standard test guideline 225 was successfully applied for use with nanomaterials. The AS sediment prepared following the OECD standard guideline was the only sediment that fulfilled the validity criteria of an 1.8-fold increase in the number of individuals, and thus only this part of the study can be considered as a standardized toxicity test. The low reproduction rate and pH-related problems in natural sediments advocate the use of artificial sediment in standard

testing. The results of the OECD toxicity tests are in line with the feeding rate test, which increases the reliability of the test. However, the following concerns may have significant effect on the results of the test and should be properly addressed in the future: 1) The spiking method of the nanomaterial may have an influence on the outcome of the test. We chose to add the dry powder of AgNP directly into the sediment, because the amount of nanoparticles was high, and the particles were unstable in water suspension in such a high concentration. More stable nanoparticle suspensions could also be spiked as suspension to avoid clumping of the material. The reduced clumping leads to a higher total surface area of the spiked component and may possibly lead to elevated toxicity. Indirect addition of the nanoparticles to the overlying water would be an environmentally more relevant way to spike the nanomaterial, but could decrease the oral uptake of the substance, since *L. variegatus* burrow into the sediment and feed below the sediment surface. 2) The characterization of nanomaterial should be carefully considered. Since we do not currently have proper methods to characterize the sediment-associated nanomaterial, characterization in this study was done in deionized water before spiking the nanoparticles into the test media. Despite the fact that the characterization in water does not correspond to the experimental conditions in the sediment, it is essential to assess the primary structure and properties of the particles in standard conditions to add comparability between the studies. The characterization of nanoparticles in the overlying water was not considered relevant, since AgNP were spiked to the sediment by direct addition and were never present in the water phase. If the indirect addition is used, the characterization in the overlying water should also be considered, as the aggregation and dissolution of coated AgNP in the water phase is differently affected by the presence of sediment (Bone et al. 2012; Unrine et al. 2012). In conclusion, there is an urgent need to develop reliable and easily achievable methods for the characterization of the

393 nanomaterials in the sediment media. However, the former concerns should not hinder the
394 toxicity testing of nanomaterials in sediment or other complex environmental matrix. Despite the
395 methodological challenges, tests give us important information on the possible toxicity of
396 nanomaterials.

5. CONCLUSIONS

The acute toxicity of Ag spiked as AgNP to *L. variegatus* was greatly decreased in sediments compared to literature-reported waterborne toxicity. Silver nitrate was significantly more toxic than AgNP in all of the test sediments, but sediment properties had a different effect on the toxicity of the two compounds. The toxicity of $AgNO_3$ was lower in the sediments with fine grain size and relatively high amount of OC. The low pH of the sediment seemed to overcome these factors when Ag was spiked as AgNP, and toxicity as reproductive failure, changes in the feeding behavior and sediment avoidance was only observed in the natural KS sediment with the lowest pH value of the tested sediments. We suggest that low pH of the KS sediment enhances the release of Ag^+ from AgNP and thus promotes the toxicity. However, nanoparticle-specific toxicity or synergistic effect of both Ag^+ and AgNP, and natural heavy metals cannot be excluded. Finally, we conclude that OECD guideline 225 “Sediment-Water Lumbriculus Toxicity Test Using Spiked Sediment” can be used for evaluating the toxicity of nanomaterials in sediments. Further studies considering AgNP in sediments should concentrate on the dissolution kinetics and the effects of sediment pH on the toxicity of AgNP. In general with nanoparticles, the development of characterization methods in complex environmental media is the most essential issue. However, the lack of characterization methods should not hinder the toxicity testing of nanoparticles in complex environmental media, since nanoparticles are constantly released into the environment where they are likely to pose a risk to the benthic ecosystems.

Conflict of Interest: The authors declare that they have no conflict of interest.

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535 **TABLES**

536

537 **Table 1** Nominal and determined silver concentrations (mg/kg dry weight) spiked as silver

538 nitrate ($AgNO_3$) and silver nanoparticles (AgNP) in test sediments

Compound	Nominal concentration	Determined concentration in sediments (mg/kg)[a]		
		Artificial	Höytiäinen	Kuorinka
$AgNO_3$	10	11.7 (0.6)	10.8 (0.0)	10.1 (0.0)
	40	36.9 (1.0)	42.8 (0.4)	41.9 (0.3)
	100	92.7 (1.7)	98.9 (0.9)	103.1 (3.6)
	200	180.6 (1.6)	199.3 (1.2)	208.9 (2.9)
	400	360.4 (7.1)	394.8 (5.2)	415.9 (4.0)
AgNP	100	90.6 (1.9)	95.7 (10.8)	77.1 (5.0)
	300	262.5 (2.3)	273.8 (18.1)	256.7 (23.5)
	600	537.4 (12.3)	471.1 (9.8)	525.8 (27.4)
	900	725.4 (23.5)	741.2 (20.1)	801.3 (10.5)
	1200	985.1 (17.8)	943.4 (164.0)	1097.9 (7.5)

539 [a] Mean and standard deviation of 3-5 replicates

Table 2 The characteristics of the test sediments. LOI% = Loss of ignition, OC% = organic carbon, BC% = black carbon, IC% = inorganic carbon, dw% = dry weight

	Sediment		
	Artificial	Höytiäinen	Kuorinka
pH [a]	6.70 (0.26)	7.10 (0.21)	5.10 (0.11)
LOI% [b]	6.30 (0.06)	10.6 (0.1)	6.03 (0.03)
OC% [b]	0.59 (0.15)	3.12 (0.31)	2.22 (0.14)
BC% [b]	0.04 (0.01)	0.05 (0.00)	0.14 (0.00)
IC% [b]	0.75 (0.37)	-	-
dw% [c]	60.0 (0.2)	18.2 (0.1)	33.2 (0.3)
Cd [d]	0.15 (0.08)	3.34 (0.18)	1.07 (0.07)
Cr [d]	3.79 (0.81)	51.9 (4.8)	20.1 (1.7)
Cu [d]	3.56 (3.15)	53.0 (4.8)	35.8 (3.5)
Ni [d]	2.49 (0.41)	44.2 (3.8)	25.0 (1.7)
Pb [d]	0.77 (0.14)	44.4 (1.9)	18.8 (1.8)
Zn [d]	3.19 (1.60)	130 (7)	56.6 (5.4)

[a] Mean (standard deviation) of weekly measures during the 28 d toxicity test (n>55).

[b] Percent of the sediment dry weight, mean (standard deviation) of 3 replicates

[c] Percent of the sediment total weight, mean (standard deviation) of 3 replicates

[d] Metal concentration as mg/kg of dry sediment, mean (standard deviation) of 24 to 26 replicates

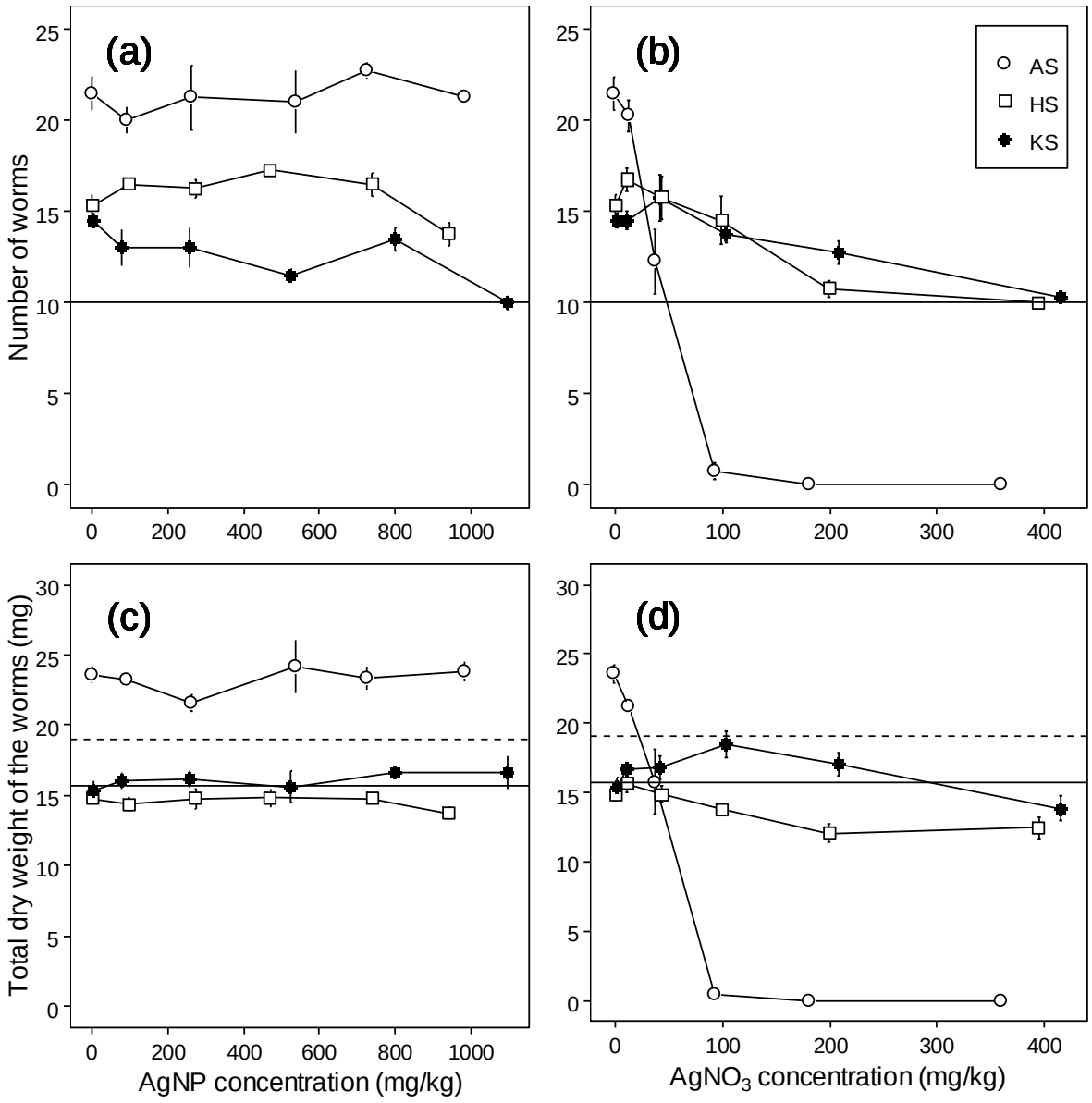
546 **Table 3** Calculated 50 % reproduction-inhibition concentrations (IC50) and 50 % effect
547 concentrations (EC50) for decrease in the number of worms compared to control for silver nitrate
548 ($AgNO_3$) and silver nanoparticle (AgNP) exposed *Lumbriculus variegatus*

			Sediment		
	End Point	Compound	Artificial	Höytiäinen	Kuorinka
Reproduction[a]	IC50	$AgNO_3$	23.9 (3.60)	129 (19.9)	213.50 (47.77)
	IC50	AgNP	-	-	442.84 (316.74)
Number of worms[a]	EC50	$AgNO_3$	38.0 (1.97)	525 (107.07)	687.59 (187.75)

549 [a] Based on three parameter log-logistic model, Ag (determined concentration) in kg of dry
550 sediment (standard deviation)

551

552 **FIGURES**



553

554 **Fig 1.** Toxicity of Ag spiked as silver nanoparticles (AgNP) and silver nitrate ($AgNO_3$) to
555 *Lumbriculus variegatus* after a 28-day exposure in spiked artificial (AS), Höytiäinen (HS) and
556 Kuorinka (KS) sediments at various Ag concentrations (mg/kg dry weight). Number of worms
557 after a 28-day exposure to AgNP (a) and $AgNO_3$ (b). Dry biomass of worms after exposure to
558 AgNP (c) and $AgNO_3$ (d). Each symbol indicates mean and standard deviation of four replicate
559 samples, except for control exposure that had six replicates. Solid line in (a) and (b) indicates the

number of worms (10) at the beginning of the experiment. Solid line in (c) and (d) indicates the starting dry biomass of the worms in the AS and HS sediments (15.69 mg), dashed line in the KS sediment (19.04 mg).

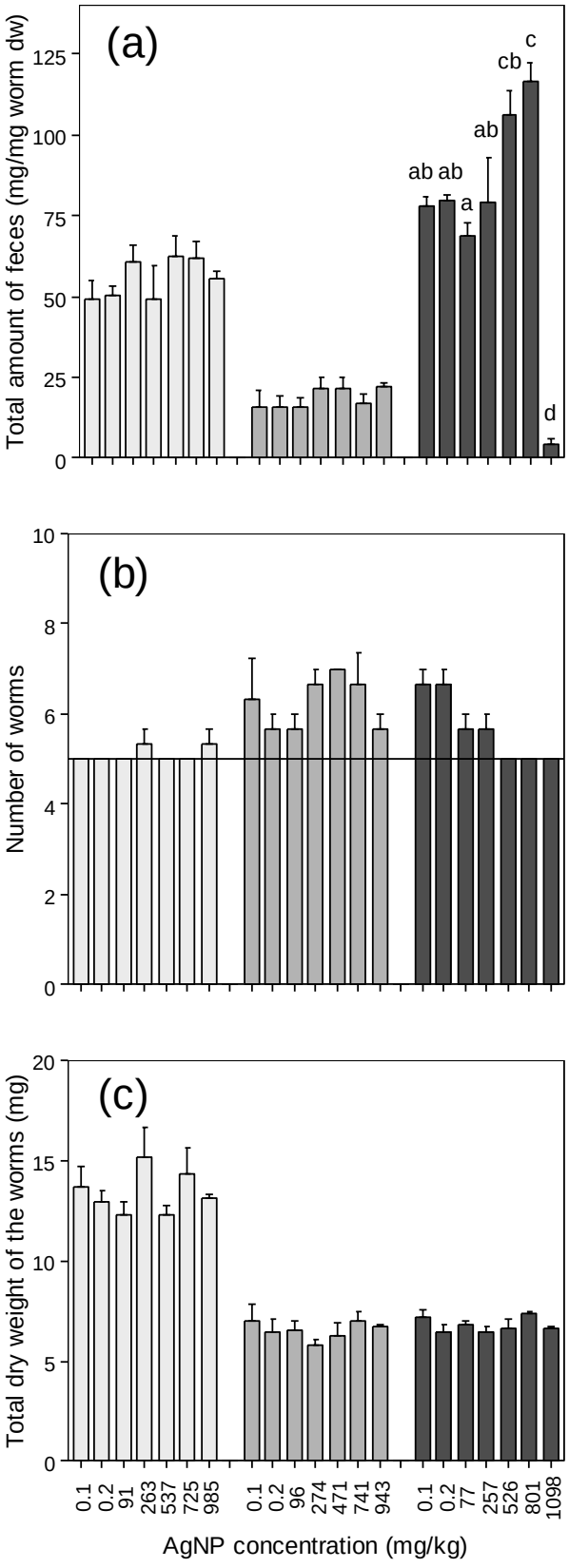


Fig 2. Effects of Ag spiked as silver nanoparticles (AgNP) on *Lumbriculus variegatus* in 14-days feeding rate test in artificial (AS), Höytiäinen (HS) and Kuorinka (KS) sediments at various Ag concentrations (mg/kg dry weight). Two control groups without AgNP are marked as 0.1 and 0.2. Mass of dry feces produced during the experiment normalized to the total dry biomass of the worms (a). Identical letters (a-d) indicate groups that do not significantly differ from each other ($p < 0.05$). Number of worms in the end of the experiment; the solid line indicates the number of worms in the beginning of the experiment (b). Total dry biomass of the worms after the experiment (c). Each bar indicates mean and standard deviation of three replicate samples.