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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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2 Toxicity testing of silver nanoparticles in artificial and natural sediments using the benthic

3 organism *Lumbriculus variegatus*

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26 **ABSTRACT**

27 The increased use of silver nanoparticles (AgNP) in industrial and consumer products worldwide

28 has resulted in their release to aquatic environments. Previous studies have mainly focused on

29 the effects of AgNP on pelagic species, while few studies have assessed the risks to benthic

30 invertebrates despite the sediments acts a large potential sink for nanoparticles. In this study, the

31 toxicity of sediment-associated AgNP was evaluated with the standard sediment toxicity test for

32 chemicals provided by the Organization of Economic Cooperation and Development. The

33 freshwater benthic oligochaete worm *Lumbriculus variegatus* was exposed to sediment-

34 associated AgNP in artificial and natural sediments at concentrations ranging from 91 to 1098

35 mg Ag/kg sediment dry weight. Silver nitrate ($AgNO_3$) was used as a reference compound for

36 Ag toxicity. The measured endpoints of toxicity were mortality, reproduction and total biomass.

37 In addition, the impact of sediment-associated AgNP on the feeding rate of *L. variegatus* was

38 studied in a similar test set-up as mentioned above. The addition of AgNP into the sediment

39 significantly affected the feeding rate and reproduction of the test species only in the highest

40 concentration (1098 mg/kg) of Ag in the natural sediment with the lowest pH. In comparison, the

41 addition of $AgNO_3$ resulted in reproductive toxicity in every tested sediment, and Ag was more

42 toxic when spiked as $AgNO_3$ than AgNP. In general, sediments were observed to have a high

43 capacity to eliminate the AgNP derived toxicity. However, the capacity of sediments to eliminate

44 the toxicity of Ag follows a different pattern when spiked as AgNP than $AgNO_3$. This study

45 emphasizes the importance of sediment toxicity testing and the role of sediment properties when

46 evaluating the environmental effects and behavior of AgNP in sediments.

47 **Keywords;** Silver nanoparticles, environmental toxicology, nanoecotoxicology, sediment

48 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

71 exposure than aqueous tests (Petersen et al. 2015). In water-only exposure AgNP with varying

72 coatings results in LC-50 (lethal concentration to kill 50 % of the test organisms in 96 h) of 0.07-

73 0.33 mg/L to the benthic organism *Lumbriculus variegatus* (Khan et al. 2015). When the same

74 species is exposed via sediment, AgNP shows no mortality upon exposure at 367 mg/kg

75 (Coleman et al. 2013). However in sediment exposure AgNP induces oxidative stress in *Nereis*

76 *diversicolor* already at concentrations lower than 10 mg/kg (Cozzari et al. 2015). Results indicate

77 that the AgNP induced toxicity is reduced when particles are introduced into sediment, but the

78 role of sediment properties has not yet been studied.

79 The aims of this study were: 1) to examine how the sediment properties influence the toxicity of

80 AgNP, 2) to compare the toxicity of Ag spiked as AgNP to dissolved Ag spiked as silver nitrate

81 ($AgNO_3$), and 3) to evaluate the suitability of the OECD standard test method guideline 225 for

82 use with nanomaterials. Artificial and two natural sediments that differed in their characteristics

83 were selected and spiked with polyvinylpyrrolidone-coated AgNP and $AgNO_3$. The OECD

84 standard test guideline 225 "Sediment-Water Lumbriculus Toxicity Test Using Spiked

85 Sediment" (OECD 2007) was followed, and mortality, reproduction and changes in biomass

86 were used as indicators of toxicity to the endobenthic aquatic Oligochaeta *Lumbriculus*

87 *variegatus*. In addition, the feeding rate of *L. variegatus* was used as an endpoint of toxicity for

88 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

111   mM/L as [Ca] + [Mg]) with a constant aeration. The light regime was adjusted to 16 hours light

112   and 8 hours dark, and temperature was held constant at 20 ± 2 °C. A layer of paper towels was

113   used as a substrate. Worms were fed twice a week with a Tetramin fish food (Tetrawerke) and

114   water was renewed once a week. Acclimation phase of 24 h was used to adapt the worms to test

115   water.

116   **2.3 Sediments**

117   One artificial sediment (AS) and two natural sediments collected from Lake Höytiäinen (HS)

118   and Lake Kuorinka (KS) were used in this study. Both natural sediments have been used as clean

119   reference sediments in similar experiments, and possible trace amounts of organic chemicals are

120   low and not believed to have an influence on the outcome of current experiments (Mäenpää et al.

121   2008; Ristola et al. 1996). The sediment AS was prepared using the OECD guideline 225

122   (OECD 2007). The exact constituent composition was 5 % peat, 74 % quartz sand (60 % < 0.2

123   mm, 40 % 0.2–1.0 mm), 20 % kaolin and 51 % water (of total dw). *Urtica dioica* powder (0.5 %)

124   was added as a food source to AS and pH was adjusted to 6.7 with $CaCO_3$.

125   For analyses, natural sediments were sieved through a 1 mm sieve to remove large particles and

126   debris. Subsamples of the sediments were dried at 105 °C over-night to measure dry weight. The

127   determination of organic carbon, inorganic carbon and black carbon were done with Analytik

128   Jena TOC analyzer with a solid sample module (Analytik Jena N/C 2100). Furthermore,

129   subsamples of the sediments were heated for 2 h at 550 °C in a muffle furnace oven (Naber 2804

130   L47) to obtain the loss of ignition percent. All analyses were done in three replicates.

131   The heavy metal concentrations of sediments were measured from two different test vessels for

132   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

155 added to the sediment in a stock solution dissolved in water (400 g/L). The sediment was treated

156 in a similar way as the AgNP-spiked sediment.

2.5 Toxicity test

158 The toxicity of AgNP was tested according to the OECD guideline 225, using $AgNO_3$ as a

159 reference for Ag^+ toxicity (OECD 2007). The test was conducted in 250 ml beakers (diameter 6

160 cm) with 4 replicates for each treatment, and 6 replicates for the control treatment. The amount

161 of the sediment was adjusted to the ratio of 1:50 (dry biomass of worms:total organic carbon of

162 the sediment, w:w). The sediment-overlying water ratio was adjusted to approximately 1:3 (v:v).

163 The water hardness of AFW was 2.5 mM/L ([Ca] + [Mg] concentration) and pH was 7.5 (OECD

164 2007). The sediments were allowed to settle for 7 days with gentle aeration before adding 10

165 similar-sized *L. variegatus* into the test vessels. The worms were not synchronized for the

166 toxicity test based on the consistent results with only low variation in reproduction and biomass

167 of the worms in the preliminary test (data not shown). During the incubation, the temperature

168 was kept constant at 20 ± 2 °C, and the light regime was 16 h light to 8 h dark. Oxygen and pH

169 were measured once a week during the test. After the 28-day exposure time, the worms were

170 removed from the sediment, counted and placed on a petri dish with a small amount of AFW. A

171 depuration time of 4 h was used to let the worms empty their gut before placing the worms in an

172 oven at 105 °C for overnight. The dry weight was measured with a microbalance (Sartorius

173 4503). Missing worms were interpreted as mortality and extra worms as reproduction in the test

174 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).

197   One-way ANOVA with Tukey's HSD (honestly significant difference) post-hoc test ($p < 0.05$)

198   was used to compare the amount of fecal pellets in the feeding rate test. The normality of the

199   data was tested with Shapiro-Wilk normality test, and the homogeneity of variances with

200   Levene's test. Due to the small sample size (n=3) the normality of the treatment groups was

201   assumed from the normally distributed control groups (n=6) in all sediments. Statistical analyses

202   and graphical illustrations were done with R version 3.0.1.

203

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

226 to *L. variegatus* and the only sediment where mortality was observed (Fig 1b). Reproduction was

227 decreasing with increasing $AgNO_3$ concentration in all of the tested sediments (Fig 1b). The

228 calculated IC50 values for the reproduction and EC50 values for the number of worms

229 (compared to control) indicate that the Ag spiked as AgNP was only toxic in KS, and Ag spiked

230 as $AgNO_3$ showed highest toxicity in AS, followed by HS, and the lowest toxicity was observed

231 in KS (Table 3).

232 No statistically significant differences in the total dry biomass of the worms were observed

233 among the exposure concentrations in any of the test sediments spiked with AgNP (Fig 1c). The

234 total dry biomass decreased with increasing $AgNO_3$ concentration in AS, but stayed constant in

235 KS and HS (Fig 1d). Furthermore, the sediment properties affected the total biomass of the test

236 species among the test sediments. In the beginning of the experiment the total dry biomass of the

237 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

238 test period the total dry biomass of worms increased in the control groups by on average 49 %

239 (23.31 mg SD 1.89) in AS but decreased by on average 7 % (14.56 mg SD 1.03) in HS and 16 %

240 (16.01 mg SD 1.60) in KS.

241 **3.3 Feeding rate**

242 Silver nanoparticle exposure had no effect on the fecal pellet production of the worms in HS and

243 AS (Fig 2a). In the KS sediment, however, the fecal pellet production increased with increasing

244 exposure concentration of AgNP with an exception that at the highest exposure concentration

245 (1098 mg/kg) the worms were avoiding the sediment and the pellet production thus decreased

246 (Fig 2a).

247   In the natural sediments HS and KS the worms reproduced during the 14-day exposure period

248   (Fig 2b). In the HS sediment the worms reproduced in each concentration somewhat evenly, but

249   in the KS sediment reproduction was observed only in the controls and in the two lowest

250   exposure concentrations (Fig 2b). In the AS sediment only few extra worms were found in

251   occasional test vessels. No significant differences were found in the total dry biomass of the

252   worms between the different Ag concentrations. The biomass gain was different among the

253   sediments, as also observed in the toxicity test. The biomass of the worms increased during the

254   14-day exposure period in AS (71 %), stayed constant in KS, and decreased in HS (17 %) (Fig

255   2c).

4. DISCUSSION

256

257 Silver nitrate and AgNP are known to be extremely toxic to the benthic organisms (Khan et al.

258 2015; Nair et al. 2013). However, the majority of the toxicity studies have been done using

259 waterborne exposures, not considering the natural environment of the benthic organisms. In

260 waterborne exposures the uptake of Ag occurs primarily over the respiratory body surface.

261 Sediment exposures are more environmentally realistic, as organisms feed on the sediment, and

262 Ag is also internalized into the organisms through the gut epithelium. Dietary uptake is

263 especially important when Ag is spiked as AgNP, as particles can be internalized directly via

264 endocytosis (García-Alonso et al. 2011). Endocytic uptake can lead to nanoparticle-specific

265 modes of toxicity, which cannot be considered in water-only exposures.

266 In this study, the toxicity of Ag spiked as AgNP and $AgNO_3$ to *L. variegatus* in sediment

267 exposures was remarkably lower compared to waterborne exposures in the literature. Khan et al.

268 (2015) reported the LC50 concentrations in the 96 h acute toxicity test to be 64.6 µg/L for PVP-

269 coated AgNP and 4.4 µg/L for $AgNO_3$ in the OECD 225 standard AFW. In the present study no

270 mortality was observed in any of the tested sediments even in the highest 1098 mg/kg (dw)

271 concentration of sediment-associated AgNP. The EC50-value for $AgNO_3$ was 38 mg/kg (dw) in

272 AS sediment, but no mortality was observed in other test sediments. The decrease of toxicity of

273 Ag in sediments compared to the waterborne exposures is dramatic, especially when spiked as

274 AgNP, despite the possible direct uptake of AgNP by endocytosis. The capacity of sediment to

275 decrease the toxicity of Ag emphasizes the need of sediment toxicity tests when evaluating the

276 environmental effects of AgNP. Our results indicate that the toxicity to benthic fauna may be

277 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-

324   normalized ingestion rate was highest among the test sediments but the biomass loss was the

325   largest. No significant difference in the total biomass was found between the treatments in

326   toxicity or feeding rate test, despite the significant decrease in the ingested amount of sediment

327   in the highest concentration of AgNP in KS. The biomass change seems not to be an applicable

328   endpoint for the acute toxicity tests in the natural sediments with poor nutritional value, as the

329   worms were losing weight also in the control groups of the HS and KS sediments.

330   The feeding behavior of *L. variegatus* has been shown to give an immediate response to the

331   exposure, and it is considered to be a more sensitive endpoint than mortality, biomass gain or

332   reproduction (Leppänen and Kukkonen 1998). Generally the ingestion rate tends to decrease

333   with increasing concentration of contaminant, but in the KS sediment *L. variegatus* ingested

334   more sediment with increasing AgNP concentration. We suggest that the antibacterial properties

335   of AgNP disturbed the microbial growth in the sediment, which impeded adequate nutrition of *L.*

336   *variegatus*, and thus worms had to compensate for the nutrient-poor food by ingesting more

337   sediment. In the highest exposure concentration, however, the AgNP-induced stress seemed to

338   become too high for the *L. variegatus*, as the worms avoided the sediment throughout the test

339   period and thus the feeding rate was minimal. The increase in the feeding rate was only observed

340   in the KS sediment. The microbes can be a more important food source in KS compared to the

341   other test sediments due to the poor nutritional value. Also the low pH of KS is believed to be an

342   intensifying factor for AgNP toxicity as discussed before.

343   The natural sediments HS and KS used in this study were selected to represent typical unpolluted

344   Finnish lake sediments from a watershed without industrial influence, and have been used in

345   studies as clean reference sediments (Mäenpää et al. 2003; Ristola et al. 1996). The geochemical

346   background level of metals is slightly elevated if compared to the consensus-based threshold

347 effect concentrations (TEC), meaning that these metals possibly cause toxic effects in a

348 freshwater ecosystem (MacDonald et al. 2000). In HS the Cd, Cr, Cu, Ni, Pb, and Zn

349 concentrations are above the TEC. In KS the metal concentrations are also elevated but

350 somewhat lower compared to HS, and only Cd, Cu and Ni are above the TEC values. The

351 background metal concentrations are however typical for the sediments in this area (Ristola et al.

352 1996). When a test sediment is amended with Ag, it is possible that Ag^+ and AgNP displace

353 sediment-bound metals and release them into the sediment pore water. Especially Zn and Ni are

354 known to be displaced by Ag (Call et al. 1999). The measured toxicity in the natural sediments

355 may therefore be a mixture effect of metals, Ag being the predominant active substance. Higher

356 concentration of background metals may therefore explain the higher toxicity of $AgNO_3$ in HS

357 sediment compared to the KS sediment. In the AgNP treatments this effect is not pronounced, as

358 the dissolution of nanoparticles is believed to be more promoted by the lower pH of KS

359 compared to HS, leading to the higher toxicity in KS. The environmental relevance is often a key

360 factor when considering the behavior of nanoparticles in the aquatic environment. As the

361 properties of the natural sediments differ greatly from the artificially prepared standard sediment,

362 we consider testing in the natural sediments highly important, despite the fact that the

363 environmental factors apart from the nanoparticle exposure may complicate interpreting the

364 results.

365 The OECD standard test guideline 225 was successfully applied for use with nanomaterials. The

366 AS sediment prepared following the OECD standard guideline was the only sediment that

367 fulfilled the validity criteria of an 1.8-fold increase in the number of individuals, and thus only

368 this part of the study can be considered as a standardized toxicity test. The low reproduction rate

369 and pH-related problems in natural sediments advocate the use of artificial sediment in standard

370   testing. The results of the OECD toxicity tests are in line with the feeding rate test, which

371   increases the reliability of the test. However, the following concerns may have significant effect

372   on the results of the test and should be properly addressed in the future: 1) The spiking method

373   of the nanomaterial may have an influence on the outcome of the test. We chose to add the dry

374   powder of AgNP directly into the sediment, because the amount of nanoparticles was high, and

375   the particles were unstable in water suspension in such a high concentration. More stable

376   nanoparticle suspensions could also be spiked as suspension to avoid clumping of the material.

377   The reduced clumping leads to a higher total surface area of the spiked component and may

378   possibly lead to elevated toxicity. Indirect addition of the nanoparticles to the overlying water

379   would be an environmentally more relevant way to spike the nanomaterial, but could decrease

380   the oral uptake of the substance, since *L. variegatus* burrow into the sediment and feed below the

381   sediment surface. 2) The characterization of nanomaterial should be carefully considered. Since

382   we do not currently have proper methods to characterize the sediment-associated nanomaterial,

383   characterization in this study was done in deionized water before spiking the nanoparticles into

384   the test media. Despite the fact that the characterization in water does not correspond to the

385   experimental conditions in the sediment, it is essential to assess the primary structure and

386   properties of the particles in standard conditions to add comparability between the studies. The

387   characterization of nanoparticles in the overlying water was not considered relevant, since AgNP

388   were spiked to the sediment by direct addition and were never present in the water phase. If the

389   indirect addition is used, the characterization in the overlying water should also be considered, as

390   the aggregation and dissolution of coated AgNP in the water phase is differently affected by the

391   presence of sediment (Bone et al. 2012; Unrine et al. 2012). In conclusion, there is an urgent

392   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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533

534

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

540   **Table 2** The characteristics of the test sediments. LOI% = Loss of ignition, OC% = organic

541   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)

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

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

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

545   [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*

	End Point	Compound	Sediment		
			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

FIGURES
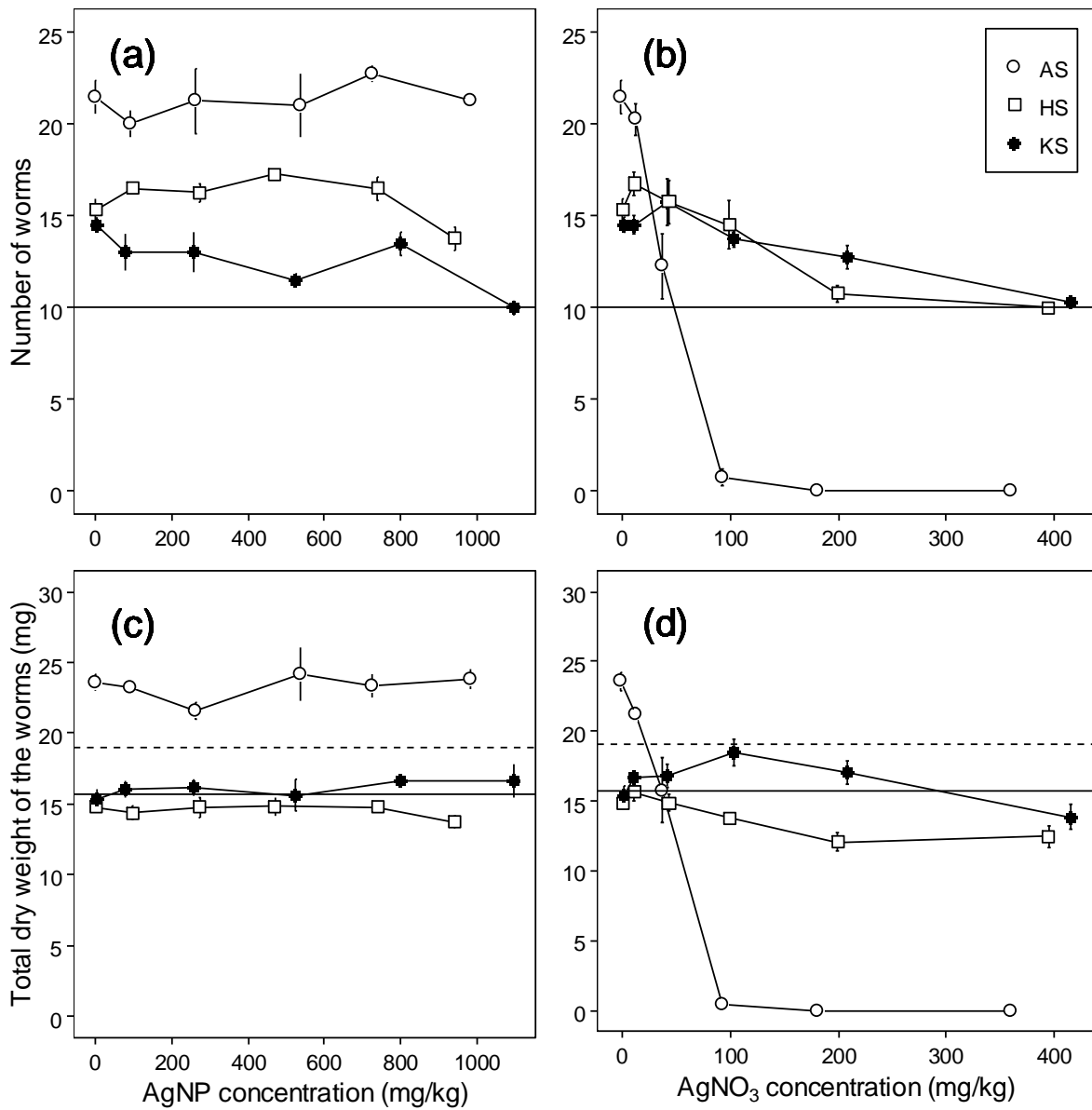
554 **Fig 1.** Toxicity of Ag spiked as silver nanoparticles (AgNP) and silver nitrate (AgNO$_3$) to

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

560 number of worms (10) at the beginning of the experiment. Solid line in (c) and (d) indicates the

561 starting dry biomass of the worms in the AS and HS sediments (15.69 mg), dashed line in the KS
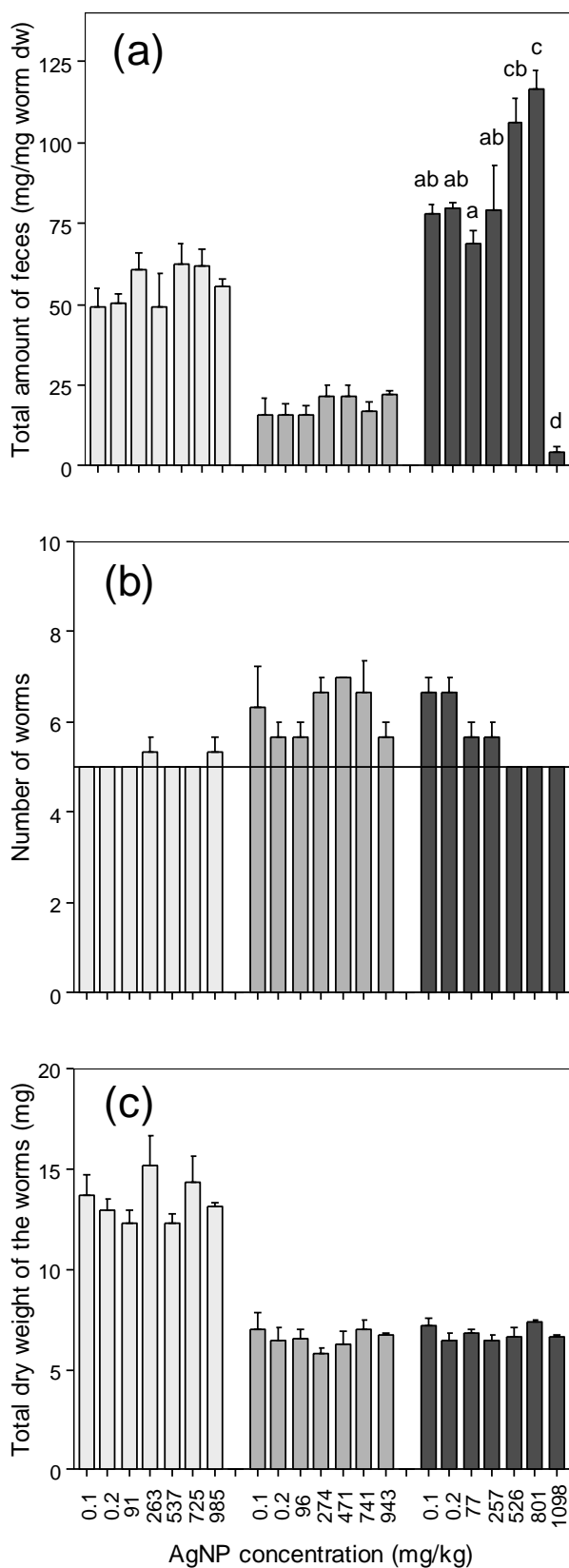
562 sediment (19.04 mg).

563

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.