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Chronic toxicity of silver nanoparticles to *Daphnia magna* under different feeding conditions

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Abstract

Despite substantial information on the acute toxicity of silver nanoparticles (AgNP) to aquatic organisms, little is known about their potential chronic effects and the applicability of current test guidelines for testing nanomaterials. The purpose of this study was to study the influence of food availability on toxicity. This was done through a series of *Daphnia magna* 21-day reproduction tests (OECD 211) using 30 nm citric acid stabilized AgNP aimed at studying the influence of food abundance on the reproductive toxicity of AgNP in *D. magna*. The experiments were carried out as static renewal tests with exposure concentrations from 10 to 50 µg Ag/L, and test animals were fed green algae *Pseudokirchneriella subcapitata* in low and high food treatments. The endpoints recorded were survival, growth of parent animals and number of live neonates produced. Detrimental effects of AgNP on survival, growth and reproduction were observed in concentrations higher than 10 µg Ag/L, whereas the animals exposed to 10 µg Ag/L had larger body length and produced more offspring than controls at both food treatments. High food treatment resulted in higher animal survival, growth and reproduction compared to result found for low food treatment.

Keywords – AgNP; Nanomaterials; Reproduction; Nanoecotoxicology

1. Introduction

Silver is used in a wide range of applications due to its antimicrobial properties, and in recent years the use of silver in nanoparticle form has dramatically increased (Moore, 2006). Data from year 2013 show that silver nanoparticles (AgNP) have become the dominant engineered nanoparticles used in consumer products (The Project on Emerging Nanotechnologies, 2013). The increasing use of nanosilver-containing products may lead to higher silver emissions into the environment, which raises concern about environmental implications (Elzey and Grassian, 2010, Hoheisel et al., 2012). Different forms of silver can have negative impacts on aquatic ecosystems by causing toxic effects to aquatic organisms (Shafer et al., 1998, Hoheisel et al., 2012). While a substantial number of studies describe the lethal toxicity of various forms of silver, including silver nitrate (AgNO_3) and silver nanoparticles (AgNP) (Allen et al., 2010, Hoheisel et al., 2012, Kennedy et al., 2010), the information on chronic toxicity to aquatic organisms is scarce. Also the mechanisms of nanosilver toxicity are poorly understood. There is an ongoing debate regarding the modes of toxicity and the contribution of silver ions to toxic effects (Poynton et al., 2012).

To the best of our knowledge, there are only four studies published regarding reproductive toxicity of AgNP to *D. magna* (Zhao and Wang, 2011, Pokhrel and Dubey, 2012, Gaiser et al., 2011, Blinova et al., 2013). Some papers state the necessity of chronic studies for better understanding of AgNP toxicity, but they also note that such studies are both time consuming and expensive (Gaiser et al., 2012, Zhao and Wang,

2011). Blinova *et al.* (Blinova et al., 2013) investigated how different coatings and test media affect AgNP toxicity, using polyvinylpyrrolidone (PVP) and collargol coated particles, and natural river and lake water as media. River water had a high content (35 mg C/L) of dissolved organic carbon (DOC). No sub-lethal effects were observed for both types of AgNP, but daphnids exposed to AgNP in lake water (5.3 mg C/L) showed significant decrease in reproduction rates at 100 µg/L. The varying toxicity is most likely attributed to the difference in DOC concentrations (Blinova et al., 2013), as DOC and other organic components in natural water can substantially influence silver speciation, toxicity and bioavailability to aquatic organisms (Bianchini and Wood, 2008, Ratte, 1999). Zhao and Wang (Zhao and Wang, 2011) also observed decrease in reproduction when daphnids were exposed to 50 µg Ag/L. They used carbonate coated AgNP in simplified M7 medium, and added cysteine to form complexes with Ag⁺ ions. As addition of cysteine did not lower the toxicity, Zhao and Wang attributed the reproductive effects found to silver in NP form (Zhao and Wang, 2011). Lower exposure concentrations of AgNP (1-2 µg Ag/L) have been reported to increase body length (Gaiser et al., 2011) and reproduction of daphnids (Pokhrel and Dubey, 2012).

The documented AgNP toxic effects show high interstudy variability, which may be partly explained by differences in particle size and surface functionality, test media or other parameters. For example, it has been reported that AgNP acute toxicity (48-h EC50) can be multiple times lower when DOC is present in the media (Blinova et al., 2013, Cupi et al., (in press)) or when food is applied during testing (Allen et al., 2010). In

addition, the influence of DOC content on AgNP chronic toxicity (21-d) was addressed in the study by Blinova et al (Blinova et al., 2013) showing a higher toxicity of AgNP with lower content of DOC. In the study by Allen et al. (Allen et al., 2010) food availability during AgNP exposure was found to alter results from acute toxicity testing. Variations in food abundance have been shown to markedly influence the fitness of *D. magna* in the absence of any toxicants (Martínez-Jerónimo et al., 1994, Porter et al., 1983). Overexploitation of resources, resulting from intraspecific competition, was observed to result in reduced survival, growth and fecundity (Miller, 1967). These resource-related mechanisms may contribute to periodic fluctuations in resource-consumer interactions that are frequently observed in both laboratory settings and natural systems (McCauley and Murdoch, 1987, McCauley et al., 1999). Fluctuations in consumer density can result in transient periods of low or high food availability that might interfere with AgNP toxicity.

The purpose of this study was, therefore, to elucidate the influence of food availability on AgNP toxicity to *D. magna*. We considered two food conditions: low food (as suggested by OECD guideline nr. 211 (Organisation for Economic Co-operation and Development, 2012)) and high food (three times the amount applied for low food conditions). The endpoints of interest were survival for 48 hour acute toxicity tests, and survival, cumulative offspring and growth of the parent daphnids for 21 day chronic toxicity tests. *D. magna* was selected as the test subject because it is very sensitive to silver (Ratte, 1999) and widely recognized as a well-established test organism in

ecotoxicology due to its ecological importance, ease of culturing, and short life span (Roemer et al., 2011). Along with other filter feeders present in zooplankton, it is an important link in the aquatic food web and can therefore have an influence on species at higher trophic levels (Baun et al., 2008).

2. Materials and methods

Organisms, medium, and chemicals

D. magna culture was maintained in modified Elendt M7 medium (with double amount of trace elements and ten times higher concentration of KCl compared to OECD recipe (Organisation for Economic Co-operation and Development, 2012)) and held in 1 L beakers at a density of one individual per 100 mL. The culture was kept in a 20 °C climate controlled room with 16:8 hour light:dark cycle. Animals in the stock culture were fed with green algae *Pseudokirchneriella subcapitata* three times a day by automatic pump. The algae for feeding were cultured in 20 °C and harvested when reaching a cell density of around 10^6 to 10^7 cells/ml. For acute and chronic tests that involved feeding the animals, the algal suspension was concentrated by settling at 4 °C for 3 to 4 days and the settled biomass was used for feeding after removing the excess medium. For chronic tests a fresh algal suspension was prepared weekly. The algal cell density was determined by Z2 Coulter Counter® (by Beckman Coulter) and the carbon content was calculated using the previously reported correlation where 10^4 cells/mL equals about 0.1 mg C/L (Halling-Sørensen et al., 1996). In all toxicity experiments, the

daphnids used were <24 hour old neonates (first brood was not used). Modified Elendt M7 medium was used for all tests and prepared according to OECD guideline 211 (Organisation for Economic Co-operation and Development, 2012).

Commercially available citric acid coated AgNP were purchased from Cline Scientific AB, Gothenburg, Sweden. The particles were suspended in Milli-Q water and had a reported diameter of 30 nm. The nominal concentration according to manufacturer was 20 mg Ag/L, which was confirmed by Inductively Coupled Plasma – Optical Emission Spectroscopy (ICP-OES) measurements. The particle suspensions were kept at 4 °C in the dark, and used in toxicity tests without prior sonication.

2.1. Chemical analysis and characterization

The total Ag concentration in the AgNP stock suspension and test suspensions before adding algae or daphnids was analyzed using ICP-OES (Varian Vista-MPX CCD simultaneous ICP-OES). The samples were digested in 16 % HNO₃ for 24-h at room temperature in the dark, then diluted with deionized water (DIW) and kept at room temperature in the dark until analysis.

Mass balance analysis was carried out for an exposure concentration of 40 µg/L by simulating the chronic test experimental setup described below. Five beakers for each food treatment were prepared, adding an adult daphnid (> 3 weeks old) to each beaker. After three days test animals were removed from the beakers with a mesh, dipped in

clean water and digested with concentrated nitric acid (65%). The content of the beaker was stirred and collected for analysis. The beakers were then carefully washed with concentrated nitric acid to remove the silver adsorbed to the glass. Samples were analyzed by ICP-OES.

The content of dissolved Ag was tested by preparing AgNP suspension in M7 medium, leaving it for 72h (to simulate the longest time between changing the media in the chronic test setup), and then separating particulate and dissolved Ag by ultracentrifugation (Beckman Coulter L8-60M). The suspensions were prepared in 6 replicates and spun at 3×10^4 rpm (154049 g) for 20 minutes. Subsequently, the supernatants were removed, acidified with nitric acid, and analyzed by ICP-MS (Agilent 7700x).

Before toxicity testing, the particle size and zeta potential in stock suspension and AgNP suspensions in M7 medium was characterized by ZetaSizer Nano ZS (by Malvern Instruments Ltd). Additionally, samples of AgNP stock solution and AgNP suspensions in M7 medium were examined by transmission electron microscope Tecnai T20 G² (by FEI). Samples were prepared by placing 4 μ L of suspension on a copper grid covered with carbon film (by Agar Scientific), which was then left at room temperature for around 4 hours for water to evaporate.

2.2. *Acute toxicity tests*

The 48 hour acute toxicity testing was carried out following the OECD Guideline 202 (Organisation for Economic Co-operation and Development, 2004) using freshly prepared, oxygen saturated M7 medium and pH ranging from 8.2 to 8.6. AgNO₃ was used as reference compound for the acute toxicity tests. Three 48 hour acute tests were performed with AgNP and *D. magna* (<24 h old): one with no feeding and, deviating from the guideline, two with feeding (low and high food levels – 2.4 × 10⁵ cells/mL and 7.2 × 10⁵ cells/mL, respectively). The exposure concentrations were 10, 25, 50, 75, 100, 150 and 200 µg Ag/L for the test with no added food, and 25, 50, 100, 150, 200, 250 and 300 µg Ag/L for tests with low and high food levels. The beakers were kept in a 20 °C temperature controlled room in the dark and immobility was recorded after 24 and 48 hours.

2.3. Chronic toxicity tests

Three chronic tests were performed – one with AgNO₃ as a reference test with low food conditions, and two with AgNP with low and high food conditions. The chronic tests were set up following the OECD 211 Guideline (Organisation for Economic Co-operation and Development, 2012). The test animals were < 24-h old *D. magna* neonates and all the test suspensions were prepared with freshly prepared, oxygen saturated M7 media with pH ranging from 8.2 to 8.6. During testing, animals were maintained individually, each in a 100 mL glass beaker with 50 mL of test suspension, each exposure treatment and control having 10 replicates. Exposure concentrations were 0.25, 0.5, 1.0 and 1.6 µg Ag/L for test with AgNO₃ and 10, 20, 30, 40 and 50 µg Ag/L for tests with AgNPs. Parent

animals were transferred to new beakers three times per week by using a clean plastic mesh. The beakers were kept in a 20 °C temperature controlled room with a 16:8-h light:dark cycle. The daphnids were fed every day using concentrated algae suspensions with amounts resulting in 2.4×10^5 cells/mL and 7.2×10^5 cells/mL concentrations for low and high food treatments, respectively.

Mortality of the test animals and production of live offspring was recorded daily. The neonates were removed from the test suspension to prevent them from consuming the food intended for the parent animal. Body length, excluding spina, was digitally measured under magnification on day 0, 5, 9, 14 and 21.

2.4. Data treatment

The 48 hour acute toxicity (48-h EC₅₀) and 95 % confidence intervals were calculated by using logistic curve fitting with inverse estimation, assuming a log-normal distribution (Christensen et al., 2009). For chronic toxicity, data were expressed as mean \pm standard deviation. Statistical analyses were carried out to determine the AgNP effects on growth, reproductive output and time to the first brood, as well as the effect of different food treatments. Data for growth and reproduction were checked for normal distribution with the Shapiro-Wilk method and Levenes' test for homogeneity of variances. If criteria were met, one-way analysis of variance (ANOVA) was used to compare the effects for different silver concentrations and two-way ANOVA for comparison of different food levels and silver concentrations. If data were not normally

distributed and had equal variances, they were rank-transformed (Akritas, 1990) before performing ANOVA. Data for time to first reproduction event were analyzed using Kaplan-Meier log-rank test. Significance for all statistical tests was assumed with $p < 0.05$. Only data from parent animals that survived until the termination of the test were used for the statistical analysis with the exception of the cumulative time to first reproduction analysis.

3. Results

3.1. AgNP characterization

The analysis of AgNP stock suspension using TEM (Figure 1), DLS and ICP-OES were consistent with the data provided by the manufacturer. Dilutions of AgNP stock suspension in Milli-Q water showed that with lower concentrations of AgNP the polydispersity index (PDI) was increasing (Table 1), which might be an indication for particle agglomeration and/or aggregation. Zeta potential measurements verified that the AgNP stock suspension was stable (-46.9 ± 1.3 mV), but diluted suspensions in M7 medium showed decreased stability. Unfortunately, good quality data from samples containing AgNP suspended in M7 medium could not be obtained, most likely due to rapid agglomeration caused by the high ionic strength of the medium. The concentration of dissolved Ag after 3 days in M7 medium was determined to account for $3.1 \pm 0.4\%$ of the nominal Ag content. Mass balance analysis revealed that after 3 days the actual silver concentration in the suspension was only $53 \pm 5\%$ and $70 \pm 6\%$ from the

nominal concentration for low and high food treatments, respectively. Measurements of silver content in daphnids were all below the quantification limit. Amounts corresponding to 11 ± 2 % (low food) and 8 ± 1 % (high food) of nominal content of silver was found on the glass beaker walls, and the remaining silver is left as unclassifiable loss. For the sake of simplicity, nominal silver concentrations have been used for all data analysis.

3.2. Acute toxicity of AgNP towards D. magna

Acute toxicity test with AgNO_3 yielded a 48 hour EC_{50} value of $0.75 \mu\text{g Ag/L}$ [95 % CI 0.61; 0.92], which is close to the values found in the literature (Bianchini et al., 2002, Kennedy et al., 2010, Allen et al., 2010). The concentration-response curves and the 48 hour EC_{50} values obtained in the acute toxicity tests with AgNP clearly showed that with more food provided the toxicity was decreased (Table 2). Daphnids exposed to AgNP in high food treatments had algal cells adhered to their exoskeleton, which resulted in some immobility in lower exposure concentrations, most likely due to the difficulties to swim around and moult.

3.3. Chronic toxicity of AgNP under different feeding conditions

The chronic test with AgNO_3 showed no significant effect ($p < 0.05$) on growth, reproduction per surviving female or days to first brood in any exposure concentration, but there was a clear effect on mortality (60 % mortality at the highest concentration of $1.6 \mu\text{g Ag/L}$). The study by Zhao and Wang (Zhao and Wang, 2011) has reported very

similar results, having no effects on growth and reproduction, but 50 % mortality at 1.6 $\mu\text{g/L}$. In our study the exposure to AgNP affected survival, growth and reproduction of *D. magna* with similar concentration-response patterns for both food treatments. In the case of low food treatment an increased mortality was observed for concentrations higher than 20 $\mu\text{g/L}$, whereas for high food treatment similar effects occurred at concentrations above 40 $\mu\text{g/L}$. Both growth and reproduction of parent *D. magna* was inhibited with increasing concentrations of AgNP (Table 3). Growth rate per surviving female is presented as difference in size of the parent animals from day 0 to day 21. As shown in Table 3, half of parent animals in controls had died at day 21. As both tests were performed simultaneously and animals were fed the same food, it is very likely that the increased mortality was due to a bacterial infection in the algal suspension used for feeding during the last days of testing. A bacterial infection could also explain why silver exposure in lower concentrations (10 and 20 $\mu\text{g/L}$) had somewhat beneficial effect on survival compared to control and that no increased mortality was observed for the exposed animals in the same time frame. However, there is a need for scientific studies to test the potential hormetic effect of low concentrations of silver. Regardless of the food treatment negative effects on growth occurred in concentrations above 10 $\mu\text{g/L}$, but it should be noted that high variations in growth rates of individual animals were observed within each exposure concentration. Both treatments also showed a slight increase in animal size at 10 $\mu\text{g/L}$ exposure, but it was statistically significant ($p = 0.004$) only for the low food treatment. The lowest concentration with a statistically significant ($p < 0.05$) inhibitory effect on growth was at 30 $\mu\text{g/L}$ for both food levels.

The growth rate of the live parent animals increased significantly ($p < 0.05$) in the high food treatment compared to low food treatment for controls and all exposure concentrations except for 30 $\mu\text{g/L}$ exposure (Table 3). The number of offspring produced per living parent daphnid (Table 3) showed concentration-response patterns similar to that of the growth rate data. However, the cumulated number of offspring per living parent *D. magna* at day 21 varied considerably between treatments and exposure concentrations. A significant difference in the cumulated number of offspring was noted at 40 $\mu\text{g/L}$ and 50 $\mu\text{g/L}$ in the low food treatment as well as at 30 and 50 $\mu\text{g/L}$ in high food treatment ($p < 0.05$). The cumulated number of offspring at 10 $\mu\text{g/L}$ was slightly higher than what was observed for the controls for both food treatments. However, due to the high variation between the individual parent animals, this difference was not statistically significant ($p > 0.05$).

The time to first reproduction showed some variation among individual animals within the different exposure concentrations and food scenarios. When analyzing time to first brood for daphnids surviving until day 21, on average a slight non-significant increase ($p > 0.05$) was observed under low food conditions (Table 3). It should however be noted that the *D. magna* mothers included in this analysis had produced at least one clutch of live neonates during the exposure period, but there were also a few parent daphnids that survived until the end of the test without producing any offspring. When including the parent animals that did not survive until the termination of the test as well as those that were alive but did not reproduce during the test, the results provide an indication

of how many daphnids were able to reproduce in a given treatment and AgNP exposure. Cumulative frequencies in the number of reproducing females over time were lower in the low food treatment compared to the higher food treatment (Figure 2). Furthermore, the cumulative frequencies decreased with increased exposure concentration from 20 $\mu\text{g/L}$ and 50 $\mu\text{g/L}$ in low and high food, respectively (Figure 2).

4. Discussion

The acute toxicity tests showed that the addition of food to *D. magna* tests can have a pronounced influence on toxicity of AgNP. The 48 hour EC50 values were approximately 4-fold and 6-fold higher for low and high food treatments, respectively compared with the EC50 obtained without addition of food. This is in agreement with the findings by Allen *et al.* (Allen et al., 2010) who also found an almost 6-fold increase in 48 hour LC50 value for *D. magna* when food was supplied during AgNP exposure. In addition, this study showed that higher food availability counteracted the observed effect on survival, growth and reproduction of *D. magna* when exposed to AgNP under low food conditions. Plausible explanations for reduced AgNP toxicity might be decreased exposure or decreased uptake of AgNPs or Ag ions when algal food is present. Allen *et al.* (Allen et al., 2010) proposed that AgNP could form complexes with organic matter and thus become unavailable for uptake. However, the decreased toxicity might also be a result of complexation between the organic material in the medium and ionic silver released from the AgNP (Allen et al., 2010). This is also supported by the fact that *P.*

subcapitata, which was used as food in this study, is known to produce exudates, which can considerably decrease toxicity of metals by complexation (Koukal et al., 2007). Moreover, experiments have shown that AgNP can adhere to algal cells (Navarro et al., 2008), which may lead to lower amounts of AgNP remaining in suspension, as a fraction of algae supplied to the test setup were sedimenting on the bottom of the beaker, making it less available for uptake by a predominantly pelagic species such as *D. magna*. Zhao and Wang (Zhao and Wang, 2010) have reported that ingestion of AgNP which are adsorbed to algae accounts for more than 70 % of silver that accumulates in *D. magna*. This indicates that food is a very important route of uptake for AgNP in daphnids. However, in our study the higher abundance of food was found to decrease toxicity thus suggesting a lower availability of particles adsorbed to algae, most likely due to the observed sedimentation of algae during incubation. In addition, daphnids may adjust their food intake rate with environmental food availability (Pop, 1991, Lampert, 1994, Plath, 1998). Increased filtration rates in the absence of food or under conditions of low food availability may thus lead to increased uptake of AgNP, either as agglomerates or attached to algal particles, into the gut.

In general, the two chronic tests performed in this study showed that increasing concentrations of AgNP affect survival, growth and reproduction of *D. magna*. Similar to our findings for daphnids exposed to 10 µg Ag/L, Gaiser *et al.* (2011) found an increase in body length at a concentration of 1 µg Ag/L. Various hypotheses have been proposed to explain this phenomenon, for example that the surviving *D. magna* may be the fittest

and therefore more likely to grow larger, or that an adaptation/tolerance of the daphnids to metal exposure includes metabolic changes favoring growth (Gaiser et al., 2011). Higher AgNP exposure concentrations lead to reduced growth and reproduction in a dose-response manner. Decreased cumulative offspring was observed also in studies by Zhao and Wang (2011) and Blinova *et al.* (2013) at AgNP exposures of 50 and 100 $\mu\text{g/L}$, respectively. Reduced reproductive output can be partly ascribed to reduced growth as egg numbers depend on the size of parent daphnids (Blinova et al., 2013). However, this can probably not explain the full magnitude of reproductive decline observed in our experiments and additional effects on reproduction have to be taken into account.

Cumulated number of females reproducing (Figure 2) illustrates that both AgNP exposure and food abundance significantly influence the daphnids' ability to survive and reproduce. At low food conditions, daphnids exposed to AgNP can still survive, but do not produce any offspring at the highest AgNP concentration (Table 3). In contrast, daphnids exposed to the same AgNP exposure concentrations in the high food treatment were still able to reproduce even at 50 $\mu\text{g/L}$, indicating that higher food availability may benefit population fitness under AgNP exposure. Surviving individuals that are not able to reproduce can be considered dead in an ecological sense, since they will not contribute to population sustainability or growth. Obviously, when either reproduction or juvenile survival ceases completely a migrationally isolated population will become extinct regardless of the effect on other individual life history traits (e.g.,

(Linke-Gamenick et al., 2000)). When juvenile mortality and reproductive inhibition is less than 100 %, the relative importance of changes in different individual life-history traits (e.g., caused by contaminants) on population growth and sustainability varies with e.g., lifecycle type. This makes it difficult to predict population level impacts of contaminants directly from individual level effects without integrating observed effects on life history traits into a population model. However, in a study by Forbes *et al.* (2010) the authors showed that for *D. magna* fecundity was consistently (i.e., in 26 out of the 26 selected cases) the least elastic life history trait. This means that even relatively large effects on reproductive output had relatively low impacts on population growth rate, whereas the opposite was the case for juvenile survival.

To the best of our knowledge, this is the first study to present AgNP chronic toxicity depending on food availability. However, the question regarding effects of food in the absence of toxicants has already been addressed in the literature by conducting experiments that were lasting the whole life cycle of *D. magna* with different food levels (Martínez-Jerónimo et al., 1994). The life span of *D. magna* is up to 100 days under favorable conditions (Preuss et al., 2009). Martínez-Jerónimo *et al.* (1994) showed that higher amounts of food supplied led to increased growth and reproduction rates. They also noted that animals exposed to high food abundance died earlier than the ones exposed to medium food treatment. The ultimate size and cumulative reproductive output ended up being the same for both food treatments. That raises the question whether 21 days are enough to reveal the actual chronic toxicity of AgNP. Performing

toxicity tests that cover the whole life cycle of *D. magna* would provide results that could possibly reveal whether the ultimate size and cumulative reproductive output change with different food treatments under AgNP exposure. However, a major drawback for conducting a whole life cycle experiment is that it is very time consuming and resource demanding.

5. Conclusion

This study aimed to investigate what effects food availability may have on chronic toxicity of AgNP to *D. magna*. For the two 21 day tests a dose-response relationship was observed for mortality, growth and reproduction. The high food treatment lowered observed detrimental effects of AgNP on animal survival, growth rate and reproductive output in comparison to low food treatment, likely due to alterations in exposure or toxicokinetics depending on food density. This study has shown that there are perspectives for future research to obtain better understanding about chronic toxicity of AgNP. For instance, additional information on bioaccumulation of AgNP in *D. magna* would be needed to understand various toxic effects.

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TABLES AND FIGURES

Table 1: AgNP hydrodynamic diameter (nm) and suspension stability (zeta potential, mV) in Milli-Q water, M7 medium, and undiluted stock suspension (n = 3). HDD: Hydrodynamic diameter; Pdl: Polydispersity index.

Table 2: Food treatments (*P. subcapitata*) and 24 and 48 hour EC50 values for AgNP acute toxicity tests with *D. magna*. 95 % confidence intervals are shown in brackets.

Table 3: Exposure concentrations and toxicity endpoints for *Daphnia magna* after exposure to different food levels for 21 days. Mean \pm standard deviations are shown for living animals (initially 10 animals were used for each concentration and controls).
*Significantly different from control ($p < 0.05$); † Significantly different from high food level ($p < 0.05$).

Figure 1: TEM image of AgNP stock solution.

Figure 2: Cumulative frequencies for *D. magna* that were reproducing as a function of exposure time for different concentrations of AgNP. Low food: Approximately 2.4×10^5 cells/mL; High food: Approximately 7.2×10^5 cells/mL.

Table 1:

	Conc. ($\mu\text{g Ag/L}$)	HDD by volume (nm)		Pdl	Zeta potential (mV)
		Peak 1	%		
Stock	20000	34.0 \pm 1.2	100	0.145 \pm 0.035	-46.9 \pm 1.3
Milli-Q	1000	35.8 \pm 0.2	100	0.192 \pm 0.010	-55.2 \pm 1.9
	100	34.5 \pm 0.5	100	0.173 \pm 0.003	-27.4 \pm 5.1
	50	33.6 \pm 0.7	98	0.250 \pm 0.034	-26.9 \pm 11.6
	10	33.8 \pm 4.7	94	0.243 \pm 0.055	-15.7 \pm 3.7
M7	100	55.4 \pm 4.5	100	0.180 \pm 0.011	-8.56 \pm 0.76
	50	48.2 \pm 2.9	100	0.248 \pm 0.006	-13.3 \pm 0.23
	10	44.5 \pm 1.2	100	0.487 \pm 0.004	-6.72 \pm 1.5

Table 2:

Treatment	Food (cells/mL)	24h EC50 ($\mu\text{g Ag/L}$)	48h EC50 ($\mu\text{g Ag/L}$)
No food	-	61.2 [61.2;61.2]	47.6 [47.6;47.6]
Low food	2.4×10^5	200 [197;204]	184 [178;191]
High food	7.2×10^5	304 [297;313]	302 [210;436]

Table 3:

Food treatment (cells/mL)	AgNP conc. ($\mu\text{g Ag/L}$)	Survival (%)	Time to first brood (d)	Cumulative offspring per living parent	Growth from day 0 to 21 (mm)
2.4×10^5 (Low)	Control	50	8.6 \pm 0.7	82.0 \pm 23.2	2.91 \pm 0.18 [†]
	10	60	8.6 \pm 0.9	110.5 \pm 18.0	3.18 \pm 0.12 ^{*†}
	20	80	9.7 \pm 1.5	58.0 \pm 39.1 [†]	2.68 \pm 0.47 [†]
	30	50	9.8 \pm 0.5	35.6 \pm 32.6	2.61 \pm 0.18 [*]
	40	20	11 [*]	1.0 ^{*†}	2.62 ^{*†}
	50	20	-	0 [*]	2.27 ^{*†}
7.2×10^5 (High)	Control	50	8.5 \pm 0.9	136.0 \pm 26.3	3.48 \pm 0.21
	10	80	7.8 \pm 0.9	153.6 \pm 28.4	3.64 \pm 0.20
	20	100	9.0 \pm 1.6	121.6 \pm 21.1	3.36 \pm 0.09
	30	90	9.8 \pm 2.0	73.6 \pm 22.5 [*]	2.82 \pm 0.20 [*]
	40	90	8.8 \pm 1.4	100.6 \pm 27.5	3.12 \pm 0.13 [*]
	50	40	9.4 \pm 1.7	31.5 \pm 29.1 [*]	2.94 \pm 0.17 [*]

Figure 1:

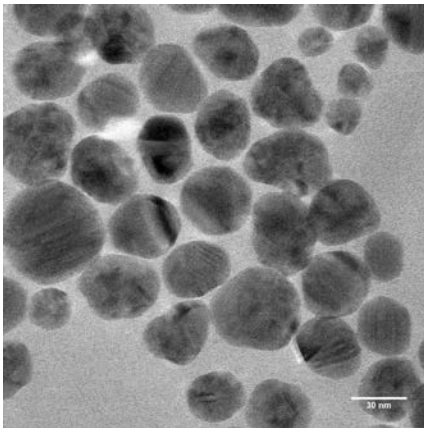


Figure 2:

