Growth inhibition in Raphidocelis subcapita – evidence of nanospecific toxicity of silver nanoparticles

Merethe Kleiven[a*], Ailbhe Macken[b], Deborah H. Oughton[a]

[a] *Norwegian University of Life Sciences, Center for Environmental Radioactivity, P.O.Box 5003 NMBU, 1432 Ås, Norway*

[b] *Norwegian Institute for Water Research (NIVA), Gaustadalléen 21, N-0349 Oslo, Norway*

[*] Address correspondence to *merethe.kleiven@nmbu.no*

ABSTRACT

Silver, known for its antibacterial properties and for its toxicity to aquatic organisms, is one of the most frequently used nanomaterials and silver nanoparticles can be found in a range of consumer products as well as medical applications. The present study investigated the toxicity of three different nanomaterials (Mesosilver, NM300K and NM302) and $AgNO_3$, in the algae *Raphidocelis subcapitata.* Exposures in the low µg L^{-1} range were combined with characterization of exposure media to determine whether differences in toxicity could be linked to changes in Ag speciation. All tested Ag compounds, except the NM302 Ag rods, reduced growth in the following order $AgNO_3$ ≥ M-Ag > NM300K > NM302 with 50 % effect concentrations of 7.09 (3.83-10.52), 9.7 (range not calculated) and 24.18 (15.66-98.16) µg L^{-1}, for $AgNO_3$, Mesosilver and NM300K, respectively. Characterization of exposure media showed that both concentration and time influenced the speciation and stability of Ag in test media, regardless of Ag source, and also affected the toxicity to *R. subcapitata*. In both $AgNO_3$ and Mesosilver exposure the toxicity was correlated with the presence of Ag(I) (< 10 kDa), however levels of Ag(I) were too low to account for the observed Mesosilver effects, indicating a nanospecific contribution. Nanospecific toxicity was also observed for NM300K after 24 h of exposure, however the algae population recovered over time probably due to changes in exposure caused by aggregation of the nanoparticles.

Key words: Nanosecific,Toxicity,Silver, Algae

1. INTRODUCTION

The antimicrobial properties of silver have been known for centuries, and is the reason why silver nanoparticles (AgNPs) are amongst the most frequently used nanomaterials on the market (Vance et al., 2015). In addition to medical applications (e.g. wound dressings, surface coatings of medical devices), AgNPs are increasingly being used in consumer products, for example in cosmetics, cloths, cleaning agents, and food additives (Echavarri-Bravo et al., 2017). With the increase in AgNP applications follows an increased risk of environmental release of AgNPs and their transformation products (e.g. Ag_2S and AgCl) potentially posing a risk to biota (Ribeiro et al., 2015).

The toxicity of Ag in aquatic environments is well-documented (Ratte, 1999), and AgNPs have been reported to induce toxicity to a range of different organisms: bacteria (Fabrega et al., 2009, Echavarri-Bravo et al., 2017), algae and invertebrates (Ribeiro et al., 2015, Sørensen and Baun, 2015), and fish (Chae et al., 2009, Bruneau et al., 2016). However, despite the attention nanoparticle toxicity has received in the last decades there still are uncertainties regarding toxicity mechanisms, particularly whether it is nanospecific (caused by the nanoparticles themselves), caused by the release of ions, or a combination of the two (Fabrega et al., 2011, Sendra et al., 2017). This will depend on the type of particle and its physicochemical properties (e.g. size, surface charge, coating) as well as exposure conditions (e.g. media composition, pH, temperature, conductivity), and highlights the importance of exposure characterization throughout the experimental test period.

As primary producers, algae play an essential role in aquatic ecosystems (Ribeiro et al., 2015, Wang et al., 2016), and alterations in these communities are likely to also influence species in higher tropic levels, and thus potentially the whole ecosystem (Ribeiro et al., 2015). In the present study the freshwater green algae *Raphidocelis subcapitata*, a commonly used species in regulatory testing and a key constituent in aquatic systems, were exposed to three different AgNPs (NM300K, Mesosilver, NM302), as well as $AgNO_3$. The nanomaterials represent different sizes, shapes and stabilizing agents, as well as including the OECD representative nanomaterials (NM300K, NM302) and a commercial cosmetic product (Mesosilver skin conditioner). The objective of the current study was to try to link differences in toxicity between the tested Ag compounds to the exposure characteristics obtained through thorough exposure characterization over time. We hypothesized that aggregation and changes in concentrations of dissolved Ag(I), which was expected to be the active component, would be the main factors influencing toxicity.

2. MATERIALS AND METHODS

2.1. Preparation and characterization of silver suspensions

The nanomaterials used in this study were OECD representative Ag nanomaterials, specifically NM300K and NM302 (provided by the Joint Research Centre Reference Nanomaterial Repository, Ispra, Italy). Both nanomaterials were supplied as aqueous dispersants, the NM300K with 4 % Polyoxyetylene Glycerol Trioleate and Tween 20, with a total Ag content of 10.16 % (w/w) and the NM302 in a dispersion with the additives Rheology modifiers (≤ 2 wt%), polymers and surfactants (≤ 1 wt%) and a total Ag content of 7.4 wt%. In addition, a commercial silver colloidal suspension, Mesosilver (M-Ag NP) (Purest Colloids, Inc, Westampton, NJ, USA) was used, and finally silver nitrate (p.a. quality, Sigma-Alrich) was included as a positive control, a reference for dissolved silver toxicity.

The NM300K and NM302 stock suspensions were prepared according to Jensen et al. (2016). Briefly, a 2.56 g Ag L^{-1} stock suspension was prepared by dispersing the original suspensions in MilliQ water (18 MΩ·cm) and sonicating for 10 min at 10 % amplitude using a 400 Watt Branson Sonifier S-450D (Branson Ultrasonics Corp., Danbury, USA) equipped with a standard 13 mm disruptor horn (Model number: 101-147-037). The M-Ag NP, delivered as a colloidal suspension from the manufacturer with a concentration of 20 mg L^{-1}, was used without any sonication. A 1 g L^{-1} stock solution of $AgNO_3$ was prepared in MilliQ water. Intermediate stock suspensions of 50 µg Ag L^{-1} were prepared from the main stock suspensions of NM300K and NM302, while for $AgNO_3$ an intermediate stock solution of 10 Ag mg L^{-1} was prepared. These intermediate stock solutions were used to prepare the exposure suspensions by direct addition.

Particle size of the AgNPs in stock suspensions were obtained by transmission electron microscopy (TEM) and dynamic light scattering (DLS). Characterization of exposure suspensions was conducted with DLS and size fractionation with respect to Ag coupled with inductively coupled plasma mass spectrometry (ICP-MS, Agilent 8800) for measurements of Ag concentrations.

2.1.1. Transmission electron microscope

The three AgNP stock suspensions were added (10 µl) to a 400-mesh Cu coated Piloform film (Agar Scientific, Essex, UK) and the specimens were left to evaporate overnight in the dark. The images were acquired, on a FEI Morgani 268 transmission electron microscope (FEI, Eindhoven, Netherlands) operating at 80 keV. The images were analyzed with the software Adobe Photoshop CS5 to obtain the average particle size.

2.1.2. Dynamic light scattering

Particle size measurements were performed on a Malvern Zetasizer ZS (Malvern instruments Ltd, Worcestershire, UK) equipped with a laser source with wavelength 633 nm. The Zeta-averaged

hydrodynamic diameters and size distributions was obtained for the main stock suspension of AgNPs (2.56 g L^{-1} for NM300K and NM302, 20 mg L^{-1} for M-Ag), as well as intermediate stocks and exposure suspensions at 0, 24, 48, and 72 h. The measurements were performed on samples pre and post centrifugation (4000 rpm, 10 min) conducted to remove the algae from suspension.

2.1.3. Total Ag concentrations and size fractionation

To obtain information on Ag concentrations and size fractions present in exposure suspensions over time, membrane filtration followed by ICP-MS were performed. For determination of total Ag concentrations samples were collected from the exposure suspensions prior to addition of algae at time zero, and then at 24, 48, and 72 h of exposure. For the samples collected during the exposure (24-72 h) an additional sample was collected for determination of total Ag concentration after algae was removed by centrifugation at 4000 rpm for 10 min, prior to fractionation and analysis. After removal of the algae, a size fractionation with respect to Ag was conducted by membrane filtration with a cut-off of 0.22 µm (membrane syringe filter, Millipore) and an ultracentrifugation filter (centrifuged at 5000xg for 15 min) with a 10 kDa cut-off (Amicon Ultra-15 centrifugal filters). The size fraction > 0.22 µm was defined as particulate, fraction < 0.22 µm and > 10 kDa was defined as nanoparticles/colloidal, while the fraction < 10 kDa was defined as low molecular mass (LMM) and assume to be dissolved Ag. All fractionated water samples for Ag analysis were acidified with 10 vol % ultrapure HNO_3. All samples were then stored in the dark at 4 °C prior to ICP-MS measurements (ICP-MS, Agilent 8800).

2.2. Test organisms, culture preparations and growth inhibition test

The freshwater algae *Raphidocelis subcapitata* (NIVA strain CHL 1 from The Norwegian Culture Collection of Algae, NORCCA, owned by NIVA, Oslo, Norway) was exposed to three different Ag NPs (NM300K, NM302, and mesosilver (M-Ag NPs)) and $AgNO_3$. The tests were conducted according to the OECD 201 (OECD, 2011), with slight modifications according to Cerrillo and Mendoza (2015).

2.2.1. Algal growth inhibition test

The algae were cultivated in 250 ml Erlenmeyer flasks (100 ml culture volume), capped with air permeable cellulose stoppers allowing gas exchange. Both pre-culturing and the experiments were conducted under test conditions: at 23 °C and at an illumination 60 µE m^{-2} s^{-1} (cool fluorescent light). The flasks were continuously shaken at 90 rpm. The duration of the tests were 72 h. The pH was measured at the beginning and end of the test. The algae were pre-cultured in OCED 201 media for 3 days before being used in the test, ensuring algae were growing exponentially. Exposure media was a modified OCED medium without Fe-EDTA. The initial algal density of all test vessels was $5x10^6$ cells L^{-1} (density demined by a cell counter, Coulter counter) in a final volume of 1 ml.

Preliminary range finding tests were conducted with all compounds to determine the range of concentrations to be used in the definitive tests. Based on these results five concentrations, arranged in geometrical series were selected for each compound. Six replicates of controls, containing only medium and algae, were included in each test together with three replicates of each exposure concentration. An additional two replicates for each concentration were added for exposure characterization after 24 and 48 h. The nominal exposure concentrations were in the range of 0.32 to 32 µg Ag L^{-1} for $AgNO_3$, 5 to 50 µg Ag L^{-1} for M-Ag NPs, 2.56 to 25.6 µg Ag L^{-1} for NM300K, and 0.26 to 25.6 mg Ag L^{-1} for NM302. In addition to the control group, a dispersant control was included for the NM300K and NM302 toxicity tests to assess the potential negative/positive effects of the dispersant used to stabilize these two nanomaterials. The concentration of dispersants was equal to the concentration present in the highest exposure concentrations of NM300K and NM302, and was found not to induce any toxicity.

The toxicity endpoint investigated was growth inhibition as a response to exposure to different silver nanomaterials and silver nitrate. The growth/growth inhibition was quantified by measuring the algal biomass as a function of time. This was conducted by chlorophyll-a-extraction in accordance to the method specified in Mayer et al. (1997). In short, 1 ml of all exposure suspensions were sampled into individual foil-wrapped plastic tubes, 0.1 ml Locust Bean Gum suspension (30 mg in 20 ml H_20), and 4.4 ml acetone (100 % with magnesium carbonate) were added to each tube. Samples were mixed well and stored in the dark at room temperature until the next day, prior to determination of chlorophyll concentration with a fluorescence spectrophotometer (Agilent Technologies. Cary Eclipse Fluorescence Spectrophotometer), excitation wavelength 430 nm and emission wavelength of 670 nm.

The growth inhibition was calculated by converting the obtained fluorescence values into algae biomass by the means of a calibration curve. The calibration curve was obtained by measuring three replicate algae inoculums with four different algae densities ranging from $3x10^3$ to $5x10^5$ cells ml^{-1} both with a cell counter (Coulter counter) and with the fluorescence spectrophotometer. The obtained calibration curve had a R^2 of 0.99.

The reference substance $K_2Cr_2O_7$ was employed as a positive control and followed the same procedure as described above (SOP reference).

2.3. Statistical analysis

The statistical testing of growth inhibition was performed with GraphPad Prism 6 (GraphPad Software, La Jolla, CA 92037, USA). Statistical analysis was carried out using a one-way analysis of

variance followed by a Tukey-Kramer means comparison test to identify significant differences compared to the controls. Statistical significance was accepted at *p* 0.05.

The EC10 and EC50 (concentration that elicits an estimated 10 and 50 % toxic effect) values for all compounds were calculated using REGTOX-EV7.0.6.xls (Eric Vindimian http://eric.vindimian.9online.fr), a curve fitting macro for Microsoft Excel. Toxicity data for all compounds were fitted to a sigmoidal curve and either the Weibull or Hill models were used to calculate the effective concentration (EC) values.

3. RESULTS AND DISCUSSION

3.1. Particle characterization

3.1.1. TEM

The M-Ag and NM300 Ag NPs were both spherical (Table 1, Figure S1 a and b) and had a primary particle size of 11±3 (n=425) and 16±5 (n=383) nm, respectively. The NM302 Ag nanomaterial mainly contained long rod-shaped Ag NPs (µm range), measuring 176±41 nm (n=30) in their smallest dimension (Table 1, Figure S1 c). Aggregates/agglomerates exceeding the size range of the primary particles were observed for all three nanomaterials. The particle sizes obtained for NM300K and NM302 are both according to the sizes given by the manufacturer (15 nm and 100-200 nm thick for NM300K and NM302, respectively). For M-Ag NPs the obtained particle size was larger than expected based on the manufacturers information (0.6 nm). The most probable cause for this discrepancy is an overestimation in the size measurements, due to the difficulties of measuring the size of the smallest particles present in the TEM images.

3.1.2. DLS

The hydrodynamic particle sizes obtained for the stock suspensions are reported in Table 1. The Z-averaged particle sizes are generally larger than particle sizes obtained by TEM, according to expectations. The Number mean particle sizes were in accordance with the manufactures specifications of 0.6 and 15 nm for M-Ag and NM300K, respectively, and for NM300K also with the average particle size obtained with TEM (Table 1). Dynamic light scattering was not a suitable method for size characterization of the NM302 Ag NPs due to their rod shape, as well as their rather large size and instability in suspension (sedimentation of the particles). For polydisperse samples or samples with distinct particle size populations, DLS tend to overestimate the mean particle due to the masking of smaller particles intensity reflection by the relative higher intensity reflected by larger particles (Handy et al., 2008a). The discrepancy between the Z-averaged particle size, the

Number mean and TEM results observed for the stock suspensions in the present study can be explained by this artefact.

As expected, the presence of algae in the exposure measurements heavily influenced the particle size measurements obtained by DLS of exposure suspensions, and DLS was not able to give a reliable Z-averaged particle diameter. Centrifugation of the samples to remove the algae did not improve the obtained results (s.d., and PDI did not decrease) (Table S1). As was seen in stock solutions, the Number mean particle size was considerably smaller than the Z-averaged particle size. However, low particle concentrations result in increasing measurement uncertainties, thus making DLS for particle characterization in exposure suspensions with low exposure concentrations (μg L^{-1}) challenging.

The Zeta potential for the M-Ag and NM300K stock suspensions (Table 1) indicate different stabilizing mechanisms for the two particles. The M-Ag seem to be electrostatically stabilized, which is in accordance with the findings of Echavarri-Bravo et al. (2017). The NM300K Ag NPs are known to be sterically stabilized through the adsorbance of non-ionic surfactants. The Zeta potential obtained for the NM300K AgNPs in the current study were close to zero and thus confirming the lack of electrostatic stabilization. These results agree with Lodeiro et al. (2017) and Kleiven et al. (2018) which both reported Zeta potentials for NM300K in MQ water to be slightly negative, but close to zero. However, other studies have reported zeta potentials of -22±3 (Echavarri-Bravo et al., 2017) and -15 mV (Hund-Rinke et al., 2017), albeit at different concentrations and preparation methods.

Table 1. Characterization of stock suspensions of the three Ag nanomaterials tested (Mesosilver (M-Ag), NM300K, and NM302) measured by TEM and DLS. Results provided as mean ± one standard deviation. NA: Not applicable.

	Stock concentration	TEM diameter (nm)	Z-Average diameter (nm)	Number mean diameter (nm)	Polydispersity index	Zeta potential (mv)
M-Ag	0.020 g L^{-1}	11±3 (N= 425)	38.3±0.3	1.0±0.4	0.6±0.04	-39±2
NM300K	2.56 g L^{-1}	16±5 (N=383)	73.6±0.5	22±16	0.284±0.006	-0.2
NM302	2.56 g L^{-1}	176±41 (N=30)	NA	NA	NA	NA

3.2. Exposure concentrations and size fractionation

Concentration and time were important factors influencing the speciation and stability of Ag in test media, regardless of Ag source, thus potentially also affecting the toxicity to *R. subcapitata*.

Measured total Ag concentrations (Table S2) at the beginning of the exposure were between 70 and 90 % of nominal concentrations in the $AgNO_3$ (0.3±0.05, 0.7±0.06, 2.4±0.6, 8±1, and 25±2 µg Ag L^{-1}) and NM300K (2.0±0.43, 3.7±0.7, 7±1, 14±3, 24±6 µg Ag L^{-1}) exposures. In the M-Ag exposures, measured concentrations were close to nominal (5.4±0.1, 10.7±0.6, 19±0.0, 35±1, and 53±2 µg Ag L^{-1}). In the NM302 Ag NP exposures the measured concentrations were far from the nominal, ranging from 79-250 µg Ag L^{-1} (at maximum 30 % at the highest concentration), and sedimentation of the NPs could be observed in the exposure vessels. Therefore, all exposure concentrations are hereafter reported as measured concentrations.

A decrease in total measured Ag in the test media (i.e., the total sample including algae) was observed for all exposures over the 72 h duration of the test (Table S2), which has also been reported in other studies with AgNPs (Echavarri-Bravo et al., 2015, Ribeiro et al., 2015). For $AgNO_3$ and M-Ag the decrease was concentration dependent, with only a 10 % decrease in the highest concentrations. While NM300K showed no concentration dependence, with an average concentration decrease of 50±10 %.

There is general agreement that the speciation of Ag is more important for the toxicity than the total concentration of Ag (Ratte, 1999, Köser et al., 2017). Thus, size fractionation with respect to Ag was performed for all exposures to assess the change in Ag size fractions over time. The trend was the same for all exposure groups and all exposure concentrations, with aggregation and complexation resulting in a shift towards larger particulate matter over time (Figure 1). This pattern of aggregation over time has also been reported in studies on other organisms (Farmen et al., 2012, Kleiven et al., 2018).

In the highest $AgNO_3$ exposure concentration (25 µg Ag L^{-1}) 98 % (24 µg Ag L^{-1}) of the Ag was present as LMM Ag at the beginning of the exposures (Figure 1). However, the LMM fraction decreased in a concentration dependent manner with a decrease in the percentage found as LMM as the initial concentration decreased, and also a relatively lower reduction over time as the initial exposure concentration increased. After 72 h, LMM Ag was only detectable in significant quantities in the two highest exposures concentrations (12 and 1 µg L^{-1} in the 25 and 8 µg Ag L^{-1} exposures, respectively).

In the M-Ag exposures NPs/colloids was the main Ag fraction present at time zero, however LMM Ag also constituted on average 33±2 % of the total measured Ag (Figure 1, Table S2), although given the range seen in the Number Mean diameter, this fraction could have included small nanoparticles. The M-Ag exposures were relatively stable over time, with the exception of the two lowest concentrations (5.4 and 10.7 µg L^{-1}) where larger particles (> 220 nm) increased with time. Farmen et al. (2012) exposed Atlantic salmon (*Salmo salar*) to this Ag NP in a low ionic strength lake water, and also observed a relatively stable NP/colloidal Ag fraction over time (48 h) with a reduction in the LMM Ag as the most significant change in exposure.

Close to 100 % of the Ag in the NM300K exposures was present as NPs at time zero, independent of concentration. With time, aggregation occurred and the particulate Ag (>220 nm) increased from zero to 80-90 % after 72 h (Figure 1). Interestingly, the LMM Ag (< 10 kDa) also increased slightly over time in a concentration dependent manner (ranging from 0.005 to 0.788 µg L^{-1} in the lowest to the highest exposure concentration) (Table S2). Köser et al. (2017) investigated the stability of the NM300K Ag NPs in different ecotoxicity media, including the OECD media used in this study. They found that the NM300K had a high dispersion and redox stability with little aggregation or dissolution over time (72 h). The dissolved Ag was reported to be 1-2 % (total Ag concentration of 7.9 mg L^{-1}) which is in accordance with the findings reported in the current study. However, Köser et al. (2017) did not observe any further increase in dissolved Ag over time (3 days), nor any evidence of aggregation. This was contradictory to the current study where an increase in dissolved Ag (LMM Ag) (from 1 % to 8 %) was detected in the two highest exposure concentrations, plus evidence of aggregation. However, both the differences in start concentrations (orders of magnitude higher in Köser et al. (2017) than in the current study), plus the removal of Fe-EDTA from the media used in the current study and the presence of algae, could explain the differences seen between the two studies.

To assess the association of the Ag to the algae, either by adsorption or accumulation, the algae were removed by gentle centrifugation. For $AgNO_3$ and M-Ag the relative fraction of Ag associated to the algae increased with decreasing Ag concentration (Figure 2). For NM300K the association to

algae seemed to be independent of exposure concentration (Figure 2). However, after 72 h larger particulate matter > 220 nm was the dominant fraction in the lower concentrations of $AgNO_3$ and M-Ag, as well as in all concentrations of NM300K, thus the results could reflect a co-removal of larger particles with the algae during centrifugation rather than association to the algae, or a combination of the two. In the higher exposure concentrations of $AgNO_3$ and M-Ag, where the colloidal/NP and LMM Ag remained the dominant fractions after 72 h, the reduction of Ag after removal of the algae most likely reflects the Ag bound to the algae. However, these results might be confounded by the fact that the amount of algae present in the highest exposure concentrations are considerably lower than in the lower concentrations due to the toxicity induced at these high exposure concentrations. Thus, less Ag would be bound to algae merely due to the lower number of algae available.

3.3 Growth inhibition

All tested Ag compounds, except the NM302 Ag rods, reduced growth in *R. subcapitata* in the following order $AgNO_3 \geq$ M-Ag > NM300K > NM302.

Increasing concentrations of $AgNO_3$, M-Ag and NM300K caused a decrease in algae growth. For $AgNO_3$ and NM300K exposures there was a concentration-dependent increase in growth inhibition after 24 h (Figure 3), with the exception of the lowest concentration (0.25 µg L^{-1}) in the $AgNO_3$ exposure where an increased growth was observed. A similar increased growth in the lowest exposure were observed in all treatments after 72 h of exposure, although only significant in the M-Ag NPs treatment ($p = 0.0005$). This is a commonly reported phenomenon, hormesis, also reported for nanomaterials (Iavicoli et al., 2014).

In the $AgNO_3$ exposure the growth inhibition observed after 24 h was still significant after 72 h ($p = 0.0146$ and <0.0001 for 8 and 25 µg Ag L^{-1}, respectively). While in the NM300K exposures, all significant growth inhibition at 24 h ($p = 0.0326$ and 0.0071 for 14 and 24 µg Ag L^{-1}, respectively) was no longer present after 48 and 72 h of exposure (Figure 3). In the M-Ag exposure significant growth inhibition ($p < 0.05$) was observed at all exposure concentrations, except the lowest (5.4 µg L^{-1}), after 24 h and remained relatively stable throughout the exposure with significant growth inhibition in the three highest exposure concentrations remaining after 72 h ($p < 0.05$).

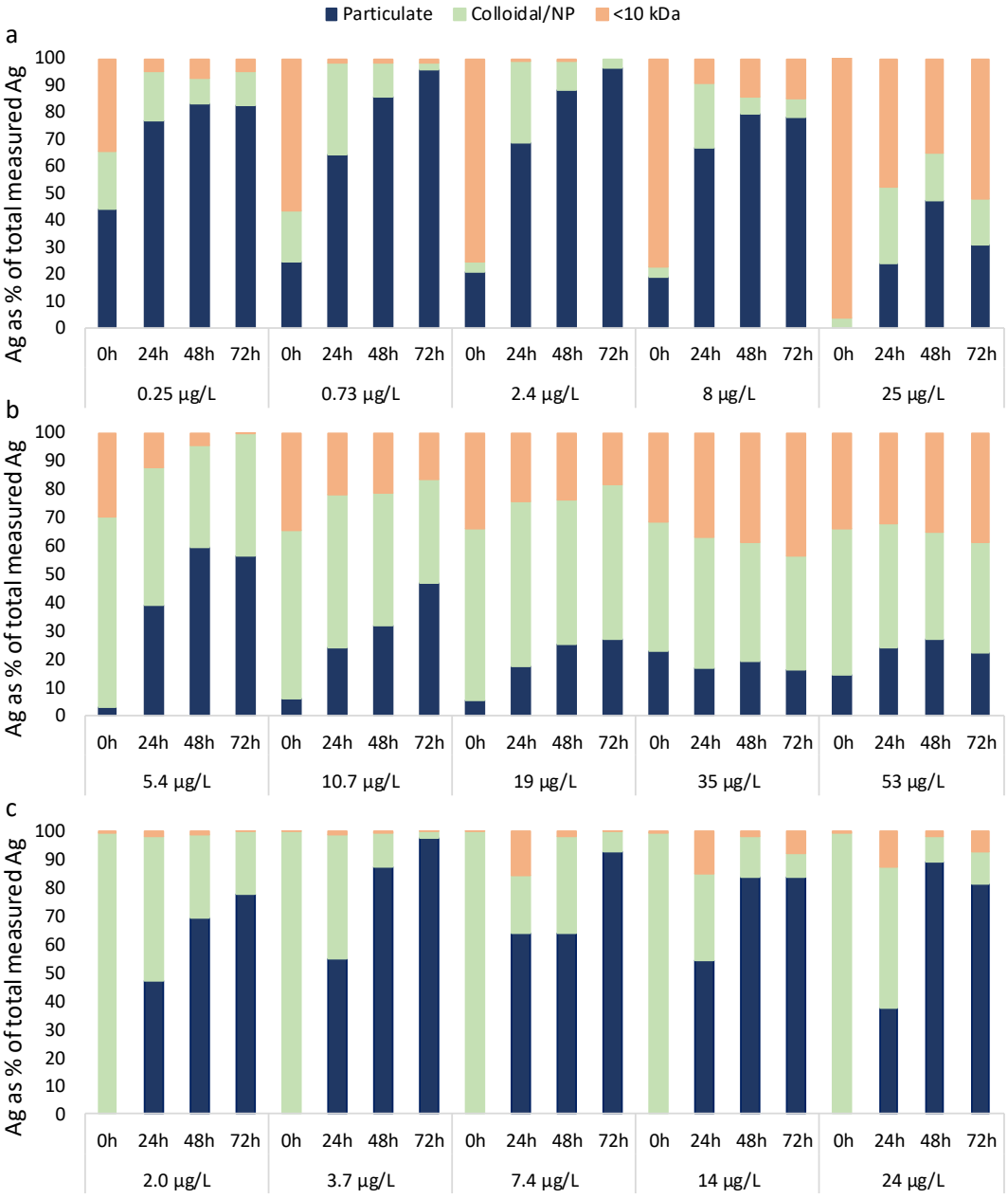

Figure 1. Changes in size fractions of Ag (as % of total measured Ag) present in the a) $AgNO_3$, b) Mesosilver (M-Ag NP), and c) NM300K Ag NP test media over time. Particulate > 220 nm, Colloidal/NP < 220 nm and > 10 kDa, LMM < 10 kDa.

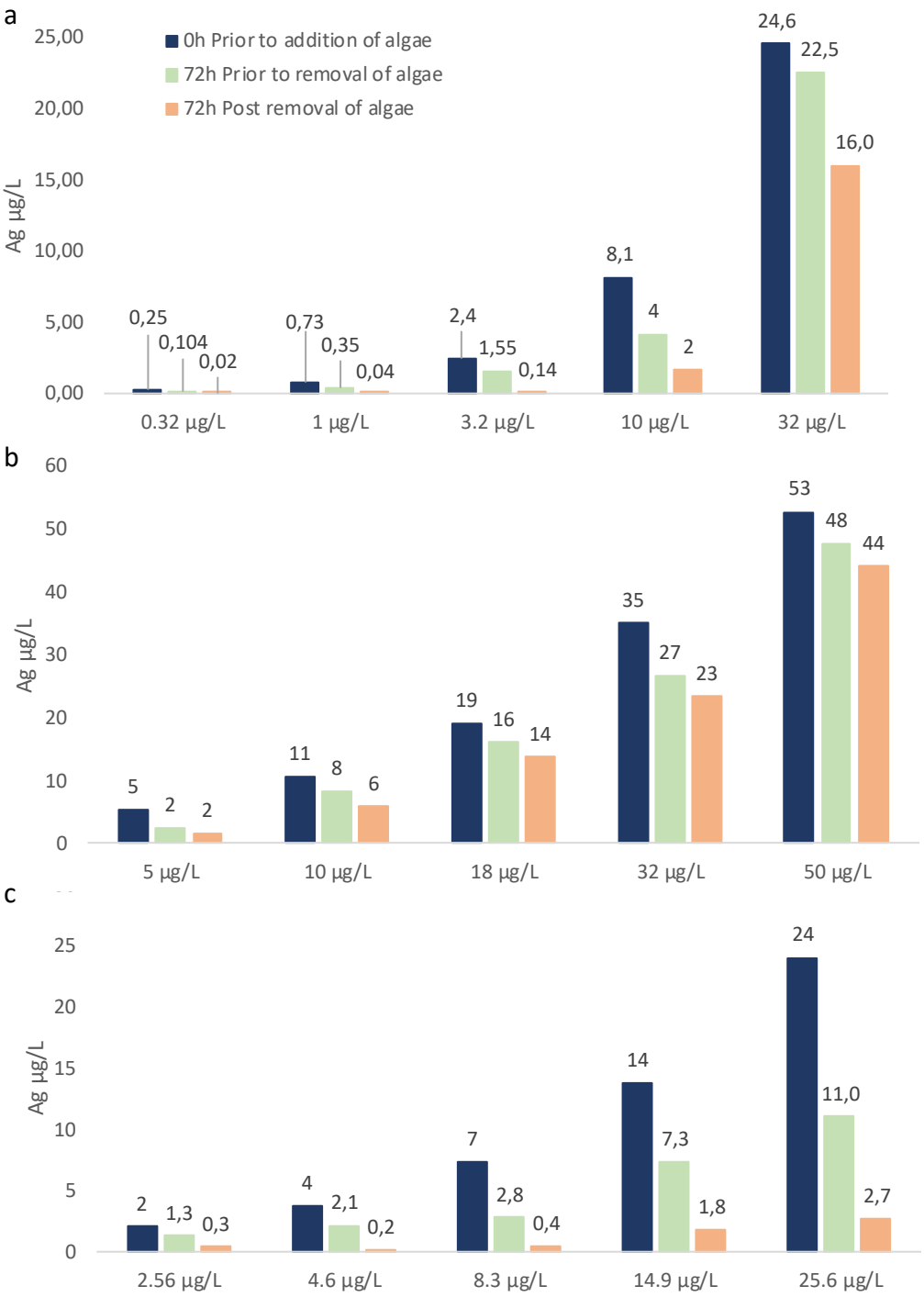

Figure 2. Measured total Ag concentration at t=0 h prior to addition of *R. subcapitata,* and after 72 h before and after removal of the algae by low speed centrifugation in a) $AgNO_3$, b) Mesosilver (M-Ag), and c) NM300K. Be aware the different scales at the Y-axis.

The EC50 values obtained in the current study (Table 2) are among the lowest reported for $AgNO_3$, Ag NPs in general and NM300K specifically. The EC50 values for *R. subcapitata* have previously been reported in the range 4.9-34 and 15-140 µg L^{-1} for $AgNO_3$ and NM300K, respectively (Ribeiro et al., 2014, Sørensen and Baun, 2015, Hund-Rinke et al., 2018). The EC50 reported for NM300K in the

current study (13-24 µg L^{-1}) agrees well with the EC50 of 15-81 µg L^{-1} reported by Hund-Rinke et al. (2018). When it comes to the M-Ag NPs there are not much in the literature to compare with. Ellegaard-Jensen et al. (2012) report LC50 4.4 mg L^{-1} for *C. elegans*, however this is a species known to be highly tolerant to a range of contaminants. Farmen et al. (2012) report NOEC of 20 µg L^{-1} for Atlantic salmon exposed for 48 h, a species known for its sensitivity. Echavarri-Bravo et al. (2017) exposed marine algae species to $AgNO_3$, Mesosilver (Mesosilver Hot tub™ cleaner) and NM300K, and reported EC50 (growth inhibition) in the range of <10 to 50, 145 to above 1000 and as low as 72 µg L^{-1} for $AgNO_3$, NM300K and Mesosilver, respectively. Compared to these studies the EC values obtained in the present study are low, and of the four Ag compounds tested in the present study, $AgNO_3$ was the most potent growth inhibitor (Table 2).

Differences in toxicity between the different AgNPs could be linked to differences in size and stability seen in stock solutions, particularly the lack of toxicity seen for NM302K. Although Mesosilver and NM300K showed a similar size range for both Z-averaged diameter and TEM (11 and 16 nm, respectively), the number mean was lower for Mesosilver (0.6 nm) which might contribute to the higher toxicity of these particles. However, these measurements to not explain all the perturbations seen toxicity, and characterization of the exposure solutions can provide additional information to better understand the changes in time and impact of Ag speciation on toxicity.

The EC50 value for the reference substance $K_2Cr_2O_7$ was 0.6 mg/L (95 % CI 0.187-1.972). In the controls for all tests, the change in pH was < 0.5 units during the test, well within the 1.5 units given as the maximum allowed change in the guideline (Table S3).

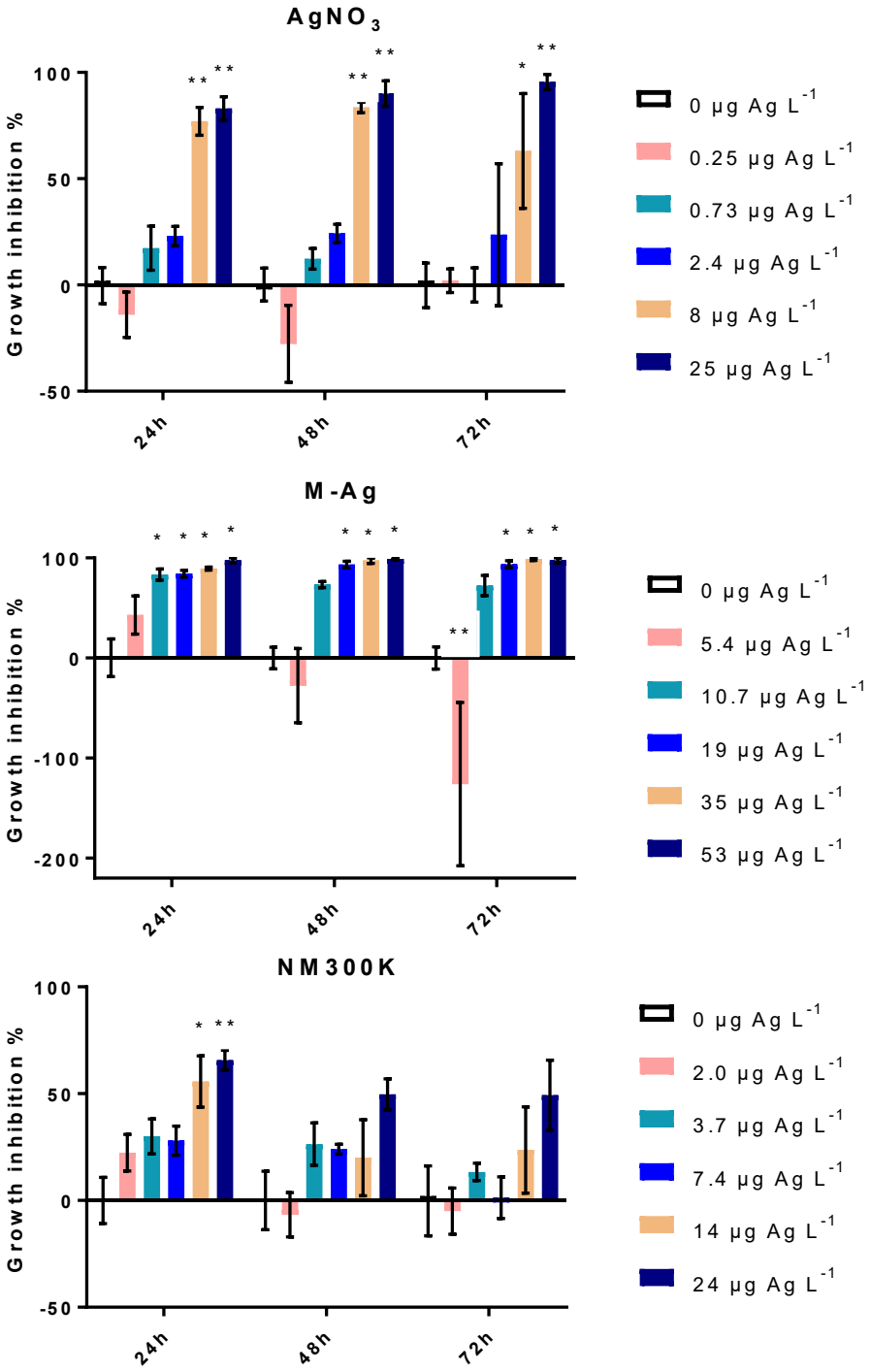

Figure 3. Growth of *R. subcapitata* during 72 h of exposure to $AgNO_3$, M-Ag NPs and NM300K Ag NPs. * $p < 0.05$, ** $p < 0.01$.

Table 2. Effect concentration (µg Ag L^{-1}) 10 % and 50 % (EC10 and EC50) on growth *R. subcapitata* exposed to $AgNO_3$, M-Ag NPs and NM300K Ag NPs. Results are provided with their 95 % confidence interval (CI) in parentheses. Parameter estimations calculated using the Hill model.

Test substance	Exposure time (h)	EC10 (95 % CI) (µg Ag L^{-1})	EC50 (95 % CI) (µg Ag L^{-1})	Model (Regtox)
$AgNO_3$	24	0.8 (0.21-2.04)	4.19 (2.55-6.68)	Hill
	48	1.03 (0.24-2.78	3.36 (2.24-6.81)	Hill
	72	3.36 (1.58-4.70)	7.09 (3.83-10.52)	Weibull
M-Ag NP	24	1.65 (6.4E-07 – 7.67)	5.77 (0.91-11.90)	Hill
	48	7.99 (NC)	9.67 (NC)	Hill
	72	8.48 (NC)	9.74 (NC)	Hill
NM300K	24	0.93 (0.06-4.45)	12.83 (7.07-30.19)	Hill
	48	2.77 (0.01-18.42)	29.51 (13.40-298.53)	Hill
	72	9.23 (0.68-21.9)	24.18 (15.66-98.16)	Hill

NC: Not Calculated

3.4 Linking toxicity to exposure characterization

The results presented in the current study provides two lines of evidence that the toxicity observed in the M-Ag and NM300K AgNP exposures cannot be explained by the presence of LMM Ag, but rather a nanospecific toxicity or a combination of the two, also reported by for example Sendra et al. (2017).

The toxicity of $AgNO_3$ was linked to the presence of LMM Ag. Toxicity was only observed in the two highest exposure concentrations, which also were the only groups still containing LMM Ag after 72 h. A concentration dependent trend in toxicity was observed at 24 and 48 hours. But by 72h no toxicity was seen in the lower concentration exposures, by which time the LMM Ag had disappeared. The LMM decreased from 6.3 and 24 µg L^{-1} in the two highest exposures at time 0 to 0.6 and 11.8 µg L^{-1} at 72 h, and the growth inhibition changed from 72 and 83 % to 63 and 96 % after 72 h.

The stability in the toxicity of M-Ag reflects the stability seen in the size fractionation results. Also here there are indications that the toxicity was linked to the presence of LMM Ag, since the significant growth inhibition disappears with the reduction of LMM Ag in the lowest concentrations. However, despite similar concentrations of LMM Ag (10 and 11 µg Ag L^{-1}) in the highest exposure (25 µg L^{-1}) of $AgNO_3$ and the 35 µg L^{-1} M-Ag exposure, respectively, the growth inhibition was much

higher in the M-Ag (89%) than in the $AgNO_3$ (53%) after 24h. This could indicate an additional NP induced toxicity in the M-Ag exposure.

In the NM300K exposure, the toxicity present after 24 h of exposure could not be linked to the presence of LMM Ag since close to 100 % of the Ag at time zero were present as nanoparticles in the two highest exposure concentrations. There was a slight increase in LMM Ag over time (a maximum LMM Ag concentration of 1.4 µg L^{-1} at 24 h), however this concentration of LMM Ag would not be high enough alone to explain the toxicity (growth inhibition of 66 %) at 24 h, strongly indicating a nanospecific toxicity.

It should however be kept in mind that in natural waters Ag will not exist as Ag^+ for long, but form complexes with inorganic (e.g. chloride and thiosulphate) and organic (e.g. natural organic matter) ligands (Hiriart-Baer et al., 2006). These ligands could influence the fate of different AgNPs in different ways, and thus also their toxicity. For example, the presence of chloride has been reported to reduce the toxicity of $AgNO_3$ towards *R. subcapitata* (Lee et al., 2005). Hiriart-Baer et al. (2006) found that Ag-thiosulphate complexation increased the uptake of Ag into the algae, but that the toxicity of these complexes was lower than for Ag^+. Components believed to influence AgNP behavior and fate in the environment (e.g. ionic strength, Total Organic Carbon, Dissolved Organic Carbon, chloride etc.) have been frequently studied. However, they are usually studied separately in controlled laboratory experiments and cannot account for the potential interactions between all of these different factors and the complexity found in natural waters (Conine et al., 2017). It is also important to consider seasonal changes in natural systems since they influence the condition of the organisms and thus potentially also their sensitivity towards contaminants (Conine et al., 2017). Despite the low EC values reported in the current study (low µg L^{-1}), they are however still higher after 72 h than expected environmental concentrations of 0.5-2 µg L^{-1}(Gottschalk et al., 2011). The acute 24 h EC10 values are however < 2 µg L^{-1} for all Ag exposures, except the NM302 Ag rods. Whether or not these AgNPs in natural environments would pose a risk to the algae communities would depend on the environmental conditions (abiotic and biotic), however the higher complexity of the exposure media in natural systems would most likely reduce toxicity rather than enhance it.

4. CONCLUSIONS

The objective of this study was to investigate the effects of several types of silver nanomaterials to the freshwater algae *R. supcapitata* in combination with characterization to investigate changes in speciation and/or aggregation state over time. The tested particles were different in shape, size and stabilizing material. Using a robust set of fractionation and characterization techniques to monitor changes in nanoparticle behavior over the exposure period, insight into the aggregation processes in

the test media that effected algal growth was possible. Our results identified a combination of factors that appeared to be responsible for the observed toxicity. The toxicity of M-Ag and NM300K AgNPs could not be explained by the presence of LMM Ag alone, but rather a nanospecific effect or a combination of the two is more likely. The results also showed the importance, and dynamic nature of exposure duration and the need for characterization in toxicity testing of nanomaterials such as silver. Overall the characterization of the test media suggests that changes in speciation can be influenced by both time and concentration, as well as the algae concentration, which can act as confounding factors for toxicity tests.

5. ACKNOWLEDMENT

The Research Council of Norway through the NANoREG (grant number 310584) and NANOCHARM (grant number 221391) projects funded this work. The authors would also like to thank Ø. Enger for assistance with the ICP-MS measurements.

6. DATA ACCESSIBILITY

Research data pertaining to this article is accessible upon request to the authors.

REFERENCES

BRUNEAU, A., TURCOTTE, P., PILOTE, M., GAGNE, F. & GAGNON, C. 2016. Fate of silver nanoparticles in wastewater and immunotoxic effects on rainbow trout. *Aquatic toxicology (Amsterdam, Netherlands),* 174**,** 70-81.

CERRILLO, C. & MENDOZA, G. 2015. Standard Operating Procedure. Toxicity Test with microalgae Pseudokirchneriella subcapitata for NANoREG core nanomaterials, NANoREG Deliverable D4.12. *http://www.nanoreg.eu/media-and-downloads/deliverable-reports/312-nanoreg-deliverables-sops*.

CHAE, Y. J., PHAM, C. H., LEE, J., BAE, E., YI, J. & GU, M. B. 2009. Evaluation of the toxic impact of silver nanoparticles on Japanese medaka (Oryzias latipes). *Aquatic Toxicology,* 94**,** 320-327.

CONINE, A. L., REARICK, D. C., XENOPOULOS, M. A. & FROST, P. C. 2017. Variable silver nanoparticle toxicity to Daphnia in boreal lakes. *Aquatic Toxicology,* 192**,** 1-6.

ECHAVARRI-BRAVO, V., PATERSON, L., ASPRAY, T. J., PORTER, J. S., WINSON, M. K. & HARTL, M. G. 2017. Natural marine bacteria as model organisms for the hazard-assessment of consumer products containing silver nanoparticles. *Marine environmental research,* 130**,** 293-302.

ECHAVARRI-BRAVO, V., PATERSON, L., ASPRAY, T. J., PORTER, J. S., WINSON, M. K., THORNTON, B. & HARTL, M. G. 2015. Shifts in the metabolic function of a benthic estuarine microbial community following a single pulse exposure to silver nanoparticles. *Environmental Pollution,* 201**,** 91-99.

ELLEGAARD-JENSEN, L., JENSEN, K. A. & JOHANSEN, A. 2012. Nano-silver induces dose-response effects on the nematode Caenorhabditis elegans. *Ecotoxicology and environmental safety,* 80**,** 216-223.

FABREGA, J., FAWCETT, S. R., RENSHAW, J. C. & LEAD, J. R. 2009. Silver nanoparticle impact on bacterial growth: effect of pH, concentration, and organic matter. *Environmental science & technology,* 43**,** 7285-7290.

FABREGA, J., LUOMA, S. N., TYLER, C. R., GALLOWAY, T. S. & LEAD, J. R. 2011. Silver nanoparticles: Behaviour and effects in the aquatic environment. *Environment International,* 37**,** 517-531.

FARMEN, E., MIKKELSEN, H. N., EVENSEN, O., EINSET, J., HEIER, L. S., ROSSELAND, B. O., SALBU, B., TOLLEFSEN, K. E. & OUGHTON, D. H. 2012. Acute and sub-lethal effects in juvenile Atlantic salmon exposed to low mu g/L concentrations of Ag nanoparticles. *Aquatic Toxicology,* 108**,** 78-84.

GOTTSCHALK, F., ORT, C., SCHOLZ, R. W. & NOWACK, B. 2011. Engineered nanomaterials in rivers - Exposure scenarios for Switzerland at high spatial and temporal resolution. *Environmental Pollution,* 159**,** 3439-3445.

HANDY, R. D., VON DER KAMMER, F., LEAD, J. R., HASSELLOV, M., OWEN, R. & CRANE, M. 2008a. The ecotoxicology and chemistry of manufactured nanoparticles. *Ecotoxicology,* 17**,** 287-314.

HIRIART-BAER, V. P., FORTIN, C., LEE, D.-Y. & CAMPBELL, P. G. 2006. Toxicity of silver to two freshwater algae, Chlamydomonas reinhardtii and Pseudokirchneriella subcapitata, grown under continuous culture conditions: influence of thiosulphate. *Aquatic toxicology,* 78**,** 136-148.

HUND-RINKE, K., SCHLICH, K., KÜHNEL, D., HELLACK, B., KAMINSKI, H. & NICKEL, C. 2017. Grouping concept for metal and metal oxide nanomaterials with regard to their ecotoxicological effects on algae, daphnids and fish embryos. *NanoImpact*.

HUND-RINKE, K., SCHLICH, K., KÜHNEL, D., HELLACK, B., KAMINSKI, H. & NICKEL, C. 2018. Grouping concept for metal and metal oxide nanomaterials with regard to their ecotoxicological effects on algae, daphnids and fish embryos. *NanoImpact,* 9**,** 52-60.

IAVICOLI, I., FONTANA, L., LESO, V. & CALABRESE, E. J. 2014. Hormetic dose–responses in nanotechnology studies. *Science of the Total Environment,* 487**,** 361-374.

JENSEN, K. A., BOOTH, A., KEMBOUCHE, Y. & BORASCHI, D. 2016. Validated protocols for test item preparation for key in vitro and ecotoxicity studies, NANoREG Deliverable D2.06.

KLEIVEN, M., ROSSBACH, L. M., GALLEGO-URREA, J. A., BREDE, D. A., OUGHTON, D. H. & COUTRIS, C. 2018. Characterizing NM300K Silver Nanoparticles Behavior, Uptake and Toxicity in Caenorhabditis elegans. *Environmental toxicology and chemistry*.

KÖSER, J., ENGELKE, M., HOPPE, M., NOGOWSKI, A., FILSER, J. & THOMING, J. 2017. Predictability of silver nanoparticle speciation and toxicity in ecotoxicological media. *Environmental Science-Nano,* 4**,** 1470-1483.

LEE, D.-Y., FORTIN, C. & CAMPBELL, P. G. 2005. Contrasting effects of chloride on the toxicity of silver to two green algae, Pseudokirchneriella subcapitata and Chlamydomonas reinhardtii. *Aquatic Toxicology,* 75**,** 127-135.

LODEIRO, P., BROWNING, T. J., ACHTERBERG, E. P., GUILLOU, A. & EL-SHAHAWI, M. S. 2017. Mechanisms of silver nanoparticle toxicity to the coastal marine diatom Chaetoceros curvisetus. *Scientific Reports,* 7**,** 10.

MAYER, P., CUHEL, R. & NYHOLM, N. 1997. A simple in vitro fluorescence method for biomass measurements in algal growth inhibition tests. *Water Research,* 31**,** 2525-2531.

OECD 2011. OECD 201 Freshwater Alga and Cyanobaceria, Growth Inhibition Test. OECD Guidelines for the testing of chemicals. Adopted:23 March 2006. Annex 5 corected: 28 July 2011. .

RATTE, H. T. 1999. Bioaccumulation and toxicity of silver compounds: A review. *Environmental Toxicology and Chemistry,* 18**,** 89-108.

RIBEIRO, F., GALLEGO-URREA, J. A., GOODHEAD, R. M., VAN GESTEL, C. A., MOGER, J., SOARES, A. M. & LOUREIRO, S. 2015. Uptake and elimination kinetics of silver nanoparticles and silver nitrate by Raphidocelis subcapitata: The influence of silver behaviour in solution. *Nanotoxicology,* 9**,** 686-695.

RIBEIRO, F., GALLEGO-URREA, J. A., JURKSCHAT, K., CROSSLEY, A., HASSELLOV, M., TAYLOR, C., SOARES, A. & LOUREIRO, S. 2014. Silver nanoparticles and silver nitrate induce high toxicity to Pseudokirchneriella subcapitata, Daphnia magna and Danio rerio. *Science of the Total Environment,* 466**,** 232-241.

SENDRA, M., YESTE, M., GATICA, J., MORENO-GARRIDO, I. & BLASCO, J. 2017. Direct and indirect effects of silver nanoparticles on freshwater and marine microalgae (Chlamydomonas reinhardtii and Phaeodactylum tricornutum). *Chemosphere,* 179**,** 279-289.

SØRENSEN, S. N. & BAUN, A. 2015. Controlling silver nanoparticle exposure in algal toxicity testing A matter of timing. *Nanotoxicology,* 9**,** 201-209.

VANCE, M. E., KUIKEN, T., VEJERANO, E. P., MCGINNIS, S. P., HOCHELLA, M. F., JR., REJESKI, D. & HULL, M. S. 2015. Nanotechnology in the real world: Redeveloping the nanomaterial consumer products inventory. *Beilstein Journal of Nanotechnology,* 6**,** 1769-1780.

WANG, S., LV, J., MA, J. & ZHANG, S. 2016. Cellular internalization and intracellular biotransformation of silver nanoparticles in Chlamydomonas reinhardtii. *Nanotoxicology,* 10**,** 1129-1135.

SUPPLEMENTARY INFORMATION

Growth inhibition in Raphidocelis subcapita – evidence of nanospecific toxicity of silver nanoparticles

Merethe Kleiven[a*], Ailbhe Macken[b], Deborah H. Oughton[a]

[a] *Norwegian University of Life Sciences, Center for Environmental Radioactivity, P.O.Box 5003 NMBU, 1432 Ås, Norway*

[b] *Norwegian Institute for Water Research (NIVA), Gaustadalléen 21, N-0349 Oslo, Norway*

[*] Address correspondence to merethe.kleiven@nmbu.no

Figures

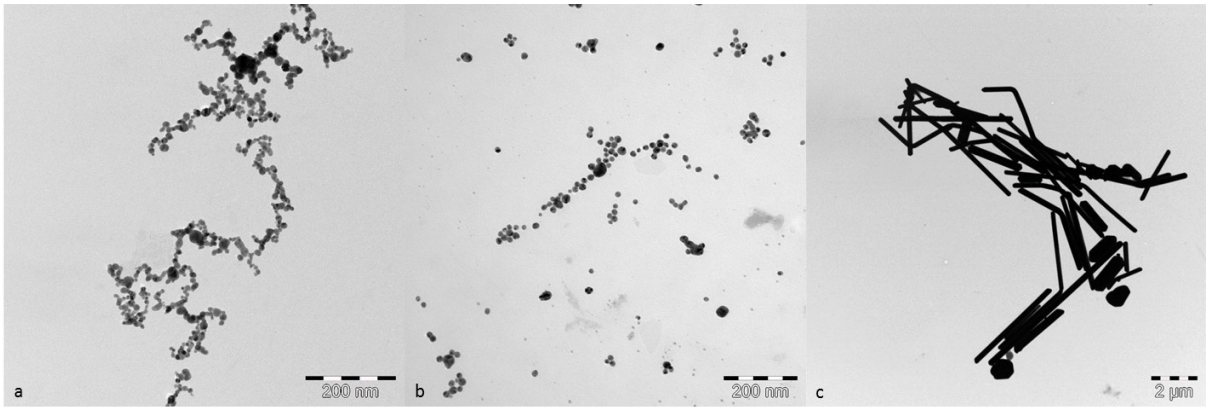

Figure S1. TEM images of stock suspension of Mesosilver (M-Ag) (a), NM300K (b), and NM302 (c) AgNPs in MQ water. The scale bare on a) and b) is 200 nm, on c) it is 2 µm.

Tables

Table S1. Particle size (mean ± one standard deviation) measured by DLS in exposure suspensions prior and post removal of algae by centrifugation (4000 rpm, 10 min). NA: not available

		Prior to removal of algae			Post removal of algae		
		Z-averaged diameter (nm)	Number mean diameter (nm)	PDI	Z-averaged diameter (nm)	Number mean diameter (nm)	PDI
$AgNO_3$	0h	NA	NA	NA	207±112	66±24	0.4±0.1
	24h	504±551	60±12	0.7±0.3	77±335	26±9	0.8±0.1
	48h	110±28	62±7	0.3±0.2	551±351	37±10	0.6±0.2
	72h	1094±1550	73±22	0.8±0.2	150±73	61±16	0.7±0.3
M-Ag	0h	NA	NA	NA	102±92	1.8±0.3	0.3±0.1
	24h	75±55	3±1	0.3±0.2	250±252	3±1	0.4±0.2
	48h	71±16	20±24	0.25±0.09	300±136	43±7	0.33±0.1
	72h	157±101	67±9	0.2±0.1	296±304	23±20	0.4±0.2
NM300K	0h	NA	NA	NA	NA	NA	NA
	24h	NA	NA	NA	119±13	88±9	0.19±0.06
	48h	1171±1171	45±12	0.9±0.2	1676±1727	48±19	0.8±0.2
	72h	250±136	55±9	0.5±0.2	347±351	38±8	0.8±0.2

Table S2. Measured Ag concentrations in $AgNO_3$, Mesosilver (M-Ag), NM300K, and NM302 exposures throughout the duration of the experiment, and for the different size fraction. LOD 0.0049 µg L^{-1}.

Measured Ag concentrations (µg Ag L^{-1})																				
		0h				24h					48h					72h				
Ag exposure	Nominal concentration	Total (prior to algae)	Particulate	Colloidal/ NP	<10 kDa	Total (prior to algae removal)	Total (post algae removal)	Particulate	Colloidal/ NP	<10 kDa	Total (prior to algae removal)	Total (post algae removal)	Particulate	Colloidal/ NP	<10 kDa	Total (prior to algae removal)	Total (post algae removal)	Particulate	Colloidal/ NP	<10 kDa
AgNO3	0,32 µg/L	0,25±0.05	0,11	0,05	0,09	0,11	0,04	0,09	0,02	<0,0049	0,065	0,021	0,05	0,01	<0,0049	0,104±0.009	0,024±0.005	0,09	0,01	< 0,0049
	1 µg/L	0,73±0.06	0,18	0,14	0,41	0,31	0,15	0,20	0,11	<0,0049	0,32	0,07	0,28	0,04	<0,0049	0,35±0.03	0,04±0.01	0,34	0,01	<0,0049
	3,2 µg/L	2,4±0.6	0,50	0,10	1,80	0,71	0,37	0,49	0,22	<0,0049	0,82	0,18	0,73	0,09	<0,0049	1,55±0.07	0,14±0.06	1,49	0,06	<LOD
	10 µg/L	8±1	1,54	0,30	6,30	4,2	1,7	2,80	1,01	0,39	6,3	2,2	5,00	0,41	0,89	4±2	2±2	3,25	0,30	0,60
	32 µg/L	25±2	0,00	1,00	24,00	21	17	5,00	6,00	10,00	21	13	10,00	3,70	7,30	22,5±0.7	16	7,00	3,75	11,75
M-Ag	5 µg/L	5,4±0.1	0,17	3,60	1,60	5,1	3,7	2,00	2,47	0,63	4,7	3	2,80	1,69	0,21	2,5±0.3	1.6±0.06	1,39	1,07	0,01
	10 µg/L	10,7±0.6	0,67	6,30	3,70	9,6	8,3	2,30	5,20	2,10	8,8	7	2,80	4,10	1,90	8±1	6±1	3,83	3,05	1,35
	18 µg/L	19±0	1,00	11,50	6,50	17	16	3,00	9,90	4,10	16	13	4,00	8,20	3,80	16±2	14±2	4,33	8,70	2,97
	32 µg/L	35±1	8,00	16,00	11,00	30	27	5,00	14,00	11,00	31	27	6,00	13,00	12,00	27±2	23±2	4,33	10,73	11,60
	50 µg/L	53±2	7,67	27,00	18,00	50	47	12,00	22,00	16,00	48	43	13,00	18,00	17,00	47±4	44±3	10,67	18,67	18,33
NM300K	2,56 µg/L	2,0±0.4	<0.0049	2,284	0,016	0,7	0,5	0,340	0,368	0,012	0,6	0,1	0,430	0,183	0,007	1,25±0.07	0,4±0.4	0,969	0,276	<0,0049
	4,6 µg/L	3,7±0.7	<0.0049	4,283	0,017	1,7	1,1	0,930	0,749	0,021	1,1	0,3	0,960	0,132	0,008	2±2	0,17±0.08	2,065	0,050	0,006
	8,3 µg/L	7±1	<0.0049	7,770	0,030	3,9	1,8	2,500	0,790	0,610	1,1	0,7	0,700	0,379	0,021	3±1	0,4±0.1	2,545	0,196	0,009
	14,9 µg/L	14±3	<0.0049	13,940	0,060	6,6	3,8	3,600	2,000	1,000	5,1	1,6	4,270	0,739	0,091	7.3±0.6	2±1	6,130	0,606	0,565
	25,6 µg/L	24±6	<0.0049	23,830	0,170	11,0	7,9	4,100	5,500	1,400	25,0	4,6	22,300	2,250	0,450	11±7	3±2	8,955	1,257	0,788
NM302	256 µg/L	82				20	0,8				36	0,7				24±6	0,8±0.3			
	800 µg/L	79				61	2,4				47	3,6				65±7	3±1			
	2500 µg/L	86				72	5,8				73	16				74±2	7±5			
	8000 µg/L	110				75	54				80	64				78±2	45±8			
	25600 µg/L	250				85	73				86	77				89±3	72,3±0.6			

Table S3. Growth rates and pH in control groups in toxicity testing with *R. subcapitata*, all replicates merged.

Exposure	pH in controls		Growth rates in controls
	0h	72h	72 h
$K_2Cr_2O_7$	8	8.3	1.8
$AgNO_3$	7.9	7.8	0.8
M-Ag	8.1*	7.8	1.0
NM300K	7.9	7.8	1.0
NM302	7.9	7.8	1.3

*pH measured at 24 h.