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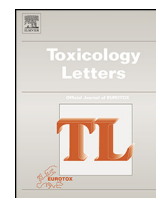
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The critical importance of defined media conditions in *Daphnia magna* nanotoxicity studies[☆]



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HIGHLIGHTS

- Dispersed citrate capped silver nanoparticles were synthesised and characterised.
- Nanoparticle aggregation was assessed in *Daphnia magna* diluted and undiluted media.
- Aggregation was assessed by TEM and AFM measurements.
- There was a significant reduction in toxicity in media1 compared to that in media10.

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ABSTRACT

Due to the widespread use of silver nanoparticles (AgNPs), the likelihood of them entering the environment has increased and they are known to be potentially toxic. Currently, there is little information on the dynamic changes of AgNPs in ecotoxicity exposure media and how this may affect toxicity. Here, the colloidal stability of three different sizes of citrate-stabilized AgNPs was assessed in standard strength OECD ISO exposure media, and in 2-fold (media2) and 10-fold (media10) dilutions by transmission electron microscopy (TEM) and atomic force microscopy (AFM) and these characteristics were related to their toxicity towards *Daphnia magna*. Aggregation in undiluted media (media1) was rapid, and after diluting the medium by a factor of 2 or 10, aggregation was reduced, with minimal aggregation over 24 h occurring in media10. Acute toxicity measurements were performed using 7 nm diameter particles in media1 and media10. In media10 the EC₅₀ of the 7 nm particles for *D. magna* neonates was calculated to be 7.46 µg L⁻¹ with upper and lower 95% confidence intervals of 6.84 µg L⁻¹ and 8.13 µg L⁻¹ respectively. For media1, an EC₅₀ could not be calculated, the lowest observed adverse effect concentration (LOAEC) of 11.25 µg L⁻¹ indicating a significant reduction in toxicity compared to that in media10. The data suggest the increased dispersion of nanoparticles leads to enhanced toxicity, emphasising the importance of appropriate media composition to fully assess nanoparticle toxicity in aquatic ecotoxicity tests.

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1. Introduction

The widely accepted definition of a nanomaterial is any substance with at least one dimension between 1 and 100 nm in size (Borm et al., 2006). At this scale, the physical and chemical

properties of a substance can differ substantially from those of their larger counterparts; a fact which is responsible for the rapid growth of nanotechnology as a discipline over the past decade. Silver nanoparticles (AgNPs) have become exploited for their potent antimicrobial properties and can be found in a multitude of products, such as antimicrobial dressings and coatings of catheters (Chaloupka et al., 2010), odour free fabrics used in clothing (Benn and Westerhoff, 2008), and multiple applications in the food industry, such as in food packaging, storage bags, and chopping boards (Chaudhry et al., 2008). As a consequence of the bacterial toxicity and easy manufacturer and handling of AgNP, it has quickly become the most widely available nanomaterial.

Due to their widespread use, the likelihood of silver nanoparticles entering the environment has increased, with recent studies

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showing that ionic and nanoparticle silver can be leached from odour-free socks through washing (Benn and Westerhoff, 2008; Geranio et al., 2009), and from surfaces coated in nano-silver paints in rain-water runoff (Kaegi et al., 2010). Once released into the environment, the mobility, bioavailability, and toxicity of AgNPs are strongly influenced by their colloidal stability (Badawy et al., 2010). Many factors can affect colloidal stability, including the type of capping agent (Tejamaya et al., 2012) and the local environmental conditions such as pH, ionic strength and specific ion effects (Römer et al., 2011; Cumberland and Lead, 2009; Baalousha et al., 2013). The evidence of their release, coupled with multiple reports showing signs of silver nanoparticle toxicity in vitro (Ahamed et al., 2008; Arora et al., 2009; AshaRani et al., 2009) and in vivo (Gaiser et al., 2011; Roh et al., 2009) shows the need for environmentally relevant toxicity testing using the appropriate test species in order to minimise and quantify any risk.

Daphnia magna is one of the recommended test species for acute (OECD, 2004) and chronic (OECD, 2012) ecotoxicity tests by the Organisation for Economic Co-operation and Development (OECD). We have shown previously, through use of flow field flow fractionation (FFF) and dynamic light scattering (DLS), that citrate-stabilised AgNPs form aggregates in standard strength *D. magna* OECD ISO test media. It was also shown that a ten-fold dilution of this standard media minimised aggregation without inducing neonate immobilisation or reducing the reproductive output of adult *Daphnia* (Römer et al., 2011) thus providing optimum conditions for nanoparticle exposures.

While it has been shown that media composition can influence particle aggregation, the effect that this has on responses of *D. magna* to nanoparticles has not been assessed. Here, we measured the stability of three different sizes of citrate-stabilised AgNPs in media1, media2 and media10, with different ionic strengths, by atomic force microscopy (AFM) and transmission electron microscopy (TEM). The influence of these conditions on the toxicity of the particles was determined through acute immobilisation studies based on OECD guidelines in undiluted media and ten-fold diluted media (media10) using the smallest nanoparticles (7 nm).

2. Materials and methods

2.1. Silver nanoparticles

The method of synthesis for citrate-capped AgNPs has been published previously (Römer et al., 2011). Three different sizes of AgNPs (labelled AgNP1, AgNP2 and AgNP3) were prepared from a standard reduction of silver nitrate in sodium citrate. Suspensions were cleaned by ultrafiltration to remove dissolved silver (Amicon, 1 kDa regenerated cellulose membrane, Millipore) using a diafiltration method to prevent drying of the particles (Cumberland and Lead, 2009). The particles were redispersed in 0.15 mM citrate solution as redispersion in water causes increase in size of the particles and aggregation. This process was repeated at least three times to remove reactants in solution. Particles were then characterised by transmission electron microscopy (TEM) and atomic force microscopy (AFM).

2.2. Transmission electron microscopy (TEM)

Samples were prepared by partially, but not fully, drying a drop of the particle solution on a copper mesh 400 holey carbon film (Agar scientific) at room temperature (Domingos et al., 2009). The grid was washed several times with high purity water (maximum resistivity 18.2 M Ω cm⁻¹) and re-dried as above. Images were obtained from a Phillips Tecnai F20 (accelerating voltage 200 kV), with Oxford ISIS energy dispersive X-ray (EDX), and recorded using Gatan Digital Micrograph software. Data were analysed using Gatan Digital Micrograph (size distribution), Image J (circularity equation shown in supporting information, Eq. S1) and Fractalyse (fractal dimension, the relationship between the projected area for two dimensions and characteristic length scales of the aggregate). Fractal dimension was calculated by using a geometric power law scaling the relationship between each dimensional geometry, which in this case was the projected area for two dimensions and characteristic length scales of the aggregate (Baalousha et al., 2008; Lee and Kramer, 2004). Image processing for fractal analyses was performed as described elsewhere (Baalousha et al., 2008; Ferretti et al., 1997; Lee and Kramer, 2004).

2.3. Atomic force microscopy (AFM)

To ensure optimal mica sheet (Agar Scientific) coverage the solutions were diluted 50 times. Ten ml of the diluted samples were ultracentrifuged onto a freshly cleaved mica sheet. To provide a flat support for the mica, PTFE caps were placed at the bottom of the tubes. Beckman L7-65 Ultracentrifuge with SW40 swinging bucket rotor was used in the preparation, the parameters were as follows, speed 10,000 rpm, relative centrifugal field at rmax: 160,000 \times g, rav: 114,000 \times g and rmin: 67,200 \times g, temperature 15 $^{\circ}$ C, run time 60 min. After centrifugation the tubes were carefully emptied, the PTFE caps removed and the mica sheets were thoroughly washed in high purity water (maximum resistivity 18.2 M Ω cm⁻¹) and dried at ambient conditions prior to AFM analysis. Images were obtained from a XE 100 AFM in non-contact mode, recorded using XEP software and analysed with XEI software. Non-contact mode silicon AFM Cantilevers PPP-NCHR were used (spring constant 42 N m⁻¹), X-Y scan sizes were 5 μ m \times 5 μ m with resolution of 250 pixels per line. Scanning rates were optimised to acquire a stable and clear image without damaging the tip or detaching particles during scanning, usually 0.3–1 Hz.

Viscosity was measured using a TA Instruments rheometer. Using the sizes measured by TEM, AFM and the viscosity of the different media dilutions, the time (*T*) for all the particles in the solution to migrate to the bottom of the fluid under gravity was calculated. The equation and calculations are shown in supplementary information (Eq. S2).

An analysis of variance (ANOVA) was applied to compare more than one set of data at one time and is the overall test used to determine whether means of multiple groups differ. A *p* value of <0.05 was used to indicate a significant difference at 95% confidence levels.

2.4. *Daphnia* culture media

Standard OECD ISO media containing CaCl₂·2H₂O (294 mg L⁻¹); MgSO₄·7H₂O (123.25 mg L⁻¹); NaHCO₃ (64.75 mg L⁻¹); KCl (5.75 mg L⁻¹) and Na₂SeO₃ (2 μ g L⁻¹) (Taylor et al., 2009) was prepared at pH 7.5. The medium was used at full ionic strength (8.84 μ M) denoted media1 alongside a two-fold dilution (media2) and a ten-fold dilution (media10).

2.5. Nanoparticle stability testing

The stability of the three different sized citrate-stabilised AgNPs, in the three OECD ISO media concentrations, was investigated by TEM and AFM. The cleaned stock solution (12 mg L⁻¹) was dispersed in the media to obtain a solution of 2.2 mg L⁻¹ and kept in the dark at room temperature for 24 h. The final pH of the solutions was adjusted to 7.5. After 24 h, the TEM and AFM images of particles in media as well as their EDX spectrum were taken.

2.6. Acute exposures of *Daphnia* to AgNP3

D. magna were cultured in media1 (Taylor et al., 2009) and in media10 (Römer et al., 2011), as outlined previously. Animals completed at least one full generation (ca. 14 days) in the appropriate media before neonates were removed for toxicity testing. Nominal exposure concentrations of 0.1, 0.5, 1, 2.5, 5, 7.5, 10, 11.25 and 20 μ g L⁻¹ of AgNP3 were employed for acute toxicity measurements in either media1 or media10. Capping agent (citrate) and negative controls were used to assess background mortality and the potential adverse effects of the capping agent. Groups of 10 *D. magna* neonates (<24 h old) were added to exposure vessels containing 250 mL of media10 or media1 and exposed for 24 h to one of the concentrations listed above (*n* = 3 exposure vessels per concentration, per media type) in accordance with OECD exposure guidelines (OECD, 2004). No food or supplements were added during the exposure period. Neonate immobilisation and/or behavioural abnormalities were assessed visually at 24 h. The 24 h EC_{50s} were calculated via the trimmed Spearman–Kärber method (Hamilton et al., 1977) when applicable and dose-response curves were plotted using GraphPad Prism4 (version 4.03, GraphPad Software Inc.).

3. Results and discussion

3.1. Characterisation of silver nanoparticles

Three batches of AgNPs were synthesised and characterised and found to have core diameter sizes of approximately 20 nm (termed AgNP1), 10 nm (AgNP2) and 7 nm (AgNP3), all stabilised with citrate. These characterisation results, using UV-Vis spectroscopy, DLS and FFF, have been reported previously (Römer et al., 2011). Size measurement with TEM and AFM are reported in the supporting information, Table S1.

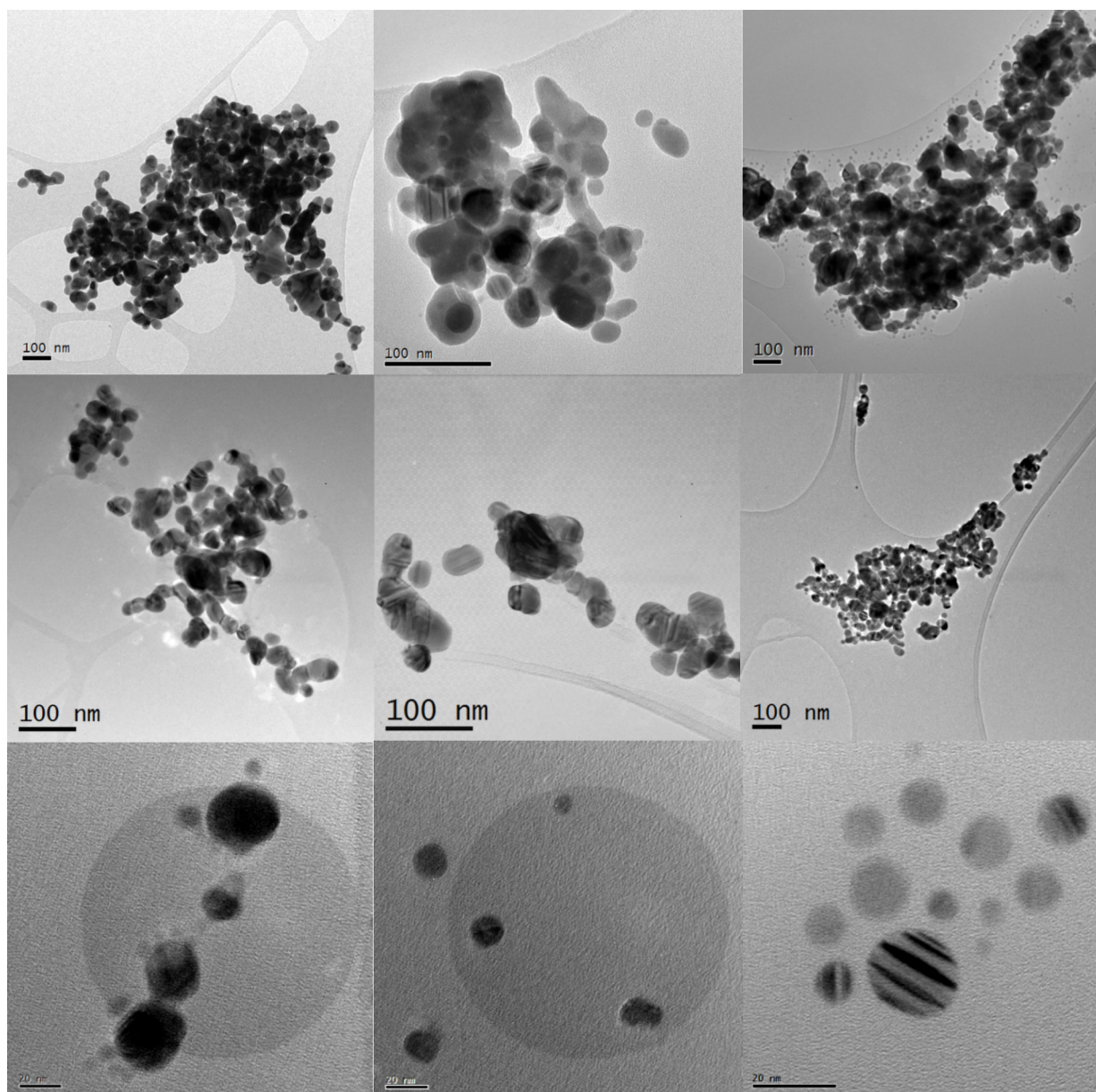


Fig. 1. TEM images for (first row from left to right) 20 nm (AgNP1), 10 nm (AgNP2) and 7 nm (AgNP3) diameter silver nanoparticles, in standard strength (media1), two-fold diluted (media2) and ten-fold diluted (media10) OECD ISO media after 24 h in solution. The scale bar for media1 and media2 is 100 nm, and 20 nm for media10.

3.2. Effect of OECD ISO test media on AgNP properties

The stability of particles AgNP1, AgNP2 and AgNP3 in OECD ISO test media was assessed by measuring the size of the particles in three different dilutions of the media by TEM and AFM. TEM images obtained for all particles in the different media dilutions (media1, media2 and media10) are shown in Fig. 1 (EDX results are in supplementary information, Figure S4) and the images obtained by AFM are shown in Fig. 2. For media1 and media2, as expected, large aggregates can be seen by both methods whereas in media10 aggregation is minimal and particles are dispersed. These results are fully in agreement with data from FFF and DLS (Römer et al., 2011), and are reported at a 24-h time point only, to correspond to the duration of the toxicity exposure studies. Longer times will lead to higher aggregation.

Table 1 shows the size of the largest and smallest aggregates in media1 and media2, and single particles for media10, as determined by TEM. For media1 and media2 it was not possible to obtain

an aggregate size distribution because of the polydispersity of the sample. In the case of media10, AFM size distribution is reported because little to no aggregation was observed in those samples after 24 h.

Particles agglomerate strongly in both media1 and media2, although some individual NPs are also observed, as seen in Figs. 1 and 2. It has been observed previously that increasing the ionic strength of the media and changing its composition can affect NP size (Römer et al., 2011; Tejamaya et al., 2012). The effects of the ionic strength also depend on particle size. Media1, which has the highest ionic strength, induces aggregation very quickly on every particle size used; very large aggregates were observed in every case, with the presence of some smaller particles. In the case of media2, with half the ionic strength of media1, particles aggregated quickly, and in the case of AgNP3 very large aggregates could still be observed. In the media with the lowest ionic strength, media10, all particles showed a significant increase of about 2–3 nm in size, compared to the as prepared particles (Table S1 in supporting

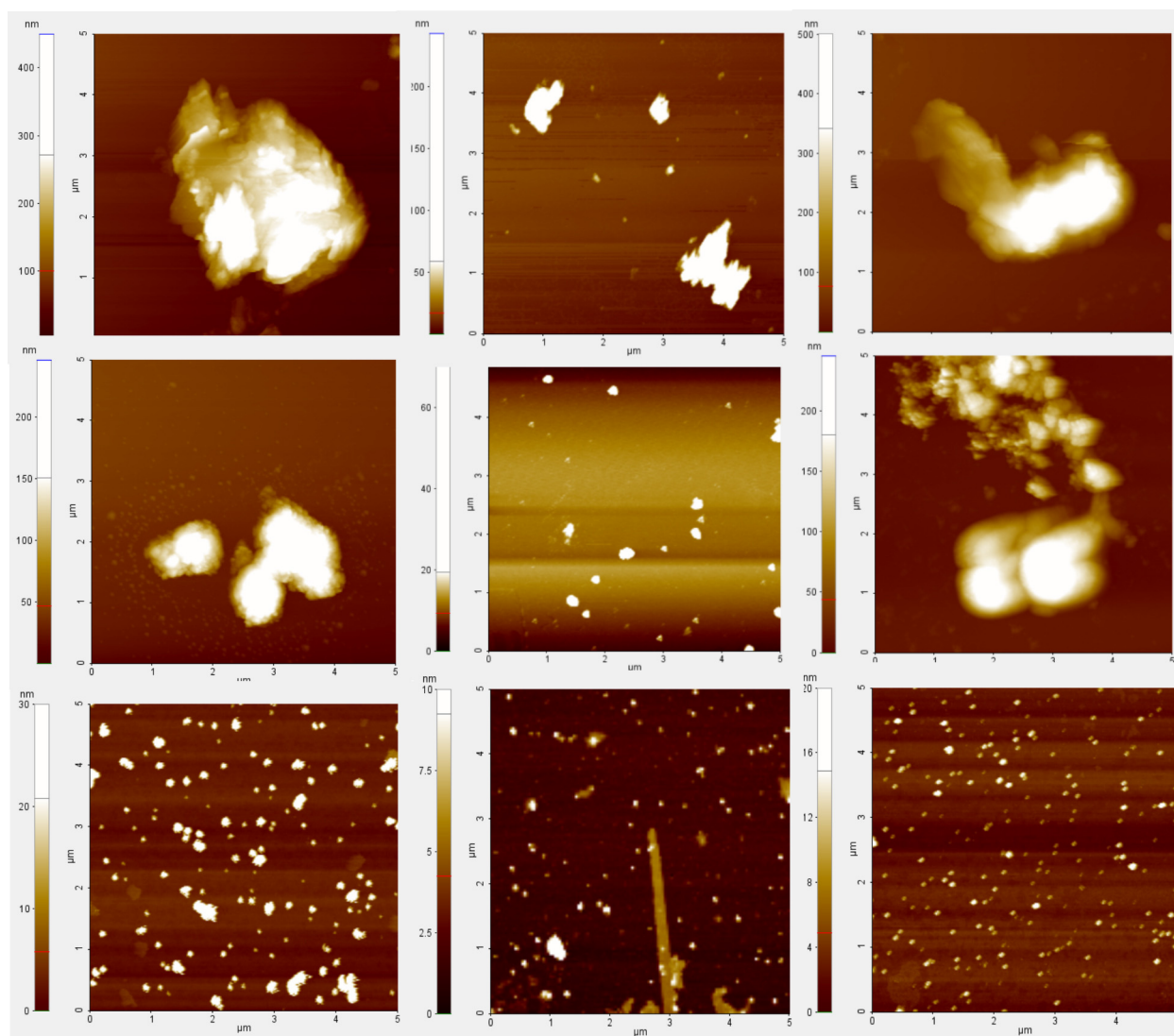


Fig. 2. AFM images for (first row from left to right) 20 nm (AgNP1), 10 nm (AgNP2) and 7 nm (AgNP3) diameter silver nanoparticles, in standard strength (media1), two-fold diluted (media2) and ten-fold diluted (media10) OECD ISO media after 24 h in solution. The AFM images are $5 \mu\text{m} \times 5 \mu\text{m}$ and the samples were prepared by centrifugation method.

information) and in media10, perhaps due to dissolution and re-crystallization (Yang, 2007; Tejamaya et al., 2012).

Fractal dimension (D) for the different aggregates was also calculated (Table 1) and results show no statistical difference ($p=0.20$)

between concentrated media. Fractal dimension is a ratio that compares statistically how fractal patterns change with the scale in which they were measured (Christian et al., 2008). In all cases the particles form compact aggregates with a fractal dimension

Table 1
Properties of the AgNPs prepared in media1, media2 and media10, measured by TEM and AFM.

Measurement	Range of aggregate sizes TEM (nm)	Single particle size TEM (nm)	Single particle size AFM (nm)	Circularity (from TEM)	Fractal dimension (TEM) (D)
<i>Media1</i>					
AgNP1	117–1774	1.7–41		0.17 ± 0.06	1.91 ± 0.11
AgNP2	100–400	4–60	n.a.	0.25 ± 0.11	1.94 ± 0.08
AgNP3	50–3300	2–40		0.28 ± 0.13	1.88 ± 0.13
<i>Media2</i>					
AgNP1	80–1200	5–55		0.31 ± 0.16	1.87 ± 0.10
AgNP2	40–510	3–80	n.a.	0.32 ± 0.13	2.05 ± 0.36
AgNP3	60–1100	3–60		0.25 ± 0.12	1.91 ± 0.16
<i>Media10</i>					
AgNP1		19 ± 9 ($n=109$)	22 ± 9 ($n=113$)	0.91 ± 0.04	
AgNP2	n.a.	13 ± 7 ($n=103$)	10 ± 3 ($n=100$)	0.93 ± 0.05	n.a.
AgNP3		10 ± 5 ($n=200$)	7 ± 3 ($n=146$)	0.95 ± 0.06	

n.a.: not available.

Table 2

Rate of sedimentation in media1 and media10, calculated from Stokes' law (calculations in SI). In the case of media1, sedimentation times were calculated for the smallest and largest aggregates found in the TEM images analysed.

Measurement	Media1						Media10		
	AgNP1		AgNP2		AgNP3		AgNP1	AgNP2	AgNP3
Sedimentation time (days)	30	0.13 (3.2 h)	19	1.2 (28.8 h)	76	0.017 (25 min)	470	1879	3836
Size of particle (nm)	117	1774	100	400	50	3300	20	10	7

indicative of a rate-limited aggregation (Buffle et al., 1998). This type of agglomeration mechanism most likely results in slower sedimentation than for diffusion limited aggregation, thus keeping the NP aggregates in solution for longer periods of time (Baalousha et al., 2008). It can be observed in Table 2 that large aggregates in media1 have a shorter sedimentation time compared to the dispersed particles in media10. In the case of AgNP3, which were used to calculate the EC₅₀ values, larger aggregates sediment within 1 h. In media10 single particles were observed and very few aggregates were seen (Figs. 1 and 2, and Table 2). This corresponds with data obtained previously with DLS and FIFFF (Römer et al., 2011).

Circularity values obtained were consistently small (Table 1), which is expected due to the change in size and shape. Circularity values range from 1 for a circular object (spherical or disc-shaped nanoparticle) to 0 for a highly elongated object or irregular shape/(rod-shaped nanoparticle), and it can be defined as the ratio of the area of a nanoparticle with its perimeter (Stevenson et al., 2012). The significant difference is between the NPs in media10, which have a circularity value near to 1, and the aggregates in concentrated media1 and media2, which have a much lower value as aggregate shape is being measured, not individual particle shape. Circularity of media10 is not significantly different to the NPs as prepared (shown in supporting information, Table S1).

3.3. AgNP3 toxicity to *D. magna* in diluted and undiluted OECD ISO media

In both media, immobilisation followed a concentration-dependent pattern with an absence of adverse effect in both the negative and capping agent controls. In media10, where NPs were largely dispersed, a lowest observed adverse effect concentration (LOAEC) of 2.5 µg L⁻¹ was observed, with immobilisation of the total population occurring at 10 µg L⁻¹. The corresponding LOAEC in media1, where aggregation occurred, was 11.25 µg L⁻¹ and (despite an increased exposure range of up to 20 µg L⁻¹) complete immobilisation was not achieved (Fig. 3). Using the Spearman–Kärber method, the EC₅₀ for media10 was calculated as 7.46 µg L⁻¹, with lower and upper 95% confidence intervals of 6.84 µg L⁻¹ and 8.13 µg L⁻¹ respectively. Although an EC₅₀ value could not be calculated from the data acquired for media1, it cannot lie below 11.25 µg L⁻¹ and is therefore higher than that observed in media10.

Clearly, the smaller, more dispersed NPs give rise to increased toxicity (Fig. 3). There are two potential causes related to (a) a change in real exposure concentration rather than added or nominal dose, due to aggregation and other changes and (b) change in the intrinsic bioavailability/toxicity (on a mass basis) of the aggregates when compared to the dispersed NPs. The sedimentation rates in Table 2 and the observed feeding behaviour of *Daphnia*, where feeding is performed throughout the water column and also at the container bottom, suggest that effective exposure concentration has not changed and supports the inference that the aggregates are of lower bioavailability and/or toxicity compared to the dispersed phase. This change may be due to a loss of specific surface area or due to lower internalisation of the NP aggregates. Although

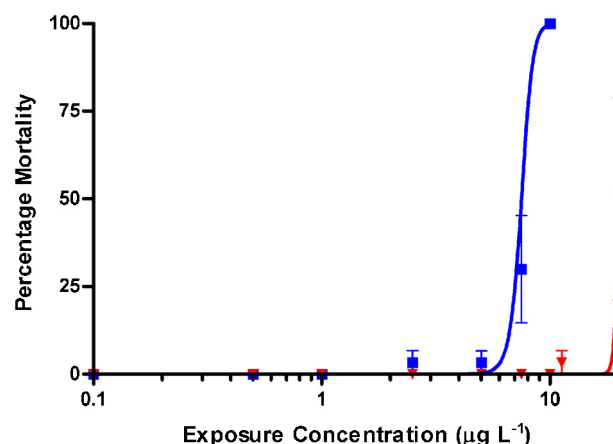


Fig. 3. A comparison of the dose response curves exhibited by *Daphnia magna* neonates exposed to 7 nm diameter silver nanoparticles (AgNP3) in standard strength OECD ISO media (media1 ▼) and a ten-fold dilution (media10 ■). Error bars represent the standard error of each exposure group. The EC₅₀ in media10 was calculated as 7.46 µg L⁻¹, with lower and upper 95% confidence intervals of 6.84 µg L⁻¹ and 8.13 µg L⁻¹ respectively. For media1, an EC₅₀ could not be calculated, but it was clearly above the lowest observed adverse effect concentration (LOAEC) of 11.25 µg L⁻¹.

aggregation can decrease the “available” surface area of materials, dependent on particle number, size distribution, and the fractal dimensions of the aggregate (Zhang et al., 2007; Hotze et al., 2010; Lowry et al., 2012), the nature of the aggregates formed suggests the effect is due to lower internalisation of aggregates rather than a reduced specific surface area.

4. Conclusions

The number of particles and the specific surface area available for interactions will both be larger in the dispersed form, leading to responses that reflect the true impact of nanoparticles on ecotoxicity test organisms. It is therefore important to ensure dispersion of AgNPs to be able to assess nanoparticle toxicity for pelagic species, while also providing a full characterisation of dispersed NPs and their aggregates prior to exposures.

Conflict of interest

None declared.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.toxlet.2013.08.026>.

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