Journal of Chromatography A, 1218 (2011) 4226–4233

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Aggregation and dispersion of silver nanoparticles in exposure media for aquatic toxicity tests

Isabella Römer[a], Thomas A. White[b], Mohammed Baalousha[a], Kevin Chipman[b], Mark R. Viant[b], Jamie R. Lead[a,*]

[a] School of Geography Earth, and Environmental Sciences, University of Birmingham, Edgbaston, Birmingham B15 2TT, UK
[b] School of Biosciences, University of Birmingham, Edgbaston, Birmingham B15 2TT, UK

ARTICLE INFO

Article history:
Available online 24 March 2011

Keywords:
Flow field-flow fractionation
FFF
Daphnia magna
Nanomaterial
Nanoscience

ABSTRACT

Silver nanoparticles (AgNPs) are currently being very widely used in industry, mainly because of their anti-bacterial properties, with applications in many areas. Once released into the environment, the mobility, bioavailability, and toxicity of AgNPs in any ecosystem are dominated by colloidal stability. There have been studies on the stability or the aggregation of various nanoparticles (NPs) under a range of environmental conditions, but there is little information on fully characterised AgNPs in media used in (eco)toxicity studies. In this study, monodisperse 7, 10 and 20 nm citrate-stabilised AgNPs were synthesised, characterised and then fractionated and sized by flow field-flow fractionation (FFF) and measured with dynamic light scattering (DLS) in different dilutions of the media recommended by OECD for *Daphnia magna* (water flea) toxicity testing. Stability of NPs was assessed over 24 h, and less so over 21 days, similar time periods to the OECD acute and chronic toxicity tests for *D. magna*. All particles aggregated quickly in the media with high ionic strength (media1), resulting in a loss of colour from the solution. The size of particles could be measured by DLS in most cases after 24 h, although a fractogram by FFF could not be obtained due to aggregation and polydispersity of the sample. After diluting the media by a factor of 2, 5 or 10, aggregation was reduced, although the smallest NPs were unstable under all media conditions. Media diluted up to 10-fold in the absence of AgNPs did not induce any loss of mobility or fecundity in *D. magna*. These results confirm that standard OECD media causes aggregation of AgNPs, which result in changes in organism exposure levels and the nature of the exposed particles compared to exposure to fully dispersed particles. Setting aside questions of dose metrics, significant and substantial reduction in concentration over exposure period suggests that literature data are in the main improperly interpreted and nanoparticles are likely to have far greater biological effects than suggested thus far by poorly controlled exposures. We recommend that the standard OECD media is diluted by a factor of ca. 10 for use with these NPs and this test media, which reduces AgNP aggregation without affecting the viability of the text organism.

1. Introduction

Nanoparticles (NPs) are usually defined as those particles that have at least one dimension between 1 and 100 nm in size [1,2]. Metal NPs are easily prepared by traditional aqueous synthesis routes and can be chemically modified by a variety of capping agents providing monodisperse, high purity NPs suitable for (eco)toxicological studies [3]. Silver nanoparticles (AgNPs) are very widely used currently in industry [1], mainly because of their anti-bacterial properties, with applications in cosmetics and as bacteriocides in fabrics and other consumer products [2,4–6]. This increasing popularity means that these particles are currently being released to the environment [7,8] and their toxicity must be assessed. They have been shown to be toxic to microbes and invertebrates although somewhat less so to fish and humans [9].

Once released into the environment, the mobility, bioavailability, and toxicity of AgNPs are dominated by their colloidal stability [10]. Many factors can affect colloidal stability, including the type of capping agent, the local environmental conditions, such as pH, ionic strength, and the background electrolyte composition [11–14]. There have been a number of studies on the stability or the aggregation of various NPs under a range of environmental conditions [1,10], but there is little information on AgNPs [10,12,15,16] and almost none investigating the impacts of ecotoxicological exposure media on stability.

* Corresponding author. Tel.: +44 121 414 8147.
E-mail address: j.r.lead@bham.ac.uk (J.R. Lead).

0021-9673/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.chroma.2011.03.034

The lack of appropriate data is of concern as the dispersion and stability of tested nanoparticles for *in vitro* or *in vivo* exposures will alter both the effective nanoparticle dose and the nature of the toxicant (dispersed and aggregated form) [15]. Such changes may lead to altered absorption, distribution, metabolism, excretion (ADME) and mechanisms of toxicity, affecting interpretation of toxicity data [17–19]. Due to these effects, it is essential for an ecotoxicology test to fully characterise the particles in the media that will be used.

The freshwater invertebrate species *Daphnia magna* is a well established test organism in ecotoxicology, and recognized by the OECD, United States Environmental Protection Agency (USEPA) and the European Union (Registration, Evaluation, and Authorization of Chemical, REACH) due to ease of culture, short life span and ecological importance. Specifically, the OECD has outlined two aquatic toxicity tests employing *D. magna* assessing both acute (48 h) immobilization and chronic reproductive (21 days) perturbation [20,21]. The acute and chronic OECD tests comprise half of the core USEPA environmental toxicity assessment, required for all new chemical substances expected to have substantial environmental release (≥1000 kg/year released to surface water after wastewater treatment) [22] and thus have a clear importance as a regulatory tool for controlling discharges of potentially toxic materials. The EU regulation REACH requires performance of the acute test on *D. magna* only for substances manufactured or imported in quantities of >10 metric tons/year; for substances produced or imported at >100 metric tons chronic data on daphnids may be required (decided on a case-by-case basis) [23].

While OECD exposure media for aquatic organisms are appropriate for traditional chemical toxicity testing, the propensity of nanomaterials to agglomerate in complex solutions [15] can lead to unexpected results. In this study, monodisperse citrate-stabilized AgNPs of well defined sizes were characterised in media used for OECD *D. magna* toxicity testing, and in dilutions of this media. Detailed information of aggregation was collected by dynamic light scattering (DLS), flow field flow fractionation (FIFFF) and by zeta potential measurement. Parallel measurements of *D. magna* viability in media and identical dilutions allow recommendation of optimised test methods for the investigation of daphnia toxicity.

2. Experimental

2.1. Silver synthesis and cleaning

AgNPs stabilized with citrate were synthesised by varying previously reported methods [1,24,25]. Three different sizes of AgNPs (labelled AgNP1, AgNP2 and AgNP3) were prepared from a standard reduction of the silver salt in sodium citrate. Solutions of 100 ml sodium citrate (0.31 mM), 100 ml silver nitrate (0.25 mM) and sodium borohydride (10 mM for AgNP1 and AgNP3, and 0.25 mM for AgNP2) were prepared in pure water and kept at 4 °C in the dark for 30 min. The silver nitrate and sodium citrate solutions were mixed together in a conical flask and vigorously stirred. Subsequently 6 ml of the solution of the reducing agent, sodium borohydride ($NaBH_4$), was added in one batch. After 10 min of stirring, the solution was heated slowly to boiling and heated for a further 90 min, left overnight and then cooled (4 °C, in the dark). To obtain the smallest nanoparticles (labelled AgNP3), the heating process was performed without $NaBH_4$, and at the end 6 ml of the 10 mM reducing agent was added and the solution was heated for 10 extra minutes. AgNPs were cleaned to remove the excess reagents before use. Suspensions were cleaned by ultrafiltration (Amicon, 1 kDa regenerated cellulose membrane, Millipore) using a diafiltration method to prevent drying of the particles. The particles were redispersed in citrate solution to avoid further growth, this process was repeated at least three times.

The AgNPs were characterised using a multi-method approach including flow field-flow fractionation (FlFFF), surface plasmon resonance (SPR), zeta potential and dynamic light scattering (DLS).

2.2. Preparation of the media for culture of D. magna

Media preparation followed the OECD guideline [21] for the culturing and exposure to xenobiotics of *D. magna*, with a minor modification as detailed previously [26]. Briefly, OECD-recommended ISO media consisted of calcium chloride (294 mg l^{-1}), magnesium sulphate (123.25 mg l^{-1}), sodium carbonate (64.75 mg l^{-1}) and potassium chloride (5.75 mg l^{-1}) with additional sodium selenite (0.002 mg l^{-1}) (Sigma Aldrich, UK). As dictated by the OECD guideline only media within pH range (6–9) was used. The media was used at full strength (ionic strength of 0.00884 M) and after dilution by a factor of 2, 5 or 10 (labelled: media1, media2, media5 and media10, respectively). All dilutions were made with deionised (DI) water and final pH was 7.5 in all cases.

2.3. Preparation of the AgNPs samples in the media

The solution of AgNPs as prepared (12 mg l^{-1}) was added to the media to obtain a solution of 2.2 mg l^{-1}. Two other solutions were prepared to use as controls with the same concentration as the particles in media, one by adding citrate and the other using DI water. The particles were left in the different solutions for 24 h and 21 days. The final pH of the solutions was adjusted to 7.5.

2.4. Flow field flow fractionation (FlFFF)

Separation was performed in an asymmetrical flow field flow fractionator (FlFFF) (AF2000 Mid Temperature, *Postnova Analytics*, Germany). The accumulation wall consisted of a 1 kDa regenerated cellulose membrane. The eluent was delivered by separate cross-flow (PN1130 Isocratic pump) and channel flow pumps (PN4020 channel) and was degassed before delivery (PN7520 solvent degaser). The eluent was 0.01 M NaCl, pH 7.5, as used in previous studies [1]. Channel flow was set to 1 ml min^{-1} and the cross flow was set between 0.3 and 0.5 ml min^{-1} to obtain good separation of the void and sample peak. Particles eluted from the FlFFF channel were detected by a UV spectrometer, the detector was set at 400 nm wavelength for detection of the AgNPs. Data acquisition was via LC solution multi PDA (version 2.1, *Postnova Analytics*, Germany). The channel volume was calculated applying FlFFF theory as previously described in [28] using 33 and 60 nm polyacrylamide beads PAA (*Duke Scientific Corp.*) [27], which were detected with the UV detector at 254 nm. Diffusion coefficients were calculated based on standard FlFFF theory and converted to size using the Stokes–Einstein relationship [27]. Between 1 ml and 2 ml of the sample was injected, 1 ml for the samples as prepared and in water (controls), and 2 ml for the samples in media, at least three independent replicates were analysed per sample and the data averaged. Excellent agreement (peak heights differing by <2%, in general) between replicates was observed. The particles in the media and in water were measured by FIFFF after 24 h.

2.5. DLS and zeta potential

Measurements were conducted with a Malvern Zetasizer 5000 in low volume disposable cuvettes and at least five concordant measurements recorded to calculate a mean *z* average size. Measurements were made on all samples after 24 h. DLS was measured for all samples over a 5 h period. Samples were also measured after 24 h and 21 days.

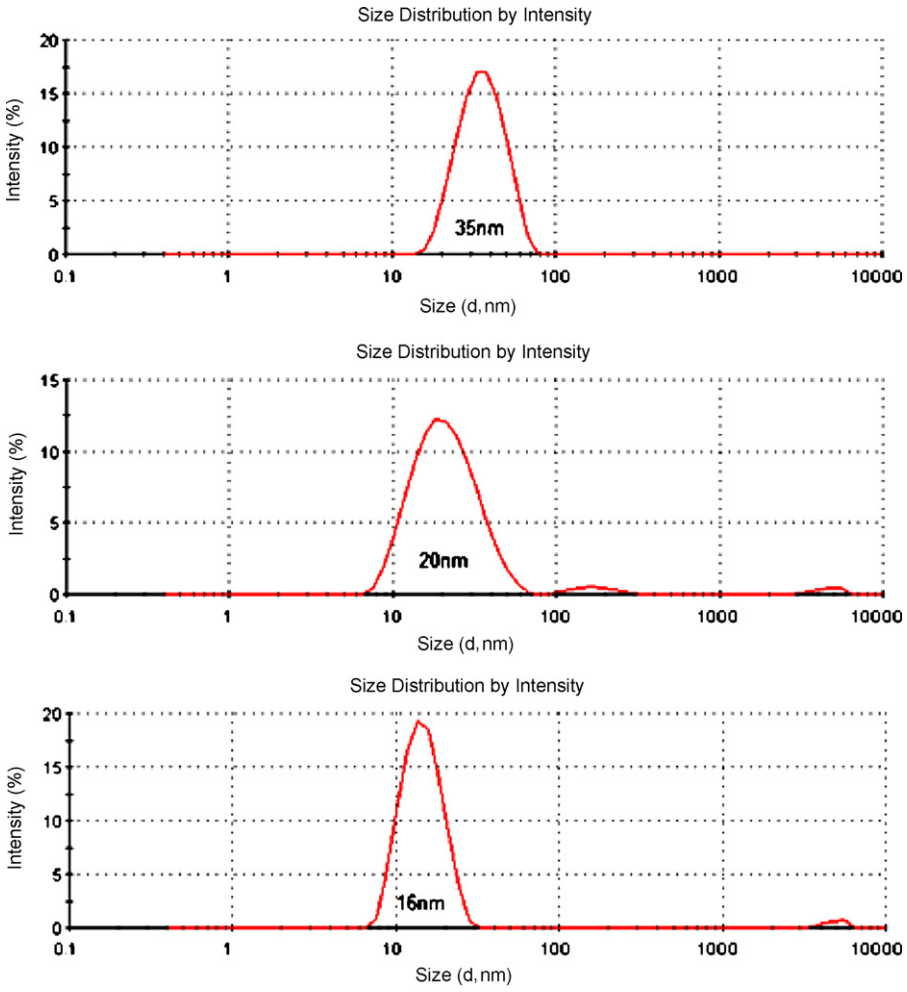

Fig. 1. Size distribution by intensity obtained with DLS for AgNP1, AgNP2 and AgNP3.

2.6. Surface plasmon resonance

Spectral scans were obtained from an ultraviolet–visible spectrometer (Lightwave) through a 1 cm pathway quartz cuvette (VWR). The instrument was referenced with a sealed water cell and wavelengths were collected from 200 to 800 nm from at least three measurements and were blank corrected.

2.7. D. magna acute toxicity testing

Acute *D. magna* toxicity tests following OECD guidelines [20] were performed using media dilutions in parallel with the NP agglomeration tests above. Groups of 10 *D. magna* neonates (<24 h old) were rinsed using DI water and immediately transferred to 250 ml beakers containing media1 (no dilution), media2 (media1 diluted by factor of 2), media5 (media1 diluted by factor of 5), media10 (media1 diluted by factor of 10), media20 (media1 diluted by factor of 20) and DI water (100 ml, $n = 4$ beakers per media concentration). Rinsing avoids contamination of the exposure media by the culture media, which is particularly important for the highly dilute exposure media studies. Immobilisation of neonates was visually assessed after 24 and 48 h and noted alongside any other irregularities (e.g. organism becoming trapped at the liquid surface). According to OECD guidelines [20] any neonate unable to swim within 15 s of gentle test vessel agitation is defined as immobilised regardless of antennae movement. As defined in these guidelines, no food or supplements were provided during the test period, and pH was recorded at 0 and 48 h.

2.8. D. magna chronic reproductive toxicity testing

Chronic *D. magna* reproductive toxicity tests following OECD guidelines [21] were performed using media dilutions suggested by the apparent no observed effect level (NOEL) in the acute toxicity tests. Single *D. magna* neonates (<24 h old) were rinsed and immediately transferred to 250 ml beakers containing media1, media10, media20 (media1 diluted by a factor of 20) and DI water (100 ml, $n = 5$ per media concentration). Animals were maintained in these beakers for 21 days, with twice weekly media replacement and daily algal feeding (*Chlorella vulgaris*, 100 μl on days 1–2, 150 μl on days 3–7, 200 μl on days 8–21) but with no additional supplements. To assess reproductive capability, the number of offspring were counted and removed from the test vessel daily. Again any other irregularities were recorded.

3. Results

3.1. Characterisation of Ag nanoparticles as prepared

The AgNPs were characterised using a multi-method approach using SPR, FIFFF, zeta potential and DLS. Fig. 1 shows the size distribution by intensity obtained with DLS for the different particles in their "as-prepared" state and Fig. 2 shows the results obtained with FIFFF. The summary of sizes (derived from maximum peak height)

Table 1
Size distributions for the monodisperse and stable AgNPs prepared, measured by DLS and FFF for the samples as prepared and in water. Zeta potential measured is also shown.

Sample name	*z* average diameter DLS (particles as prepared) (nm)	*z* average diameter DLS (particles in water) (nm)	Weight average diameter FFF (as prepared) (nm)	Zeta potential as prepared (pH 5.4)
AgNP1	35	42	20.8 ± 0.4	−46
AgNP2	20	25	9.6 ± 0.6	−40
AgNP3	16	27	7.2 ± 0.2	−45

for the AgNPs obtained by both methods is shown in Table 1. The size obtained by DLS is considerably larger than the size obtained by FIFFF. We observed small increases in size of all particles in water after 24 h measured by DLS, where the smallest particles, AgNP3, doubled in size and the other particles showed small increases of about 5 nm. The NPs were subsequently stable in pure water after a 21 days period.

Zeta potential was determined for the different particles in citrate and also in water with changing pH values (from 2 to 12) to find the point of zero charge. All particles had a zeta potential of around −40 when in citrate (Table 2), which was less negative in water alone at relevant pH values. The point of zero charge is at a pH value lower than 2, as full protonation of the citrate stabiliser occurs at this low pH value (data not shown). The lower *z* after dilution by water alone compared to dilution using a citrate solution is likely due to the desorption of weakly bound citrate after dilution in water as the system re-equilibrates.

All of the AgNPs suspensions have a yellow colouration due to the surface plasmon band (SPB) at around 414 nm, which is typical for silver [2,25]. All spectra are very similar with no shifts or peak broadening and were also symmetrical.

3.2. The effect of D. magna toxicity testing media on AgNP properties

The stability of particles AgNP1, AgNP2 and AgNP3 in daphnia media was assessed by measuring the size of the particles in different dilutions of the media by DLS and FlFFF, while zeta potential and SPR were also measured. The dilutions of the media were made by adding water, which we have previously shown [1] reduces stability by desorption of citrate of the particles due to re-equilibration between solution phase and surface-bound citrate.

After dilution, DLS was measured every hour for up to 5 h and then after 24 h and 21 days, all samples showed aggregation immediately after being added to the media, increasing with time until they reached stability at about 5 h. Size distribution by intensity measured by DLS for media1 and media10 are shown in Fig. 3. In media1 we can see aggregates of AgNPs of between 100 and 200 nm in size after 24 h, depending on the particles used initially. Visual observation showed that the solution went from yellow to colourless almost immediately; a similar loss of the 414 nm peak as measured by UV–vis spectroscopy was also observed due to loss of material from suspension by aggregation and settling. Similar results were obtained for media2, although there was some extra stability associated with AgNP2, where aggregates of about 60 nm in size were measured and remain stable, even after 21 days (data not shown). However, for samples AgNP1 and AgNP3 the solution also went colourless after an hour, showing a high level of aggregation. The media was further diluted to obtain media5 and media10. The particles of sample AgNP1 were largely stable in both dilutions but some aggregation was observed after 24 h, which increased over the next 21 days. In the case of AgNP2 there was a slight increase in size in both dilutions (increasing from 20 nm without media to 35 nm after 24 h in media10), and some larger aggregates were also observed by DLS. For AgNP3, the DLS data showed quite extensive aggregation with both of the dilutions and high polydispersity of the particles. Aggregation in media10 is less pronounced for all samples, as we can see in Fig. 3.

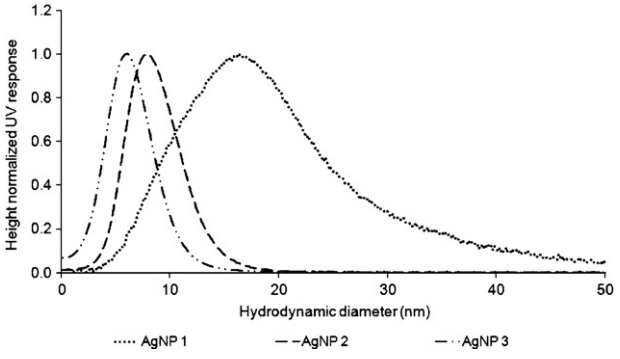

Fig. 2. Size distribution results obtained with FFF for the nanoparticles in citrate.

Zeta potential data from the same samples are shown in Table 2. Clearly these values become less negative in all media dilutions compared to the particles in water, which qualitatively agrees with the results obtained by DLS, although the zeta potential measurements were insufficiently sensitive to discriminate between media dilutions.

Fig. 4 shows the FFF results obtained for the three particles with the *D. magna* exposure media and in media dilutions and water. The peak (d_p), number (d_n), and weight (d_w) average hydrodynamic diameters and sample polydispersity were calculated for each AgNP sample in the different media applying Eqs. (1)–(3), and results are summarized in Table 3.

$$d_n = \frac{\sum_i C_i x_i}{\sum_i C_i} \quad (1)$$

$$d_w = \frac{\sum_i C_i x_i^2}{\sum_i C_i x_i} \quad (2)$$

$$\text{polydispersity} = \frac{d_w}{d_n} \quad (3)$$

where C_i is the concentration of the particles in each FlFFF slice and x_i is the measured property (i.e., hydrodynamic diameter by FlFFF) [28].

In media1 the samples were highly polydisperse and no fractogram was observed, possibly due to particle aggregation and sedimentation. In media2 a fractogram for AgNP2 only was observed, showing a stable peak at ca. 60 nm (Fig. 4b), i.e., only small aggregates were present; data could not be collected for the other NPs due to aggregation. Media5 and media10, showed peaks of similar size to the particles as prepared, with some broadening and tailing. We can see that in general peaks have right shifted, and were broader and flatter than when in a citrate solution or water (Fig. 4a–c). However, there are no obvious reductions of peak area, as we have seen elsewhere [28] in the presence of substantial aggregation. Recovery values are around 65% for all samples, but it decreases in the case of the samples which show some aggregation (data from DLS), i.e., AgNP3 in media5 and media10. Taken

Table 2
Electrophoretic mobility for the particles at different dilutions of the media. pH of the solutions was 7.5 (the same as media1).

Sample name	Zeta potential in water	Zeta potential in media1	Zeta potential in media2	Zeta potential in media5	Zeta potential in media10
AgNP1	−24.5	−16	−10.5	−10.1	−11.9
AgNP2	−20.5	−8.6	−7.9	−7.7	−11.0
AgNP3	−23	−8.5	−9.7	−10.0	−10.0

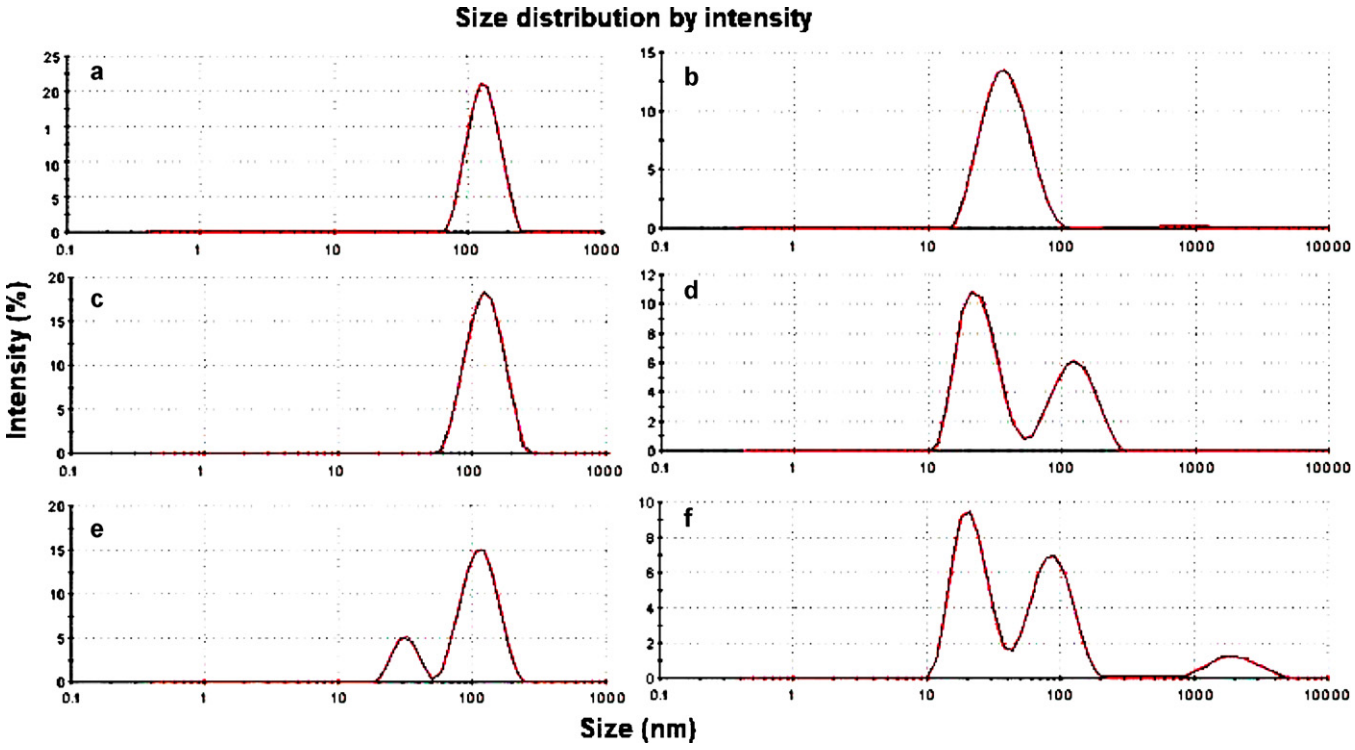

Fig. 3. Size distribution by intensity obtained with DLS in media1 and media10 (left and right) for AgNP1 (a, b), AgNP2 (c, d) and AgNP3 (e, f).

Table 3
Size distribution measured by FFF for the different AgNPs in different dilutions of Daphnia media.

Media	Sample	AgNP1	AgNP2	AgNP3
As prepared	d_p	20.8	9.6	7.2
	d_n	20.07	9.2	7
	d_w	20.9	11.9	11.6
	d_w/d_n	1.19	1.3	1.6
	Recovery (%)	64	67	64
DI water	d_p	20.4	11.7	8.4
	d_n	55.2	16.9	22.7
	d_w	86.6	24	39.6
	d_w/d_n	1.6	1.4	1.7
	Recovery (%)	62	66	64
Media2	d_p		64	
	d_n		77.6	
	d_w	n.m	206.5	n.m
	d_w/d_n		3.14	
	Recovery (%)		64	
Media5	d_p	20.5	12.6	8.7
	d_n	37.1	14	35.7
	d_w	59	18.9	45.6
	d_w/d_n	1.6	1.4	1.7
	Recovery (%)	64	64	61
Media10	d_p	21.5	10.7	9.9
	d_n	38.7	18.8	25.7
	d_w	59.1	26.3	34.1
	d_w/d_n	1.5	1.4	1.3
	Recovery (%)	64	65	62.5

d_p = hydrodynamic diameter (nm) corresponding to the peak maximum; d_n = number average hydrodynamic diameter (nm); d_w = weight average hydrodynamic diameter (nm); d_w/d_n = sample polydispersity. Particles in media1 were not measurable by FIFFF.

together, the data indicate that aggregation is occurring but is relatively minor in the media5 and media10. We can see in Table 3 that polydispersity values are lower for media10 than for media5, as well as d_n and d_w values.

3.3. Acute effect of media dilution on D. magna

D. magna neonates (<24 h) did not become immobilised when cultured in any of the diluted media for 24 or 48 h. However, in DI water there was significant ($p < 0.001$) immobilisation of 25 ± 6% of the neonates after 24 h, which increased to 35 ± 4% after 48 h (Table 4). There was also some evidence of neonates becoming trapped at the surface in media2 and media5 groups, although this was not observed in the highest dilution (media10) and there was no consistent trend.

3.4. Chronic effect of media dilution on D. magna reproduction

When maintained in DI water, all the organisms became immobilised (see Table 5) within 96 h, considerably earlier than the production of the first brood of neonates; therefore the number of offspring produced was zero. In the other media dilutions there was a complete lack of immobilisation across the 21-day period. In media20 neonate output was significantly ($p < 0.01$) lower than that of media10 and media1, however, there was no significant difference between the latter two dilutions. In addition to low fecundity in media20 there was also evidence of surface trapping and production of immobilised neonates.

Table 4
Acute effect of media dilution on *D. magna*.

Media	Starting pH	Ending pH	24 h immobilisation (%)[a]	48 h immobilisation (%)[a]	Comments
Media1	7.33 ± 0.02	7.28 ± 0.02	3 ± 2	3 ± 2	–
Media2	7.01 ± 0.03	6.98 ± 0.04	0	0	Some surface trapping
Media5	6.72 ± 0.04	6.65 ± 0.03	0	0	Some surface trapping, one example of irregular swimming
Media10	6.37 ± 0.03	6.37 ± 0.03	0	0	–
DI water	6.05 ± 0.04	6.09 ± 0.03	25 ± 6*	35 ± 4*	–

* $p < 0.001$.
[a] Significance determined from ANOVA across all 5 media concentrations.

4. Discussion

Three AgNPs of different sizes were synthesised using citrate as a capping agent. Characterisation showed highly monodisperse and stable particles. The size obtained by DLS is considerably larger than the size obtained by FlFFF (Table 1) and this result was consistent and agrees with the results we have previously obtained [1]. DLS provides intensity weighted size (hydrodynamic diameter), whereas FlFFF-UV provides a mass weighted size (hydrodynamic diameter). We note that the FFF size is mass based and when converted to particle number based value is expected to be somewhat smaller [28]. Our previous results have also shown that FIFF agrees well with AFM, TEM and fluorescence correlation spectroscopy (FCS) [29] indicating that the DLS overestimates particle size, as it measures intensity weighted size. Zeta potential results showed that the particles prepared are stable in a range of pH values in water (data not shown). A SPB at around 414 nm confirmed the presence of silver.

Clearly, the standard OECD test media caused substantial aggregation of AgNPs and media dilution reduced this effect. This increased stability with reduced ionic strength is, in accordance with expectations, based on DLVO theory [30], where increased ionic strength shields charges, reduces the diffuse layer thickness and allows NPs to come into contact sufficiently closely to aggregate. Zeta potential changes between the different dilutions of the media were not observed, possibly due to addition of Cl^- via the media [10]. Silver halides, such as AgCl, are well known to form insoluble precipitates in solution with net negative charge [11]. Thus, the measured zeta potential values, and potentially some of the DLS data, are a combination of the information on the AgNPs and the AgCl particles, making interpretation more difficult. Indeed, the effect of the conditions on AgNP aggregation has been previously studied [10] using environmentally relevant conditions. Aggregation in solutions with high ionic strength, in particular those containing divalent cations (Ca^{2+}), was found, although elevated levels of Cl^- also increase stability of citrate stabilized AgNPs at low ionic strength conditions. Other research [1] used FFF and DLS to test the stability of citrate stabilized AgNPs in solutions with added sodium, calcium and humic substances (HS). Addition of sodium and calcium caused aggregation of the AgNPs, even at low concentrations, but the addition of low and environmentally relevant concentrations of HS stabilized the NPs and reduced losses by aggregation.

If media is diluted with water, in addition to reducing salt concentration, the surface citrate which is weakly bound to the NPs, desorbs due to re-equilibration between surface and solution, and this reduces NP stability (Tables 1 and 3). This result is shown in this work and previously [1] that dilution with citrate maintains stability and minimizes aggregation. Stability of the particles in the *D. Magna* exposure media could be potentially improved by adding citrate, but possibly with the addition of extra complexity in interpreting subsequent toxicity data. This requires testing on a case by case basis.

According to FIFFF measurements, sample AgNP1 remains stable in the diluted media (as seen in Table 2) but there is broadening and tailing of the peaks (Fig. 4a). It has a peak maximum of 20 nm as prepared, and it remains constant in water, media5 and media10. This peak maximum is not identical to the weight or number average diameter (Table 3). AgNP1 have a larger size distribution than the other AgNPs (Fig. 2) in part due to greater tailing observed at larger size as size control is more difficult during synthesis. It can also be because the conditions were optimised for the elution of the smaller NPs.

Sample AgNP2 shows stable peaks indicative of dispersed NPs in the diluted exposure media (media5 and media10), with a small amount of peak broadening and tailing. The fact that we obtain a fractogram for AgNP2 in media2 is not fully understood and requires further work to ascertain. Presumably, the stability is related to the relative strength of the citrate-NP interactions which are stronger in the case of AgNP2 giving greater stability. The hydrodynamic diameter, the weight and number average diameter obtained for the particles in media2 have very different values, and polydispersity is quite high (Table 3).

Although FFF fractograms were available for the AgNP3 in the diluted media, a clear right shift (larger sizes) in maximum peak heights was observed, along with tailing and broadening of the peaks, and also a decrease in the recovery (from 64% as prepared to 61% in media5 and 62.5% in media10). We can also see the broadening of the hydrodynamic diameter, but no significant change in the polydispersity. We can conclude that aggregation and stability of the particles is size dependent, with smaller particles being less stable than larger ones. Small particles are more mobile, interact more quickly and have a greater surface energy, so will be more

Table 5
Chronic effects of media dilution on *D. magna* reproduction (over 21-day study).

Media	Immobilisation (%)[a]	No. of offspring produced[a]	Comments
Media1	0	61.8 ± 4.0	–
Media10	0	56.8 ± 12.3	–
Media20	0	10.6 ± 5.1*	Dead neonates and surface trapping
DI water	100**	0**	–

* $p < 0.01$.
** $p < 0.001$.
[a] Significance determined from ANOVA across all 4 media concentrations.

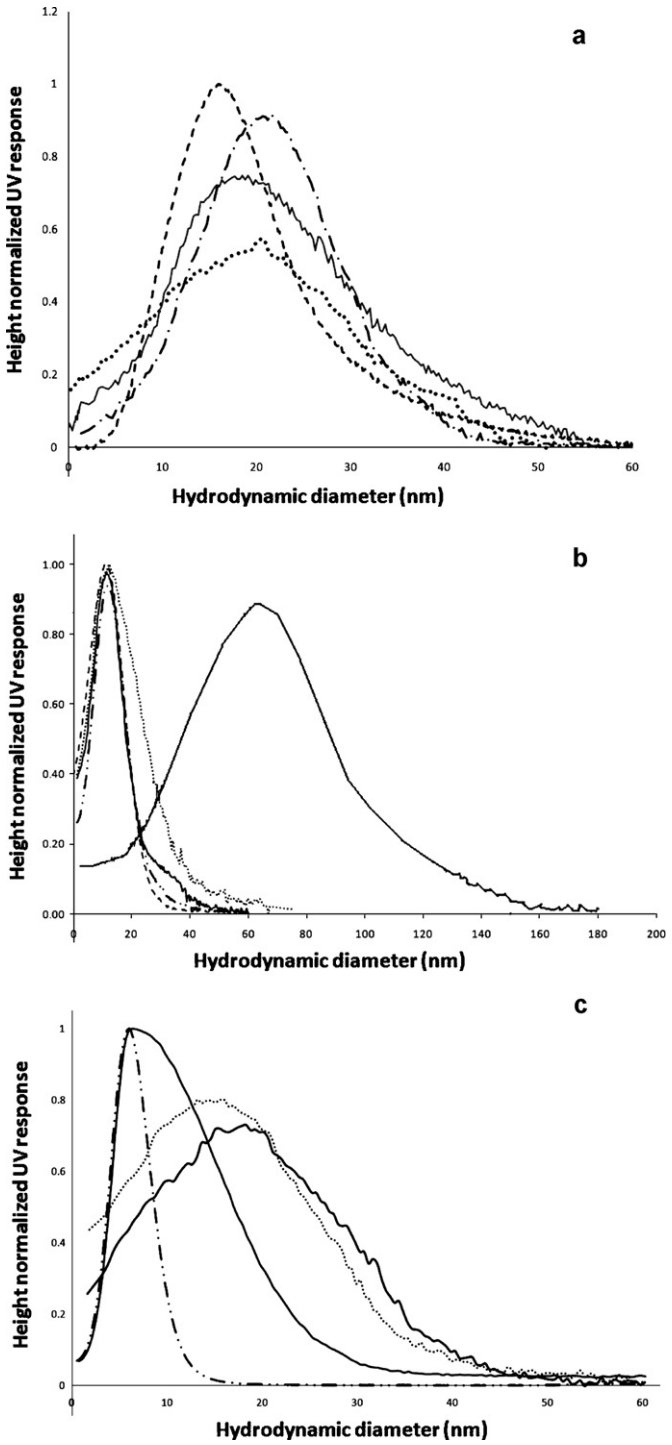

Fig. 4. Size distribution results obtained with FFF for the AgNPs in different dilutions of Daphnia media. Results for AgNP1 are shown in (a) where - - - shows the particles as prepared, ···· shows the particles in water, -·-·-· shows the particles in media5 and the gray line shows the particles in media10. AgNP2 is shown in (b) where - - - shows the particles as prepared, ···· shows the particles in water, the black line shows the particles in media2, the gray line in media5 and -·-·-· shows the particles in media10. AgNP3 is shown in (c) where -·-·-· shows the particles as prepared, the gray line shows the particles in water, the black line shows the particles in media5 and, ···· shows the particles in media10.

prone to adhere on contact, assuming the same citrate coverage and stabilisation effect. In such cases, extra stability might be provided by further dilutions and maintaining stable citrate concentrations after dilutions.

Table 3 shows that all the AgNPs as prepared have a narrow hydrodynamic diameter distribution as measured by FlFFF (Fig. 4), which seems to be similar to the weight and number average diameter, and polydispersity values are low. When the particles are dissolved in DI water we see a change in this, the weight and number average diameter increase in value, and we can see a change in the fractogram (Fig. 4), polydispersity still has low values, increasing slightly; recovery values seem to be slightly lower than the value obtained for the particles in citrate. When the particles in media5 and media10 were measured we observe that the weight and number average diameter are very different from the hydrodynamic diameter, and recovery remains relatively stable, except for AgNP3. Polydispersity values do not change substantially in the media compared to the particles in DI water. We use the hydrodynamic diameter corresponding to the peak maximum, because the number and weight average are affected by the overall aggregation (i.e., by values at large sizes or the tailing in the FlFFF fractograms) [28]. The broadening of the hydrodynamic diameter distribution fractogram indicates the increase in aggregation and formation of large aggregates. Recovery agrees with this effect, especially for AgNP3, in which we expect a higher aggregation, based on DLS data.

Comparison of the DLS and FFF data was informative. DLS could detect large aggregates (when the *z* average size was calculated) that FFF was not able to measure; these aggregates remained in the FFF channel, which turned black over time, due to the formation of oxides and sulfides, in suspensions containing large aggregates. With FFF, small unaggregated particles were measured that were not visible with DLS. Analysis of FFF (Table 3) and DLS (Table 1) data indicate that, where FFF fractograms were observed, loss by aggregation was not extensive. We recommend the use of other microscopy methods to measure particle size and fully understand suspensions on a single particle basis. Our future work includes use of other methods such as microscopy.

The minimisation and quantification of aggregation is clearly important in certain nanotoxicology testing. Aggregation will affect NP properties and toxicity by (a) reducing the nominal (added) dose to which the organisms are exposed and (b) altering the nature of the toxicant to which the organisms are exposed. Alteration of 'effective' concentration has been measured previously, where in some cases <1% of added dose is still present in suspension after the exposure period [31] and such changes clearly have large implications for interpretation of ecotoxicology data. Setting aside questions of dose metrics, significant and substantial reduction in concentration over the exposure period suggests that literature data are in the main improperly interpreted and nanoparticles are likely to have far greater biological effects than suggested thus far by poorly controlled exposures. In addition, changes in the nature of the toxicant from dispersed to aggregated form have separate but related and important effects which require consideration. Ideally, the quantification of effects on pelagic species requires the use of dispersed materials and we suggest that this is the case with *D. magna*; aggregation alters the nature of toxicant complicating interpretation. For instance, nano-aggregates may be more efficiently ingested by *D. magna* [32] while bioavailability of the dispersed material is likely to be more efficient. In addition, the specific surface area available to interact with biota will be larger in the dispersed form and there may be greater effects of the dispersed material. It is therefore important to ensure dispersion for pelagic species, while also providing a full characterisation of dispersed NPs and their aggregates prior to exposures. In addition, it is essential to measure dose and toxicant nature more exactly over

the time of the exposure, in order that interpretation of data be controlled far more precisely.

Our results suggest that media10, i.e., a dilution of standard OECD media conditions by a factor of 10, provides optimal conditions for these NPs, minimising aggregation while avoiding immobilisation and maintaining fecundity of *D. magna*. Clearly such media changes must be performed on a case by case based on understanding requirements for both NP and test organism.

5. Conclusion

We have synthesised and characterised citrate-stabilised, monodisperse silver nanoparticles at three different sizes. The effect of adding the particles to *Daphnia* exposure media at standard OECD conditions was aggregation of the particles, however, relatively small reductions of ionic strength allowed the larger particles in particular to remain stable for the period of time needed for Daphnia exposures, and the smallest particles, AgNP3, showed reduced aggregation. Under acute conditions, *Daphnia* immobilisation was evident in DI water only, in contrast to all tested media dilutions in which neonates showed only slight signs of distress. However, the more sensitive chronic reproductive toxicity test revealed absolute immobilisation in DI water and a highly significant reduction in fecundity in media20. Our results therefore suggest that media10, i.e., a 10-fold dilution of standard OECD media, provides optimal conditions for these NPs, minimising aggregation while avoiding *D. magna* immobilisation and maintaining normal reproductive output. However, for AgNPs, specifically, further work is required to investigate the effect of chloride on aggregation, precipitation and surface passivation, while further work is required to understand more subtle stress effects when using diluted media and the role of acclimation. Finally, we recommend that optimisation is required on a case by case basis. For instance, uncapped particles may be more sensitive to media effects, while sterically stabilised NPs may be less affected.

Acknowledgements

The Natural Environment Research Council are acknowledged for their support (NE/D004942/1) I.R. thanks the University of Birmingham (School of Geography, Earth and Environmental Sciences) for financial support of a studentship; T.A.W. thanks NERC for a PhD CASE study (NE/H52502X/1). Both I.R. and T.A.W. thank Cefas for a PhD case studentship, Seedcorn funding through project DP247.

References

[1] S.A. Cumberland, J. Lead, J. Chromatogr. A 1216 (52) (2009) 9099.
[2] Y. Ju-Nam, J. Lead, Sci. Total Environ. 400 (2008) 396.
[3] P.C. Lee, D. Meisel, J. Phys. Chem. 86 (1982) 3391.
[4] S.L. Luoma, PEN Report, Woodrow Wilson International Center for Scholars and The Pew Charitable Trusts, Washington, DC, USA, 2008.
[5] S.A. Blaser, M. Scheringer, M. MacLeod, K. Hungerbuhler, Sci. Total Environ. 390 (2008) 396.
[6] T.M. Benn, P. Westerhoff, Environ. Sci. Technol. 42 (2008) 4133.
[7] N. Mueller, B. Nowack, Environ. Sci. Technol. 42 (2008) 4447.
[8] F. Gottschalk, T. Sonderer, R.W. Scholz, B. Nowack, Environ. Toxicol. Chem. 29 (2010) 1036.
[9] S.J. Klaine, P.J.J. Alvarez, G.E. Batley, T.F. Fernandes, R.D. Handy, D. Lyon, S. Mahendra, M.J. McLaughlin, J.R. Lead, Environ. Toxicol. Chem. 27 (2008) 1825.
[10] A.M. El Badawy, T.P. Luxton, R.G. Silva, K.G. Scheckel, M.T. Suidan, T.M. Tolaymat, Environ. Sci. Technol. 44 (2010) 1260.
[11] T. Cosgrove, Colloid Science: Principles, Methods and Applications, Blackwell Publishing, UK, 2005.
[12] K.L. Chen, M. Elimelech, Langmuir 22 (2006) 10994.
[13] M. Tielemans, P. Roose, P. De Groote, J. Vanovervelt, Prog. Org. Coat. 55 (2006) 128–136.
[14] A.R. Petosa, D.P. Jaisi, I.R. Quevedo, M. Elimelech, N. Tufenkji, Environ. Sci. Technol. 44 (2010) 6532.
[15] J. Jian, G. Oberdorster, P. Biswas, J. Nanopart. Res. 11 (2009) 77.
[16] K.L. Chen, M. Elimelech, J. Colloid Interface Sci. 309 (2007) 126.
[17] L.C. Renwick, K. Donaldson, A. Clouter, Toxicol. Appl. Pharmacol. 172 (2001) 119.
[18] P.J.A. Borm, D. Robbins, S. Haubold, T. Kuhlbusch, H. Fissan, K. Donaldson, R. Schins, V. Stone, W. Kreyling, J. Lademann, J. Krutmann, D. Warheit, E. Oberdorster, Part Fibre Toxicol. 3 (2006) 11.
[19] H.S. Choi, W. Liu, P. Misra, E. Tanaka, J.P. Zimmer, B.I. Ipe, M.G. Bawend, J.V. Frangion, Nat. Biotechnol. 25 (2007) 1165.
[20] OECD, Guidelines for Testing of Chemicals, No. 202—*Daphnia* sp. Acute Immobilisation Test, Organisation for Economic Cooperation and Development, Paris, 2004.
[21] OECD, Guidelines for Testing of Chemicals, No. 211—*Daphnia magna* Reproduction Test, Organisation for Economic Cooperation and Development, Paris, 1998.
[22] J.V. Nabholz, R.G. Clements, M.G. Zeeman, Ecol. Appl. 7 (1997) 1094.
[23] C. Rudén, S.O. Hansson, Environ. Health Perspect. 118 (2010) 6.
[24] A. Henglein, M. Giersig, J. Phys. Chem. B 103 (1999) 9533.
[25] R.C. Doty, T.R. Tshikhudo, M. Brust, D.G. Fernig, Chem. Mater. 17 (2005) 4630.
[26] N.S. Taylor, R.J.M. Weber, A.D. Southam, T.G. Payne, O. Hrydziuszko, T.N. Arvantis, M.R. Viant, Metabolomics 5 (2009) 44.
[27] F. Dondi, M. Martin, in: M. Schimpf, K. Caldwell, J.C. Giddings (Eds.), Field-Flow Fractionation Handbook, Wiley-Interscience, New York, 2000, p. 103.
[28] M. Baalousha, A. Manciulea, S. Cumberland, K. Kendall, J. Lead, Environ. Toxicol. Chem. 9 (2008) 1875.
[29] R.F. Domingos, M.A. Baalousha, Y. Ju-Nam, M. Reid, N. Tufenkji, J.R. Lead, G.G. Leppard, K.J. Wilkinson, Environ. Sci. Technol. 43 (2009) 7277.
[30] J. Lead, E. Smith, Environmental and Human Health Impacts of Nanotechnology, Blackwell Publishing, UK, 2009.
[31] R.J. Griffitt, J. Luo, J. Gao, J.-C. Bonzongo, D.S. Barber, Environ. Toxicol. Chem. 27 (2008) 1972.
[32] P. Rosenkranz, Q. Chaudhry, V. Stone, T.F. Fernandes, Environ. Toxicol. Chem. 28 (2009) 2142.