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Quantifying the adsorption of ionic silver and functionalized nanoparticles during ecotoxicity testing: Test container effects and recommendations

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Abstract

Silver nanoparticles (Ag-NPs) are used in a wide variety of products, prompting concerns regarding their potential environmental impacts. To accurately determine the toxicity of Ag-NPs it is necessary to differentiate between the toxicity of the nanoparticles themselves and the toxicity of ionic silver (Ag) released from them. This is not a trivial task given the reactive nature of Ag in solution, and its propensity for both adsorption and photoreduction. In the experiments reported here, we quantified the loss of silver from test solutions during standard ecotoxicity testing conducted using a variety of different test container materials and geometries. This sensitive ^{110m}Ag isotope tracing method revealed a substantial underestimation of the toxicity of dissolved Ag to the green algae *Pseudokirchneriella subcapitata* when calculated only on the basis of the initial test concentrations. Furthermore, experiments with surface-functionalized Ag-NPs under standard algal growth inhibition test conditions also demonstrated extensive losses of Ag-NPs from the solution due to adsorption to the container walls, and the extent of loss was dependent on Ag-NP surface-functionality. These results hold important messages for researchers engaged in both environmental and human nanotoxicology testing, not only for Ag-NPs but also for other NPs with various tailored surface chemistries, where these phenomena are recognized but are also frequently disregarded in the experimental design and reporting.

Introduction

Silver is a naturally occurring metal that has been widely used throughout history in the form of ornaments, coins and utensils (Purcell and Peters, 1998, Chernousova and Epple, 2013). In the modern era, it has found extensive applications in the photographic, electronic and medical industries and more recently, in a rapidly expanding range of silver nanotechnology based 'antibacterial' consumer products. According to The Project on Emerging Nanotechnologies (Woodrow Wilson Institute, 2005, rev. 2013), silver nanoparticles (Ag-NPs) are the most commonly found NPs in consumer products; with a wide diversity of marketed applications in clothing, household appliances and personal care products for example. Silver in its ionic form (Ag^+) is known to be highly toxic to both prokaryotes and simple eukaryotes, and this property has been exploited in both soluble and particulate forms in biomedical applications to prevent microbial infections (Klasen, 2000, Alexander, 2009). Toxic responses to Ag have also been demonstrated in other organisms such as invertebrates and fish species (see review by Ratte, 1999). Life cycle analysis shows that a significant fraction of the Ag contained in commercial products will inevitably be released to the environment (e.g. via wastewater systems) and may subsequently pose a threat to organisms in environmental compartments such as surface waters, soils and sediments (Geranio et al., 2009, Gottschalk and Nowack, 2011, Benn and Westerhoff, 2008). While previous research has significantly advanced our understanding of silver compounds in the environment (e.g. see editorials (Andren and Armstrong, 1999, Gorsuch and Klaine, 1998) and references therein), renewed concerns have been raised due to the increasing commercialization of Ag-based engineered nanomaterials (ENMs), particularly in relation to the potential for nanoparticle-specific toxicity (Navarro et al., 2008).

One method of assessing the impact of ENMs in the environment is through ecotoxicity testing using target species with a probable risk of exposure and vulnerability to the toxicant, such as bacteria, algae or small invertebrates. Several reviews (Crane et al., 2008, Klaine et al., 2012) with practical

recommendations and guidelines for ecotoxicity testing (Handy et al., 2012, The Organisation for Economic Co-operation and Development, 2012) have been published which discuss sample preparation and dosimetry for testing ENMs. Nevertheless, the quantitative assessment of the toxicity of ENMs remains a challenging task, subject to a large array of potential artefacts and misinterpretations that are not routinely accounted for in existing standard ecotoxicity tests which were generally developed to test the toxicity of dissolved substances (Petersen et al., 2014).

In the case of Ag-NPs, a significant focus has been placed on separating the toxic effects arising from dissolved Ag and Ag-NPs, and their respective effective concentrations. This has proven challenging and remains a contentious issue (Ivask et al., 2013, Xiu et al., 2012), partly because direct quantification of dissolved Ag at relevant concentrations is not always analytically feasible or affordable, and also due to differences in the inter-laboratory application of standard ecotoxicity test protocols (e.g. use of different test containers). An initial study by Navarro et al. (2008) used cysteine, which binds to free Ag^+ ions, to account for the contribution of free ions to the overall toxicity and minimize this potentially confounding factor, while others have used ion selective electrodes (Sotiriou et al., 2012, Wang et al., 2012), ultrafiltration (Liu and Hurt, 2010) or dialysis (Newton et al., 2013, Zhao and Wang, 2011) to selectively detect or remove one of the phases in order to distinguish between the two. In many of these studies, dissolved Ag is suggested to be the major factor explaining the observed toxicity of Ag-NPs; however, the results are subject to varying levels of uncertainties. For example, added cysteine may also interact with Ag-NPs (Gondikas et al., 2012), and the removal of dissolved Ag from the system may promote further dissolution of Ag^+ from Ag-NPs. Thus, in such dynamic systems, an accurate assessment of Ag concentrations (dissolved and particulate) at each time point is critical in order to de-convolute and accurately quantify the contributions of each to the overall toxicity of Ag nanomaterials.

In a review of the literature reporting Ag, CuO and ZnO nanoparticle toxicities, Bondarenko et al. (Bondarenko et al., 2013) commented on the variability of published Ag-NP toxicity data both within

and across various test organisms, including mammalian cells. Many of these results cannot be directly compared because the test durations varied. However, after supplementing the toxicity data referenced in the review with more recently reported Ag-NP data, the reported EC_{50} values for the standard green algae test organism *Pseudokirchneriella subcapitata* were found to differ by over 600-fold from 32.4 µg/L (72 h) (Ribeiro et al., 2014b) to 21202 µg/L (4.5 h) (Wang et al., 2012), and over 40-fold even under similar test conditions (US EPA medium, 96 h) (McLaughlin and Bonzongo, 2012, Griffitt et al., 2008). This is explained in part by the different surface coatings that are typically applied to Ag-NPs (in contrast, the CuO and ZnO NP data reported in the review were all for uncoated particles). Toxicity will certainly be affected by surface coatings as differing surface functionality can directly result in differing interactions with the target organisms (El Badawy et al., 2010). However, it may also indirectly affect other relevant factors such as the proportion of dissolved Ag to total Ag (Ivask et al., 2013) or, as we demonstrate in this paper, differences in the degree of interaction with the surfaces of the test containers.

Several authors have pointed out that dissolved Ag can adsorb strongly to solid (particularly inorganic) surfaces (West and West, 1966, Wingert-Runge and Andren, 1993, Garnier and Baudin, 1989) and that this may lead to a reduction in the solution concentration of Ag during ecotoxicity testing. However, despite this effect being recognized, it is not often systematically quantified or reported for commonly used containers under relevant ecotoxicity test conditions. Polycarbonate containers have been specifically selected for ecotoxicity testing by some authors in order to minimize the adsorption of dissolved Ag to the container during the test period (Lee et al., 2004, Hiriart-Baer et al., 2006, Chen et al., 2013). However, other recent studies have evaluated Ag toxicity using a wide range of containers (see Table S1), many of them making no reference to the issue of sorption. In fact, it appears that some authors have overlooked the importance of this effect entirely, as in some articles the type of container used has not even been specified. Furthermore, in some cases it is unclear whether the total and/or ionic Ag concentration was measured during the exposure period or if the derived EC values were based solely on nominal concentrations.

The purpose of this article is to clearly demonstrate the importance of sorption effects in the testing of Ag toxicity, in both dissolved and nano-particulate forms, by: i) precisely quantifying the potential error in dissolved Ag toxicity results when applying a common standard ecotoxicity test protocol, and ii) demonstrating that the loss of Ag-NPs from solution during the test period is dependent on their surface functionality. These effects were investigated using several different container materials and geometries to carry out the standard OECD freshwater algae ecotoxicity test (The Organisation for Economic Co-operation and Development, 2002, Rev. 2006). To increase the sensitivity of the experimental design, isotope tracing using the ^{110m}Ag γ-emitting isotope was used to facilitate the direct quantification of Ag sorption to the vials.

Experimental methods

Algal growth inhibition test procedure

The OECD freshwater algae growth inhibition test was used to study the toxicity of ionic silver (or dissolved Ag; as $AgNO_3$, 99.9999% trace metals basis, Sigma-Aldrich, Castle-Hill, Australia) according to the OECD Guideline 201 for Testing of Chemicals (The Organisation for Economic Co-operation and Development, 2002, Rev. 2006), using an established mini-scale test (Arensberg et al., 1995). Eleven nominal Ag concentrations ranging from 0.45 to 10.01 µg/L were tested in a dilution series (*ratio* = 1.35) prepared with fresh standard OECD test media. Potassium dichromate ($K_2Cr_2O_7$, Sigma Aldrich) was used as the reference toxicity standard for quality control, with eleven nominal concentrations from 0.25 to 5.07 mg/L also prepared as a dilution series (*ratio* = 1.35). The initial (*t* = 0) concentrations of all Ag solutions were measured using an Agilent 8800 Triple Quadrupole ICP-MS. The sensitivity of this instrument was indispensable for the analysis of low Ag concentrations, whereas $K_2Cr_2O_7$ solutions were measured using an Agilent 7500ce ICP-MS. ICP-MS samples were

analyzed in OECD media acidified to 2% HNO_3 (v/v). Once the samples had been acidified and placed in an ICP-MS sampling tube, the Ag concentration did not change over time (i.e. Ag from the acidified samples was not adsorbed). Standard solutions were prepared in ultrapure water (2% HNO_3), and were checked for matrix effects by comparison with blanks and standards diluted in media (0.8, 2.5, 5.0 and 10.0 µg/L for Ag, and 1.8 and 3.5 mg/L for $K_2Cr_2O_7$).

The algal stock culture of *Pseudokirchneriella subcapitata* was purchased from the Marine and Atmospheric Research Division of CSIRO, Hobart, Australia. The inoculum culture in OECD test medium was grown for 48 to 72 h to ensure that the culture was in the exponential growth phase. Test cultures were grown in 4 mL of media containing the relevant concentration of test substance in 20 mL borosilicate glass incubation vials at 23 ± 2 °C. The vials were closed with caps, but these contained a hole to allow for adequate O_2/CO_2 mass transfer between the test solution and the surrounding air. An orbital shaker was used to provide constant shaking (130 rpm) and the vials were illuminated continuously from below with cool white light from 2 × 18W Crompton tri-phosphor fluorescent tubes (9150 lx, SD = 16 %). Algal cell counts were determined by *in vivo* fluorescence from 200 µL aliquots taken at 0, 24, 48 and 72 h, using a BIO-TEK Synergy HT multi-detection microplate reader (λ_{exc} = 440 nm, λ_{em} = 680 nm). All tests were performed in triplicate and run in parallel with six controls (i.e. growth media without the test compound) with initial algal cell counts of 0.5 to 1×10^4 cells/mL. EC_{50} values as well as their associated 95% confidence intervals (95% CI) were calculated using a logistic sigmoidal curve in Microsoft Excel and are reported as an average EC_{50} ± the standard deviation of the independent tests.

Dissolved Ag: monitoring concentrations using isotope tracing

An isotopic tracing experiment (using the 20 mL borosilicate vials but without algae) was conducted in parallel to monitor changes in dissolved Ag and $K_2Cr_2O_7$ concentrations using ^{110m}Ag (as $^{110m}AgNO_3$

in 0.1N HNO_3, Cerca Lea, France) and ^{51}Cr (as $Na_2{}^{51}CrO_4$ in saline solution, PerkinElmer, USA), respectively. It should be noted that CrO_4^{2-} and $Cr_2O_7^{2-}$ are equivalent at the pH used in this experiment as they are in equilibrium (Moore et al., 1966). For these purposes, three $AgNO_3$ (9.9, 3.1 and 1.0 µg/L Ag) and three $K_2Cr_2O_7$ (6.8, 2.1 and 0.8 mg/L) concentrations were freshly prepared in OECD media, representing 100% inhibition, near-EC_{50}, and no-response concentrations of the relevant test substances. The activity concentrations of all ^{110m}Ag-spiked Ag solutions and ^{51}Cr-spiked $K_2Cr_2O_7$ solutions were 0.78 kBq/mL and 1.25 kBq/mL, respectively. Additionally, a dual-spike solution of Ag and $K_2Cr_2O_7$ at a nominally equi-molar concentration (10 µg/L and 13.6 µg/L, respectively) was used to test for any adsorption of Cr species. Each solution containing Ag was tested under both light (L) and dark (D) conditions, where D samples were tested in exactly the same manner as the L samples, except that the vials for D were coated with aluminium foil throughout the experiment. All solutions were tested in triplicate. The pH was estimated with a universal pH indicator paper to avoid contaminating the pH probe with radioactive metals, and was between 7 and 8 for all solutions.

200 µL aliquots were taken at *t* = 0, 24, 48 and 72 h and transferred directly into 4 ml gamma counting tubes. After the 72 h sampling, the test vials were emptied and rinsed three times with 1 mL of ultrapure water to desorb any lightly adhered ^{110}Ag or ^{51}Cr and the empty vials were also analysed directly with the gamma counter to quantify the ^{110m}Ag or ^{51}Cr remaining on the walls. The samples were measured with a Perkin Elmer Wizard^2 automatic gamma counter. ^{110m}Ag emissions were integrated over 5 minutes between 810 and 1120 keV and ^{51}Cr emissions were integrated over 250 – 380 keV. Background counts were measured with a blank solution of the media and were subtracted from the sample counts.

Correction factors for Ag concentrations at *t* = 24, 48 and 72 h were calculated using the proportion of Ag determined in solution by ^{110m}Ag activity relative to the initial activity of the respective samples. The working range was divided into 2 and the correction factors were defined separately

for the two ranges: those for the upper range was determined by a linear regression using 9.86 and 3.14 µg/L, while those for the lower range were determined by using the proportions at 3.14 and 1.04 µg/L. Effective concentrations for each measurement at time *t* were calculated by averaging the concentration determined at *t* and at (*t*-24) h.

Container variations

Additional Ag tracing experiments were carried out under the lighting conditions described above using four alternative containers to represent a range of different laboratory protocols. The extra containers tested were: a) 50 mL glass Erlenmeyer flasks (50.8 mm base diameter), b) 100 mL glass Erlenmeyer flasks (63.5 mm base diameter), c) 50 mL polypropylene (PP) centrifuge tubes and d) 30 mL polycarbonate (PC) vials (Thermo Fisher Scientific Australia). The experiments in PP and PC were performed in exactly the same manner described above for the standard tests, whereas those in the Erlenmeyer flasks were performed using 25 mL of the Ag solutions spiked at 0.15 kBq/mL, and 1 mL aliquots were used for gamma counting at each time point.

Surface functionalized silver nanoparticle adsorption tests

A series of tests were performed to investigate the potential adsorption of surface functionalized silver nanoparticles (Ag-NPs) to container walls. In the dissolved Ag case, polycarbonate vials were shown *not* to adsorb ionic Ag (see Results section later). For this reason, they were selected for use with Ag-NPs to test whether this was also the case for surface-functionalised Ag-NPs.

Ag-NPs with two different surface functionalities – negatively charged tannic acid coating (TA) and positively charged branched-polyethyleneimine (bPEI) – were used to demonstrate potential differences in Ag-NP adsorptive behavior as a function of surface chemistry. The particles were

purchased from nanoComposix (BioPure, San Diego CA, USA) and were re-characterized using both scanning electron microscopy (SEM, Quanta 450 FEG-ESEM, FEI Company) and dynamic light scattering with ζ-potential measurements (DLS, Nicomp 380 ZLS, Particle Sizing Systems). The DLS samples were diluted in ultrapure water. Ag-NP characterization results are summarized in Table 1. Due to the range of EC_{50} values reported in the literature, two representative target concentrations were selected; 50 and 180 µg/L, designated as Low and High concentrations respectively. These adsorption tests were performed without algae; however we note that both starting concentrations were confirmed to be in the toxic range for *P. subcapitata* in a separate exposure test measured over 72 h.

In contrast to the experiments with the dissolved Ag where aliquots were taken at each assessment time from the same vial, for this experiment separate vials were set up for each assessment time (0, 24, 48 and 72 h), under both light (L) and dark (D) conditions. This was necessary to ensure that sufficient sample volume was available to reliably quantify Ag in all sampling compartments without using radioisotopes. 4 mL of Ag-NP suspension was added to each vial and incubated in the same manner as for the dissolved Ag experiments. After the required time, the solutions were lightly shaken to re-suspend any potentially sedimented Ag and 3.75 mL was sampled into a tube suitable for ICP-MS analysis. The sample was immediately acidified to 2% HNO_3 (v/v), and designated as "suspended Ag" or Ag_{-sus}. The adsorbed fractions were quantified by adding 4 mL of 4% (v/v) to the used vials and shaking in the end-over-end shaker for 24 h to dissolve the Ag-NPs. The resulting solution was diluted by adding 4.25 mL of ultrapure water and these samples designated as "adsorbed Ag-NPs" or Ag_{-ads}. An extra set of samples were incubated in PC vials for 72 h, after which time, aliquots were centrifuged through a 1kDa ultrafiltration filter in order to investigate the extent of dissolution at 72 h. The filtrate was acidified and designated "filtered Ag", or Ag_{-fil}.

Finally, a similar adsorption test was performed in 20 mL borosilicate glass vials to demonstrate any differences related to the container type. A total of 420 samples were analysed by ICP-MS with 5 and

25 µg/L standards (in both ultrapure water and OECD media) also distributed throughout the sample set in order to check for any matrix effects.

Results

$K_2Cr_2O_7$ and dissolved Ag ecotoxicity tests

The *P. subcapitata* ecotoxicity test results for $K_2Cr_2O_7$ and dissolved Ag determined following the standard OECD test protocol are shown in Figures S1 and S2, respectively. After 72 hours of exposure, the EC_{50} concentrations calculated for the $K_2Cr_2O_7$ reference substance (1.9 ± 0.2 mg/L) were in good agreement with the published reported value (Arensberg et al., 1995), indicating reliable functioning of the test apparatus and OECD test protocol. Standards prepared in acidified OECD media (2% HNO_3 (v/v)) deviated by < 2% from the standards prepared in acidified water, which is within instrumental error. All blanks on the triple-quadrupole ICP-MS returned a measured Ag "concentrations" of < 3 ng/L, more than 2 orders of magnitude below the lowest Ag concentration used. Blanks for $K_2Cr_2O_7$ were all < 0.5 µg/L.

Toxicity of $K_2Cr_2O_7$ increased over the test duration from 24 h to 48 h and 72 h as expected, i.e. due to the longer exposure of cultures to the toxicant (Figure S1). However, a reversed trend was observed for Ag, as evidenced by an increase in the average EC_{50} values over time from 2.3 µg/L at t = 24 h, to 3.0 µg/L at 48h, and 3.6 µg/L at 72 h (Figure S2). This trend is indicative of a reduction in the bioavailable fraction of the test compound by, for example, precipitation or adsorption occurring during the test. No visible precipitation was observed for either substance at the concentrations used and this was supported by simulations using the GEOCHEM-EZ program, where only precipitation of iron hydroxides were predicted (Table S2) (Shaff et al., 2010). In the case of Ag, several forms of chloride ($AgCl_x^{1-x}$) were predicted but the concentrations were sufficiently low for this species to remain in solution.

Quantification and correction of Ag adsorption to container walls

The ^{110m}Ag isotopic tracing experiments presented in Figure 1 clearly show the significant change in solution Ag concentration in 20 mL borosilicate glass vials over the 72 h test period, under a) light, and b) dark conditions. It is evident that the concentration of dissolved Ag in solution decreases substantially over the time frame of the test; with larger decreases observed at higher concentrations and under illuminated conditions. ^{110m}Ag gamma activities measured directly from the rinsed vials indicated that at least 20% of the dissolved Ag was adsorbed onto the glass container walls in the 9.9 µg/L treatment and nearly 60% in the lower concentration treatments (Figure S4). These results are in agreement with the observed losses from solution, and result in an overall mass balance of 85 – 95 %.

Application of simple adsorption correction factors calculated using the results above produced a notable shift in the dose-response curves (Figure 2), resulting in the lowering of the EC_{50} value for the test shown from 3.3 to 1.7 µg/L at t = 72 h, and similarly from 3.6 to 1.9 µg/L averaged across the tests in Figure S3. This indicates an underestimation of Ag toxicity (measured by EC_{50}) by a factor of almost 2. The extent of underestimation is time dependent, with the effect increasing at longer exposure times. These results largely explain the observed decrease in Ag toxicity over the test period reported above.

In contrast, the dual spiking experiment with ^{110m}Ag and ^{51}Cr solutions spiked with ^{51}Cr showed no significant decrease in the ^{51}Cr activity measured from the solution (Figure 3a), indicating that the $K_2Cr_2O_7$ concentration in the solutions remained constant throughout the tests. This clearly shows that the decreasing solution concentration throughout the test that resulted in an apparent decrease in Ag toxicity is specific to Ag and its solution chemistry.

Container material and geometry affect the extent of adsorption

It is also important to note that these losses are strongly dependent on the container material and geometry. Figure 3b demonstrates the different profiles in Ag concentration in 4 different container test cases over the 72h test period, with initial nominal dissolved Ag concentration of 10 µg/L. The two glass Erlenmeyer flasks resulted in substantial decreases in solution Ag concentration, with the larger flask causing relatively greater solution Ag loss. This is rationalized by the greater surface area the solution is exposed to in the larger flask, which also led to greater measurement uncertainties as indicated by the larger error bars. On the other hand, solutions in polycarbonate (flat bottom) and polypropylene (V-shape) tubes do not exhibit such significant changes, with polypropylene only showing a slight decrease in solution Ag concentration. It should be noted that the polycarbonate vial was slightly colored (translucent yellow), which may have affected the potential for photoreduction. Nevertheless, it is clear that polycarbonate and polypropylene tubes exhibit remarkably low adsorption of Ag on to the walls, leading to near-constant solution concentration of silver in the media over the test period.

Surface-functionalised Ag-NPs under test conditions

The Ag-NPs size and ζ-potential reported by the manufacturer and confirmed in the laboratory are summarized in Table 1. The two sets of data agree well, with only minor differences in the characterisation results observed. Figure 4 shows the changes in the Ag_{-sus} (containing any dissolved Ag + Ag-NP) from surface-functionalised Ag-NP suspensions in polycarbonate vials relative to the two initial concentrations, under two contrasting lighting conditions. It is clear that TA- and bPEI-coated Ag-NPs behave very differently under these conditions. While the Ag_{-sus} was largely unchanged in TA-Ag-NPs treatments, bPEI-Ag-NPs exhibited substantial decreases in Ag_{-sus} to below 10 % over the 72 h period, except for the 10 nm High treatment which showed a smaller decrease, most likely as a

result of adsorption site saturation (Table 2). After accounting for the Ag-NPs recovered from the container walls (Ag_{-ads}, Figure S6), mass balances between 80 – 120 % were obtained. Matrix effects arising from the media were negligible (generally < 2%) calculated using the additional 5 and 25 µg/L standard solutions dispersed throughout the ICP-MS sample run.

Similar trends were observed on glass at 72 h, where bPEI-Ag-NPs lost over 75% of the Ag_{-sus}, while greater proportion of TA-Ag-NPs remained in suspension. An exception to this was TA-Ag-NPs with 50 µg/L starting concentration, where over 30% was lost from suspension.

Silver was also detected from the 1 kDa filtered fraction from TA-Ag-NPs incubated for 72 h (Figure S5), indicating that 23.1 ± 1.1 % and 4.0 ± 0.6 % of Ag in 10 and 60 nm TA-Ag-NPs, respectively, were in a form smaller than the filter pore size (i.e. Ag_{-fil}, estimated between 1 – 2 nm). GEOCHEM-EZ simulation of Ag in the filtrate indicated a mixture of free Ag and soluble forms of $AgCl_x^{1-x}$ (Table S3).

Discussion

For the experiments with $AgNO_3$, at least two mechanisms associated with dissolved Ag loss are plausible under these test conditions: a) photoreduction of Ag(I) to Ag(0), and b) adsorption on to the vial walls. Adsorption of silver on to borosilicate glass and other materials has been reported previously (West and West, 1966). This is likely to be the primary mechanism of Ag loss from solution samples kept under dark conditions (relevant to other ecotoxicity tests such as those using *Daphnia* species). As expected from surface equilibria, the loss is concentration dependent, with higher concentrations of Ag leading to greater adsorption (8.9 ± 0.6, 3.8 ± 0.1 and 1.7 ± 0.1 ng at 9.9, 3.1 and 1.0 µg/L, respectively). Greater losses were observed in light exposed samples suggesting that light-induced loss mechanisms are also involved; this is likely to be photoreduction on to the vial walls. Such a mechanism may have consequences when comparing inter-laboratory results for example, where lighting differences may result in different extents of photo-reduction. As

demonstrated, container material and geometry can also contribute to a miscalculation of toxicity due to the differences in Ag interactions with the container material and the available surface area for a given amount of Ag. These losses are also likely to be influenced by other test conditions such as pH and temperature, and may even be affected by trivial factors such as the rotation speed of the shaker (which changes the surface area of the container in contact with the testing solution). In the case of glass containers, estimates of the Ag adsorption normalized to the surface area is included in the supplementary information (Table S4). It is also important to note that while this article focused on the adsorptive behavior of dissolved Ag and Ag-NPs, Ag solution chemistry and kinetics within the media in the presence of test organisms will also be critical in determining the ultimate speciation and toxicity mechanisms (Ribeiro et al., 2014a, Sørensen and Baun, 2014, Chen et al., 2013).

This study demonstrates that the best containers for testing dissolved Ag alone (i.e. polycarbonate labware) does not provide a simple solution to the issue when testing Ag-NP toxicity. The two Ag-NPs used in this study have similar metallic core properties but contrasting surface potentials: tannic acid (TA) is a negatively charged, macromolecular stabilizer while bPEI is a positively charged, highly branched polymeric stabilizer. Both TA and bPEI are hydrophilic but both surface functionalities led to Ag-NP loss from the suspension on to the container walls during the test, albeit with different rates and overall extent of loss. It is possible that the dissolution of Ag from Ag-NPs (e.g. TA-Ag-NPs) and/or adsorption of dissolved Ag mediated by the stabiliser may contribute to the observed differences over time. However, the observed differences are significantly greater than the extent of dissolution estimated by Ag_{-fil} (maximum of 23.1 % for 10 nm TA-Ag-NPs). Since dissolved Ag does not adsorb to polycarbonate, these tests unequivocally show that the Ag-NPs themselves are adsorbed on to the container walls and that this is dependent on concentration and surface functionality as expected based on adsorptive mechanisms. Dissolved Ag adsorption *can* occur in glass vials, however, and the noticeable decrease in Ag_{-sus} of 10 nm TA-Ag-NPs may also be partly due to the adsorption of the dissolved Ag, as the 10 nm TA-Ag-NP treatment had the highest proportion of Ag_{-fil}. Although it was not directly addressed in this study, it is expected that this is

time-dependent, indicating that the differentiation of dissolved Ag and Ag-NPs may be necessary throughout the duration of ecotoxicity tests.

This behaviour is analogous to protein adsorption to glass and plastic containers (Bull, 1956, Petty et al., 1974) or functionalized Ag-NP adsorption to polymer coated substrates (Sekine et al., 2013, Goreham et al., 2011), and it is likely that different materials will exhibit varying adsorptive behaviors. However, ecotoxicity tests of dissolved Ag or Ag-NPs with various organisms have been conducted using a range of different container materials and geometry/configurations, and alarmingly, several studies fail to even report these details (see Table S1). In particular, where microwell plates are used, considerable care is required due to the large surface area to volume ratio of each well, and the fact that Ag quantification at relevant concentrations is not trivial with such small volumes. While the results from these studies can still contribute knowledge to the field, they are limited in providing accurate mechanistic information for Ag-NP toxicity unless Ag losses during the test duration are either eliminated or accurately quantified and accounted for. For example, pre-soaking the apparatus with the free stabilizer (e.g. TA or bPEI) may effectively minimize the adsorption of Ag-NPs by pre-occupying the available adsorption sites. However, this may in turn aid the adsorption of dissolved Ag since Ag^{+} would interact with, for example, bPEI. Furthermore, given that test organisms themselves have a surface potential, any pre-soaking material that can effectively minimize Ag-NP adsorption may also have an affinity for the organisms and thus render further artefacts in the results. The use of flow cells can be effective in maintaining Ag concentration, but care is required to minimize accumulation of Ag in the test chamber, particularly on the walls.

Therefore, we reiterate the need for care when collecting quantitative Ag toxicity data due to the extent of Ag and Ag-NP sorption during toxicity testing with most commonly used test containers and vials. Due to the recent focus on toxicology of Ag-NPs and the vast numbers of recently published papers on the topic it appears that this is a very substantial issue. As the test results will

depend heavily on the specifications of the testing setup in each laboratory, it is recommended that each study be accompanied by a thorough assessment of the concentration at every time point of the test or at least by thorough reporting of all relevant test conditions. Isotope tracing was selected as a quantifiable, cost-effective tracer method in this study to achieve high accuracy and precision at low Ag concentrations, but appropriate mass spectroscopic methods can also now achieve the relevant detection levels. For instance, in our case, the high sensitivity and superior interference correction of the Agilent triple quadrupole ICP-MS enabled analysis at these levels, whereas the performance of conventional ICP-MS did not provide sufficient sensitivity. Alternatively, polycarbonate or polypropylene vials could be used in order to minimize the potential for adsorption of ionic Ag, although we also note that appropriately broad spectral transmission is required to ensure that adequate light reaches the test organisms in these containers. As we have demonstrated here, these materials may also cause significant sorption of Ag-NPs, therefore further corrective protocols will need to be developed and implemented for the analysis of Ag-NPs with different surface functionalities.

Conclusions

In this article, we successfully quantified the extent of dissolved Ag loss using ^{110m}Ag isotope tracing. This revealed a substantial underestimation of the dissolved Ag toxicity when *P. subcapitata* growth inhibition tests were performed in glass vials. This loss was determined to be due to contributions from both adsorption and photoreduction on to the container walls; the extent being dependent on both container geometry and material. In the case of dissolved Ag, polycarbonate containers were shown to be the most suitable material for performing this test. However, when used in the analysis of Ag-NPs, analysis of total Ag concentrations over the test period demonstrated a substantial loss of Ag-NPs from suspension that was also dependent on their surface-functionality. This has clear and important implications for both environmental and human nanotoxicology testing, not only for Ag-

NPs but also for other ENMs with specifically tailored surface chemistries. These results highlight and reconfirm the often overlooked need to quantify the concentration of test substances with complex solution chemistry throughout the duration of the test.

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Declaration of Interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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Table 1. Size and ζ-potential (ζ-V) of Ag-NPs used in the adsorption tests. Laboratory tests were used to confirm the characteristics of the Ag-NPs provided by the manufacturer.

	Manufacturer			**Laboratory**		
	TEM/SEM (nm)	DLS* (nm)	ζ-V (mV)	SEM (nm)	DLS* (nm)	ζ-V (mV)
Tannic Acid 10 nm	10.0 ± 1.8	15.3	-36.2	--	12.4 ± 0.7	-22.1
Tannic Acid 60 nm	60.8 ± 6.6	70.6	-64.1	49.7 ± 7.7	56.3 ± 1.0	-33.1
bPEI 10 nm	8.8 ± 2.2	27.7	+12.4	--	15.8 ± 2.3	+20.6
bPEI 60 nm	60.8 ± 6.6	104.0	+49.0	55.8 ± 7.1	76.8 ± 2.4	+34.4

* Manufacturer DLS measurements were performed on the Malvern Zetasizer Nano ZS, whereas the Laboratory DLS measurements were performed on the Nicomp 380 ZLS

Table 2. Initial total Ag concentration (C_0) and the percentage of Ag suspended in solution at time *t* during a test period.

	Ag C_0	Ag concentration (% of C_0)			
	(µg/L)	*t* = 0	*t* = 24	*t* = 48	*t* = 72
10 nm, Low conc.					
TA, Light	40.7 (1.2)	100 (2.8)	82.0 (8.0)	82.0 (8.0)	86.9 (5.3)
TA, Dark	40.7 (1.2)	100 (2.8)	78.7 (2.2)	80.3 (5.1)	73.8 (2.1)
bPEI, Light	51.3 (3.1)	100 (6)	27.3 (5.5)	6.5 (4.9)	5.8 (5.5)
bPEI, Dark	51.3 (3.1)	100 (6)	6.5 (4.9)	7.8 (0.5)	6.5 (4.9)
10 nm, High conc.					
TA, Light	137.3 (3.1)	100 (2.2)	106.3 (4.9)	106.8 (7.9)	105.8 (12.7)
TA, Dark	137.3 (3.1)	100 (2.2)	106.8 (5.4)	99.5 (8.8)	109.2 (3.9)
bPEI, Light	188.0 (2.0)	100 (1.1)	69.5 (2)	50.4 (6.4)	31.9 (13.1)
bPEI, Dark	188.0 (2.0)	100 (1.1)	68.8 (2)	63.5 (2.3)	55.9 (1.3)
60 nm, Low conc.					
TA, Light	51.3 (1.2)	100 (2.2)	96.1 (4.4)	96.1 (6.7)	97.4 (6.1)
TA, Dark	51.3 (1.2)	100 (2.2)	94.8 (4.4)	100 (6.7)	89.6 (12.3)
bPEI, Light	54.0 (2.0)	100 (3.7)	6.8 (4.9)	5.6 (5.1)	5.6 (5.1)
bPEI, Dark	54.0 (2.0)	100 (3.6)	2.4 (0.1)	1.2 (1.1)	0.8 (0.4)
60 nm, High conc.					
TA, Light	138 (5.3)	100 (3.8)	94.2 (9.9)	92.3 (15.7)	130.9 (18.2)
TA, Dark	138 (5.3)	100 (3.8)	102.4 (8.4)	97.6 (13.9)	115 (13.3)
bPEI, Light	169.3 (6.1)	100 (3.6)	2.4 (0.1)	1.2 (1.1)	0.8 (0.4)
bPEI, Dark	169.3 (6.1)	100 (3.6)	1.6 (0.7)	2 (0.8)	2.8 (0.8)

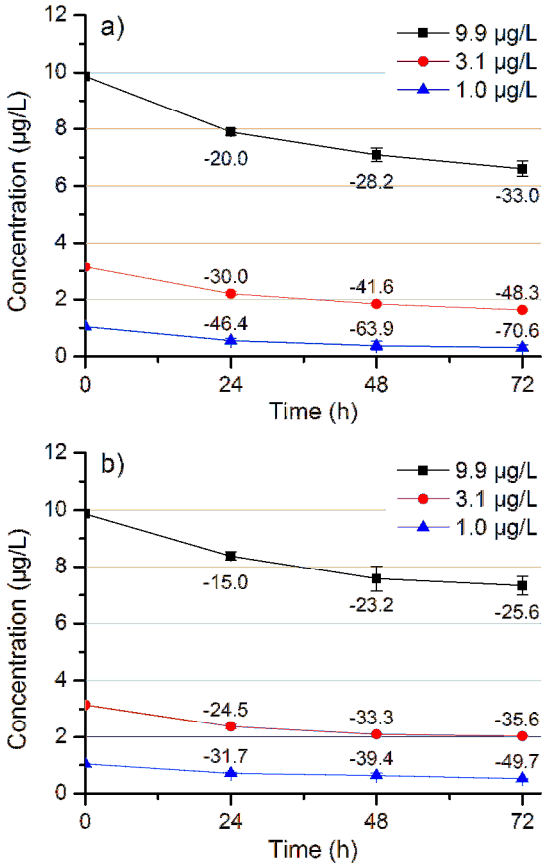
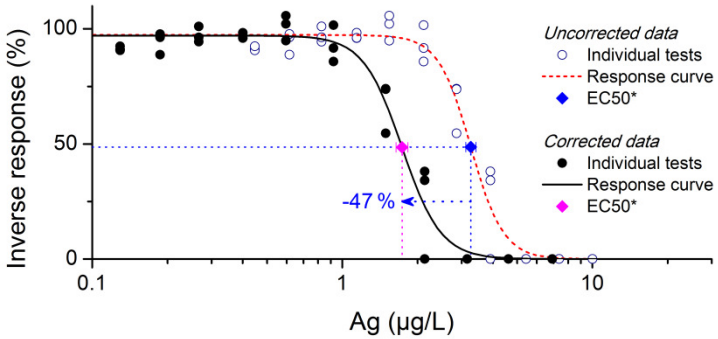


Figure 1. Solution concentrations of silver traced by ^{110m}Ag in OECD media shaken as per the usual test conditions in 20 mL borosilicate glass vials, under a) light conditions, and b) in the dark, over 72 h. Labels indicate the percentage change in Ag concentration with respect to the initial concentration at t = 0, error bars indicate ±1 SD.



t (h)	ECx	Unc. (µg/L)	Corr. (µg/L)	% Error
24	EC50	2.293	1.853	-19.2
48	EC50	2.288	1.773	-38.6
72	EC50	3.265	1.733	-46.9
	EC10	2.351	1.172	-50.1
	EC20	2.654	1.354	-49

Figure 2. An example dose-response curve and calculated EC_x values before (Unc.) and after (Corr.) application of the ^{110m}Ag correction factors. The uncorrected fit of the curve (dotted line) is also shown in the figure for comparison with the corrected curve (solid line). *Error bars on the EC_{50} data point indicate the 95% confidence interval.

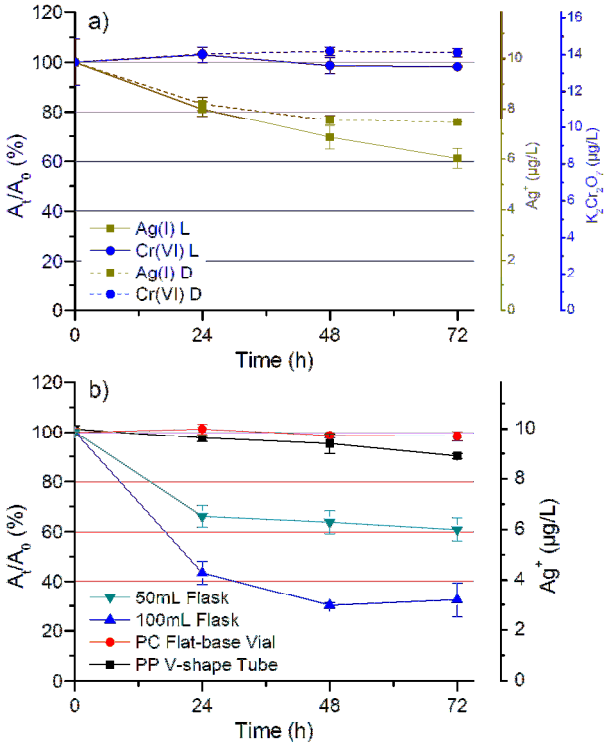


Figure 3. a) Dual isotope tracing of ^{110m}Ag(I) and ^{51}Cr(VI) under light (L) and dark (D) conditions, demonstrating the specific loss of dissolved Ag during the 72 h testing period, whereas $Cr_2O_7^{2-}$ remains in solution; b) Variability of Ag concentration in solution observed with vials of different composition and geometry.

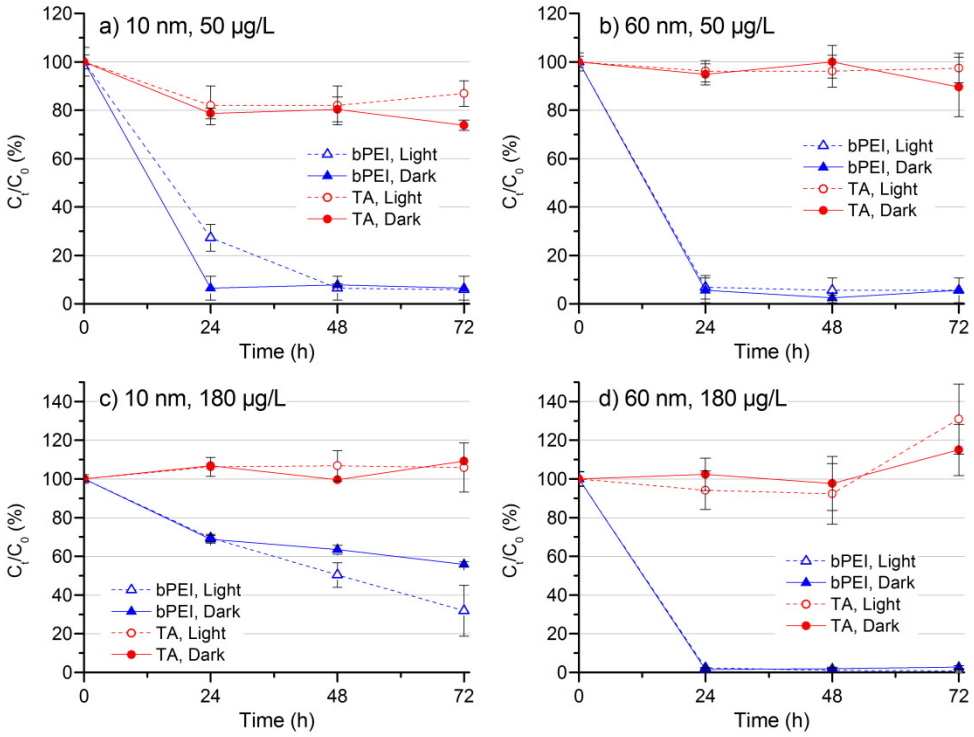


Figure 4. Comparison of the different loss profiles of Ag concentration (as Ag + Ag-NPs) from TA- and PEI-Ag-NP treatments in PC vials, where the vertical axis represents concentrations at time *t* (C_t) as a percentage of the initial (C_0).