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The influence of natural organic matter and aging on suspension stability in guideline toxicity testing of Ag, ZnO, and TiO₂ nanoparticles with *Daphnia magna*.

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Abstract— This study investigated changes in suspension stability and ecotoxicity of engineered nanoparticles (ENPs) by addition of Suwannee River natural organic matter (SR-NOM) and aging of stock and test suspensions prior to testing. Acute toxicity tests of Ag, ZnO, and TiO₂ ENPs with *Daphnia magna* were carried out following OECD test guidelines. *D. magna* was found to be very sensitive to Ag ENPs (48-hr EC50 of 33 µg L⁻¹) and aging of the test suspensions in M7 medium (up to 48 hours) did not decrease toxicity significantly. Conversely, the presence of SR-NOM (20 mg L⁻¹) completely alleviated Ag ENP toxicity in all testing scenarios, and did not aid in stabilizing suspensions. In contrast, addition of SR-NOM stabilized ZnO ENP suspensions and did not decrease toxicity. Aging for 48 hours generated monotonous concentration-response curves in the presence and absence of SR-NOM. TiO₂ ENPs did not cause immobilization of *D. magna* under any of the tested conditions in concentrations up to 100 mg L⁻¹. Presence of SR-NOM caused agglomeration in stock suspensions. Our results suggest that aging and presence of SR-NOM are important parameters in standard toxicity testing of ENPs, which in some cases may aid in gaining better control over the exposure conditions, however, in other cases might contribute to agglomeration or elimination of ENP toxicity. Therefore, modifications to the current guidelines for testing ENPs should be evaluated in a case-by-case basis.

Key words: Nanoecotoxicology, Nanoparticles, Aquatic invertebrates, Standard testing, Agglomeration.

INTRODUCTION

The advancements in the field of nanotechnology during the last two decades have enabled the production and use of various engineered nanoparticles (ENPs). It is inevitable that some of these ENPs will be discharged into the aquatic environment during the cycle of production, use and disposal [1]. Having different physico-chemical properties than their bulk counterpart, these particles need to be tested, ranked and labeled according to their toxicity [2,3]. Numerous test conditions can affect ENP stability. For example, NOM has been shown to adsorb to the surface of the ENPs creating a coating around it [4], and media composition and ionic strength can affect agglomeration of ENPs [5-9]. This is of great importance for interpretation of test results since surface coatings and aggregation of the nanomaterials will affect the fate, distribution and bioavailability towards organisms [10]. Organizations such as Organisation for Economic Co-operation and Development (OECD) and International Organization for Standardization (ISO) provide protocols for testing soluble chemicals and stable suspensions for the purpose of hazard identification. Most guidelines for standard acute tests, such as OECD 202 [11] utilizing *Daphnia magna*, have a test duration of 48 hours with immobilization being the main endpoint investigated. However, it is still not very clear if these tests are feasible to test ENPs [12], since their unique properties require proper addressing of characterization and toxicological properties. In the OECD Guidance Notes on Sample Preparation and Dosimetry for the Safety Testing of Manufactured Nanomaterials, it is stated that stability of nanoparticle suspensions should be measured and characterized over time (e.g. using techniques such as DLS) at intervals sufficient to quantify

exposures [13]. This might be an important step not only for stock suspension preparation and storage, but also for test suspensions in medium as ENPs may undergo alterations during incubation. Another recent OECD test guideline on Ecotoxicology and Environmental Fate of Manufactured Nanomaterials states that testing should include several tiers where dissolution and dispersability, agglomeration state and dispersion stability, as well as testing of transformed and aged nanomaterials should be taken into account, since there is not enough information on particle behavior over time. This should cover the particle kinetics over the whole test duration time. This guideline also states that there is a general need for using standard approaches for preparing stock suspensions and that there currently the regulatory practice for chemicals is not applicable to nanomaterials [14].

In the numerous reviews addressing the challenges of ecotoxicology testing of nanomaterials, the preparation of stock suspensions has been identified to be of high importance [12] as different preparation methods will influence the final nanoparticle sizes [2] which in turn are likely to influence toxicity outcomes [15]. There are several potential artifacts and misinterpretations in testing of nanomaterials, including agglomeration and sedimentation, altered dissolution rate and ion effects. These artifacts can occur during stock suspensions and test suspensions in medium. Therefore, dynamic changes to ENPs during testing can lead to inaccurate results [16]. Challenges in preparing stable suspensions may not only involve physical and chemical alterations of aqueous suspensions (mentioned above), but also timing considerations in preparation of stock and test suspensions relative to the onset of testing. An important factor to keep in mind for preparing stable ENP dispersions is the kinetics of ENP transformation processes in aqueous suspension which may greatly affect the reproducibility of test results. Allowing the dispersions to stabilize before dosing, could be a possible method to improve this issue.

Generally, there is no consensus on how to prepare test suspensions of ENPs for ecotoxicology studies [13]. Few studies have indicated the need to compare “aged” and “fresh” samples of ENP suspensions [17-19], highlighting the importance of investigating the dynamics of transformation processes throughout the duration of the test. While various studies have investigated the influence of NOM on the toxicity of the selected ENPs [20-23], to our knowledge, there is a lack of studies that systematically investigate the stability of stock suspensions and test suspensions in the presence of NOM and its implications on the outcome of toxicity tests. Likewise, “aging” of ENP test suspensions prior to testing remain to be studied in detail. Different ENPs behave differently under the same test conditions. TiO₂ ENPs tend to agglomerate in aquatic suspensions [24], and ZnO [5,9] and Ag [25,26] undergo dissolution. Therefore, a case-by-case consideration of possible alterations to the OECD guideline protocols is required in order to optimize suspension stability while minimizing test artifacts arising from the preparation procedure.

The Ag, ZnO and TiO₂ ENPs were chosen since they are commonly used commercially and are included in OECD prioritization list of nanomaterial testing [27]. Ag ENPs are normally used as a antimicrobial agent in various consumer products, food packaging and medical devices [17]. Some TiO₂ ENP applications involve photovoltaic devices, water treatment, and degradation of pollutants [28]. Due to their photocatalytic properties, both TiO₂ and ZnO ENPs are used in sunscreens and cosmetics [29]. Keeping in mind OECD recommendations for guideline testing, the aim of this study was to investigate the influence of two modifications to the *D. magna* acute toxicity test procedure (OECD 202) for Ag, ZnO and TiO₂ ENPs by 1) addition of natural organic matter (SR-NOM) to stabilize stock suspensions and test suspensions and 2) introduction of a 24 and 48 hour “aging” of test suspension prior to testing, in order to understand the dynamics of ENP processes during the OECD 202 48-hour test duration. It was hypothesized that these two parameters would improve the stability of the test system and lead to reproducible and quantitative toxicity test results. To test this hypothesis, series of systematic acute

tests were carried out where suspension stability and toxicity was measured simultaneously. A parallel series of tests with AgNO_3 and ZnCl_2 was included to investigate the influence of test alterations on the toxicity of the relevant dissolved species.

MATERIALS AND METHODS

Preparation stock and test solutions of metal salts

ZnCl_2 and AgNO_3 salts (Sigma Aldrich, Germany) were used as reference substances for the toxicity and behavior of dissolved Ag and Zn species. Stocks of metal salts were prepared in MilliQ water. Suwanee River natural organic matter (SR-NOM) (1R101N) containing 15% FA and 85% HA was purchased from International Humic Substances Society (MN, USA) and prepared in a stock solution at a concentration of 200 mg L^{-1} in MilliQ water. The pH was increased to 9-10 using 0.1 M NaOH and the solution was stirred overnight. During this time, the pH stabilized to that of the M7 medium (8.2-8.5), and the SR-NOM stock solution was vacuum filtered with a $1.5 \mu\text{m}$ particle retention glass microfiber filter (purchased from VWR). Test solutions were prepared by spiking media with appropriate amounts of metal salts stock solutions and SR-NOM stock solution. Four solutions of metal salts containing 20 mg L^{-1} SR-NOM (corresponding to 10.5 mg C L^{-1}) were prepared in 100 mL volumetric flasks and used freshly prepared (aged 0 hours) or aged 1.5 hours, 3 hours, and 24 hours. Aging was performed by storing the flasks in the fume hood at $20 \pm 2^\circ \text{C}$.

Nanoparticle characterization

Representative nanomaterials were acquired from the depository of the European Commission Joint Research Centre (JRC) (Ispra, Italy), which provides nanoparticle samples for the OECD WPMN sponsorship program. Ag ENPs (NM-300K) aqueous suspension dispersed in stabilizing agents (4% w/w each of Polyoxyethylene Glycerol Trioleate and Polyoxyethylene (20) Sorbitan mono-Laurate (Tween 20)) contained 10.16% Ag (w/w) (manufacturer's information). The vehicle stabilizing agent was acquired in a separate vial (NM-300K DIS Ag-dispersant) and used as a control. ZnO ENPs (NM-110 and TiO_2 ENPs (NM-104) (rutile, surface modification hydrophilic) were received in powdered form. Transmission electron microscope (TEM) data on the primary particle size of Ag ENPs was received from ENPRA (EU FP7 Project), revealing a size of 17.5 nm with a typical range of 8-47 [30]; and data on TiO_2 ENPs from MARINA project (EU FP7 Project), primary particle size $25.0 \pm 1.7 \text{ nm}$ and more information is given elsewhere [31]. Mean Feret's diameter from scanning electron microscope (SEM) images for ZnO ENPs was 151 nm with broadness of size distribution 55.6 nm [32]. Size and zeta charge of all ENPs in suspensions (stock and test suspensions) were measured using Zeta Sizer Nano Series (Malvern Instruments) according to manufacturer's instructions. Additionally, 5 μL samples from each treatment including ENP Stock I, ENP Stock II and their respective test suspensions in the highest concentration (see below), were suspended on copper grids with carbon films (Agar Scientific). The grids were covered with a petri dish and left at room temperature until water evaporated (3-4 hours). These grids were then examined by scanning electron microscope QUANTA 200F SEM (by FEI).

Preparation of ENP stock suspensions and test suspensions

Stock suspensions of Ag, ZnO, and TiO_2 ENPs concentrations were 10, 200, 1000 mg L^{-1} , respectively. The stock suspensions were prepared by mixing the weighed amount of each ENP with either MilliQ water (ENP Stock I) or with SR-NOM stock suspension with subsequent addition of MilliQ water (ENP Stock II). The final concentration of SR-NOM in Stock II was 20 mg L^{-1} (10.5 mg C L^{-1}). These suspensions were then sonicated for 20 minutes using a Branson Digital Sonifier Model S-250D at 10% Amplitude.

The ranges of concentrations of Ag, ZnO, and TiO₂ ENPs used in tests with *D. magna* were 5-100 µg L⁻¹, 2.5-20 mg L⁻¹ and 12.5-100 mg L⁻¹, respectively. The different test suspensions were prepared either in M7 medium (using ENP Stock I) or in M7 medium containing 20 mg L⁻¹ SR-NOM (using ENP Stock I and ENP Stock II, respectively) (see Figure 1). Each test suspension was prepared in triplicates and used aged 0 hours (freshly prepared), aged 24 hours and aged 48 hours. Upon preparation, size distribution and zeta potential of the freshly prepared test suspensions were measured using the Malvern Nano Zeta Sizer (see below). The test suspensions aged 24 and 48 hours were stored in a fume hood at 20±2°C for 24 and 48 hours, respectively. Before starting the test, the flasks of the aged suspensions were shaken manually for 5 seconds to resuspend the NPs, and size distribution and zeta potential were measured by DLS.

Measuring of nominal concentrations and ENP dissolution

The ENP stock and test suspensions were prepared as described above. Dissolution was measured by separating ENPs from their dissolved counterpart by ultracentrifugation, where the required centrifugation time and speed was calculated based on the diameter of the ENPs and the rotor dimensions. The suspensions were loaded in ultra-clear centrifuge tubes (Beckman Coulter, CA, USA), inserted into a swinging bucket rotor (SW 41 Ti Rotor, Beckman) and spun at 3x10⁴ rpm (154049 g) for 20 min using an L8-60M ultracentrifuge by Beckman. Subsequently, half the amount of the supernatant (4 mL) was removed from the centrifuge tubes. All samples were digested in concentrated HNO₃ (65%) and placed in a DB-3D heating bath (Holm & Halby, Denmark) up to 4 hours at 100°C. ENP stock suspensions and the highest test concentrations were analyzed by ICP-MS 7700 series (Agilent Technologies).

Culturing conditions

Daphnia magna were cultured in the laboratory in accordance with OECD recommendations [11]. The culture was grown under controlled conditions in 1L glass beakers containing 700-800 mL media (10-15 animals per beaker) at 20 ± 2° C with 16/8 hour light/dark cycle. Modified Elendt M7 medium (containing twice the amount of trace elements and a ten times higher concentration of potassium chloride compared to the OECD M7 recipe [11]) was prepared every week and used for both culturing and toxicity testing at pH 8.2 ± 0.3 and O₂ concentration of 9.0 ± 0.6 mg L⁻¹. Media in the cultures was renewed two times a week by replacing 300-400 mL of the old media with fresh M7 medium. The culture was fed daily with algae *Pseudokirchneriella subcapitata* at 1-2 mg C L⁻¹ day⁻¹.

Acute immobilization toxicity tests

The OECD 202 [11] guideline was followed for measuring *Daphnia magna* acute immobilization in response to the exposure to metal salts and ENPs. Potassium dichromate validity test revealed a 48 hour EC50 of 0.73 mg L⁻¹, which falls within the acceptable range values. Neonates of ≤ 24 hours were used for each test which involved four to six test concentrations, and control groups containing M7 medium only, M7 +SR-NOM medium, or Ag-dispersant for Ag ENP tests. Each concentration utilized 20 neonates, in four replicates with five neonates each in a 100 mL glass beaker containing 25 mL test solution/suspension. The glass beakers were covered and incubated for 48 hours at 20 ± 2 °C in the dark. After 24 and 48 hour exposure the number of immobilized organisms was counted. The animals were considered to be immobilized if they were unable to swim freely for more than 15 seconds and did not respond to gentle movement by means of a metal needle.

Speciation calculations and statistical data treatment

Visual MINTEQ (ver 3.0) was used to assess speciation of silver (added as AgNO_3), and zinc (added as ZnCl_2) in the test medium (M7) at pH 8.2. The binding of metals to SR-NOM was taken into account by including the Stockholm Humic Model at a concentration 20 mg L^{-1} (10.5 mg C L^{-1}) with specification 15% FA and 85% HA. ToxCalc v5.0.23 Software was used with maximum likelihood logit regression to generate concentration-response curves and effect concentrations with corresponding 95% confidence intervals.

RESULTS AND DISCUSSION

Acute toxicity of AgNO_3 and ZnCl_2 - influence of aging and SR-NOM

Toxicity testing of metal salts was carried out to serve as a toxicity reference for the ENP tests, as well as experimentally investigate the influence of aging and SR-NOM interaction with metals in test solutions. In a series of preliminary experiments, it was observed that the addition of 5, 10 and 20 mg L^{-1} SR-NOM slightly decreased toxicity of Zn and Ag salts with increasing SR-NOM concentration (Supplemental Data, Table S1). The next set of experiments used SR-NOM concentration of 20 mg L^{-1} , corresponding to approximately 10.5 mg C L^{-1} , which is within the range for NOM concentrations found in surface waters [33]. However, since the process of SR-NOM interaction with dissolved species is time-related, we investigated the influence of SR-NOM on metal bioavailability over time, for which changes in toxicity give an indirect measure. Test solutions were aged for 1.5, 3, and 24 hours and their toxicity was compared with freshly prepared solutions (aging 0 hours) (Figure 2, Table S2). For AgNO_3 , the 48-hr EC50 value of $2.0 [1.8-2.2]_{95\%} \mu\text{g Ag L}^{-1}$ (parenthesis representing 95% confidence intervals) was in agreement with previously reported values [34]. This value increased to $3.3 [3.1-3.5]_{95\%} \mu\text{g Ag L}^{-1}$ (1.6 fold) with the addition of 20 mg L^{-1} SR-NOM (Figure 2A). Aging of AgNO_3 test solutions in the presence of 20 mg L^{-1} SR-NOM shifted the dose-response to the right, indicating a significant decrease of toxicity over time (up to 24 hours). This was even more pronounced after 3 hour aging (Figure 2A d) and after 24 hour all animals remained mobile throughout the incubation time for all concentrations tested. Visual Minteq speciation calculations with M7 medium components revealed that for AgNO_3 , the major silver species were Ag^+ , AgCl (aq) , and AgCl^{2-} (data not shown). According to the Minteq speciation calculations carried out on dissolved silver taking into account media constituents, and given the aerobic conditions maintained throughout the incubation, we can conclude that sulfidation processes are not likely to occur. The species distribution did not change with increasing concentration or addition of NOM. Although these speciation calculations reveal that only a small amount of the dissolved silver will bind to the NOM at equilibrium, SR-NOM is a complex substance which might not be best represented by the Stockholm Humic model used here. Additionally, it has previously been hypothesized that the decrease in toxicity of elemental Ag in the presence of NOM might be due to the Ag-chloride formation, rather than Ag-NOM complexation [35]. Previous studies have shown that humic substances are able to bind toxic metal ions and render them unavailable to organisms [36] and this speciation may be changing during the toxicity testing period. Another study observed a decrease in toxicity of Ag with an increase in equilibration time [37]. Thus, longer aging time might be necessary to enable NOM binding to the Ag^+ metal ions and complexation with other ions in the solution. On this basis, it can be hypothesized that SR-NOM will also have an effect on Ag ENP toxicity by interacting with the released Ag^+ ions and reducing the Ag ENP toxicity due to ionic form.

Toxicity of ZnCl_2 to *D. magna* revealed 48-hr EC50 values of $2.5 [2.1-3.1] \text{ mg Zn L}^{-1}$ (Figure 2B). Presence of 20 mg L^{-1} SR-NOM in the freshly prepared solutions, slightly decreased toxicity (not significantly ($p < 0.05$)), and aging of the test solutions did not increase this effect. Visual Minteq speciation

calculations revealed a complexation of the Zn^{2+} ion with NOM up to 28% for the low concentrations. This complexation however decreased to <10% for highest concentrations (data not shown). These results provide some insight into the effect of SR-NOM on the metal ions released from the ENPs, showing that SR-NOM might reduce the metal-ion induced toxicity of Ag but not of ZnO ENPs.

Characterization and behavior of Ag, ZnO, TiO₂ ENPs in suspensions

The size distribution and zeta potential of the tested ENPs in aquatic suspensions under different scenarios measured by DLS are provided in Table 1, and SEM images are presented in supplemental data (Figure S1). The hydrodynamic diameter, poly-dispersivity index (PDI), and zeta potential values of the nanoparticles in the different test suspensions (concentrations) within the same testing scenario /condition were combined across concentrations and given as a range in Table 1. This was done as no specific concentration-dependent agglomeration trends were observed for any of the tested ENPs in M7 or M7 + SR-NOM medium. Results below will be discussed in the order of freshly prepared suspensions and then compared to aged suspension for general observations.

For Ag ENPs in the test condition of 0 hours aging (freshly prepared), Ag ENP Stock I revealed a size distribution with z-average of 63 nm (PDI 0.24), whereas the different test suspensions prepared from Ag ENP Stock I in M7 and M7 + SR-NOM medium aggregated reaching a size of 461-821 nm and 278-616 nm, respectively. This was accompanied by a great increase in PDI reaching levels close to 1 invalidating quantitative size distribution determinations, and a decrease in absolute zeta charge value clearly showing the increased instability of the test suspensions. For Ag ENP Stock II the hydrodynamic diameter was smaller (z-average: 29 nm; PDI 0.43), but the high PDI makes the absolute value of z-average less reliable. Similar to what was observed for Ag ENP Stock I, the test suspensions prepared from Ag ENP Stock II in M7 + SR-NOM medium aggregated to 385-896 nm and the PDI value increased to 0.85-1. Ag ENP Stock I did not considerably increase in z-average, PDI or zeta potential value. However, after 48 hours, test suspensions prepared from this stock in M7 and M7 + SR-NOM medium aggregated greatly reaching 390-1360 nm and 296-1789 nm, respectively. Ag ENP Stock II slightly increased in size over time despite a decrease in PDI and an increase in zeta charge absolute value. The test suspensions prepared from this stock also agglomerated reaching sizes of 515-987 nm after 48 hour aging. The high PDI-values and decreasing zeta potential indicate that the samples were highly unstable.

It has previously been shown that different ENP preparation methods can influence toxicity outcomes [15,38]. Amounts of <10 mg L⁻¹ TOC (SRHA) have shown to increase the total amount of Ag in suspension due to increased dispersion, while greater amounts caused agglomeration and settling [20]. Our DLS data revealed that Ag ENPs of 17.5 nm primary particle size, agglomerated in stock suspensions but aging had little effect on size distribution (Table 1). Agglomeration exacerbated when stock suspensions were diluted in M7 medium (with or without SR-NOM) for all tested concentrations. Previous studies have shown that the presence of salts in solution/increased ionic strength can cause aggregation of nanoparticles [6,7] and that divalent cations such as Ca^{2+} and Mg^{2+} cause more aggregation than monovalent cations [7,8]. In accordance with this, the SEM images (Supplemental Data, Figure S1) revealed the presence of larger agglomerates in test suspensions in medium compared to stock suspensions. It should however be noted that SEM sample preparation may introduce artificial aggregation and these images were therefore only used in a comparative analysis of agglomeration, not to obtain qualitative data on particle agglomeration. NOM has been shown to have a stabilizing effect on Ag ENP suspension [6,7], however, at higher ionic strength of the medium NOM does not help the stability of the system [39], most likely due to intermolecular bridging [8]. This could explain the increase in size over time (measured by DLS), especially pronounced for the 48 hour aging where the hydrodynamic diameter of the test suspensions reached μm levels. Based on the experiments of the

present study, it can be concluded that independent of the method the stock suspensions and test suspensions were prepared and aged, the addition of 20 mg L⁻¹ SR-NOM did not help in the stabilization of Ag ENP suspensions.

For ZnO ENPs in the test condition of 0 hours aging (freshly prepared), the z-average hydrodynamic diameter of ZnO ENP Stock I was comparable with ZnO ENP Stock II with sizes of 132 nm (PDI 0.09) and 133 nm (PDI 0.18), respectively (Table 1). The low PDI and the zeta potentials of -30 mV > ζ p > 30 mV indicate that both freshly prepared stock suspensions were in the stable range. Preparation of the suspensions with different concentrations of ZnO ENPs in medium from ZnO ENP Stock I caused aggregation, accompanied by an increase in PDI and a decrease of zeta charge absolute value. This trend was more pronounced for test concentrations prepared in M7 medium (380-638 nm; PDI 0.24-0.54), than those prepared in M7 + SR-NOM medium (136-138 nm; PDI 0.18-0.31). The set of test suspensions prepared from ZnO Stock II in the presence of SR-NOM, revealed slight agglomeration (133-198 nm; PDI 0.18-0.31). These results indicate a stabilizing effect of SR-NOM in both stock suspension and test suspensions. Regarding the influence of aging, it was found that agglomeration in the ZnO ENP Stock I slightly increased over the 48 hours, reaching a hydrodynamic diameter of 240 nm (PDI 0.56). The test suspensions prepared in M7 medium from this stock suspension, also agglomerated over time and sedimentation of particles was observed (as shown by the indicative sizes > 1000 nm; PDI 1.0). For test suspensions in M7 + SR-NOM prepared from ZnO ENP Stock I, agglomeration also occurred with aging, but to a lower extent, reaching sizes of 177-208 nm (PDI 0.32-0.44) after 48 hours. ENP Stock II remained stable over time, and the test suspensions prepared from this stock in M7 + SR-NOM medium only agglomerated slightly over time (144-162 nm; PDI 0.26-0.40).

ZnO ENPs in this study had a tendency to agglomerate in aqueous suspension (Table 1). Stock I prepared in MilliQ had a tendency to agglomerate over time, whereas the presence of SR-NOM in Stock II had a stabilizing effect. Similar trends were also observed by SEM (Supplemental Data, Figure S1), and are in agreement with other studies where SR-NOM is reported to stabilize aqueous suspensions of ZnO ENPs [9,40]. Further agglomeration was observed in the test suspensions with prepared by dilution in M7 medium where particles reached μ m sizes. This is most likely due to the presence of ions in the medium and in particular the prevalence of divalent cations [41]. Overall, the presence of SR-NOM assisted in stabilizing ZnO ENP suspensions.

For TiO₂ ENPs, ENP Stock I suspension revealed a hydrodynamic diameter z-average of 83 nm; PDI 0.24. Test suspensions prepared from TiO₂ ENP Stock I in M7 medium agglomerated greatly reaching values > 1 μ m and PDI 0.26-0.41; whereas those prepared in the presence of SR-NOM seemed to be more stable (101-109 nm; PDI 0.25-0.33). Preparation of TiO₂ ENP Stock II in SR-NOM caused large agglomeration with z-average size > 2000 nm. Large agglomerates were also observed by SEM (Supplemental Data, Figure S1). Similar trends were seen in the suspensions prepared from this stock, with sizes in the range of 455-2827 nm (PDI 0.22-0.63). Aging of the suspensions did not majorly affect the size distribution and zeta potential. Generally, test suspensions prepared using M7 medium agglomerated greatly over time, reaching μ m range. Less agglomeration was observed in the presence of SR-NOM in M7 medium. Nonetheless, all test suspensions had zeta charge values were between -30 mV > ζ p > 30 mV indicating instability (Table 1).

While various studies have reported a stabilizing effect of NOM on nanoparticles in aqueous suspensions [42,43], in our study, the most stable stock suspension with limited agglomeration was TiO₂ ENP Stock I prepared in MilliQ water. On the other hand, the preparation of TiO₂ ENP Stock II with SR-NOM, caused immediate agglomeration/sedimentation. A tendency to more stable test suspensions was however seen when for M7 + SR-NOM medium prepared from TiO₂ ENP Stock I (in MilliQ water). As a result of

media composition (especially the presence of divalent cations) TiO_2 ENPs spontaneously agglomerated upon transfer to medium (Table 1), as previously shown by other studies [24,44]. Interactions of media constituents can modify the chemistry and behavior of TiO_2 ENPs in aqueous suspension as agglomeration has been reported for CaCl_2 concentrations over 0.1 mM, and pH above 5 [45] which are conditions fulfilled by the M7 medium. These results question the use of 20 mg L^{-1} NOM in preparation of TiO_2 ENP stock suspensions in MilliQ water.

Influence of NOM and aging on acute toxicity of Ag ENP towards D. magna

ICP-MS analysis showed Ag NP stock recovery of 81 and 83% for Stock I and Stock II respectively. Recovery in the test suspensions varied between 60-72% (Supplemental Data, Table S3), showing that recovery of Ag NPs is difficult and probably more problematic at lower concentrations where a small loss has greater influence. Measuring of ENP dissolution has shown to be particularly challenging [16]. Low recovery of Ag is most likely due to losses during the handling of suspensions rather than analytical-chemical difficulties. The serial dilutions performed prior to measuring could contribute to loss of Ag (e.g. sorption to the glass walls in the measuring flasks and vials), however attempts to recover sorbed Ag by acid digestion of glassware did not disclose the cause of the relatively low recovery.. Ag-dispersant and 20 mg L^{-1} SR-NOM were non-toxic to *D. magna*, therefore, toxic effects were attributed to the presence of nanoparticles in suspension. Ag ENPs test suspensions from ENP Stock I caused immobilization of daphnids within the first 24 hours of exposure, and this immobilization did not increase within 48 hour of exposure. The immobilization of the daphnids increased from 10 % to 90% over a narrow concentration range of $23\text{-}45 \mu\text{g L}^{-1}$ and the calculated 48-hr EC_{50} was $32 [28\text{-}38]_{95\%} \mu\text{g L}^{-1}$ (Table 1). Aging of test concentrations in M7 medium did not significantly affect toxicity, whereas addition of 20 mg L^{-1} SR-NOM under all conditions completely alleviated the toxicity of Ag ENPs showing no immobilization in any of the concentrations tested.

It has been previously been found that Ag ENPs are able to undergo dissolution in suspension [25,26] and that Ag^+ ion release reaches equilibrium within 48 hours, but the amount of dissolution will depend on type of Ag ENP [25]. The acute toxicity data from our study showed that similar to experiments with AgNO_3 , Ag ENPs caused most immobilization within 24 hours of exposure. This could suggest that dissolved Ag species formed as a result of dissolution will influence toxicity as previously seen by Newton et al. [23], and that the initial contact period with the organism will dictate this toxicity. Exposure to Ag^+ has been shown to cause significant inhibition of Na^+ uptake and an increase in $\text{Na}^+ \text{K}^+ \text{ATPase}$ activity within 2 hours and complete by 12 hours [46], and this mechanism could explain the sudden immobilization of the daphnids. Though recent studies have described that aged Ag ENPs releasing more Ag^+ ions were more toxic than freshly added ENPs [21], this was not seen in our study. Dissolution of the three freshly prepared Ag NP test suspensions was quite similar, between 3-4%. Dissolution increased at 24-hour aging, even in the presence of SR-NOM (Table S3). Other studies have also reported significant increases in ion release during storage [19]. The 48-hour exposure to the freshly prepared and aged Ag ENP suspensions gave very similar results.

Addition of 20 mg L^{-1} SR-NOM eliminated the acute toxicity of Ag ENPs in the concentrations tested with *D. magna*. Previous studies have shown that presence of NOM is able to mitigate toxicity [20,21] and this effect becomes more visible with increasing amounts of DOC [19], which is also the case in this study. A decrease in immobilization leading to a complete elimination of toxicity after 24 hour aging was also seen in our tests involving AgNO_3 (Table S2). Adsorption of NOM onto Ag ENPs is believed to inhibit ion release [26], however, this was not seen in our study since dissolution increased after 24 hours. Elimination of toxicity in the presence of 20 mg L^{-1} SR-NOM might rather be explained by formation of Ag^+ -NOM complexes that are non-toxic to *D. magna*. In our study, addition of SR-NOM did not help

stabilize Ag ENP suspensions and resulted in underestimation of toxicity. This has a very important impact on regulatory toxicity testing since the addition of NOM may contribute to lowering of Ag ENP toxic effects. Therefore, based on the results presented here, addition of 20 mg L⁻¹ SR-NOM is not recommended for testing of Ag ENPs for regulatory purposes.

Influence of NOM and aging on acute toxicity of ZnO ENP towards D. magna

ICP-MS measurements revealed high recovery (close to 100%) for ZnO NPs in both stock and test suspensions. For ZnO ENPs, the number of immobilized animals increased during the 48-hr exposure period, yielding 48-hr EC50 of 6.7 [4.9-8.9]_{95%} mg L⁻¹ (Table 1). In the 0 hours aging condition, ZnO ENPs prepared from ENP Stock I in M7 medium revealed an increase in toxicity with increase in concentration, whereas no monotonous concentration-response relationships were observed for ZnO ENPs in M7 + SR-NOM media prepared from ENP Stock I or ENP Stock II (Figure 3). In these freshly prepared suspensions, higher 48 hour immobilization was observed for ZnO ENPs suspensions prepared from Stock I in regular M7 medium compared to those in the presence of SR-NOM despite the dissolution being lower (see Table S3). All test suspensions aged for 24 hours, gave a scattered response with similar 48 hour immobilization (> 50%), but low 24 hour mortality (~10%). However, the test systems seemed to stabilize after 48 hours of aging, as concentration-response curves were achieved for all testing scenarios with an overall increase in immobilization both 24 and 48 hour exposure, especially in the presence of SR-NOM. This might be explained by a trend of increasing dissolution over time as observed for suspensions from Stock I in M7 medium and suspensions from Stock II in M7+SR-NOM medium (Table S3).

In our study, there was a difference in 48 hour immobilization between ZnO ENPs (EC50 6.7 mg L⁻¹) and ZnCl₂ (2.5 mg Zn L⁻¹). However, the EC-values were in the same order of magnitude, and since ZnO ENP toxicity is believed to be linked to Zn²⁺ ion release [47]. In the freshly prepared test suspensions (0 hour aging), the presence of SR-NOM caused a scattered response in toxicity and unlike what was found in experiments with Ag ENPs, did not alleviate toxicity. It has been shown before that the presence of NOM can either enhance or reduce Zn²⁺ release from ZnO ENP depending on their concentration and chemical composition [48]. Nonetheless, our earlier experiment employing ZnCl₂ showed that SR-NOM did not ameliorate the toxic effect of Zn²⁺ ion (Table S2), and speciation calculations also revealed a decrease in Zn-NOM complexation with increase in ZnCl₂ concentration. This suggests that NOM might have different effects on toxicity of different metals, as previously hypothesized that metals bind to different moieties within the NOM [37].

While in general higher immobilization to *D. magna* was observed with 24 hour aged test suspensions, the responses did not follow monotonous concentration-response patterns. Kinetics studies have shown that ZnO ENPs (at pH 7.5) can reach dissolution steady state in 24 hours [49]. However, in our study (pH 8.2), stable testing conditions were not reached until 48 hour aging, revealing clear dose-response relationships (Figure 3) which corresponded to the increase in dissolution (Supplemental Data, Table S3). Similar to our findings, Blinova et al. [22] found that the presence of DOC did not decrease the toxicity of ZnO to freshwater crustaceans. In our study, the presence of SR-NOM along with the aging contributed to stabilization of the test suspensions and might lead to increased standard tests reproducibility.

Influence of NOM and aging on acute toxicity of TiO₂ ENP towards D. magna

High recovery (> 90%) was achieved for TiO₂ ENP stock and test suspensions by ICP-MS. TiO₂ ENPs tested in concentrations up to 100 mg L⁻¹ under the different testing conditions did not cause immobilization of *D. magna*. This is also supported by earlier studies which have revealed TiO₂ ENPs to be generally non-

toxic to *D. magna* in dark conditions [50,51]. However, it was observed that at higher concentrations TiO₂ ENPs formed a layer on the carapaces of the daphnids, affecting their inability to move freely. This could be regarded as an indirect effect due to physical restraints.

Test design implications

This study aimed to provide recommendations for developing and harmonizing standard methods for testing the aquatic toxicity of engineered nanoparticles. Our results suggest that preparation of stock dispersions in MilliQ water resulted in relatively stable suspensions with low aggregation for all three tested ENPs. It was also shown that for some ENPs, aging of test suspensions and the presence of NOM may be important variables to achieve constant exposure conditions during incubation, leading to improved reproducibility of guideline testing of ENPs. However, while this pattern was established for ZnO ENPs, the presence of SR-NOM did not stabilize Ag ENP suspensions. Additionally, the presence of 20 mg L⁻¹ SR-NOM completely eliminated toxicity, leading to an underestimation of toxicity and hence, making SR-NOM addition not suitable as a general recommendation for aquatic test guidelines for Ag ENPs. Furthermore, it was found that aging of the Ag ENP test concentrations did not appear to affect toxicity. The addition of SR-NOM to TiO₂ ENP in stock suspensions introduced agglomeration and settling. On this basis, it is recommended that SR-NOM addition to OECD guideline testing of ENPs should be introduced only in certain cases. Additionally, the influence of aging on suspension stability should be evaluated on a case-by-case basis.

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Figures and Tables:

Figure 1: Experimental test set-up and preparation scheme of test suspensions of Ag, ZnO and TiO₂ ENPs for characterization and toxicity testing. ENP Stock I was prepared in MilliQ water at concentrations of 10 mg L⁻¹ for Ag ENPs, 200 mg L⁻¹ for ZnO ENPs, and 1000 mg L⁻¹ for TiO₂ ENPs. ENP Stock II was prepared in MilliQ water containing 20 mg L⁻¹ Suwanee River Natural Organic Matter (SR-NOM) in the same ENP concentration as ENP Stock I. The different test suspensions were prepared in triplicates, either in M7 medium, or M7 medium containing 20 mg L⁻¹ SR-NOM. One set of test suspensions was tested freshly prepared (aged 0 hours), whereas the other two sets were tested aged 24 and 48 hours, respectively.

Figure 2: Immobilization of *Daphnia magna* relative to controls after 48 hours exposure to (A) AgNO₃ and (B) ZnCl₂ solutions in M7 medium aged 0 hours (a); in M7+SR-NOM medium (20 mg L⁻¹ SR-NOM) aged 0 hours (b), aged 1.5 hours (c), aged 3 hours (d), and aged 24 hours (e). Dotted lines represent 95% confidence intervals.

Figure 3: Immobilization of *Daphnia magna* neonates in response to 48-hour exposure to different concentrations of ZnO ENPs prepared from Stock I (MilliQ) and Stock II (MilliQ + SR-NOM) in the presence and absence of SR-NOM and with the additional effect of aging. Dotted lines represent 95% confidence intervals.

Table 1: Results of dynamic light scattering (DLS) measurements of stock suspensions (ENP Stock I and ENP Stock II) of Ag, ZnO, and TiO₂ ENPs (10, 200, 1000 mg L⁻¹ respectively) and test suspensions aged for 0, 24 and 48 hours (see Figure 1 for test setup) The table displays zeta-average hydrodynamic size (z-avg) and zeta-potential (z-potential) measurements along with polydispersity index (PDI). The separate readings from test suspensions (4-5 different concentrations) of a given ENP under the same scenario are combined and given as a range.

Aging (h)	Stock suspensions and respective test concentrations	Ag ENPs (NM-300K)				
		z-avg (nm)	CV ^c	PDI	z-potential (mV)	CV ^c
0	ENP stock I	63	14.1	0.25	-19	4.2
24	ENP stock I	44	11.8	0.23	-22.4	4.5
48	ENP stock I	52	10.4	0.24	-20.5	2.4
0	M7 medium	461–821	5.2–28.8	0.88–1.00	-6.9 to -9.73	2.4–8.9
24	M7 medium	347–1032	10.1–30.3	0.77–1.00	-9.7 to -10.1	4–9.2
48	M7 medium	390–1316	0.8–27.2	0.86–1.00	-7.3 to -14.4	0.6–6.4
0	M7+SR-NOM medium	258–616	10.4–48.5	0.73–0.90	-6.2 to -7.2	0.6–3.8
24	M7+SR-NOM medium	543–738	7.3–39.7	0.93–1.00	-6.0 to -7.7	7.1–14.5
48	M7+SR-NOM medium	296–1789	15.3–42.6	0.72–1.00	-7.8 to 10.3	2.6–9.8
0	ENP stock II	29	5.2	0.43	-35	1.7
24	ENP stock II	39	0.5	0.19	-32.4	2.2
48	ENP stock II	47	13.6	0.17	-36.2	4.9
0	M7+SR-NOM medium	385–896	6.9–26.5	0.85–1.00	-5.7 to -6.8	3.7–11.6
24	M7+SR-NOM medium	657–667	4.1–63.5	0.90–1.00	-7.2 to -8.1	11–28.9
48	M7+SR-NOM medium	515–987	12.4–38	1	-6.9 to -9.0	1.2–3.5

Table 1 - continued: Results of dynamic light scattering (DLS) measurements of stock suspensions (ENP Stock I and ENP Stock II) of Ag, ZnO, and TiO₂ ENPs (10, 200, 1000 mg L⁻¹ respectively) and test suspensions aged for 0, 24 and 48 hours (see Figure 1 for test setup) The table displays zeta-average hydrodynamic size (z-avg) and zeta-potential (z-potential) measurements along with polydispersity index (PDI). The separate readings from test suspensions (4-5 different concentrations) of a given ENP under the same scenario are combined and given as a range.

ZnO ENPs (NM-110)				
z-avg (nm)	CV ^c	PDI range	z-potential (mV)	CV ^c
132	1.2	0.09	33.6	0.9
145	1.2	0.24	27.8	0.4
240	3.3	0.56	23.3	3.4
380–638	0.84–5.4	0.24–0.54	–2.1 to –6.4	8.1–32.2
1644–3092	2.2–9.3	1	–2.9 to –5.9	6.5–25.5
1403–3506	1.9–14.6	1	–3.6 to –8.1	1.7–16.4
136–138	0.4–3.2	0.16–0.30	–17.5 to –18.0	3.2–10.7
140–145	0.2–5.4	0.20–0.35	–14.4 to –17.1	0.8–11.3
177–208	0.1–11	0.32–0.44	–12.9 to –14.7	0.5–5.1
133	1.9	0.18	–35.6	0
135	1.2	0.15	–25.5	4.7
133	0.9	0.14	–21.9	5
133–198	0.4–2.6	0.18–0.31	–18.0 to 19.3	1.9–8.7
133–188	0.2–4.2	0.20–0.32	–14.6 to –17.4	0.4–17.5
144–162	0.7–4	0.26–0.40	–14.4 to –16.0	2.6–6.2

Table 1 - continued: Results of dynamic light scattering (DLS) measurements of stock suspensions (ENP Stock I and ENP Stock II) of Ag, ZnO, and TiO₂ ENPs (10, 200, 1000 mg L⁻¹ respectively) and test suspensions aged for 0, 24 and 48 hours (see Figure 1 for test setup) The table displays zeta-average hydrodynamic size (z-avg) and zeta-potential (z-potential) measurements along with polydispersity index (PDI). The separate readings from test suspensions (4-5 different concentrations) of a given ENP under the same scenario are combined and given as a range

TiO ₂ ENPs (NM-104)				
z-avg (nm)	CV ^c	PDI	z-potential (mV)	CV ^c
83	2.3	0.24	17.4	22.4
89	0.7	0.28	13.6	25.7
90	1.0	0.29	18.4	3.8
592–1119	0.4–3.2	0.26–0.41	5.9–8.0	3.2–10.7
909–1325	2.2–13.9	0.29–0.88	5–8.5	1.4–25.5
737–865	1.9–14.6	0.3–0.52	4.5–6.9	1.7–16.4
101–109	0.9–5.4	0.25–0.33	–13.5 to –14.4	8–32.2
116–172	0.2–5.4	0.24–0.38	–14 to –15.2	0.8–11.3
115–154	0.1–10.9	0.29–0.39	–14.8 to –15.3	0.5–5.1
2503	6.6	0.34	–8.9	40.4
2598	16.4	0.33	–2.9	13.8
2358	0.02	0.42	–2.98	23.5
455–2827	0.5–3.7	0.22–0.63	–10.5 to –14.4	1.9–8.7
415–620	0.2–4.2	0.26–0.5	–14.1 to –15.7	0.4–17.5
444–759	0.7–4	0.2–0.47	–14.5 to –14.9	2.6–3.2

Table 2: 10% and 50% effect concentrations (EC) and corresponding 95% confidence intervals for 48-hr *Daphnia magna* immobilization tests of Ag and ZnO ENP test suspensions prepared from ENP Stock I and ENP Stock II and aged for 0, 24 and 48 hours.

Aging	Engineered nanoparticle stock I				Engineered nanoparticle stock II	
	M7 medium		M7 + SR-NOM medium		M7 + SR-NOM medium	
	EC10	EC50	EC10	EC50	EC10	EC50
Ag ENPs						
0 h	23.5 (17.8–27.4)	32.6 (28.1–38.2)	>100	>100	>100	>100
24 h	27.9 (20.5–33.0)	39.3 (33.2–45.0)	>100	>100	>100	>100
48 h	24.7	41.3	>100	>100	>100	>100
ZnO ENPs						
0 h	1.8 (0.6–2.9)	6.7 (4.9–8.9)	ND	ND	ND	ND
24 h	0.05	1.7	0.2 (9 ⁻⁶ –0.8)	1.1 (0.09–2.3)	ND	ND
48 h	1.5 (0.5–2.4)	4.7 (3.3–5.9)	0.3 (0.02–1)	1.5 (0.1–2.6)	0.4 (0.01–1.2)	2.2 (0.5–3.5)

^aPrepared from ENP stock I and ENP stock II and aged for 0 h, 24 h, and 48 h. For both ENPs, estimations of effect concentration values were based on nominal concentrations.

EC10= 10% effect concentration; EC50 = 50% effect concentrations; ENP = engineered nanoparticle; SR-NOM = Suwannee River natural organic matter; ND = not determined (effect concentration values could not be calculated as no concentration–response curves were achieved because of the scattered response).