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Influence of pH and media composition on suspension stability of silver, zinc oxide, and titanium dioxide nanoparticles and immobilization of *Daphnia magna* under guideline testing conditions

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ABSTRACT

In aquatic toxicity testing of engineered nanoparticles (ENPs) the process of agglomeration is very important as it may alter bioavailability and toxicity. In the present study, we aimed to identify test conditions that are favorable for maintaining stable ENP suspensions. We evaluated the influence of key environmental parameters: pH (2–12) and ionic strength using M7, Soft EPA (S EPA) medium, and Very Soft EPA (VS EPA) medium; and observed the influence of these parameters on zeta potential, zeta average, and acute immobilization of *Daphnia magna* for three different ENPs. Despite being sterically stabilized, test suspensions of silver (Ag) ENPs formed large agglomerates in both VS EPA and M7 media; and toxicity was found to be higher in VS EPA medium due to increased dissolution. Low-agglomerate suspensions for zinc oxide (ZnO) could be obtained at pH 7 in VS EPA medium, but the increase in dissolution caused higher toxicity than in M7 medium. Titanium dioxide (TiO_2) ENPs had a point of zero charge in the range of pH 7–8. At pH 7 in VS EPA, agglomerates with smaller hydrodynamic diameters (~200 nm) were present compared to the high ionic strength M7 medium where hydrodynamic diameters reached micrometer range. The stable suspensions of TiO_2 ENPs caused immobilization of *D. magna*, 48-h EC50 value of 13.7 mg L^{-1} (95% CI, 2.4 mg–79.1 mg L^{-1}); whereas no toxicity was seen in the unstable, highly agglomerated M7 medium suspensions, 48-h EC50 > 100 mg L^{-1}. The current study provides a preliminary approach for methodology in testing and assessing stability and toxicity of ENPs in aquatic toxicity tests of regulatory relevance.

1. Introduction

The increasing number of engineered nanoparticles (ENPs) used in products and applications, along with their novel characteristics and properties has placed a demand on screening and ranking of their ecotoxicity for hazard identification purposes. In the traditional risk assessment paradigm, the hazard identification step leads to classification of compounds and labeling of products, as well as provides information that feeds into the next steps of risk assessment. The identification of environmental hazards relies on standardized ecotoxicity testing methods. Presently, this procedure has applied to "regular" chemicals, as well as nanomaterials. However, the behavior of nanomaterials in aquatic test systems differs greatly compared to conventional water soluble chemicals, for which these tests were originally intended. Nanomaterials have a range of physico-chemical properties that are different from those of soluble chemicals, and their dispersion stability depends on intrinsic properties, as well as media parameters such as ionic strength, pH and dissolved organic carbon (Baalousha et al., 2013; Bian et al., 2011; Ottofuelling et al., 2011; Sharma, 2009; Handy et al., 2008). This makes it challenging to define and maintain constant exposure conditions during testing (Hartmann et al., 2010). As a result, most standard and guideline test protocols provided by organizations such as Organization for Economic Co-operation and Development (OECD) and International Organization for Standardization (ISO) have shown some shortcomings in testing nanomaterials. The OECD 202 guideline on immobilization of *D. magna* states that pH conditions can be between 6–9 and that dilution water of varying composition can be used, but that an optimal water hardness is 140–250 mg/L $CaCO_3$, and that the total organic carbon content must be below 2 mg/L (Organisation for Economic Co-operation and Development, 2004). The ISO 6341 method for immobilization of *D. magna* defines more strict testing conditions: "The dilution water thus prepared shall have a pH of 7.8 ± 0.5, a hardness of (225 ± 50) mg/l (expressed as $CaCO_3$), a molar Ca/Mg ratio close to 4:1 and a dissolved oxygen concentration above 7 mg/l" (International Organization for Standardization, 2010). These statements raise

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questions on the validity of current use of standard test guidelines in testing nanomaterials, since processes like dissolution, agglomeration, and sedimentation that are ongoing during the incubation period are not taken into consideration.

Based on these challenges, the OECD Working Party for Manufactured Nanomaterials is currently working on improving testing guidelines and they have released a guidance document on testing of manufactured nanomaterials (Organisation for Economic Co-operation and Development, 2010a), and on sample preparation and dosimetry for the safety testing of manufactured nanomaterials (Organisation for Economic Co-operation and Development, 2012). In the first guidance document, it is stated that the water exposure conditions of nanomaterials (such as pH and ionic strength) should be considered as they may affect both solubility and dispersibility (Organisation for Economic Co-operation and Development, 2010a). The second guidance document states that pH can affect agglomeration of charged nanomaterials (Organisation for Economic Co-operation and Development, 2012), highlighting the importance of test condition considerations in nanoecotoxicology. Another technical report by ISO states that the modifications that need to be implemented in the test system will depend on the type of medium, test organism and the properties of the nanomaterials. However, standard methods could be a good starting point, followed by careful consideration of methods for nanomaterial suspension preparation (The International Organization for Standardization, 2014).

In aquatic toxicity testing of ENPs, the process of agglomeration is very important as it affects the stability of the test system and the reproducibility of the tests outcomes. Different ENPs have different surface charge values which can affect their behavior in water, since electrostatic repulsion and attraction are determinant factors in agglomeration (The Danish Environmental Protection Agency, 2014; Handy et al., 2008); and dependent on the composition of the suspension, as well as the pH (Organisation for Economic Co-operation and Development, 2012). The point of zero charge (pzc) of a particle in suspension is the point where no electrostatic repulsion occurs and the suspension is most prone to agglomeration. Non-agglomerated primary particles sizes are difficult to maintain when introducing non-functionalized ENPs to aqueous suspensions. This is the case for ultrapure water (e.g. MilliQ water) and it becomes even more challenging for media used in toxicity testing, especially for those of high ionic strength. A variety of different synthetic freshwater media are used for toxicity tests; US EPA media ranging from Very Soft (VS) to Very Hard (VH), M4, M7 and ISO daphnia media with ionic strength ranging from 0.57 mM to 15.8 mM and hardness of 2.8–133.3 mg L^{-1} $CaCO_3$. Identifying some of the issues related to media content and taking into account the pH for suspension stability prior to testing, could lead to more appropriate test conditions with regards to reproducibility and reliability of results.

Therefore, the aim of this study was to investigate and indicate whether there exists a “window of opportunity”, i.e. a certain set of test parameters for each of the employed ENPs, under which relatively stable aquatic suspensions can be obtained, with lowest possible hydrodynamic diameter of ENP agglomerates before and during standard testing. The main deviation from the OECD 202 test guideline, however, is the lower range of media hardness compared to what is considered optimum conditions in the guideline. This “window of opportunity” was studied by employing changes in pH in media of different ionic strengths (under physiologically relevant conditions), while observing the effect of these parameters on dispersion stability, hydrodynamic diameter, zeta potential, and acute immobilization of *D. magna*. Our hypotheses is that based on information on zeta potential measurements and point of zero charge (pzc), a pH range and media composition can be identified, where each ENP suspension can remain stable and have low agglomerate hydrodynamic diameters during toxicity testing. A series of experiments were performed to determine pzc of each ENP in MilliQ water and three types of test media, and identify the optimal range of parameters which was then used to test their respective acute toxicity. Since properties of ENPs in aquatic media are particle specific, we employed 3 different ENPs having different behavior in test medium: AgNPs which were sterically stabilized and expected to undergo only limited dissolution, pristine ZnO NPs which undergo dissolution, as well as highly agglomerating TiO_2 NPs (Cupi et al., 2015). In addition to being some of the most used ENPs in products and applications, the particles studied are part of the OECD prioritization list of nanomaterial testing (Organisation for Economic Co-operation and Development, 2010b).

2. Materials and methods

2.1. Nanoparticle characterization

The Ag, ZnO and TiO_2 ENPs employed in this study were received from the European Commission Joint Research Centre. Ag ENPs (NM-300K) composed of 10.16% Ag (w/w) were received in aqueous suspension containing stabilizing agents, 4% w/w each of Polyoxyethylene (POE) Glycerol Trioleate and POE Sorbitan mono-Laurate (Tween 20) (manufacturer's information). ZnO (NM-110) and TiO_2 (NM-104) ENPs were received in powder form. Data on the primary size of the ENPs were provided by ENPRA and MARINA EU FP7 projects, and JRC reports. TEM data showed a primary particle size of 17.5 nm for Ag NPs, with a range of 8–47 (Jensen et al. 2011a, 2011b); 25.0 ± 1.7 nm for TiO_2 ENPs (Rasmussen et al., 2014); and for ZnO NPs, mean Feret's diameter of 151 nm with broadness of size distribution 55.6 nm as measured by scanning electron microscope (SEM) (Singh et al., 2011). Hydrodynamic diameter and zeta potential values for all suspensions (prepared as described in 2.2 were measured by Zeta Sizer Nano Series (Malvern Instruments) according to manufacturer's instructions. The data was analyzed by checking for inconsistencies of the hydrodynamic diameter in relation to volume and number of particles, according to the functions of the ZetaSizer.

2.2. Stock suspension preparation and point of zero charge measurements

Stock suspensions of Ag (10 mg L^{-1}), ZnO (100 mg L^{-1}), and TiO_2 ENPs (100 mg L^{-1}) were prepared by weighing the ENPs and adding MilliQ water, followed by sonication. Sonication of all stock suspensions followed the NANOGENOTOX protocol (Jensen et al., 2011a, 2011b) by sonicating using a Branson Digital Sonifier Model S-250D for 16 minutes at 10% Amplitude. The only modification was the placement of the beaker in a water bath instead of an ice-water bath as this helped avoid any increases in temperature that can occur from sonication, while at the same time avoiding the cooling effect of ice. Measuring of pzc involved preparation of two identical stock suspensions for each of the NPs; in the first one the pH was adjusted to acidic by HNO_3 and in the other one to basic by NaOH addition. Elendt M7 medium (Organisation for Economic Co-operation and Development, 2004), Very soft (VS) EPA medium and Soft (S) EPA (United States Environmental Protection Agency, 2002) medium were prepared following the respective recipes. VS EPA and S EPA media were prepared in 10x concentration due to the low amount of salts. A 1/10 dilution was then performed and the media was stirred overnight for the pH to equilibrate. 3-(N-morpholino) propanesulfonic acid (MOPS) buffer (Fluka) was prepared in concentration 8.9 mM in MilliQ water. DLS measurements were conducted right after sample preparation.