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Complete List of Authors:	Hartmann, Nanna; Technical University of Denmark, Department of Environmental Engineering Jensen, Keld; The National Research Centre for the Working Environment, Baun, Anders; Technical University of Denmark, Department of Environmental Engineering Rasmussen, Kirsten; European Commission - Joint Research Centre, Instutute for Health and Consumer Protection Rauscher, Hubert; European Commission - Joint Research Centre, Instutute for Health and Consumer Protection Tantra, Ratna; National Physical Laboratory, Cupi, Denisa; Technical University of Denmark, Department of Environmental Engineering Gilliland, Douglas; European Commission - Joint Research Centre, Instutute for Health and Consumer Protection Pianella, Francesca; European Commission - Joint Research Centre, Instutute for Health and Consumer Protection Riego Sintes, Juan; European Commission - Joint Research Centre, Instutute for Health and Consumer Protection
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Techniques and protocols for dispersing nanoparticle powders in aqueous media – is there a rational for harmonization?

Nanna B. Hartmann[1,2*], Keld Alstrup Jensen[3], Anders Baun[1], Kirsten Rasmussen[2], Hubert Rauscher[2], Ratna Tantra[4], Denisa Cupi[1], Douglas Gilliland[2], Francesca Pianella[2], Juan M. Riego Sintes[2]

[1]Technical University of Denmark (DTU), Department of Environmental Engineering, Miljoevej, DK-2800 Kgs. Lyngby, Denmark.

[2]European Commission - Joint Research Centre, Via E. Fermi, 21027 Ispra, Italy

[3]The National Research Centre for the Working Environment (NRCWE), Lersø Park Allé 105, DK-2100 Copenhagen Oe, Denmark

[4]National Physical Laboratory (NPL), Hampton Road, Teddington, Middlesex TW11 0LW, United Kingdom

*Corresponding author. Current affiliation: DTU Environnement, Miljøvej, DK-2800 Kgs. Lyngby, Denmark. Email: nibh@env.dtu.dk

ABSTRACT

Appropriate ways of bringing engineered nanoparticles (ENP) into aqueous dispersion is a main obstacle for testing and thus for understanding and evaluating their potential adverse effects to the environment and human health. Using different methods to prepare (stock) dispersions of same ENP may be a source of variation in the toxicity measured. Harmonization and standardization of dispersion methods applied in mammalian and ecotoxicity testing are needed to ensure a comparable data quality and to minimize test artefacts produced by modifications of ENP during the dispersion preparation process. Such harmonization and standardization will also enhance comparability between tests, labs and studies on different types of ENP. The scope of this review was to critically discuss the essential parameters in dispersion protocols for ENP. The parameters are identified from individual scientific studies and from consensus reached in larger-scale research projects and international organizations. A step-wise approach is proposed to develop tailored dispersion protocols for ecotoxicological and mammalian toxicological testing of ENP. The recommendations of this analysis may serve as a guide to researchers, companies, and regulators when selecting, developing and evaluating the appropriateness of dispersion methods applied in mammalian and ecotoxicity testing. However, additional experimentation is needed to further document the protocol parameters and investigate to what extent different stock dispersion methods affect ecotoxicological and mammalian toxicological responses of ENP.

INTRODUCTION

The amount of information in literature on health and environmental safety of engineered nanoparticles (ENP) is steadily increasing. However, guidance is lacking on appropriate ways of performing tests with ENP in biological test systems (referring to toxicological or ecotoxicological tests using whole organisms or cells to determine potential adverse effects, here both are described as (eco)toxicity) and subsequently interpreting the results. This remains a barrier for understanding and evaluating potential adverse effects of ENP to humans and the environment. The lack of guidance is related to a limited understanding of the dynamic and complex behavior of ENP in different testing matrices making it difficult to provide appropriate scientific advice on the best testing practices (Cupi et al., 2015; Magdolenova et al, 2012; Snyder-Talkington et al, 2012).

Tests for ecotoxicity and mammalian toxicity generally require the preparation of particle dispersions where solid particles are dispersed in liquid media. Dispersions of ENP have also gained high interest for dosing in animal screening studies and for providing better control of the delivered dose as well as a faster and less expensive test procedure (Roursgaard et al., 2010). Testing dispersions of different concentrations are typically prepared by adding aliquots of a stock dispersion into a test medium. Unless the test substance is provided by the producer/supplier in the form of a stable stock dispersion, a stock dispersion will have to be prepared by dispersing the dry powder into a suitable dispersion medium. It has previously been shown that different suspension preparation methods can influence toxicity outcomes in ecotoxicity tests (Handy et al., 2008; Jo et al., 2012) and human toxicity tests where dispersion status plays a critical role for example in the fibrogenicity of single-walled carbon nanotubes (SWCNT) in human lung fibroblasts (Wang et al 2010b). It should be mentioned that, even if dispersion status is successfully controlled, other factors, such as administration route and adsorption affinity of the ENP to biomolecules and cell surfaces, is likely to cause variations in toxicity test outcomes. For example, differences in

toxicokinetics were observed between dietary and intravenous exposure of ZnO ENP in rats (Choi et al., 2015) as well as between dietary and aqueous exposure in zebrafish (Skjolding et al., 2014). Also, adsorption of ENP onto cells and biomolecules was found to be variable depending on ENP type (Hartmann et al., 2014; Lee et al., 2015).

Many ENP powders are not easily dispersed in aqueous media and their tendency to form agglomerates is a complicating factor which must be tackled in the preparation of stable stock dispersions. Dispersibility may be improved by adjustment of pH and/or ionic strengths or addition of solvents or dispersants combined with a de-agglomeration energy using various procedures for ultrasonication, stirring, or shaking (OECD, 2012; Jensen et al., 2014; Cupi, 2015). Once nanoparticle (NP) dispersion has been prepared its stability depends, among other things, on parameters related to the type of dispersion achieved, such as electrostatic, steric, polymeric or electrosteric, and the suspended particle concentration (Jensen et al., 2014).

Farré et al. (2009) and Godymchuk et al. (2011) provided an overview of some of the advantages and limitations of different dispersion preparation methods including mechanical, ultrasonication and chemical processes such as stirring or addition of dispersants. Based upon the evidence it is apparent that the choice of dispersion preparation method is often a trade-off between dispersion stability and risk of influencing test outcomes by introduction of toxic additives or changes to particle characteristics inducing significant or unknown changes in reactivity, solubility, and toxicity (Handy et al. 2008b; 2012a). . These possible testing artefacts may in part be responsible for the diverse results for biological effects. A well-known example is the false-positive inflammatory effects of fullerenes in the brain of juvenile sea-bass (Oberdörster, 2004). Effects were since attributed to γ-butyrolactone, a highly toxic oxidation product of tetrahydrofurene (TFH) that was used as a dispersant in the preparation of fullerene dispersions (Henry et al. 2007). More

recently Menard et al. (2011) reviewed the literature on in vivo ecotoxicity of titanium dioxide (TiO_2) ENP and observed large variations which could not be clearly attributed to differences in test species or particle characteristics. It may be speculated that dispersion preparation methods might also play a role in this observed scatter in ecotoxicological effects of TiO_2 (Hartmann, 2011). Thus, appropriate ways of bringing ENPs into dispersion is a critical and intensely debated topic in the scientific literature, research projects and international organizations such as the Organization for Economic Co-operation and Development (OECD), National Institute of Standards and Technology (NIST) and International Organization for Standardization (ISO).

Identification and further development of appropriate dispersion protocols is important both from a scientific and a regulatory point of view. Stock dispersion methods should ideally increase both accuracy and precision when adverse effects of ENP are tested by 1) minimizing artefacts produced by undesirable modifications of the ENP, 2) facilitating a link between observed effects and the physico-chemical properties of pristine ENP and 3) producing sufficiently stable and homogenous stock dispersions that enable precise and representative sampling when diluted into test dispersions also referred to as working dispersions. Harmonization of dispersion protocols signifies consistency between dispersion procedures and serves to minimize variations between testing systems, labs and nanomaterials. Finally, adherence to a validated technical guidance document for dispersion protocols infers that quality and validity criteria can be established with regard to stock dispersion stability and state of dispersion.

Several protocols for preparation of dispersions of ENP for toxicity testing have already been proposed through research projects such as ENPRA (Jacobsen, 2010) PROSPEcT (PROSPEcT, 2010), NANOGENOTOX (Jensen et al, 2011a), and NANOMMUNE (Nanommune, 2011) as well as organizations and institutes for guidance and standardized methods, such as NIST and the Center for the Environmental Implications of NanoTechnology (CEINT) (Taurozzi et al 2012a; 2012b;

2012c; 2012d; 2013). Current protocols, as listed above and in Table 1, represent a development and refinement in dispersion methods over the last years as knowledge on ENP behavior and transformations during the different steps of dispersion has emerged. Hence, increasing focus is being placed on appropriate ways of dispersion ENP without altering the particle properties or creating test artefacts. However, the current protocols are limited in scope by being focused on specific test types such as *in vitro* testing and/or certain ENP, and may have been optimized according to specific criteria including dispersion stability and/or particle size distribution. This limitation in scope may in some cases limit the direct applicability of existing protocols to other test systems and their applicability to other ENP types. At the same time different labs may have different 'traditions' for using specific dispersion media and procedures, which are likely to be adapted to the specific purpose of the study and availability of equipment combined with past practice. Labs may wish to adhere to already applied procedures for the sake of internal comparability. However, this may limit inter-lab comparisons.

From a regulatory point of view, harmonization and standardization of protocols and methods applied in (eco)toxicity testing is needed to ensure repeatability and reproducibility as well as a comparable high quality of data, resulting in data upon which classification, labelling, and hazard assessments can be based. The OECD plays a key role in the international harmonization of regulatory guidelines for testing of chemicals (OECD, 2013). The agreement on mutual acceptance of data (MAD), aimed at reducing testing efforts (OECD, 1981), is based on such harmonization. Within the OECD Working Party on Manufactured Nanomaterials (WPMN) work is ongoing on issues related to testing of nanomaterials, with one output being a "Guidance on Sample Preparation and Dosimetry for the Safety Testing of Manufactured Nanomaterials" (GSPD) (OECD, 2012). This guidance has a generic nature and outlines considerations relevant to physicochemical characterization and biological tests based on available scientific knowledge. The lack of specific

guidance is explained by the fact that "…best methods for sample preparation, dosimetry, and safety testing do not yet have full consensus within the field…"(OECD, 2012). An OECD WPMN expert meeting on ecotoxicology and environmental fate was held in Berlin in January 2013 to discuss the applicability and further development of OECD test guidelines and guidance documents for nanomaterials. One recommendation from the meeting was to amend the OECD GSPD to include more detailed information on stock dispersion preparation (Kühnel & Nickel 2014). For all guidance update and development, the current challenge is to integrate the state-of-the art scientific knowledge on dispersion techniques with the regulatory requirements for a harmonized approach.

The initial stock dispersion preparation is identified as a fundamental step in obtaining meaningful results in subsequent (eco)toxicity testing. Therefore, the present study aimed to contribute to development of appropriate protocols for preparation of aqueous stock dispersions of ENP powders. This was achieved through a critical review of 5 available dispersion protocols from NANOGENOTOX, PROSPEcT, NANOMMUNE, ENPRA and NIST/CEINT. Basis upon our observations key steps in the in dispersion procedures were identified. Combined with a review of published scientific papers this provided background knowledge in a subsequent discussion on how these parameters influence the resulting (eco)toxicity and dispersion characteristics. Special emphasis was placed on sonication procedures and parameters as these were identified as a key step in the dispersion preparation. This investigation was undertaken to identify research and documentation needs and potential areas of harmonization for the preparation of stable aqueous stock dispersions from powders of ENP. The provided information is intended to minimize the risk that the preparation methods produce undesirable modifications of ENP by inducing testing artefacts. The subsequent dilution and characterization in testing media is addressed when relevant, but a detailed description hereof is outside the main scope of this review (for additional information on this the reader is referred to Jensen et al. 2014; Seitz et al. 2013; Handy et al. 2012a; 2012b).

With the proposal of a tailored dispersion protocol this investigation is intended as a starting point for development of a guidance document on stock dispersion protocols for (eco)toxicological testing assisting the production of reliable and reproducible (eco)toxicity data for MAD purposes under the Globally Harmonized System of Classification and Labelling of Chemicals (GHS) (in Europe this corresponds to the REACH (EC, 2006) and CLP (EC, 2008) regulations, respectively).

STOCK DISPERSION PREPARATION – REVIEW OF KEY PARAMETERS

Case study protocols: overview, commonalities and differences

For the purpose of this review 5 specific dispersion protocols were selected as case studies. An overview of these is provided in Table 1. Although the protocols vary greatly in some parameter values, the parameters listed are comparable. Based on information in the protocols, the following parameters were identified as key considerations in a stock dispersion preparation protocol:

I) ENP properties

II) ENP stock concentrations

III) Volume of dispersion medium

IV) Dispersion media / water quality

V) Stabilizing / dispersing agents

VI) Pre-wetting of ENP powders

VII) Dispersion procedure (mechanical and ultrasonication)

VIII) Temperature control

IX) Maintaining stability prior to dosing

X) Performance or quality assurance

All of these parameters are described for the individual protocols in Table 1 below and frame the discussions and recommendations in the remaining part of this review. Additional critical

parameters, not listed in Table 1, include the type of vials, immersion depth of the sonifier in probe dispersion protocols and position of vials in the case of ultrasound bath sonicators. Moreover, the importance of considering the *sequence* of dispersion preparation steps was pointed out by Byrne et al. (2010).

Considerations related to nanomaterial properties

An initial step in any dispersion preparation is a consideration of the specific properties of the ENP to be dispersed. Basic information on the physicochemical properties needs to be obtained by compiling existing data from the producers' technical data sheets and verifying and supplementing this information by additional physicochemical characterization as relevant. The required information includes information on particle composition, chemical surface properties, water solubility and hydrophobicity. Finally, morphological characterization of the aggregate size (-distribution) and types of agglomerates in the ENP powder is valuable for setting expectations for hydrodynamic size-distributions in dispersion. The dispersibility of ENP depends on the mechanisms underlying agglomeration in the ENP powder, which often occurs due to electrostatic forces. Other agglomeration mechanisms include physical interlock, electric, magnetic, and soft bridging (Schneider & Jensen 2009; Jensen et al., 2014). ENP agglomerated due to specific subcategories such as entanglement, bridging due to organic coatings and stickiness, and ferromagnetic properties may be particularly difficult to disperse as compared to the typically considered van der Waals forces. In such cases, acceptance of larger agglomerates in the dispersion may be necessary as separation into single particles or primary aggregates may not be feasible and agglomeration is inevitable.

The protocols reviewed in the present study have been documented mainly for metal oxides (cerium oxide (CeO_2), zinc oxide (ZnO), TiO_2 and silicon dioxide (SiO_2)) although three also apply

(one with adaptations) to carbon nanotubes (CNT), one to silver (Ag) Ag ENP and one to iron (Fe) ENP. On an operational level, ENP with similar surface characteristics may in principle be dispersed using the same procedure. This means that if a dispersion method has already been applied to one hydrophilic ENP it may also be appropriate to other hydrophilic ENP. What is understood by 'similar' is largely a matter of interpretation, and is also related to the broader on-going discussions on grouping of nanomaterials which is needed for testing, risk assessment and 'safety-by-design' purposes. In this regard, it is important to note that grouping based on chemical composition like metal oxides, metals, and carbon-containing nanomaterials will not necessarily be operational for identification of appropriate dispersion protocols. For example uncoated ZnO, SiO_2 and TiO_2 ENPs typically have iso-electric points around neutral, acidic and acidic/neutral pH levels, respectively (Komulski, 2009), resulting in highly different dispersibility and stability in pure water systems, when no additional steps are taken to stabilize the dispersion. These differences may be more pronounced in pure water compared to biological media. A range of metal oxide ENP, with varying zeta potential in deionized water ranging from −29.2 mV to 57.2 mV, displayed similar very similar zeta potentials ranging from−26.6 mV to −19.8 mV in cell medium containing 5% fetal bovine serum (Lee et al., 2015). Hence, depending on the stock dispersion media composition, differences in zeta potential and stability between different ENP may even out.

Concentrations of ENP in stock dispersions

The required ENP concentration in stock dispersions is likely to vary with the intended use. Ecotoxicologists add aliquots of stock dispersion into synthetic aquatic environmental media, whereas toxicologists prepare test dispersions cell media or perform *in vivo* studies using lung instillation, ingestion, and intravenous injection. These different exposure methods may require different minimum concentrations in the stock dispersion. Conversely an upper limit for stock

concentrations may be determined by concentrations at which extensive agglomeration occurs for specific ENP and medium. In the evaluated dispersion protocols presented in Table 1 the stock dispersion concentrations varied from 0.015 to 20 g/L. The NIST protocol prescribed concentrations from 0.5 – 20 g/L (Taurozzi et al, 2012c) with subsequent dilution to achieve test dispersions of 0.1 g/L in phosphate buffered saline solution (PBS) and Dulbecco's Modified Eagle Medium containing 10 % fetal bovine serum (DMEM-FBS) (Taurozzi et al, 2012d) or synthetic environmental medium (Taurozzi et al, 2013). The NANOGENOTOX and ENPRA protocols prescribe a stock concentration of 2.56 g/L, whereas the concentrations in the NANOMMUNE and PROSPEcT protocols are particle-dependent and vary from 0.015 g/L (CeO_2, PROSPEcT) and 0.25 g/L (CNT in the NANOMMUNE protocol) to 1 g/L (general concentration in the NANOMMUNE protocol) and 2.56 g/L (for coated ZnO, in the PROSPEcT protocol). The 2.56 g/L in the ENPRA protocol, and since adopted in the NANOGENOTOX and PROSPEcT protocols, were selected based on (1) dose requirements from the toxicologists and (2) to obtain a simple dilution scheme for *in vitro* toxicological dosing without diluting the test medium significantly while at the same time enabling direct instilling or injection of sufficiently low volumes in test animals.

Increasing the material concentrations in dispersions enhances the likelihood of collision, agglomeration and aggregation. Agglomerates are clusters of weakly bound particles, which may be separated again, whereas aggregates consist of strongly bonded or fused primary particles (ISO, 2008). Hence, if aggregation (irreversible process) occurs in the stock dispersions no subsequent steps in the dispersion preparation procedure are successful in separating the aggregates and this ultimately results in testing of large particle aggregates rather than smaller (primary) particles.

Since the critical maximum concentration that induces agglomeration depends on the ENP properties such as whether they are charged/uncharged, hydrophilic, hydrophobic, magnetic, conductive, soluble/insoluble, initially aggregated or agglomerated and the type of stabilization

such as electrostatic, steric or electrosteric, it is necessary to consider the critical concentrations for agglomeration with respect to the specific ENP to be dispersed. In a generic dispersion protocol the fixed ENP concentration may exceed the particle saturation level for some ENP, and therefore represents a trade-off when using generic dispersion protocols with fixed conditions. To counteract concentration-induced agglomeration one can either try to adjust the preparation techniques and ratios between ENP and dispersants as illustrated by modifications of the NANOGENOTOX dispersion protocol (Guiot and Spalla, 2012). The use of dispersants will be discussed further later.

The relationship between concentration and aggregation in stock dispersions was investigated for TiO_2 (NM-105) by Tantra et al. (2014). This study investigated the influence on the final TiO_2 dispersion properties as a result of systematic changes in different steps within one dispersion protocol (e.g. dispersion ageing, sonication time (20 sec – 15 min), sonication power (in W), pulsed operation mode, amplitude, sonication in the presence/absence of an ice bath, material subsampling, particle concentration). It was concluded that TiO_2 particle concentration in the stock dispersion was the most influencing factor for dispersion properties. Six concentrations were tested, ranging from 0.015 to 2.6 g/l and a concentration-dependent shift in particle size distributions was observed with higher particle concentrations resulting in a greater degree of aggregation/agglomeration. Studies by Hartmann et al. (2012) and Ji et al. (2010) found that dispersions of TiO_2 (P25 Evonik) ENP in ultrapure water prepared by ultrasonication were stable for minimum of 6 and 24 hr respectively at particle concentrations of 2-100 mg/L as evaluated by visual inspections, UV-Vis, DLS, and zeta potential. Previously Tantra et al (2010) noted a relationship between particle concentration, zeta potential and stability of ENP systems for multi-walled carbon nanotubes (MWCNT), gold and silica ENP. The samples used were commercially bought (highly stable) colloidal suspensions; the dispersion medium of the gold nanoparticles was not specified by the supplier (Tantra et al., 2010), whereas the other three were suspended in deionized water based

media. The study reported that there is a distinctive region (referred to as the 'stable region') in the plots of zeta-potential versus particle concentration, in which the zeta-potential value is independent of nanoparticle concentration. Results from the study showed that all samples were highly stable, as indicated by their large negative zeta-potential values, with average mean ranging from – 43 to - 56 mV. The average standard deviation of measurement within the stable region was reported to be within +/-4 mV; it was only at extreme dilutions (referred to as the 'unstable region) that the mean value of the zeta-potential changed. The 'stable region' for hydrodynamic diameter and zeta potential with a lower limit for particle concentration between 10^{-4} and 10^{-2} wt% depending on particle type (corresponding to approximately 0.1-10 g/L). Clearly, it was not the intent of such studies to give specific recommendations on particle concentrations, as case-specific interplay between concentrations, ionic strength, stabilizing agents, pH, has to be taken into account. However, such studies are indicative of how easy it is to vary dispersion quality as a result of small changed made to the protocol e.g. particle concentration.

The resulting hydrodynamic size in the stock dispersions and in test media is not only relevant for having control of the initial exposure characteristics, but also for potential mechanisms and observed biological effects (Jensen et al., 2014). In ecotoxicological tests greater toxicity was observed for Ag and Au ENP when they were less agglomerated in a diluted exposure medium for *D. magna* (Römer et al., 2013) and zebra fish embryos (Truong et al., 2012). As lower ENP concentrations and lower ionic strength of the media is likely to causes less agglomeration it is therefore plausible that a higher relative toxicity may be observed at lower particle concentrations in low ionic strength media (Baun et al., 2008; Römer et al., 2013; Cupi, 2015). The agglomeration and aggregation state in the stock dispersion is therefore crucial for subsequent exposure and effects in (eco) toxicological testing. Data suggest that sufficiently stable stock dispersions often can only be prepared at concentrations in the mg/L range if made in de-ionized water. Assuming that fully

dispersed ENP powders are a worst case scenario for hazard testing, the stock concentrations need to ideally be as low as possible to avoid/minimize particle agglomeration and as close to the highest tested concentration as possible without producing artefacts due to dilution of test media. However, 'stable' non-agglomerating particle concentration regions need to be established on a case-by-case basis. If dispersions are prepared using stabilizing agents and/or pH/ion-strength optimization, then sufficiently stable dispersions may be made for notably higher concentrations. Achieving a good dispersion at the highest possible concentrations would naturally be facilitated by tailoring each medium and preparation technique for the specific ENP. However, such an approach does not result in harmonization. Although pure water could often be the immediate preferred choice of medium, it may not enable the required doses for neither *in vitro* nor *in vivo* toxicological testing.

Pre-wetting of ENP powders

Hydrophobic ENP present a challenge in the preparation of aquatic dispersions. A 'pre-wetting' step is included in some dispersion procedures to facilitate dispersions of these ENP. The hydrophobicity of a ENP may either stem from surface chemistry such as coatings or functionalization or from its inherent atomic surface structure such as honeycomb structure of carbon nanotubes (CNT) and is generally enhanced by nano-scale surface roughness (Li et al., 2002).

A pre-wetting step (i.e. making a paste of the powder ENP by mixing it with a liquid) is prescribed in many of the reviewed dispersion protocols. Pre-wetting has the purpose to overcome the hydrophobicity of the 'native' ENP by changing its surface properties. In the PROSPEcT protocol pre-wetting with DI water is recommended for CeO_2. This procedure might be advantageous for ENP, which are hydrophilic, but appear as dense agglomerates, or form soft bridging, or have large and maybe even reactive surface areas. In these cases, increased dispersion

may possibly be reached by increasing reaction time with water before sonication. In other reviewed protocols the pre-wetting is undertaken with a 0.5 vol% ethanol solution (Table 1). Different techniques, water and/or solvents might be applied depending upon the material. The purpose in the initial step is to assist de-agglomeration of specific ENP. This process may require a hydrophilic solvent and/or a solvent with a lower surface tension compared to that of water. The surface tension of ethanol is σ=0.02 N/m as compared to that of water 0.07 N/m and ethanol is also hydrophilic. General use of ethanol would therefore in general improve the initial dispersion of both agglomerates and hydrophilic compounds. When subsequently dispersed in water the pre-wetted particles may be more easily dispersed, and ethanol can be evaporated if sufficient heating, sonication or time is applied. However, if evaporation is incomplete, the presence of ethanol in the dispersion might potentially affect subsequent experimental results by producing adverse effects on the test organisms (Caro & Cederbaum, 2004; Brown & Brown, 2012). To prevent this it is thus important to ensure proper evaporation of the solvent.

In the ENPRA protocol pre-wetting is applied as a standard procedure for ZnO ENP, independently of hydrophobicity of the tested ENP, for the sake of full comparability in comparative testing. In the more generic NANOGENOTOX dispersion protocol it is applied to all ENP to ensure full comparability across a wider set of test materials. This represents a trade-off between comparability and minimizing modifications of test material and thereby potential artefacts. It may, however, be a necessary inclusion for harmonization across different ENP.

It is still difficult to conclude whether general pre-wetting using chemicals other than water should be recommended for harmonized dispersion protocols. The critical issues are whether the chemical used for pre-wetting significantly changes relevant physicochemical ENP properties or the compound in itself or by degradation products may induce important biological side-effects. For the 0.05 vol% ethanol concentrations used in the ENPRA, NANOGENOTOX and PROSPEcT, such

effects are still not observed or reported. One could decide, as in the ENPRA procedure, that pre-wetting is only applied when necessary. In any case it is important, and already normally applied in toxicological studies, to include media control to incorporate such effects into account. This procedure, however, does not enable control over potential changes to the test material where a specific concern would be changes in chemical surface coatings.

Dispersion medium composition

Given the diversity of aquatic media that can be used for dispersion preparation, specification of the individual ingredients in the stock dispersion media and their qualities is a key for harmonization. Media composition is known to influence ENP behavior in a complex manner, for example as a result of ion composition and ionic strength (Ottofuelling et al., 2011). The requirements for a stock dispersion media include (1) simplicity for predictability of particle behavior, and (2) compatibility with biological assays. As the stock dispersion is subsequently diluted into a test media or test matrix the stock dispersion media does not need to contain the nutrients necessary for survival of the test organisms. However, the constituents of the stock media should not have a negative biological effect.

Pure water was selected as the foundation in all reviewed protocols presented in Table 1. The two main criteria are specified with regards to purity, as determined by resistivity, and bacterial contamination as indicated by the presence of endotoxins. The resistivity criteria have been defined to ensure high purity of the water as water-resistivity depends on its ion content. Higher ion content, caused by impurities, leads to a higher conductivity and hence lower resistivity. Water is considered pure when its resistivity is above 18.2 MΩ·cm at 25◦C. High purity water does usually not improve the dispersion of ENP, but is requested to avoid unpredicted/uncontrolled variations in stability and toxicological effects due to variable water chemistry.

Microbial purity is another highly important criterion in dispersion protocols. Bacterial contamination, or presence of other bio-colloids, may be a critical factor in ENP dispersion, due to the potential hetero-aggregation of particles and bacteria (Hotze et al., 2010), production of exudates, and biological degradation of organic surface coatings , which in turn affect dispersion properties. However, more importantly for toxicological testing, presence of bacteria might initiate strong toxicological responses. The reviewed protocols (Table 1) specify the absence of bacterial contamination which is indicated by the concentration of lipopolysaccharides / endotoxins as given in EU/ml , where 1 EU/ml is approximately 0.1-0.2 ng endotoxin/ml (Ryan, 2004). It should be noted that, while bacterial and other microbial contamination may be removed by filtration, endotoxins could still be present and would require additional treatment. Some protocols provide specific limit values for levels of lipopolysaccharides / endotoxins in water. Three protocols (ENPRA, NANOGENOTOX and PROSPEcT) prescribe filtration through a filter with a $\leq$0.45 μm pore size, which is considered to retain microorganisms and produce sterile water. To reduce changes in dispersion properties due to water chemistry or bacterial contamination, and to make stock dispersion compatible with a subsequent use in a variety of biological tests, it is generally recommended to use high quality, ultrapure water with a resistivity of above18.2 MΩ·cm in ENP stock dispersion protocols.

pH and medium composition

ENP dispersions can also be stabilized through increasing the electrostatic stabilization as a result of the charges from the electric double layer. These charges might either be negative or positive, depending on the pH of the medium they are found in. There also exists a pH at which these charges are neutral and this point is called the isoelectric point, or point of zero charge. It is here where the electrostatic repulsion is non-existent and that ENP are more prone to agglomeration. Therefore, pH is an important parameter in dispersion stability.

While various studies (Badawy et al., 2010; Domingos et al., 2013; Zhu et al., 2014) used the parameter of pH to stabilize suspensions in media, few studies employed pH to control dispersion of ENP stock suspensions for toxicological studies (Cupi, 2015), although similar principles apply. Stability of TiO_2 P25 ENP dispersed in high purity water water was assessed at pH 4, 6 and 8. The isoelectric point was around 5, and most stable suspensions with regards to particle sizes and zeta potential were those at pH 8, which was the furthest away from the isoelectric point (von der Krammer et al. 2010). Similar results were observed for TiO_2 P25 where the isoelectric point was around 6, when pH was adjusted to 3, 5.9, 7, 9 and 11. The smallest agglomerates occurred in pH 9 and 11 (Horst et al 2012). Cupi (2015) observed the stability of Ag, ZnO and TiO_2 ENP in high putity (MilliQ-filtered) water, Elendt M7 medium, Very soft (VS) EPA medium and Soft (S) EPA over pH values 2-12. For all three ENP, lowest sizes could be achieved in high purity water, but pH values where the suspensions were more stable depended upon the particle. Agglomeration increased with a rise in ionic strength of the media and was highest for Elendt M7 medium. For suspensions to be used for toxicological studies, Guiot and Spalla (2013) determined that measuring the isoelectric point of 4 different TiO_2 ENP is important, but in case of addition of a stabilizing agent, pH of that agent also needs to be taken into account. Addition of BSA at neutral pH might initiate rapid aggregation due to interactions of almost neutral ENP and negatively charged BSA.

Identification of the isoelectric point for the each suspension seems to be a fundamental step when preparing stock suspensions, allowing for an evaluation of particle behavior over a range of pH values and identification of most stable and physiologically relevant conditions.

Ultrasonication procedure

The dispersion of dry ENP powder in aqueous medium is most often facilitated by sonication, applying sound energy with ultrasonic frequencies to the dispersion. Other methods include magnetic stirring, vortexing, and shaking. While these are less aggressive methods, they are also less efficient in dispersing ENP. A variety of sonicator types are available, which differ in efficiencies and in the manner in which energy is delivered to the sample. Some of the most common types are probe sonicators (direct), bath sonicators (indirect), and less commonly used cup horn sonicator. Some advantages and disadvantages of different sonication apparatus are summarized in Table 2. Sample contamination may occur when probe sonicating different samples consecutively or from trace metal release due to erosion of the probe tip (usually made from titanium). Using a silica (glass) probe tip (sonotrode) might reduce this risk. This, however, requires a reduced amplitude (IMLAB, 2013) and results in a reduction of delivered effective energy and sonication efficiency. It is also noteworthy that dispersion protocols specify careful cleaning of the probe-sonicator between samples and that the potential cross-contamination is low when the stock dispersion are present in g/L concentrations. A cross-contamination of 10 µg into 2.56 mg/ml in 6 ml would result in less than 0.065 wt% contamination, which usually would be further diluted in the experiment. Bath sonication, on the other hand, has a major drawback from a harmonization point of view, as it is practically impossible to accurately control the effective energy delivered to the sample as it is not delivered directly into the sonicated sample. The effective energy delivered to the sample is lower for bath sonicators compared to probe (and cup horn) sonicators, making them less efficient from a dispersibility point of view (Jiang et al. 2009; Franklin et al. 2007; Caneba et al. 2010; Mejia et al. 2012).

In a sonication device the input power, i.e. the electrical energy consumed by the device, is converted to high frequency energy pulses usually given in Hz, number of pulses per sec, which is then transformed into mechanical vibrations as in a probe with a certain amplitude equal to the

distance of probe movement resulting in the formation of microscopic waves. This process results in an output of acoustic power (W) with an certain intensity (W/cm^2) per ultrasonic source surface unit such as probe tip surface area (Capelo-Martínez, 2008). This can again be described as a local energy density (W·sec/ml), defined as "the amount of delivered energy per unit of suspension volume", meaning that at equal particle concentration and power, "higher energy densities (i.e., lower dispersion volumes) will result in a greater disruptive effect" (Taurozzi et al, 2012b). As indicated by Taurozzi et al. (2011) the efficiency of the energy transformation from the electrical input power to the acoustic power effectively received by the sonicated dispersion depend predominantly on the specific sonication device. Therefore, simply reporting the displayed input power and sonicator settings does not accurately reflect the actual energy delivered to the sample and hence accounting for lack of reproducible results (Taurozzi el al., 2011). However, as illustrated by the case studies presented in Table 1, sonication power, settings and probe dimensions are the parameters normally specified in the dispersion protocols considering that similar types of probe sonicator would be used. In reality many different types of sonicators are available in different probe designs, powers, frequencies, and range in amplitudes. The challenge is to develop a calibration method that is widely applicable. In preparation of the final NANOGENOTOX protocol, however, the consumed energy at the fixture was used for calibrating the delivered energy of different types of sonicators. The resulting average particle size was found to decrease as a power function of sonication time for all applied sonication instruments, resulting in comparable particle sizes at the same delivered energy (Jensen et al., 2011b).

Calibration of different probe sonicators is a key issue. Some probe sonicators read out the delivered energy dose given to the sample, but this feature is not available in all sonicators and data may not be directly comparable. Different methods exist to calculate the effective energy delivered to the sample including calorimetric methods based on temperature increase (Taurozzi et al., 2012b)

and methods based on measured power consumption during working and no-load operation such as sonication of dispersion and air, respectively (Bihari et al., 2008). In the ENPRA project harmonization was attempted by using the same brand and make of sonicator. In the NANOGENOTOX project different sonicators were used and calibrations were performed to determine the amplitude and durations based on the consumed energy measured using a Watt-meter at the wall-fixture combined with performance testing on an internal common material and benchmark data on hydrodynamic size-distributions on all test materials (Jensen et al. 2011c). Other and more specific methods for harmonization include measurement of the amount iodide to iodine (E_0 = -0.615V) conversion by oxidation according to the Weissler reaction during sonication. However, this procedure also has some quantitative limitations due to sensitivity to temperature and some reactivity without hydrodynamic cavitations (Morison and Hutchinson 2009), which need to be understood with respect to specific use.

Reproducibility in preparing ENP dispersions by sonication requires consistency at least in effective energy delivered to the sample[1], the vials used, temperature, medium viscosity, particle concentration and sample/vessel volume. For probe sonication also the shape and diameter of the probe, as well as probe immersion depth is important. Smaller sample vessel diameters and an immersion depth of 2-5 cm was recommended for standard probes (Taurozzi et al., 2011). Based upon existing data, it appears that comparable and reproducible results can be made using probe sonication and that this is a practical, accessible and pragmatic choice for harmonization of dispersion protocols, whilst acknowledging that further optimization and guidance is needed.

To reach one or a set of harmonized dispersion protocols it is necessary to establish standard procedures for determining and calibrating the specific delivered energy and de-agglomeration efficiency of sonicators combined with a standardized reporting requirement. Development of such

[1] A sample is here defined as dispersion of nanomaterials including any additives

a method for calibration, combined with detailed reporting of sonication procedure information, would greatly improve interpretability, comparability and reproducibility. As a starting point for reporting requirements the list of sonication parameters in (Taurozzi et al., 2011; 2012a) could be consulted, where the latter also includes more general reporting requirements for preparation of ENP dispersions.

During sonication the reactive species produced during cavitation and heating of the sample may directly produce modifications and degradation of ENP, dispersants, coatings and/or media components. Heating would have additional importance in evaporation if high energies or long sonication times are used. A cooling coil might be mounted in the bath sonicators water bath. For probe sonication cooling might be done by placing the sample in an ice-water bath (or ice-salt bath) (Table 1) which the transfer of heat (cooling of the sample) might be optimized by increasing the vessel wall-surface-to-volume ratio. In addition, pulsed mode sonication, in which the sonicator operates at alternated on/off intervals, might aid in minimizing heating of the sample and thus improve temperature control (Taurozzi et al, 2012b). However, this mode is not available in all sonicators, which has been a limiting factor in development of the procedures discussed here.

If the ENP dispersion is to be used in biological testing then knowledge of possible influences of sonication procedures on toxicity is of vital importance for interpretation of experimental results. As indicated by Taurozzi et al. (2011) sonication leads to formation of reactive species ('sonic activation') during cavitation. An increased toxicity of ENP dispersions after sonication may theoretically be explained by formation of radical species such as thermal dissociation of water into •OH radicals and •H atoms (Riesz and Kondo, 1992) and subsequent recombination into hydrogen peroxide (Brown and Goodman, 1965), which may interact with ENP and change its surface chemistry (Taurozzi et al. 2011). Oxidation is practically inevitable during sonication and may produce some limitations in testing acute effects of materials with elements in reduced state.

Further, sonication may generate the formation of toxic degradation products of dispersants or other media constituents (Wang et al., 2012), change surface coating chemistry (Taurozzi et al., 2011), enhanced release of metal ions or increased toxicity due to smaller particle sizes (Cronholm et al., 2011). A few studies compared the biological effects of ENP dispersions when prepared by use of stirring, bath sonication and probe sonication (Table S1 in SI). The general trend is an enhanced toxicity of bath and probe sonicated ENP dispersions compared to non-sonicated dispersions such as stirred. However, as studies vary in test organism, test material as well as sonication type and settings, such as frequency or time, it is not possible to make any direct comparison. Overall, due to known possible sonication-induced modifications to ENP and their biological reactivity it is recommended to only apply the minimum energy input required to obtain a disperse particle dispersion.

Dispersants

A challenge for accurate exposure and dosing in (eco)toxicological testing of dispersed ENP powders is related to their different agglomeration and aggregation (or 'bundling' in the case of CNT). In principle, the interactions between ENP in dispersion (or in general between two interfaces in a dispersed system) may be described by the DVLO theory, named after Derjaguin, Landau, Verwey, and Overbeek (Chen and Elimelech, 2007; Feiler et al., 2000). In brief, the overall interaction energy between two interfaces is the net balance of the repulsive electrostatic Coulomb (double layer interaction) forces and the attractive van der Waals forces. The overall force determines whether ENP in aqueous media form stable dispersions or agglomerates (Salager, 1994). In order for CNT bundling to occur strong van der Waals interactions are required along the tube hampering their dispersibility (Edri and Regev, 2008). In order to enhance the dispersibility and stability of ENP in aqueous media various dispersants might be added to overcome the attractive

forces through increased steric repulsion. A division may be made between natural dispersants including proteins and humic acids and synthetic dispersants such as poloxamers or other non-ionic surfactants (Handy et al., 2012a). When adding dispersants, modifications of biological response(s) is a general concern resulting from inherent toxicity or antioxidant properties of the surfactant as well as hampering the direct interactions between the ENP and biological surfaces. Wang et al. (2010a) noted a dispersant should not induce toxic effects in itself and at the same time neither mask nor enhance the biological activity of the ENP. Biological relevancy and testing regime are also important factors to consider. It is of interest that Thomas et al. (2011) reported that serum protein and natural organic matter (NOM) have been suggested as the most promising choice of dispersants for human and environmental toxicity studies, respectively. This has already been implemented in the CEINT / NIST dispersion protocol (Table 1).

Serum proteins

In *in vitro* and *in vivo* toxicity testing, biological dispersants are often highly favored over synthetic chemicals. Certain serum proteins, such as bovine serum albumin (BSA), were found to efficiently aid dispersion of some ENP in toxicity testing media. Kim et al. (2011) examined CNT dispersion efficiency and biocompatibility as evaluated by toxicity testing using trypan blue dye exclusion, lactate dehydrogenase (LDH) leakage, and neutral red assays using following dispersants: 0.5% BSA, dimethylsulfoxide (DMSO), 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) and 1% Tween 80. All 4 dispersants were found to be biocompatible in the sense of not inducing cytotoxic effects. Their efficiency as dispersants was observed to depend on the type of CNT, i.e. SWCNT or MWCNT. The stability of the dispersions was monitored over 16 weeks and the following descending order from more stable to less stable was established: BSA > Tween 80 > DPPC > DMSO for MWCNT and BSA > DPPC > Tween 80 > DMSO for SWNCT (Kim et al., 2011). Although this indicates that dispersants may have to be chosen based on the material to be

dispersed, data also indicate that BSA seems to be an efficient dispersant for CNT. BSA also efficiently improved the dispersibility of TiO_2 ENP in different cell culture media, for which phosphate concentration was determined to be a key factors governing variations in ENP dispersion between different media (Ji et al., 2010). Conformation of the BSA protein, which is pH dependent, was found to influence CNT dispersion efficiency (Edri and Regev, 2008). To enable dispersion of TiO_2, a required ratio between serum albumin and ENP concentrations was determined by Bihari et al. (2008): TiO_2 in a concentration < 0.2 mg/ml might be stabilized by addition of 1.5 mg/ml serum albumin from human, mouse or bovine, corresponding to approximately 0.15 vol% depending on serum density. The method was found to be applicable resulting in dispersions with agglomerate average diameter < 290 nm for the following ENP: TiO_2 (rutile), ZnO, Ag, SiO_2, SWNT, MWNT, and diesel SRM2975 particulate matter. In comparison, 2 vol% serum is prescribed for the PROSPEcT and ENPRA protocols for dispersing ENP at a concentration of 2.56 mg/ml, whereas only 0.05 wt% sterile-filtered BSA-water is recommended for the same particle concentration in the NANOGENOTOX protocol (Table 1). In both ENPRA and NANOGENOTOX dispersion protocols, the amounts required were based on titration to identify the common best dispersant concentration for TiO_2 (NM-101) and MWCNT (NM-400) and subsequently documented for other ENP to be used in these projects. In addition to BSA other types of serum proteins used for dispersing ENP have been investigated or used, including human serum albumin, mouse serum albumin, mouse serum (Bihari et al., 2008), fetal bovine serum (Ji et al., 2010) and Survanta® natural lung surfactant (Wang et al., 2010a), 10% BAL (bronchoalveolar lavage) fluid from sibling mice mixed into MilliQ-filtered high purity water with 0.9 % NaCl (Jacobsen et al., 2009).

Natural organic matter

Natural organic matter (NOM) is a complex matrix of organic materials that plays an important role in the aquatic environment (Sillanpää, 2015). The composition of NOM varies depending on

environmental factors and biological processes but generally contain a large fraction of hydrophobic acids such as humic acids (HA) and fulvic acids (FA). HA and FA differ in water solubility, molecular weight, functional group distribution and elemental composition (Sillanpää, 2015). These substances are large molecules containing hydrophilic and hydrophobic parts including aromatic rings and functional groups such as carboxyls and hydroxyls (Kördel et al. 1997). These structures give NOM a good complexation capacity where various pollutants/metals may bind. Therefore, NOM play a major role in mobility of contaminants through the process of adsorption, aggregation and sedimentation (McCarthy and McKay, 2004), and in bioavailability of metals (Buffle and van Leeuwen, 1992). Due to this property, NOM were shown to act as a stabilizing agent for colloids under certain conditions (Tiller & O'Melia 1993; Wilkinson et al. 1997), and during the last decade, various forms of NOM were also employed to stabilize and control dispersions of ENP. The most commonly used forms of NOM in ecotoxicological research of NP include commercial and isolated HA and FA, and NOM containing different amounts of HA and FA such as the Suwannee River natural organic matter (SR-NOM). NOM addition to ENP dispersion before sonication has been the common practice in most studies.

NOM has been widely used to stabilize a variety of ENP in test medium (Akaighe et al., 2012; Baalousha et al., 2013; Domingos et al., 2013) but only a few studies have used it to stabilize stock suspensions (Cupi, 2015). Manier et al. (2011) investigated the effect of HA on the stability of CeO_2 ENP in stock dispersions prepared in high purity water and different synthetic freshwater media. NOM has been used to stabilize stock suspensions of metal (Ag) and metal oxide (ZnO, TiO_2) ENP (Cupi et al., 2015). Coating of the ENP with NOM leads to steric and electrostatic repulsion as well as formation of a negative surface charge on the NP, which assist in stabilizing NP dispersions. However, at high NOM concentrations bridging might occur which result in

sedimentation. HA adsorption to metal oxide ENP has mostly been attributed to electrostatic interaction and ligand exchange (Yang et al. 2009). In test media, SR-NOM and SR-HA (15 mg/L) (Akaighe et al. 2012), and SR-FA (5 mg/L) (Baalousha et al. 2013) were found to produce stabilization of Ag NP at low ionic strength media. Despite the stabilization effect, an important parameter to keep in mind is the implication of NOM presence in the biological effects of the ENP. Presence of NOM reduced the toxicity of Ag NP towards Japanese medaka embryos in dispersion most likely due to coating of NP and decreasing the release of ions (Kim et al. 2013) , and/or complexation with Ag^{+} present in the solution (Kim et al. 2013; Gao et al. 2012a). Increasing amounts of NOM were also associated with reduced amounts of Zn^{2+} released from ZnO ENP (Li et al. 2013). Previous studies revealed that agglomeration on ZnO ENP was highly dependent on NOM concentration (Zhou and Keller 2010, Domingos et al. 2013). SR-NOM concentrations of 20 mg/L were able to decrease agglomeration in ZnO nanoparticles (Domingos et al. 2013). Zhou and Keller (2010) noted that SR-NOM in concentrations >10 mg/L increased the stability of the test system. Humic substances such as SR-HA at 0.2-5 mg/L (Domingos et al. 2009) or 10 mg/L (Thio et al 2011) were also shown to stabilize TiO_2 ENP dispersions. Four mg/L SR-NOM was found to reduce aggregation of TiO_2 in dispersion (Zhang et al. 2009). Similar to test suspensions NOM might also exert an influence on stability of stock suspensions. The affinity of NOM to different ENP depends on chemical composition, chemical structure and present capping agents. Stabilization depends upon type of NOM and concentration; therefore, use of NOM for stabilizing dispersions of different ENP needs to be performed in a case-by-case basis.

Poloxamers

Polaxamers is a group of non-ionic polymers which are widely used as ENP dispersants (Wang et al., 2012). In the NANOMMUNE stock dispersion protocol 160 ppm Pluronic F126 is added to facilitate dispersion of CNT but not for other ENP (Table 1). The implications of poloxamer

degradation during sonication and subsequent consequences of potential degradation products for biological effects was examined by Wang et al. (2012). This study focused on dispersions of MWCNT using two representative Pluronic surfactants (F-68 and F-127), which are commonly used in dispersing carbon-based ENP. As an alternative to the Pluronic surfactants, the study compared results to dispersions prepared with BSA as dispersant. It was found that the dispersions prepared with Pluronic surfactants became highly cytotoxic after probe as well as bath sonication both in the presence and absence of MWCNT, and depended on sonication time, power, and frequency. This was not the case when BSA was used in dispersing the MWCNT (Wang et al., 2012). Evidence indicates that if poloxamer dispersants are used they need to be added after the sonication step to avoid potential production of toxic degradation products; however, the use of alternative to poloxamers dispersants needs to be considered when possible.

Other dispersants

In addition to the already mentioned dispersants other materials have also been added to increase the dispersibility of ENP in aqueous media. Gao et al. (2012b) investigated dispersion methods for carbon-based ENP for ecotoxicity testing. The toxicity of a number of surfactants and tetrahydrofuran (THF) were assessed for their ecotoxicity to green algae *P. subcapitata* and crustacean *C. dubia*. The surfactants include polyvinylpyrrolidone (PVP), gum arabic (GA), sodium dodecylbenzenesulfonate (SDBS), sodium dodecyl sulfate (SDS), sodium cholate (Na-cholate), Triton X-15 and Triton X-100. Based on ecotoxicity studies it was found that Triton X-15, PVP, GA exerted no significant negative impacts on growth of *P. subcapitata* in concentrations up to 1,000 mg/L. Lack of adverse effects to *C. dubia* was only seen for PVP and GA. Hence from an ecotoxicological point of view PVP and GA might be proposed as appropriate surfactants for general ENP dispersion protocols. However their suitability over a range of particle types and toxicity towards different organisms requires further clarification. At the same time potential

stimulating effects of surfactants also need to be considered. GA is a complex plant exudate with antioxidant properties and was found to enhance growth of *P. subcapitata* compared to control (Gao et al., 2012b). In past studies the use of THF in ENP dispersions for ecotoxicity studies was found to be controversial (Oberdörster, 2004; Zhu et al., 2007; Henry et al., 2007). It has been established that toxic transformation products are formed from THF oxidation causing toxicity artefacts (Henry et al., 2007). Data indicate the need for appropriate controls to monitor negative as well as positive effects of dispersants on test organism responses.

TOWARDS HARMONIZED DISPERSION PROTOCOLS

In the quest of selecting or further developing stock dispersion protocols for ENP it is important to clarify the intended use and purpose of such protocols. For *in vivo* studies, it is important that ENP is well-dispersed and that the vehicle is biologically relevant for the intended exposure route and toxicological evaluations whether they be *in vivo* or *in vitro*. For *in vitro* and aquatic ecotoxicological studies, it could be argued that reaching stable stock dispersions is not essential, because stock dispersions are often diluted in complex media prior to biological testing. Here ENP behavior will anyway change dramatically and stability achieved in the stock dispersion is lost. However, it is postulated that use or further development of appropriate and harmonized stock dispersion protocols is important to:

- Minimize test artefacts: if ENP are heavily agglomerated in the stock dispersion, the agglomeration state may be 'transferred' to the test media where agglomeration may increase further. Further, inappropriate dispersion methods might induce other test artefacts by modifying ENP properties.
- Ensure quality control: by developing appropriate and transparent dispersion protocols test artefacts may be further minimized and retrospectively evaluated.

- Facilitate data comparability: comparability in initial dispersion characteristics may be attained and issues related to stock dispersion preparation is not a confounding factor when comparing results between tests. By not harmonizing stock dispersion procedures an additional level of uncertainty is added.
- Enable future quantitative structure–activity relationship (QSAR) model developments: using common dispersion protocols for testing ENP properties including physico-chemical properties, fate and (eco)toxicity might provide more coherent datasets with the possibility of making direct links between outcomes of different tests and ENP properties.
- Allowing for establishment of quality criteria: development of technical guidance for ENP dispersion infers that quality and validity criteria are established for the properties of the resulting dispersion such as dispersion stability and state of dispersion.

It needs to be recognized that reaching harmonization and standardized protocols will always be a compromise between optimum dispersion on one hand and optimum biological/ physiological and material compatibility of the medium and concentrations required in the stock dispersion on the other hand. The advantages and disadvantages of strict harmonization and full flexibility are illustrated in Figure 1. Generic protocols, such as ENPRA and NANOGENOTOX dispersionprotocols, are highly relevant for regulatory testing providing the ability to compare test results more directly, whereas tailored dispersion protocols may be more applicable to scientific research questions where the ability to compare between different test results may be of less concern.

The extent of harmonization, case-by-case adaptation and flexibility are issues that require attention when a specific dispersion protocol is selected or developed, to avoid the situation that a generically optimized protocol is applied to a specific particle type, for which it is unsuitable producing either false positive or false negative toxicological results. Hence, some mechanism is

needed within a general protocol to detect and deal with exceptions through specification of performance criteria. Therefore, there is an urgent need to critically evaluate already existing protocols for their general applicability or domain of applicability (type(s) of ENPs, all or specific bioassays).

Based on the information collected through this review, a step-wise approach for developing a tailored dispersion protocol is proposed in Figure 2. This figure highlights the information required and typical points of decision to establish a new or evaluate an existing ENP dispersion protocol. In the first step, the purpose of the experiment/test and intended assay (testing regime such as ecotoxicological or mammalian test system) needs to be clarified with information on the intended exposure route (Figure 2). In addition, performance criteria need to be considered (for example what is considered an acceptable criterion for stability). This is the basis for the choices made in the development of the dispersion protocol. While the step-wise approach shown in Figure 2 describes development of a tailored dispersion protocol for one ENP or a group of ENP, the same steps are required to be considered in a more generic dispersion protocol. However, where a tailored dispersion protocol may be driven by specific performance criteria such as whether stable and well-dispersed dispersions are obtained or no further dispersion efforts are needed, a generic protocol is driven by general performance criteria that all ENP, for which the protocol is considered applicable, will be relatively well dispersed.

As shown in Figure 2, the first steps in development of a tailored dispersion protocol involve collecting basic information on ENP including data particle composition, coating, solubility and hydrophobicity. This information enables informed choices in the subsequent steps of the dispersion protocol. A choice of suitable stock concentration is then made. Ideally this needs to be low as possible to minimize particle agglomeration while at the same time taking into account the concentration requirements in the subsequent tests, for which the dispersion is being prepared. . The

hydrophobicity of the material determines the need for pre-wetting, which is usually done with ethanol although other pre-wetting solutions may also be considered. In case of pre-wetting relevant controls need to be considered and complete evaporation of the pre-wetting agent is required to be ensured. As illustrated in Figure 2 ENP is now mixed into water for which certain quality criteria are defined in the protocol. It is recommended that high quality, ultrapure water with a resistivity of 18.2 MΩ·cm in ENP stock dispersion protocols be employed. The mixing procedure needs to be described explicitly in the protocol as it may influence the resulting dispersion. If the dispersion is not stable upon mechanical mixing, sonication needs to be applied, where probe sonication is considered the most practical, accessible and pragmatic choice from a reproducibility and harmonization point of view. To avoid heating of the sample it is required to be placed in a water bath or ice-water bath during sonication with temperature to be monitored to ensure stable conditions. Sonication time needs to be selected to minimize the energy input, reducing risk of ENP modifications, while still obtaining a disperse particle dispersion. Appropriate sonication controls need to be included to ensure that the ENP is not modified due to this procedure and that no toxic sonication products are formed. Reporting requirements are needed for a detailed description of the sonication procedure including information on specific energy delivered to the sample. If this is still not sufficient to ensure a stable dispersion, different dispersants are required to be considered. The specific choice of dispersant may depend on the testing regime and biological relevancy with serum protein and NOM as common choices for human and environmental toxicity studies, respectively. However, other alternatives exist. An ideal dispersant would aid dispersion stability over a range of particle types, be non-toxic and neither mask or enhance biological activity of the ENP. A suitable dispersant concentration needs to be specified while taking into account that higher concentrations may be counter effective on dispersibility due to bridging. As for pre-wetting

and sonication, it is important to include controls that capture possible test artefacts induced by dispersants as a result of ENP transformations or modifications.

DISCUSSION

Several dispersion protocols have already been developed and are used for specific ENP or groups of ENP and biological test systems. However, to ensure the appropriateness of a harmonized dispersion protocol several issues were identified that urgently require additional clarification. Sonication plays critical role in the dispersion preparation, but also represents a key challenge. It is not feasible to give one general recommendation on the sonication procedure that applies to all ENP. It is evident that the higher sonication energy the greater risk of partial oxidation of the ENP and degradation of organic coatings, functionalization and maybe even changes in the structure of compounds such as CNT. From a reproducibility and harmonization point of view it is proposed to use moderate probe sonication as a practical, accessible and pragmatic choice, whilst acknowledging that optimization and additional guidance is needed to optimize and harmonize the procedure between labs. Some guidance on this is offered by Taurozzi et al. (2011; 2012a), which also include suggestions for reporting procedures. However, ways of measuring and reporting the energy effectively delivered to the dispersion and control the effective de-agglomeration efficiency need to be further elaborated to make them practically and routinely applicable. Theoretically, minimizing energy input from sonication may serve to minimize artefacts in biological tests. However, no studies have been identified, which systematically investigate links between sonication procedures and biological effects of the resulting dispersion. Hence, before a final conclusion can be made on the most appropriate sonication methods – especially from a (eco)toxicity testing point of view - well designed studies are needed to investigate the influence of sonication procedures and settings, such as frequency and time, on dispersibility as well as

(eco)toxicity. This would include an examination of the cause of any sonication-dependent changes to ENP properties linked to the observed biological effects.

The addition of dispersants is a somewhat controversial issue as it may either mask or enhance the biological activity of the ENP (Cupi et al. 2015). A thorough evaluation of available dispersants is needed to better understand their influence and mode of influence on ENP biological effects, behavior during sonication, including formation of (toxic) degradation products, and during biological exposure such as potential bio-modifications. If addition of dispersants cannot be avoided, methods are needed to take into account modifications of ENP by addition of dispersants when performing biological tests.

In the suggested step-wise approach to develop tailored stock dispersion (Figure 2) the underlying principles were to minimize changes to the pristine ENP in order to reduce the risk of testing artefacts and to obtain a stable dispersion. The actual quantitative (or qualitative) criteria for characterizing a dispersion as 'stable' need to be defined on a case-by-case basis. The diagram is flexible in the sense that other aims or quality criteria could be established based on specific protocol requirements. For example criteria may be included with respect to (mono/poly)dispersibility, particle size distributions, and acceptable physiochemical changes. It could also be argued that, from a regulatory perspective, mono-dispersibility around a small average peak size needs to be included as a criterion reflecting a worst-case situation with higher probability of nano-specific effects. With this in mind the step-wise approach shown in Figure 2 is proposed as a starting point for further discussions and developments on this topic.

CONCLUSIONS

This study provides an overview of current practice in selected dispersion protocols as well as potential implications for mammalian and ecotoxicity testing. Based on an identification of critical issues and parameters to be taken into account in protocol development for stock dispersion

preparation, a step-wise approach for tailored dispersion protocols is presented describing the key protocol parameters: particle concentration, pre-wetting, dispersion media, sonication and dispersants. The developed approach provides an adaptable framework which may serve as a starting point for further work towards developments of harmonized dispersion protocol for ENP. It needs to be emphasized that appropriate controls needs to be included in all steps in the dispersion procedure that are likely to entail modifications of the material properties or surface chemistry. Through the analysis, leading to the suggested step-wise approach, a number of issues were identified that require clarification and guidance development, of which the more critical issues relate to the development of:

- Measuring and reporting schemes for effective sonication energy input
- Links between sonication procedures and biological effects
- Methods of taking into account modifications of ENP properties and effects when adding dispersants to ENP suspensions.

A common approach to preparation of stock dispersion prior to toxicity testing may improve the possibility of defining meaningful quality control and validity criteria and hence facilitate data comparability, and ultimately enable future QSAR developments by providing more coherent datasets. It is our intention that the proposed step-wise approach be used for development and reporting of future development of protocols for both scientific, regulatory and standardization activities on ENP dispersion protocols.

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Table 1. Overview of some existing protocols for dispersing ENPs in aquatic media.

Project / organisation	**CEINT / NIST (1200-3)**	**NANOGENOTOX**	**NANOIMMUNE**	**PROSPEcT**	**ENPRA**
Year of publication	2012	2011	2011	2010	2010
Testing regime	Toxicity (in vitro) and ecotoxicity (acute) testing	Toxicity testing (in vivo and in vitro)	Immunotoxicity testing (in vivo and in vitro)	Ecotoxicity (and toxicity) testing	Toxicity testing (in vivo and in vitro)
ENP(s) used in protocol development	TiO_2	SiO_2, TiO_2, MWCNT	e.g. TiO_2, ZnO, SiO_2, CNTs and various Fe ENPs	CeO_2, ZnO	TiO_2, ZnO uncoated, ZnO coated, Ag, MWCNT
Stock concentration (mg/ml)	0.5 – 20 mg/ml	2.56 mg/ml	1 mg/ml (general), 0.25 mg/ml (CNTs)	1 mg/ml diluted to 0.015 mg/ml (CeO_2), 2.56 mg/ml (coated ZnO)	2.56 mg/ml
Volume of dispersion medium	50 mL	6 mL	-	Approximately 15 mL (CeO_2) 4-6 mL (coated ZnO)	6 mL
Pre-wetting	No	Yes, generically with 0.5 vol% ethanol for all ENPs	No	Yes, with DI water for CeO_2, and with 0.5 vol% ethanol for coated ZnO	Yes, with 0.5 vol% ethanol for both coated and uncoated ZnO
Dispersion media	Type 1 biological grade (sterile) DI water	Purest water available (e.g. Nanopure and MilliQ water)	Ultrapure water	DI water (CeO_2), Highest standard DI water available and filtration through a filter ≤ 0.45 µm (coated ZnO)	0.45 µm filtered DI water or higher quality

Resistivity	18 MΩ·cm	Up to 18.2 MΩ·cm, (Nanopure Diamond UV Water), 18.2 MΩ·cm (MilliQ water)	-	~ 18 MΩ·cm	-
Pyrogens	Absence of endotoxin contamination	< 0.001 EU/ml (Nanopure Diamond UV Water), < 0.02 Endotoxin Units/ml (MilliQ water)	Lipopolysaccharides < 0.25 ng/ml	-	-
Sonication	Yes	Yes	Yes	Yes	Yes
Means of sonication	Probe sonication	Probe sonication	Probe sonication (SiO_2 and metal oxides). Ultrasonic bath for CNTs	Probe sonication	Probe sonication
Sonicator settings	50 W, pulsed operation mode (80% on / 20% off)	400 W at preferably 10% amplitude	-	130 W at 90% amplitude (CeO_2) 400W (coated ZnO)	400 W at 10% amplitude
Time	15 min	16 min (12 min for instruments that cannot go below 20% amplitude)	3x20 sec (5 sec break) (SiO_2), 2 min (metal oxides), 2x10 min w/ vortex (CNTs)	20 sec (CeO_2), 16 min (coated ZnO)	16 min
Cooling	On ice water bath	On ice water bath	On ice (SiO_2 and metal oxides)	On ice water bath (optional for CeO_2)	On ice water bath
Dispersant in stock dispersion	No	0.05 %w/v Bovine Serum . It is recommended that the	160 ppm Pluronic F126 (CNTs), not added for	For toxicity testing: 2 vol% mouse serum (in vivo) or 2%	2 vol% serum (e.g. bovine or mouse)

		albumin type is adjusted according to the species tested	other materials.	serum of choice (in vitro)	
Subsequent addition of stabilising / dispersing agents	Bovine Serum Albumin (Toxicological testing, NIST 1200-4) or Humic Acid (Environmental Testing, NIST 1200-5r1).	-	-	-	-
Recommended maximum time between stock preparation to use in test	48 hours (stability validated)	1 hour (stability validated). Can be re-dispersed by shaking	Max. 1 day. If the time between stock preparation and use in test exceeds 30 min. then the stock should be sonicated prior to dilution in test media	Stable for 1 hour but immediate use is recommended	1 hour (stability validated). Can be re-dispersed by shaking
Quality assurance	LDS/DLS/XDC and pH	DLS and qualitative support from optical microscopy	Analytical ultracentrifugation/laser diffraction/DLS, zeta potential, and measurements of dissolved ions	DLS	DLS and qualitative support from optical microscopy

Table 12. Advantages and disadvantages of common sonicator types.

Sonicator type	Relative Advantages	Relative Disadvantages
Bath (indirect)	Low risk of contamination Larger volumes can be handled Good thermal control is possible	Low rate energy output Poor control of delivered energy
Probe (horn) sonication (direct)	High rate of energy output Good control of delivered energy Good thermal control is possible	Contamination from probe (sonotrode) tip erosion Potential risk if cross-contamination Smaller volumes can be processed
Cup horn (indirect)	Less risk of contamination High rate of energy output High level of thermal control possible	Availability in general and cost Smaller volumes can be processed

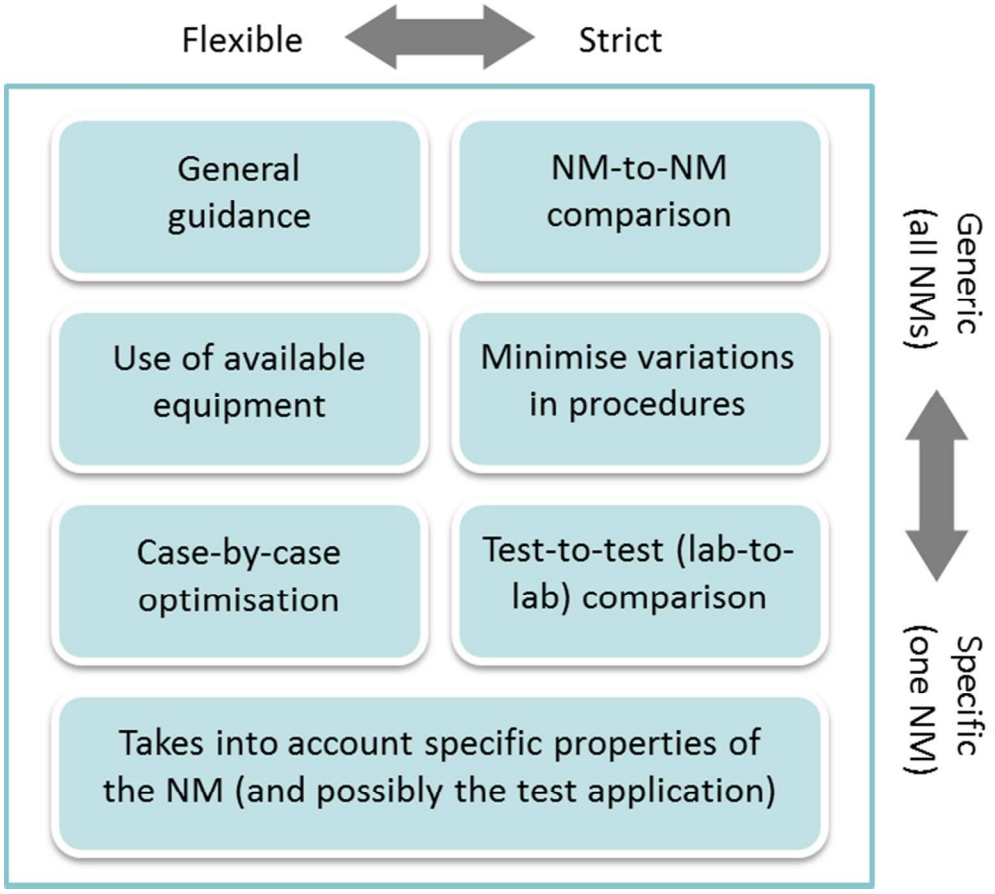
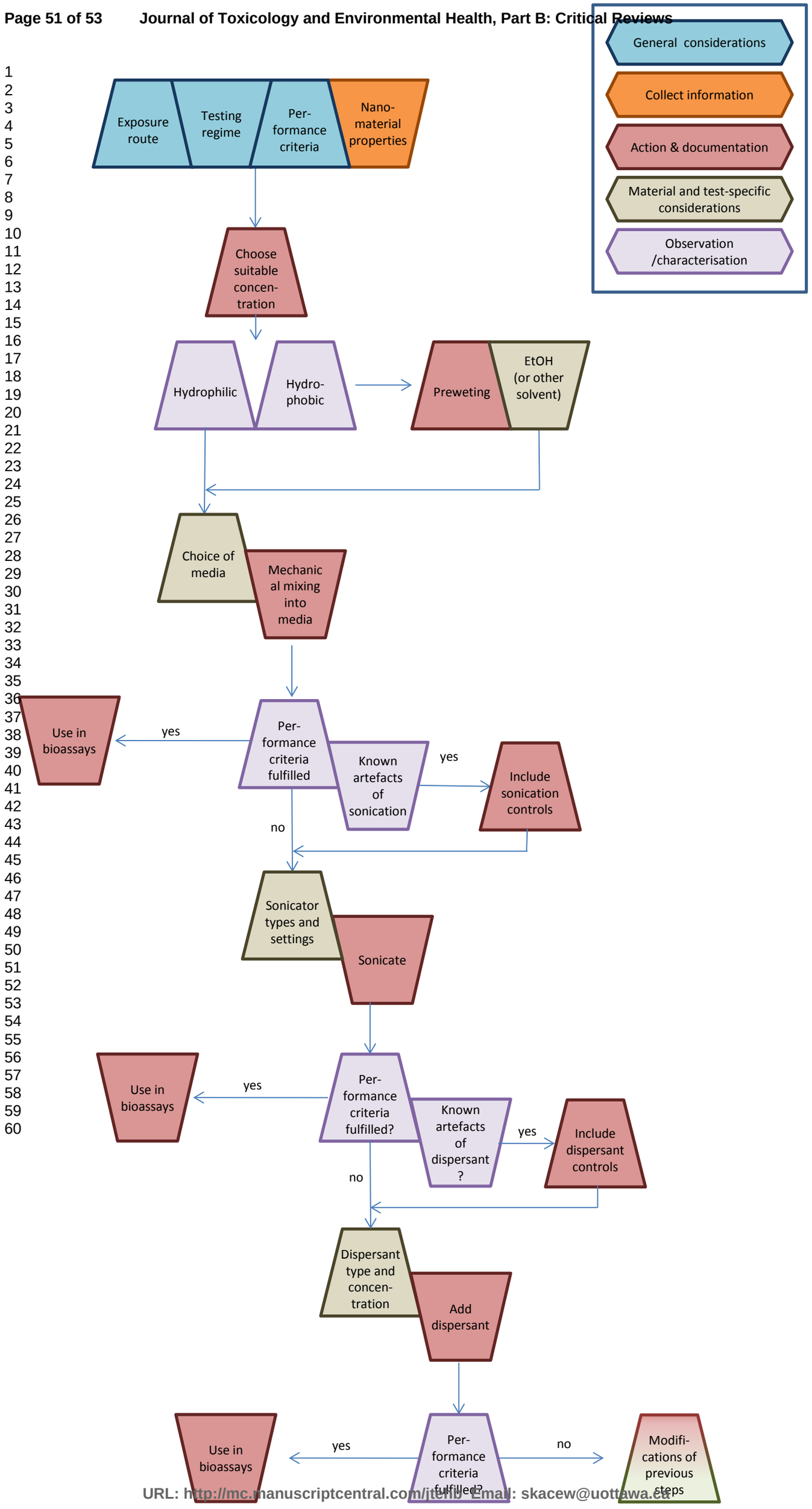


Figure 1. Illustration of different approaches to harmonisation of dispersion protocols. The protocol can either be prescriptive giving strict specifications (e.g. ENP concentrations, sonicator type, model and setting, mandatory pre-wetting step) or it can provide the overall framework but allow more flexibility and individual choices. At the same time, a protocol can be more general (with a specific range of applicability given) or it can be tailored to a specific ENP (or a group of ENPs). Harmonization and standardisation of dispersion protocols will always be a compromise between optimum dispersion for specific ENPs on one hand and compatibility between test systems and ENPs on the other hand.


General considerations
Collect information
Action & documentation
Material and test-specific considerations
Observation /characterisation
Exposure route
Testing regime
Per-formance criteria
Nano-material properties
Choose suitable concen-tration
Hydrophilic
Hydro-phobic
Preweting
EtOH (or other solvent)
Choice of media
Mechanic al mixing into media
Use in bioassays
yes
Per-formance criteria fulfilled
Known artefacts of sonication
yes
Include sonication controls
no
Sonicator types and settings
Sonicate
Use in bioassays
yes
Per-formance criteria fulfilled?
Known artefacts of dispersant ?
yes
Include dispersant controls
no
Dispersant type and concen-tration
Add dispersant
Use in bioassays
yes
Per-formance criteria fulfilled?
no
Modifi-cations of previous steps

SUPPORTING INFORMATION

Table S1. Examples of the influence of sonication on biological effects in ecotoxicity (and toxicity) testing

Nanoparticle type	Type of sonication	Type of organism / cell	Influence of sonication	Other observations	Reference
CuO	Bath (Intersonic, IS-2, 300W, 35 kHz)	*Nitellopsis obtusa* (macrophytic algae) *Chlorella* (microphytic algae) *Thamnocephalus platyurus* (shrimp) *Brachionus* calyciflorus (rotifer)	No substantial differences in toxicity between sonicated and non-sonicated dispersions. However sonicated dispersion was more toxic than non-sonicated dispersion in the case of *N. obtusa*, and the opposite situation was found for *B. calyciflorus*)	Re-agglomeration was observed immediately after sonication within 15 s, and the particle size distributions of nonsonicated and sonicated 30-mg/L nCuO dispersions practically did not differ after 5 min	**[1]**
Ag	Bath (Branson Ultrasonics, 2510 Ultra sonic cleaner, 15 kHz for 1.5 h)	*P. promelas* embryos	A short sonication period (5 min) just prior to testing resulted in a significant increase in mortality compared to exposure to particles that were just stirred. Similar amounts of dissolved Ag were released from the Sigma Ag NPs tested regardless if solutions were stirred or sonicated ($p > 0.05$), but with concentrations increasing significantly with increasing concentrations (all comparisons $p > 0.05$)	Sonication resulted in a decrease in the formation of aggregates compared to solutions that were only stirred, regardless of NP size.	**[2]**
TiO2	Probe sonication (QSonica, Sonicator 4000, Newton, CT) for 5 minutes at 20 kHz, 20mm, 0.5 inch Ti horn)	*H.azteca*	Sonication of TiO_2 NP stock solutions increases toxicity compared to stirred solutions. Also, in general, animals exposed to sonicated TiO_2 showed significantly lower dry weight than stirred groups.	-	**[3]**
CeO_2	Probe (1 minute / 70 watts) Versus magnetic stirring (vigorous, 24h)	*Daphnia magna* *Ceriodaphnia dubia* *Pseudokirchneriella subcapitata*	For C. dubai: higher toxicity after exposure to the probe sonicated dispersions compared to stirred dispersions. For P. subcapitata: although different dispersal protocol leads to different agglomerate size of $nCeO_2$, the algae growth inhibition were similar For D. magna: no inhibition of the mobility was recorded independent of media and dispersion method	The use of probe sonication as a dispersal methods and the addition of HA (2 mg.L-1, TOC), were the optimal protocol to produce small and consistent particles sizes in dispersions, with a reasonable stability over the exposure period. Moreover this protocol appears to be the most reproducible methods to disperse the ceria nanopowder in the different aqueous media.	**[4]**

Cu	Probe (Sonifier B12 power supply with converter and standard microtip from Branson Sonic Power Company (USA). Approximate output of 14 W in a 2 mL dispersion)	A549 cells (lung epithelial cells)	Significant difference ($p > 0.001$) in release of Cu from Cu ENPs. All particles were dissolved at sonicated conditions, whereas ca. 65% of the particles were dissolved in the non-sonicated particle dispersion. Sonication of the particle dispersion also influenced the induced toxicity when compared to the non-sonicated samples ($p > 0.001$). NB! A higher extent of released Cu for sonicated particle dispersions compared to nonsonicated dispersions does not explain well observed differences in toxicity. The differences in size of particles and agglomerates is a more plausible explanation	Visual observations clearly revealed that sonication rapidly changed the appearance of the cell medium containing visible large particle agglomerates to a dark brownish appearance with no visible large particle agglomerates	**[5]**

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