

Research paper

Regulatory adequacy of aquatic ecotoxicity testing of nanomaterials

Rune Hjorth*, Lars M. Skjolding, Sara N. Sørensen, Anders Baun

Department of Environmental Engineering, Technical University of Denmark, 2800 Kgs. Lyngby, Denmark



ARTICLE INFO

Keywords:

Nanomaterials
Nanoparticles
Ecotoxicology
Regulatory adequacy
Decision-making

ABSTRACT

Nanoecotoxicology as a discipline has matured significantly over the last years, from the first paper in 2004 to close to a thousand studies published today. We are therefore no longer facing a scarcity of data as severe as only a few years ago. From a regulatory standpoint, it is timely to question whether ecotoxicity testing is now able to facilitate regulatory decision-making on manufactured nanomaterials (MNs). In this paper, we review the state of aquatic ecotoxicity testing of MNs as well as the overarching issues that challenge the reliability and relevance of such testing. We conclude that within the field there is an increased focus on *characterization* of the exposure rather than *controlling* exposure as it is traditionally done in guideline testing of chemicals. However, the lack of characterization options under actual testing conditions makes it difficult to make meaningful comparisons between studies, which question the regulatory reliability of the data currently available. Accordingly, lack of data suited for regulatory decision-making is still a pressing issue in nanoecotoxicology even though the data availability has increased. Nevertheless, we emphasize that by deliberately directing test method developments towards increased regulatory reliability and acknowledging the implicit limitations in the dual purpose of guideline testing for chemical risk assessment (i.e. for hazard identification and for hazard assessment) it is possible to generate data sufficient for regulatory needs.

1. Introduction

The literature on the ecotoxicological effects of manufactured nanomaterials (MNs) has expanded significantly since the first paper published in this field (Oberdörster, 2004), i.e. from 89 studies identified in the ENRHES project (Stone et al., 2010a) to the about 770 studies included in the NanoE-Tox database (Juganson et al., 2015). We are therefore no longer facing a scarcity of data as severe as only a few years ago. From a regulatory perspective, the question then becomes whether the availability of such data now enables regulatory decision-making on MNs.

It is common regulatory practice that an ecotoxicological test result is considered more valid for regulatory use if it is obtained according to accepted and validated guidelines, e.g. OECD technical guidelines (TGs) or ISO standards. It is reasonable to establish and follow such guidelines, as it will reduce costs, use of experimental organisms and will in turn enable regulatory bodies to trust and accept previously derived ecotoxicological effect data according to the principles of mutual acceptance of data. At the same time, the use of standardized guidelines increases reproducibility of the test and comparability across substances (Ågerstrand et al., 2011). The existing aquatic ecotoxicological TGs are developed for testing of chemicals that dissolve in water and the test setups are as such not expected to influence the exposure

concentration or bioavailability of the tested chemical. This situation is different for MNs as MNs are physical entities (most often particles) that may undergo a range of transformation processes before and during testing (Baun et al., 2017; Skjolding et al., 2016). The applicability of the OECD TGs to ecotoxicity testing of MNs has therefore been questioned (e.g., Hansen et al., 2017a) and the need for adapting the OECD TGs for ecotoxicity testing has been emphasized several times (Rasmussen et al., 2016; Petersen et al., 2015; Skjolding et al., 2016; Hund-Rinke et al., 2016; Kühnel and Nickel, 2014). Historically, similar concerns about the suitability of TGs for testing so-called difficult substances led to the OECD's guidance for testing of difficult substances (OECD, 2000) and work has been undertaken by the OECD Working Party on Manufactured Nanomaterials (WPMN) and several EU projects (e.g., MARINA, NANoREG and NanoValid) to redress this situation for MNs (Lynch, 2016). This has resulted in OECD guidance for sample preparation and dosimetry of MNs (OECD, 2012) and the OECD WPMN has initiated a guidance document on aquatic toxicity testing of MNs, which will be available in the near future. While this is highly relevant and urgently needed to increase the regulatory adequacy of data generated using existing and modified TGs the question remains whether the currently available data are adequate for regulatory decision-making.

As shown in Table 1, the terms regulatory reliability, relevance and

* Corresponding author.

E-mail address: ruhj@env.dtu.dk (R. Hjorth).

Table 1

Definition of regulatory reliability, relevance and adequacy of toxicological data for hazard and risk assessment (Klimisch et al., 1997).

Reliability	Evaluating the inherent quality of a test report or publication relating to preferably standardized methodology and the way the experimental procedure and results are described to give evidence of the clarity and plausibility of the findings.
Relevance	Covering the extent to which data and tests are appropriate for a particular hazard identification or risk characterization.
Adequacy	Defining the usefulness of data for hazard/risk assessment purposes. Where there is more than one study for each endpoint, the greatest weight is attached to the studies that are the most relevant and reliable.

adequacy were defined by Klimisch et al. (1997) and have since been adopted by e.g. the OECD and European Chemicals Agency (OECD, 2005; ECHA, 2008). It should be noted that the definitions applied by different stakeholders differ slightly, but generally the adequacy of data to inform regulatory decision-making is described in terms of the relevance and reliability of the data. Whereas reliability refers to the intrinsic quality and reproducibility of data, the relevance of the data differs depending on the scope of the risk assessment (Hartmann et al., 2017).

To answer the question on the feasibility of regulatory decision-making based on currently available data we must first address the adequacy of such data for decision-making and clarify how and where it fits into risk assessment paradigms. In contrast to existing reviews on nanoecotoxicology, the focus in the present paper is therefore the regulatory adequacy of the current testing to inform environmental risk assessment as well as highlight how testing can be made more relevant for regulatory decision-making.

We start out by reviewing the reliability and relevance of aquatic ecotoxicological and bioaccumulation testing as well as discuss the use of available data. Lastly, we provide recommendations for how to improve the regulatory adequacy of such testing.

2. The state of scientific and regulatory aquatic ecotoxicity testing of nanomaterials

A number of reviews have recently been published on aquatic nanoecotoxicology all highlighting the challenges associated with testing MNs (Skjolding et al., 2016; Petersen et al., 2015; Bour et al., 2015a; Juganson et al., 2015). Likewise, several major European FP7 projects have chosen to assess the current guidelines and evaluate the applicability and/or possible adaptations needed for MNs (Lynch, 2016). For instance, the MARINA project aimed at providing “an overview of the progress on ecotoxicity testing protocols with a focus on the formation requested by regulatory bodies for safety assessment of MNs” (Hund-Rinke et al., 2016). The project proposed specific modifications of e.g. OECD TG 201 (freshwater algae and cyanobacteria growth inhibition), TG 202 (*Daphnia* acute toxicity) and TG 210 (Fish early life stage). However, general OECD guidance for nanoecotoxicity testing is still in the making. The latest drafted version (OECD, 2017) shows that the work in progress is positive and will assist future work. In parallel, ECHA has sent out two draft documents for consultation (ECHA, 2016a; ECHA, 2016b) which provide substantial revisions to the recommendations for ecotoxicological endpoints for MNs. The general issues highlighted for consideration during test planning include defining representative controls, dissolution rate and potential ion release, agglomeration behavior, degradation and transformation, selection of the exposure regimes, frequency of concentration measurements, use of mass-based metrics and nano-specific measurements. While the areas highlighted are indeed important, there is an ongoing debate as to whether all measurements have to be conducted for all MNs or if they can be considered on a case-by-case basis. These proposed changes are aligned with the findings in the literature although as described by

Hansen et al. (2017b) the guidance could be further improved. The OECD draft guidance document on aquatic toxicity testing of MNs also addresses several of these concerns (OECD, 2017).

Overall, there are two major issues concerning reliable ecotoxicity testing: 1) creating and maintaining stable suspensions and 2) appropriately characterizing suspensions. Lastly, aquatic ecotoxicity testing of MNs has shown to involve a range of potential testing interferences that makes the data interpretation difficult and questions the reliability of the test outcomes (Skjolding et al., 2016; Petersen et al., 2014). In the following, all three topics will be covered.

2.1. Sample dispersion and stability

An overarching issue in the reproducibility and reliability of ecotoxicity testing of MNs relate to the initial dispersion and the resulting variance in the aqueous suspensions and consequently the stability of the suspension. While several international projects have prepared specific dispersion protocols for toxicity testing (e.g. ENPRA, PROSPECt, NANOGENOTOX; MARINA) general guidelines have not been harmonized. According to Hartmann et al. (2015), the general problem of such harmonization is that “...harmonization and standardized protocols will always be a compromise between optimal dispersion on one hand and optimal biological/physiological and material compatibility of the medium and concentrations required in the stock dispersion on the other” (see Fig. 1).

A range of dispersions approaches exists to obtain stock suspensions e.g. sonication (probe or bath) and addition of natural organic matter (NOM). Several studies have investigated the effects of different environmental matrix components such as NOM on the behavior and bioavailability and therefore the toxicity of different MNs in aquatic test systems. A major motivation behind these studies is the attempt to stabilize the dispersion of MNs during aquatic toxicity testing by adjusting various physical or chemical properties of the media. While the presence of NOM may increase MN dispersion stability, this approach also has limitations and complicating factors, which hampers standardization. Thus, the determination of the methods used relies heavily on expert judgement or careful review of the existing literature.

The majority of studies have indeed shown the presence of NOM to increase the stability of MN dispersions (Grillo et al., 2015), including MNs of Ag, TiO₂ and CNTs (Baalousha et al., 2013; Kennedy et al.,

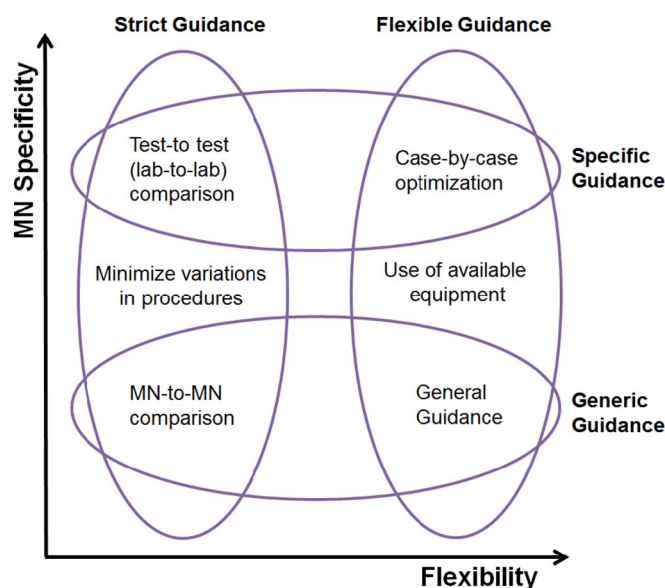


Fig. 1. Different approaches to harmonization of dispersion protocols. The approach chosen will be a compromise between conditions optimal for dispersing specific MNs and the testing conditions that need to be met. (Adapted from Hartmann et al., 2015.)

2009; Belen Romanello and de Cortalezzi, 2013). This is, however, not always the case as demonstrated for Ag nanoparticles (Cupi et al., 2015). The interaction between MNs and NOM is complex as it is influenced by various mechanisms, including the presence of divalent cations (e.g. Ca^{2+} and Mg^{2+}), the characteristics of the NOM, MN and medium constituents (Grillo et al., 2015). The majority of studies that have investigated the influence of NOM on different MNs also find NOM to reduce the MN toxicity, for example in algae and daphnids (Angel et al., 2013; Cupi et al., 2015). Theoretically though, increased MN stability is expected to extend the MN residence time in the water column, and thereby the exposure time, leading to a possibly increased toxicity (Grillo et al., 2015). A number of hypotheses have been suggested to explain the observed reduction in MN toxicity, and these include formation of NOM-ion complexes of low bioavailability, changes in MN surface charge or chemistry due to NOM-MN interactions, and antioxidant effects of NOM scavenging MN-generated ROS (Grillo et al., 2015). In contrast, Cupi et al. (2015) found that NOM addition did not affect the toxicity of ZnO nanoparticles. However, the risk that MN toxicity may be underestimated by allowing addition of NOM to guideline tests must be considered the most problematic scenario for hazard identification testing purposes. The complexity of these interactions are further illustrated by studies of e.g. Cupi et al. (2016) and Miao et al. (2015), showing how lowering pH or adding NOM decrease agglomeration/aggregation of Cu- and ZnO MNs, respectively, but in turn increase their dissolution, which may also influence toxicity.

It is evident from these studies and reviews that environmental matrix components influence toxicity by different mechanisms, most likely dependent on the media, biological species, and the type of NOM and MN. Therefore, while some of the parameters that affect toxicity of a given nanomaterial for a given environmental receptor are known, comprehensive protocol standardization of environmental matrix components is not feasible at the present state of knowledge. Thus, it can be considered premature to recommend addition of NOM in testing guidelines for aquatic ecotoxicity (Wickson et al., 2014) given that results (e.g. stabilization and altered toxicity) may depend on the type of MN and NOM and that no scientific consensus has yet been reached.

The process of agglomeration affects the stability of the test system and for aquatic toxicity testing this challenges the reproducibility of the testing outcomes. In general, OECD recommends ensuring that the concentration of the tested MN remains within $\pm 20\%$ of the initial concentration. As different MNs have different surface charge dependent on the MN and the testing media composition non-agglomerated primary particles sizes are difficult to maintain when testing uncoated MNs in testing media recommended by OECD TGs. Even for ultrapure water, this is challenging and the situation becomes critical for media of high ionic strength (Cupi et al., 2016). Only a few studies have investigated the stability of MNs in different ionic strength media relevant for regulatory ecotoxicity tests (Cupi et al., 2016; Römer et al., 2011, 2013; Tejamaya et al., 2012). In the study by Römer et al. (2013), undiluted media caused the most agglomeration in a standard test setup for Daphnia testing, whereas less agglomeration was found when using diluted media. A similar finding was reported by Tejamaya et al. (2012) using unmodified OECD M7 medium, ten times diluted M7 medium, and modifications to the medium such as replacement of chloride with nitrate or sulfate conducting tests with Ag nanoparticles. Based on their observation Tejamaya et al. (2012) found that the use of high ionic strength media should be avoided. Besides ionic strength, the concentration of divalent ions like Ca^{2+} and Mg^{2+} will influence the stability of test dispersions (Baalousha et al., 2013). The study by Cupi et al. (2016) followed up on these recommendations and found that guideline testing of MNs could be improved by measuring of the point-of-zero-charge (or isoelectric point) in relevant testing media prior to toxicity testing to identify the optimal parameters (a “window of opportunity”) such as pH and media composition/ionic strength. In Cupi et al. (2016) such a methodology in testing and assessing stability and toxicity of MNs is exemplified for the OECD TG 202 Daphnia test, where

dispersions proved more stable when the zeta potential was above $+30$ mV. If the corresponding pH is within the physiological range of the test organism this pH should be preferred. Likewise, media with low ionic strength can potentially affect the health of the test organism and sensitivity testing should be performed with the modified media to exclude stress imposed by the media.

The concentration dependent agglomeration may strongly influence the bioavailability of MNs in test systems (Petersen et al., 2015; Baalousha et al., 2016; Skjolding et al., 2016), which questions the applicability of limit tests for MNs. Even though no effects are observed at limit test concentrations (often 100 mg/L) effects may occur at lower concentrations. This will, by definition, invalidate the limit test approach for MNs. If a strong dependency of dispersion stability on the concentration is observed it is recommended to prepare the test dispersions for each concentration individually. Test concentrations of 100 mg/L are scientifically questionable but may be necessary to test because of classification and labelling requirements. Similar considerations led Hund-Rinke et al. (2015) to recommend testing multiple concentrations to obtain information about the dose–response relationship.

Alternatively, a modified test system could be used to maintain a constant exposure throughout the test phase (Boyle et al., 2015). Such a test system generally aims at maintaining a circulation of the tested MNs throughout the testing period. This has been found successful when using e.g. OECD TG 210 (Shaw et al., 2016). However, it should be noted that such a method is unsuitable for organisms sensitive to turbulence e.g. daphnids. Similarly, semi-static test setups (with media renewal every 1–3 days) or using a hydrostatic pressure flow-through system as proposed by Bundschuh et al. (2012) could prove feasible for more stable MN dispersions.

2.2. Exposure characterization

As described above, generating reliable exposure conditions has proved difficult, however putting that issue aside, the challenge readily becomes how to adequately characterize the tested suspensions. Although the last decade of nanoecotoxicological research has seen a great improvement concerning the characterization of MNs, the number of relevant characterization parameters and the importance of each one has been cause for debate in the scientific literature. One clear message remains: In order to yield proper scientifically justified results from ecotoxicity tests, an exposure characterization has to be performed. Consequently, particle size determination has been of high priority in many studies as potential novel effects of MNs are often attributed to the particle size. Several techniques to obtain such distributions are widely used and give information on particle characteristics in aqueous media (e.g. DLS, NTA) or on dry powder (or analysis of dried stock suspensions) through electron and atomic force microscopy. Although techniques and instrumentation also are available to study aqueous samples with these imaging techniques, their usage is not widespread. In general, all techniques have shortcomings and multiple pitfalls exist in sample preparation and data analysis for MN characterization which potentially can lead to erroneous conclusions in the exposure characterization.

For some MNs the potential release of metal ions must be characterized as especially the dissolution kinetics related to aquatic media is crucial for determining the ecotoxicity. Quantification of the dissolution kinetics as well as losses before, during and after incubation is key to determine the actual exposure concentrations for MNs and the released ions and thus obtaining reliable concentration-response relationships needed for regulatory purposes (Sørensen and Baun, 2015; Sekine et al., 2015). However, it can be debated how well the ion concentration in a dispersion correlates to the actual exposure mediated through uptake and adsorption.

According to the review by Skjolding et al. (2016) it is of very high importance to account for and describe the influence of agglomeration

for most, if not all, MNs during aquatic toxicity testing. Agglomeration is especially important for TiO₂ and CeO₂ (Hartmann et al., 2010; Gaiser et al., 2012; Cupi et al., 2016; Van Hoecke et al., 2008) since it is difficult to maintain stable suspension for these MNs in test media. Thus, sedimentation of TiO₂ and CeO₂ is often reported and physical effects on test organisms cannot be ruled out. Skjolding et al. (2016) furthermore concluded that dissolution in the test medium and release of ionic metal species for Ag, ZnO, and CuO MNs often can explain the observed toxicity (Notter et al., 2014). The dissolved metal ion has in many cases been found to dominate the ecotoxic effects, although some studies found that the ion alone cannot fully explain the observed toxicity (e.g. Sørensen et al., 2016). It is no simple task to quantify the dissolution rate of MNs and even more difficult under actual test conditions. Studies that fail to report high recoveries of MN and complete mass balances will generally have low regulatory relevance with respect to disclosing the existence of a nanoparticle specific effect (Skjolding et al., 2016). Furthermore, it should be stressed that dissolution is a dynamic process that is ongoing from preparation of the stock suspensions before testing as well as during the incubation period in the toxicity tests (Sørensen et al., 2015).

In 2010, Stone et al. (2010b) proposed using six main physico-chemical parameters for characterization; 1) Aggregation/agglomeration/dispersibility, 2) size, 3) dissolution, 4) surface area, 5) surface charge and 6) surface composition/surface chemistry. Similar parameters have been proposed by the OECD as important for characterization in a regulatory context (OECD, 2016). While the OECD document clearly specifies which parameters to consider, it is important to note that not all the parameters are necessarily relevant for every MN. Consequently, the choice of which parameters to monitor and quantify must be evaluated on a case-by-case basis.

It is important to underline that today there is not a full scientific understanding of the importance of any of the parameters or the interactions between them for the toxicity endpoints in current TGs. Thus, the current recommendation is that as much characterization data as possible should be reported for each MN in order to be able to look back and re-evaluate results at a later stage. This represents a move from the traditional focus on controlling exposure in TGs applied to dissolved chemicals towards a focus on describing exposure through a range of different techniques (Sørensen et al., 2016).

While focus in numerous papers has been on initial characterization before ecotoxicity testing, it is recognized that quantification of the actual exposure during testing is needed to increase both the scientific value and the regulatory adequacy of ecotoxicological studies (OECD, 2014; Lützhöft et al., 2015). This was also highlighted in a critical review of current ecotoxicological testing of MNs by Skjolding et al. (2016), which discuss possible strategies for coping with these challenges. Adapting testing guidelines has been the focus of numerous European research projects (e.g. MARINA, NanoValid and NANoREG) and several national projects. The overview articles published from these projects (e.g. Bondarenko et al., 2016; Hund-Rinke et al., 2015; Hund-Rinke et al., 2016) show that there is no consensus on which parameters to measure or more generally how testing should be performed. This is also exemplified in the scientific literature highlighted in a review on physico-chemical parameters reported in ecotoxicological studies from 2006 to 2015 showing that while particle size was reported in approximately 90% of all published papers, whereas parameters such as coating, surface area and shape were only reported in 30–40% of the published papers (Juganson et al., 2015). However, these numbers are related to the characterization of the pristine MNs prior to testing. For studies that carried out characterization in testing media/environmental conditions, size determination was carried out in approximately 60% of the studies and dissolution in approximately 30% (Juganson et al., 2015). This lack of characterization under actual testing conditions makes it difficult, if not impossible, to make meaningful comparisons between studies – even if they are carried out in accordance with OECD TGs. This raises serious questions about the

regulatory reliability and relevance of the data currently available (Lützhöft et al., 2015; Hartmann et al., 2017).

2.3. Test interferences

Besides the issues with controlling and describing exposure conditions, an additional challenge has proven to be MN induced test interferences. Current OECD hazard identification toxicity TGs were designed to reflect the direct toxic effects of a chemical compound on the test organism. However, MNs are shown to inhibit the algal growth rates and affect the mobility of daphnids via seemingly non-toxicological mechanisms, sometimes referred to as “physical effects” (Sørensen et al., 2015). Case in point, in crustaceans, adsorption of CeO₂, Pt and TiO₂ MNs on the exoskeleton, cuticle and antenna is reported to influence mobility, molting, and swimming velocity (Artells et al., 2013; Cupi et al., 2015; Dabrunz et al., 2011; Gaiser et al., 2011; Noss et al., 2013). Thus, the use of immobility as an endpoint in the OECD guideline for acute daphnia toxicity testing may be problematic in cases where immobility reflects physical impairment rather than toxicity. The inclusion of both lethality and immobility as endpoints has been suggested, as well as a mesh bottom inserted beaker, restricting daphnids from contact with larger clusters of MNs at the beaker bottom (Sørensen et al., 2015). Recently, Hjorth et al. (2017a) documented the technical challenges of conducting ecotoxicity testing of Fe MNs, with issues present in guidelines testing with bacteria, crustaceans, worms and algae. Other issues associated with gut blockage and surface effects, such as effects on fish gills and other respiratory surfaces (e.g. Petersen et al., 2011) have not yet been adequately addressed in the literature.

Furthermore, the presence of organisms may hamper the MN characterization during testing, by interfering with the characterization techniques. For example, using dynamic light scattering to determine the MN size distribution “in situ”, i.e. at the end of an algal or daphnia acute toxicity test is hampered by the samples extracted containing algae or daphnia exudates in addition to the MNs, which interferes with dynamic light scattering analysis (Sørensen, 2016).

In algal growth rate inhibition tests, MNs may scatter light from reaching algal cells and thereby reduce the growth rate, also termed “shading”, rather than or in addition to any toxic effect. Shading effects are reported for CNTs, Au and Pt MNs (Schwab et al., 2011; Sørensen et al., 2016; Van Hoecke et al., 2013), while for ZnO, CuO and TiO₂ MN shading is found to be negligible (Aruoja et al., 2009; Hartmann et al., 2010; Hund-Rinke and Simon, 2006). For Au and Pt MNs, shading alone could not explain the growth rate inhibition determined, indicating additional toxic and/or physical effects of these MNs (Sørensen et al., 2016; Van Hoecke et al., 2013). With the exception of the study by Schwab et al. (2011), all other studies attempting to quantify the influence of shading have used setups, in which the algae and MN-suspensions are physically separated. In these setups, the MN-suspensions are placed between the algae and the light source to expose the algae only to the light passing through the MN-suspensions. This approach however, only reveals shading from MNs distantly located from algal cells, and not shading caused by MNs adsorbed to the algal cells (Hjorth et al., 2016). In general, algal cells can overcome temporary shading (e.g. from distant MNs) without necessarily experiencing growth reduction, but MN cell adhesion can result in permanent shading as well as other physical effects such as limitation of nutrient availability. Another limitation to this approach is that a lowered algal growth rate due to shading may mask toxicity, as slow growing algae may be less sensitive to toxic MNs (Cleavers and Weyers, 2003).

Analysis of changes in the algal pigment composition has been suggested as a potential qualitative measure for true shading, i.e. shading as it is experienced by the algae (Hjorth et al., 2016). The approach relies on algal photo-acclimation, causing algae to rapidly adapt their pigment composition in response to changing light conditions and hence a quantification of these changes can serve as an endpoint to quantify shading effects (Hjorth et al., 2016). This approach

is however currently under development and at present not yet applicable for standardization purposes.

At the current state-of-knowledge, it is recommended to include a test for shading effects for MNs that form dark or turbid suspensions in media, adhere to algal surfaces, and have relatively low toxicity, as this entails exposure concentrations in the upper end of the classification range (10–100 mg/L) (Sørensen, 2016). Shading effects are most easily investigated through a separation setup, despite its shortcomings. Though special vials/plates are required, these are relatively easy to obtain and the incubator and analysis methods are the same as those for the algal guideline test.

Interference of MNs with algal growth quantification techniques has also been reported as a potential source of error (Handy et al., 2012a, 2012b; Hartmann et al., 2013). The most common biomass quantification methods are based on cell counting using microscopy and fluorescence measurements of extracted algal pigments. In algal growth inhibition tests with MNs, high background particle numbers may disturb the biomass measurements (Hartmann et al., 2013), therefore background corrections using test suspensions without algae are recommended by ISO (2012). Recent research finds that CNT may adsorb on algal cells very rapidly and absorb the fluorescent light of chlorophyll (Booth and Farkas, 2017). Therefore, a pre-test with comparison of algal fluorescence with extracted chlorophyll could be included to ensure that this influence on biomass determination is recognized. This interference further stressed the previous recommendations to check and validate the quantification method against traditional microscopy as shown by e.g. Hartmann et al. (2013), Handy et al. (2012a, 2012b) and Kalman et al. (2015).

In general, it is still debatable what constitutes ‘true’ toxic effects and what should be deemed physical effects or testing artefacts. Separating different types of effects caused by either physical interactions or e.g. by dissolved ions is not only necessary to elucidate the toxic mechanisms, but also to address concentration dependent behavior (Baalousha et al., 2016) and effects which do not align with the dose-response paradigm. Essentially, these types of effects are induced due to high concentrations and/or lack of stability during incubation and can be considered testing artefacts. The reasoning for this is that these effects only occur at a given (too high) concentration and thus cannot be extrapolated to no-effect concentrations. However, if physical effects persist with documented dispersion stability, then these should not be disregarded.

3. Data relevance and environmental compartments

Although the literature reporting aquatic toxicity data for MNs is expanding rapidly, the adequacy of these data for regulatory risk assessment and decision-making purposes has been questioned. In a recent review by Lützhøft et al. (2015) the open scientific literature was searched to identify studies concerning the nine selected MNs deemed regulatory relevant (Ag, CB, CeO₂, CNTs, CuO, QDs, TiO₂, ZnO and nZVI) and reporting endpoint data relevant for risk assessment, such as NOEC/LOEC and/or EC/IC/LC₅₀. Lützhøft et al. (2015) concluded that although 1200 studies were identified only a few of them provided data adequate for regulatory risk assessment purposes.

The immediate recipient of MNs will in many cases be the water compartment. From here, the residence time of MNs in the water column is highly diverse and depends on both the intrinsic properties of the MNs as well as the properties of the receiving water compartment (Baun et al., 2017). However, when reviewing the literature it is evident that there is still uncertainty as to which environmental compartment will be most important for each specific MN. Selck et al. (2016) argues that the settling behavior of MNs is more likely to lead to an exposure of benthic organisms and sediment systems. This statement is supported by the fact that the modelled average concentration of MNs in sediment often exceeds that in the water phase by several orders of magnitude (Gottschalk et al., 2013). It is also reasonable to expect MNs with low

dispersibility or stability in environmentally relevant media be found in sediments (Baun et al., 2008). A pragmatic approach for selecting MNs that should undergo sediment testing, could be to use the tiered agglomeration behavior scheme in the draft TG for agglomeration (OECD, 2016). MNs that by this method is found to be non-dispersible or show a condition-dependent stability < 50% would be obvious candidates for sediment testing.

A test setup using a pre-exposure step where MNs are suspended, allowed to settle and then both the overlaying phase as well as the bottom phase is assessed separately could be useful for dissolving MNs, such as Ag, Cu and Zn (Petersen et al., 2015). This would allow for potential separation of effects due to the dissolved and particulate fractions. Currently, there are no data to support this general approach and very little practical work has been conducted to assess which method of suspension should be used and what timescales should be involved.

With regards to choice of test organisms, Petersen et al. (2015) indicated that the biological receptors chosen should be selected based on material fate in the test system, to avoid testing that cannot assess worst-case scenarios (maximum exposure). However, as outlined by Skjolding et al. (2016) it is not necessarily the maximum exposure concentration (on a mass basis) that creates the “worst case scenario”. For example, concentration mediated agglomeration/aggregation may occur (Baalousha et al., 2016) whereby higher concentrations can give rise to a lower exposure.

For the testing of chemicals, as well as of MNs, tests with algae, daphnids and fish constitute the base set of organisms for which data must be available to complete the different parts of risk assessment (e.g., for classification and labelling, PBT assessment and estimation of predicted no-effect concentration (PNEC)). Tests with these organisms are regarded per se of regulatory relevance as representative organism groups at different trophic levels with relevant toxicity endpoints (like mortality, immobilization, behavior, reproduction) and they are considered as regulatory reliable due to standardization and inter-laboratory testing. The regulatory reliability of current test methods as described in detail above is challenged when MNs are tested, but this is almost exclusively due to the difficulties in keeping stable exposure conditions as well as characterizing the exposure and is not related to the choice of test organism.

Classically, chronic endpoints are more sensitive than acute ones and as such have also a higher weight in the hazard assessment. For MNs, the long-term exposure to low concentrations could be of high relevance (Baun et al., 2008) and identification and quantification of chronic effects of MNs is therefore of high regulatory relevance. In general, far fewer tests for chronic effects have been reported in the literature compared to the number of studies reporting on acute effects (Lützhøft et al., 2015; Juganson et al., 2015). Not only are longer-term tests for chronic effects cost and labor intensive, but it is also more difficult to maintain stable exposure conditions during incubation. Furthermore, “confounding factors” may have to be introduced to the test system e.g. the addition of food. The study by Mackevica et al. (2015) reported on the influence of different amounts of food on the outcome of daphnia reproduction tests (OECD TG 211) with Ag MNs. They found that the addition of higher food levels resulted in higher animal survival, growth and reproduction compared to tests with lower food levels (Mackevica et al., 2015). It has also been shown that the uptake of MNs in daphnids is influenced by the presence of food (Skjolding et al., 2014), which may influence the chronic effects found in long-term exposure tests (Sakka et al., 2016). Furthermore, since the interaction of MNs with algal exudates may affect the bioavailability of MNs, the presence of algae (as food) in daphnia reproduction tests (OECD TG 211) may inadvertently affect the observed toxicity. It is worth noting that currently there is no evaluation of such effects in higher tier tests with vertebrates.

Bioaccumulation and biomagnification may enhance the internal exposure concentrations in organisms and thus increase the risk

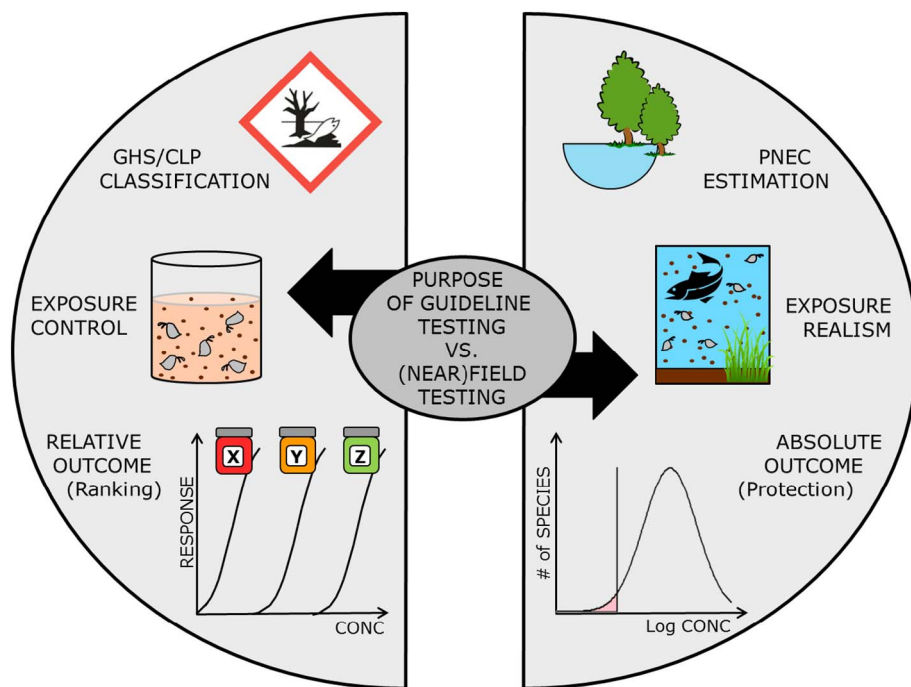


Fig. 2. Overview of the regulatory purpose of guideline testing and (near)field testing. Whereas the latter is better suited for setting 'absolute' environmental quality standards, guideline testing supports a more relative use of data for hazard ranking and labelling.

potential. As such, the assessment of the bioaccumulation potential plays a major role at different levels in the chemical safety assessment (e.g. PBT assessment). Conventional methods based on the determination of equilibrium-based partition coefficients are generally not regarded as valid for MNs. MNs may accumulate without reaching equilibrium between the organism and the surrounding medium and higher internal concentrations may be found through dietary exposure feeding (Handy et al., 2012a, 2012b; Skjolding et al., 2014). This may even result in biomagnification in the food chain if depuration of incorporated MNs is negligible (Skjolding et al., 2014; Croteau et al., 2011; Unrine et al., 2012). This phenomenon is not entirely new and thus testing principles for dietary exposure has been included in the OECD TG 305 for Bioaccumulation in Fish (OECD, 2012). This type of exposure is recommended for "difficult substances" where a constant water phase concentration is difficult to maintain (OECD, 2000). It has been recognized that the estimation of a bioconcentration factor is invalid for MNs and other "difficult substances" and has hence been replaced with a biomagnification factor (BMF). While this still remains to be implemented, it should also be kept in mind that such endpoints do not fit into the guidance for e.g. PBT or vPvB assessments in REACH where the BCF value is used as the criterion for assessing bioaccumulation (ECHA, 2014). For a further discussion of MN bioaccumulation see Skjolding (2015). MN persistence is covered in this issue by Baun et al. (2017).

Tests with *D. magna* for quantification of MN bioaccumulation have been mentioned as promising candidates for development of guideline tests though some concerns has been raised with regards to relevant exposure scenarios, e.g. the risk of overestimating of the biomagnification potential due to the specific feeding traits of daphnids (OECD, 2014). Furthermore, there are technical difficulties in determining whether ingested MNs are genuinely taken up or merely residing in the alimentary canal of the daphnids (Tangaa et al., 2016). It is therefore possible to investigate MN uptake and subsequent depuration of MNs in *D. magna*, however 'true bioaccumulation' (i.e. tissue uptake) have proven difficult to adequately study (Jensen et al., 2016). It was concluded by OECD (2014) that for the assessment of biomagnification the focus should be on the whole body burden, rather than differentiating between MN uptake by organisms and MN attached to organisms, since all MNs are likely to be ingested by the next trophic level organism. This assumes that MNs remain associated with the lower trophic level

organism over a significant duration of time. The trophic transfer of MNs has been documented in both aquatic and terrestrial tests (e.g. Skjolding et al., 2014b and Unrine et al., 2012).

As described in the recent review by Tangaa et al. (2016) sediments are expected to be the main starting point for trophic transfer of MNs in the aquatic ecosystem due to the expected agglomeration and sedimentation of particles in natural waters. This in combination with the feeding traits of sediment-dwelling organisms highlights the relevance of developing guidelines for bioaccumulation studies in sediments. It was concluded at the OECD Expert Meeting on Ecotoxicology and Environmental Fate in Berlin in 2013 that the OECD TG 315 (Bioaccumulation in Sediment dwelling Benthic Oligochaetes) as well as OECD TG 317 (Bioaccumulation in Terrestrial Oligochaetes) in principle are applicable for testing of MNs (Kühnel and Nickel, 2014), but that the spiking procedures have a high influence on the outcome of the tests similar to the conclusion in Section 2.1.

Whether the relevance of the base set can be considered high is, from a scientific point of view, debatable as the guideline tests are not optimized for environmental relevance in the sense of realism. Furthermore, it should be highlighted that studies with high reliability does not necessarily contain data with high reproducibility, as normally would be implied. In fact, reproducibility is seldom obtained in the field of nanoecotoxicology, primarily due to the issues addressed in this paper. However, from a regulatory point of view, relevance is linked to the fact that relevant representative species are tested for relevant effects.

Lastly, data assessed to be of little regulatory relevance, does not necessarily imply bad data. In fact, scientific studies without a regulatory focus or regulatory compliance have value in themselves and are still needed to further the field of nanoecotoxicology (Hjorth et al., 2016; Wickson et al., 2014).

4. Regulatory use of ecotoxicity data

Within a regulatory context, ecotoxicity data are normally used in two distinctly different ways: 1) The "classification use", i.e. for classification, labelling and determination of the toxicity (T) criterion in a PBT assessment, and 2) The "protective use", i.e. the derivation of predicted no-effect concentrations (PNECs). This also matches the distinction within ecotoxicology between anticipatory laboratory

ecotoxicity testing for hazard identification and assessment testing for environmental impact (Calow, 1997; Hjorth, 2016). As such, guideline testing should support regulatory hazard ranking and labelling, whereas field or 'near-field' testing is better suited for setting more 'absolute' environmental quality standards (see Fig. 2). However, for most chemicals and materials both the classification use and the protective use of data tend to be based on guideline testing, partly due to data availability. Quality measures of relevance and reliability will most often also favor studies carried out according to guidelines and standards for which international consensus has been gained. This gives a strong focus on tests with the base-set organisms that, combined with a set of defined criteria, allows for fulfilment of the regulatory double purpose of ecotoxicity data.

For conventional chemicals the reliability and relevance of the tests have been evaluated and a precedent has been established over the last decades. The use of the same test results at different stages in the risk assessment procedure therefore relies on agreed-upon cut-off values and extrapolation methods. However, for MNs the double use of guideline testing for both classification and protective purposes remains unevaluated at this time. As we have previously pointed out extrapolation from guideline testing may indeed be inadequate for PNEC determination.

It should be fully recognized that nanoecotoxicology tests serve different purposes and different tests are needed to fulfill different regulatory needs. For hazard identification the ideal test offers controlled exposure conditions which combined with a high degree of MN characterization allows for reliable and reproducible benchmarking. For hazard assessment, testing of environmentally realistic concentrations and under more realistic conditions may yield results that are more relevant for deriving at no-effect concentrations. New tests and endpoints may be needed to facilitate this and the extrapolation methods used to obtain no-effect concentrations should be scrutinized as their validity is questioned (Lützhøft et al., 2015; Hjorth, 2016; Aitken et al., 2011; Palmqvist et al., 2015; Syberg and Hansen, 2015).

4.1. From lab to real world extrapolation

Going from single species in vitro and in vivo toxicity data to predicted no-effect concentrations in various complex environmental ecosystems requires a solid data foundation in conjunction with well-established methods for extrapolation. Recently nanoecotoxicology, along with toxicology in general, have seen a trend towards more high throughput in vitro screening and testing to generate more comprehensive datasets for MNs. Although in vitro testing provides interesting insights into mode of action of MNs, there is a paucity of data offering a comparison between in vivo and in vitro systems, and thus little validation of such tests for environmental risk assessment purposes. Whereas the progress in this area is promising there is still further need for comparisons between in vitro and in vivo systems to evaluate the validity of in vitro environmental assays in regulatory testing (Hjorth et al., 2017b).

Replacing whole animal models requires a thorough understanding of adverse outcome pathways (AOPs) to facilitate accurate in vitro to in vivo extrapolation based on a mechanistic understanding (Gerloff et al., 2016). However, as argued by Hjorth et al. (2017b), in vitro testing is not likely to replace in vivo models for environmental risk assessment of MNs. Instead, in vitro testing should complement higher-tier testing, for instance by providing mechanistic information as well as verifying and screening novel endpoints.

Holden et al. (2016) and Bour et al. (2015a) provide an overview of the current status on the use of mesocosms approach in the assessment of the effects of MNs. Most of the studies assess fate and bioaccumulation but few assess trophic transfers, mechanisms of toxicity and mode of action. The approaches are varied and range from relatively simple laboratory studies to field experiments. It is clear that the issues associated with the use of mesocosms are the same as applied to

conventional chemicals (mainly that they have increased ecological complexity and ecosystem relevance and reduced system control). Given the importance of MN transformations and fate in the determination of effects, consideration of developing mesocosm assays should not be discouraged on ground of the high complexity. However, this development should be followed with careful characterization and following the fate of MNs throughout the testing period. This in itself is not a trivial matter especially in the complex environmental matrices introduced in mesocosm studies. With regards to the regulatory adequacy of these approaches, the publications of Tella et al. (2014, 2015) demonstrate that mesocosm tests with MNs should be further developed for regulatory purposes. In the current risk assessment paradigm mesocosm studies do play a role in defining the PNEC value, but mesocosm studies will typically only be conducted for higher tier risk assessment, e.g. for high production volume substance or substance of very high concern, due to the very costly nature of these testing setups. Only few of the currently used MNs fall in these categories and it is therefore, at present, likely that mesocosm tests will play a limited role in the regulatory risk assessment of MNs.

However, whereas currently micro- and mesocosms experiments (i.e. community and ecosystem testing) remain almost unexplored for MNs (Bour et al., 2015a, 2015b; Minetto et al., 2016), there is no doubt that their use would be beneficial to nanoecotoxicology as a scientific discipline since a better understanding of ecotoxicity, in general, is obtained by using laboratory studies in conjunction with field-based studies (Chapman, 1995). Establishing dose-response relationships for MNs is difficult, e.g. due to their concentration dependent and dynamic behavior (Baalousha et al., 2016), making it hard to extrapolate NOEC levels and correspondingly estimate accurate PNEC values. Higher-level ecosystem tests offer a platform to limit extrapolation by testing MNs in systems more closely related to the environment as well as offering a more realistic exposure regime, as also illustrated in Fig. 2 (Hjorth, 2016).

5. Conclusion

When ecotoxicity and bioaccumulation tests are carried out in accordance with the current OECD testing guidelines, a number of technical challenges arise from the inherent differences between MNs and conventional dissolved chemicals for which the tests were originally developed. The reliability of test outcomes depends on extensive characterization of the tested MN, the procedure of the preparation of test dispersions, and the description of the observed biological responses in the test systems. However, biological responses observed in ecotoxicity testing obtained with current OECD TGs are most often difficult to link to MN properties and exposure or dose.

It is important to underline that there is not currently a full scientific understanding of the importance of each parameter or the interactions between them for the toxicity endpoints in current guideline tests. Thus, the current recommendation is that as much data on the characterization of MN as possible should be reported in order to be able to look back and re-evaluate results at a later stage. This represents a move from the traditional focus on controlling exposure in TGs applied to dissolved chemicals towards a focus on describing exposure through a range of different techniques. This is identified as the way forward to obtaining data, which on the one hand are adequate for regulatory purposes and on the other hand may disclose nanoparticle-specific effects. Based on the literature review carried out, our major recommendations for improving the regulatory adequacy of aquatic ecotoxicity testing in are summarized below:

- Dissolution and especially dissolution kinetics is one of the key parameters to consider for certain MNs. However, it is important to note that the dissolution kinetics ideally should be measured in the presence of the test organisms, in order to account for the effect of exudates or similar artefacts that would not be accounted for by

doing a parallel dissolution test.

- Recommendations on methods for determining MN dissolution in testing media should be aligned with the methods recommended in the draft OECD TG dissolution rate in aqueous media (OECD, 2014).
- Test setups with modified media should be considered to comply with the current requirement of OECD TGs maintaining at least 80% of the initial test concentration in suspension. Furthermore, clarification to the 80% requirement should be stated, i.e. whether the requirement relates to initial size, agglomeration/aggregation and/or ongoing dissolution.
- It is evident from the reviewed literature that the addition of organic matter may mask toxicity and it is generally discouraged (OECD, 2017). However, on a case-by-case basis the possibility of using NOM to stabilize MNs during testing may be considered, but if this is chosen a range of appropriate controls must be included to document the influence of OM on the MN toxicity.
- The influence of the addition of food has to be clearly specified since literature reviewed has shown different toxicity and uptake dependent on food levels applied.
- More information on other endpoints (e.g., genotoxicity, neurotoxicity, immunotoxicity, indication of oxidative stress, haematology) than those traditionally used in guideline tests (e.g., immobility, lethality, growth rate inhibition) should be collected (Hund-Rinke et al., 2015), since the literature reviewed proposes that current endpoints may not be suited for identifying all nanoparticle effects.
- Different test approaches serve different regulatory purposes and the use of testing in regulatory nanoecotoxicology should be reexamined.

In general, guidance on separating different types of effects caused by either physical interactions or by dissolved ions is necessary to elucidate the toxic mechanisms, but also to address concentration dependent effects and behavior which do not align with the dose-response paradigm. Physical effects must be accounted for, by e.g. including shading controls in algal tests.

The literature reviewed shows that the lack of characterization under actual testing conditions makes it very difficult, if not impossible, to make meaningful comparisons between studies – even if they are carried out in accordance with OECD TGs – and this questions the regulatory reliability of the data currently available.

As noted by Klaine et al. (2012) ‘A consensus view exists that the paucity of usable data on the environmental hazard of nanomaterials has created unacceptable uncertainty in risk analysis from the regulatory decision-making perspective’. As shown in this paper, this point is unfortunately still valid even though the data availability has increased. However, the ongoing adaptations OECD testing guidelines and the development of a technical guidance document for aquatic testing of MN represent significant steps in alleviating this situation. It is recommended that test developments directed towards increased regulatory reliability acknowledge the dual purpose of generating data for chemical risk assessment (i.e. for hazard identification and for hazard assessment) which calls for an increased focus on MN characterization in one set of tests and increased environmental realism in another.

Acknowledgements

This research was funded by the European Union's Horizon 2020 project ProSafe (promoting the implementation of Safe by Design), grant agreement 646325. Rune Hjorth and Sara N. Sørensen have received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement no 686239. We would like to thank Philip Sayre and Klaus Günter Steinhäuser for guiding us through the ProSafe reviews and for their highly qualified feedback. Furthermore, we thank the participants of the breakout group

discussion (chaired by Rebecca Klaper and Nanna B. Hartmann) at the Joint scientific conference of NANoREG, ProSafe & OECD (29 Nov–1 Dec 2016; Paris, France) for constructive critique and suggestions to an earlier version of the paper.

References

- Ågerstrand, M., Küster, A., Bachmann, J., Breitholtz, M., Ebert, I., Rechenberg, B., Rudén, C., 2011. Reporting and evaluation criteria as means towards a transparent use of ecotoxicity data for environmental risk assessment of pharmaceuticals. *Environ. Pollut. (Barking, Essex: 1987)* 159 (10), 2487–2492. <http://dx.doi.org/10.1016/j.envpol.2011.06.023>.
- Aitken, R.A., Bassan, A., Friedrichs, S., Hankin, S.M., Hansen, S.F., Holmqvist, J., Peters, S.A.K., Poland, C.A., Tran, C.L., 2011. Specific Advice on Exposure Assessment and Hazard/Risk Characterisation for Nanomaterials under REACH (RIP-oN 3) - Final Project Report. European Commission, Brussels, Belgium.
- Angel, B.M., Batley, G.E., Jarolimek, C.V., Rogers, N.J., 2013. The impact of size on the fate and toxicity of nanoparticulate silver in aquatic systems. *Chemosphere* 93, 359–365. <http://dx.doi.org/10.1016/j.chemosphere.2013.04.096>.
- Artells, E., Issartel, J., Auffan, M., Borschneck, D., Thill, A., Tella, M., Brousset, L., Rose, J., Bottero, J.-Y., Thiéry, A., 2013. Exposure to cerium dioxide nanoparticles differently affect swimming performance and survival in two daphnid species. *PLoS One* 8, e71260. <http://dx.doi.org/10.1371/journal.pone.0071260>.
- Aruoja, V., Dubourguier, H.-C., Kasemets, K., Karjalainen, A., 2009. Toxicity of nanoparticles of CuO, ZnO and TiO₂ to microalgae *Pseudokirchneriella subcapitata*. *Sci. Total Environ.* 407, 1461–1468.
- Baalousha, M., Nur, Y., Römer, I., Tejamaya, M., Lead, J.R., 2013. Effect of monovalent and divalent cations, anions and fulvic acid on aggregation of citrate-coated silver nanoparticles. *Sci. Total Environ.* 454–455 (2013), 119–131.
- Baalousha, M., Sikder, M., Prasad, A., Lead, J., Merrifield, R., Chandler, G.T., 2016. The concentration-dependent behaviour of nanoparticles. *Environ. Chem.* 13, 1–3. <http://dx.doi.org/10.1071/EN15142>.
- Baun, A., Hartmann, N.B., Grieger, K., Kusk, K.O., 2008. Ecotoxicity of engineered nanoparticles to aquatic invertebrates: a brief review and recommendations for future toxicity testing. *Ecotoxicology* 17, 387–395.
- Baun, A., Sayre, P., Steinhäuser, K.G., Rose, J., 2017. Regulatory relevant and reliable methods and data for determining the environmental fate of manufactured nanomaterials. *NanImpact* (submitted, this issue).
- Belen Romanello, M., de Cortalezzi, M.M., 2013. An experimental study on the aggregation of TiO₂ nanoparticles under environmentally relevant conditions. *Water Res.* 47, 3887–3898. <http://dx.doi.org/10.1016/j.watres.2012.11.061>.
- Bondarenko, O.M., Heinlaan, M., Sihtmaa, M., Ivask, A., Kurvet, I., Joonas, E., Jemec, A., Mannerstrom, M., Heinonen, T., Rekulapelly, R., Singh, S., Zou, J., Pyykkö, I., Drobne, D., Karhu, A., 2016. Multilaboratory evaluation of 15 bioassays for (eco) toxicity screening and hazard ranking of engineered nanomaterials: FP7 project NANOVALID. *Nanotoxicology* 10, 1229–1242. <http://dx.doi.org/10.1080/17435390.2016.1196251>.
- Booth, A., Farkas, J., 2017. Are fluorescence-based chlorophyll quantification methods suitable for algae toxicity assessment of carbon nanomaterials? *Nanotoxicology (in review)*.
- Bour, A., Mouchet, F., Silvestre, J., Gauthier, L., Pinelli, E., 2015a. Environmentally relevant approaches to assess nanoparticles ecotoxicity: a review. *J. Hazard. Mater.* 283, 764–777. <http://dx.doi.org/10.1016/j.jhazmat.2014.10.021>.
- Bour, A., Mouchet, F., Cadars, S., Silvestre, J., Verneuil, L., Bagné, D., Chauvet, E., Bonzom, J.-M., Pagnout, C., Clivot, H., Fourquaux, I., Tella, M., Auffan, M., Gauthier, L., Pinelli, E., 2015b. Toxicity of CeO₂ nanoparticles on a freshwater experimental trophic chain: a study in environmentally relevant conditions through the use of mesocosms. *Nanotoxicology*.
- Boyle, D., Boran, H., Atfield, A.J., Henry, T.B., 2015. Use of an exposure chamber to maintain aqueous phase nanoparticle dispersions for improved toxicity testing in fish. *Environ. Toxicol. Chem.* 34, 583–588. <http://dx.doi.org/10.1002/etc.2840>.
- Bundschuh, M., Seitz, F., Rosenfeldt, R.R., Schulz, R., 2012. Titanium dioxide nanoparticles increase sensitivity in the next generation of the water flea daphnia magna. *PLoS One* 7. <http://dx.doi.org/10.1371/journal.pone.0048956>.
- Calow, P., 1997. General principles and overview. In: Calow, P. (Ed.), *Handbook of Ecotoxicology*. Blackwell Scientific Publications, Oxford, England.
- Chapman, J.C., 1995. The role of ecotoxicity testing in assessing water quality. *Aust. J. Ecol.* 20 (1), 20–27.
- Cleuvers, M., Weyers, A., 2003. Algal growth inhibition test: does shading of coloured substances really matter? *Water Res.* 37, 2718–2722. [http://dx.doi.org/10.1016/S0043-1354\(03\)00060-5](http://dx.doi.org/10.1016/S0043-1354(03)00060-5).
- Croteau, M.-N., Dybowska, A.D., Luoma, S.N., Valsami-Jones, E., 2011. A novel approach reveals that zinc oxide nanoparticles are bioavailable and toxic after dietary exposures. *Nanotoxicology* 5, 79–90. <http://dx.doi.org/10.3109/17435390.2010.501914>.
- Cupi, D., Hartmann, N.B., Baun, A., 2015. The influence of natural organic matter and aging on suspension stability in guideline toxicity testing of silver, zinc oxide, and titanium dioxide nanoparticles with daphnia magna. *Environ. Toxicol. Chem.* 34, 497–506. <http://dx.doi.org/10.1002/etc.2855>.
- Cupi, D., Hartmann, N.B., Baun, A., 2016. 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. *Ecotoxicol. Environ. Saf.* 127, 144–152. <http://dx.doi.org/10.1016/j.ecoenv.2015.12.028>.
- Dabrunz, A., Duester, L., Prasse, C., Seitz, F., Rosenfeldt, R., Schilde, C., Schaumann, G.E.,

- Schulz, R., 2011. Biological surface coating and molting inhibition as mechanisms of TiO₂ nanoparticle toxicity in *Daphnia magna*. PLoS One 6 <http://dx.doi.org/10.1371/journal.pone.0020112>. (e20112–e20112).
- ECHA, 2008. Guidance on information requirements and chemical safety assessment chapter R.10: characterisation of dose [concentration]-response for environment. Available from: http://echa.europa.eu/documents/10162/13632/information_requirements_r10_en.pdf.
- ECHA, 2014. Guidance on Information Requirements and Chemical Safety Assessment Chapter R.7c: Endpoint Specific Guidance, Version 2.0 November 2014.
- ECHA, 2016a. Guidance on Information Requirement and Chemical Safety Assessment Appendix R7-1 Recommendations for Nanomaterials Applicable to Chapter R7b - Endpoint Specific Guidance.
- ECHA, 2016b. Guidance on Information Requirement and Chemical Safety Assessment Appendix R7-2 Recommendations for Nanomaterials Applicable to Chapter R7c Endpoint Specific Guidance Draft (Public) Version 2.0.
- Gaiser, B.K., Biswas, A., Rosenkranz, P., Jepson, M.A., Lead, J.R., Stone, V., Tyler, C.R., Fernandes, T.F., 2011. Effects of silver and cerium dioxide micro- and nano-sized particles on *Daphnia magna*. J. Environ. Monit. 13. <http://dx.doi.org/10.1039/c1em10060b>.
- Gaiser, B.K., Fernandes, T.F., Jepson, M.A., Lead, J.R., Tyler, C.R., Baalousha, M., Biswas, A., Britton, G.J., Cole, P.A., Johnston, B.D., Ju-Nam, Y., Rosenkranz, P., Scown, T.M., Stone, V., 2012. Interspecies comparisons on the uptake and toxicity of silver and cerium dioxide nanoparticles. Environ. Toxicol. Chem. 31, 144–154. <http://dx.doi.org/10.1002/etc.703>.
- Gerloff, K., Landesmann, B., Worth, A., Munn, S., Palosaari, T., Whelan, M., 2016. The adverse outcome pathway approach in nanotoxicology. Comput. Toxicol. 1–9.
- Gottschalk, F., Sun, T., Nowack, B., 2013. Environmental concentrations of engineered nanomaterials: review of modeling and analytical studies. Environ. Pollut. 181, 287–300. <http://dx.doi.org/10.1016/j.envpol.2013.06.003>.
- Grillo, R., Rosa, A.H., Fraceto, L.F., 2015. Engineered nanoparticles and organic matter: a review of the state-of-the-art. Chemosphere 119, 608–619. <http://dx.doi.org/10.1016/j.chemosphere.2014.07.049>.
- Handy, R.D., Cornelis, G., Fernandes, T., Tsyko, O., Decho, A., Sabo-Attwood, T., Metcalfe, C., Steevens, J.A., Klaine, S.J., Koelmans, A.A., Horne, N., 2012a. Ecotoxicity test methods for engineered nanomaterials: practical experiences and recommendations from the bench. Environ. Toxicol. Chem. 31, 15–31. <http://dx.doi.org/10.1002/etc.706>.
- Handy, R.D., van den Brink, N., Chappell, M., Muehling, M., Behra, R., Dusinska, M., Simpson, P., Ahtaiainen, J., Jha, A.N., Seiter, J., Bednar, A., Kennedy, A., Fernandes, T.F., Riediker, M., 2012b. Practical considerations for conducting ecotoxicity test methods with manufactured nanomaterials: what have we learnt so far? Ecotoxicology 21, 933–972. <http://dx.doi.org/10.1007/s10646-012-0862-y>.
- Hansen, S., Hjorth, R., Skjolding, L., Bowman, D., Maynard, A., Baun, A., 2017a. A critical and in-depth analysis of the dossiers from the OECD sponsorship programme - has the OECD failed the nanosafety-community. Environ. Sci.
- Hansen, S.F., Sørensen, S.N., Skjolding, L.M., Hartmann, N.B., Baun, A., 2017b. Revising REACH guidance on information requirements and chemical safety assessment for engineered nanomaterials for aquatic ecotoxicity endpoints: recommendations from the EnvNano project. Environ. Sci. Eur. 29 (1), 14. <http://dx.doi.org/10.1186/s12302-017-0111-3>.
- Hartmann, N.B., Von der Kammer, F., Hofmann, T., Baalousha, M., Ottofuelling, S., Baun, A., 2010. Algal testing of titanium dioxide nanoparticles-testing considerations, inhibitory effects and modification of cadmium bioavailability. Toxicology 269, 190–197. <http://dx.doi.org/10.1016/j.tox.2009.08.008>.
- Hartmann, N.B., Engelbrekt, C., Zhang, J., Ulstrup, J., Kusk, K.O., Baun, A., 2013. The challenges of testing metal and metal oxide nanoparticles in algal bioassays: titanium dioxide and gold nanoparticles as case studies. Nanotoxicology 7, 1082–1094. <http://dx.doi.org/10.3109/17435390.2012.710657>.
- Hartmann, N.B., Jensen, K.A., Baun, A., Rasmussen, K., Rauscher, H., Tantra, R., Cupi, D., Gilliland, D., Pianella, F., Riedo Sintes, J.M., 2015. Techniques and protocols for dispersing nanoparticle powders in aqueous media-is there a rationale for harmonization? J. Toxicol. Environ. Health B Crit. Rev. 18, 299–326. <http://dx.doi.org/10.1080/10937404.2015.1074969>.
- Hartmann, N.B., Ågerstrand, M., Lützhöft, H.C.H., Baun, A., 2017. NanoCred: a transparent method to assess the regulatory adequacy of ecotoxicity data for nanomaterials – relevance and reliability revisited. NanImpact (accepted).
- Hjorth, R., 2016. Environmental Risk Assessment and Management of Engineered Nanomaterials – The Role of Ecotoxicity Testing. (PhD Thesis) Technical University of Denmark, Kgs. Lyngby, Denmark.
- Hjorth, R., Sørensen, S.N., Olsson, M.E., Baun, A., Hartmann, N.B., 2016. A certain shade of green: can algal pigments reveal shading effects of nanoparticles? Integr. Environ. Assess. Manag. 12, 200–202. <http://dx.doi.org/10.1002/ieam.1728>.
- Hjorth, R., Coutiris, C., Nguyen, N., Sevcu, A., Baun, A., Jøner, E., 2017a. Ecotoxicity testing and environmental risk assessment of iron nanomaterials for sub-surface remediation – recommendations from the FP7 project NanoRem. Chemosphere 182, 525–531. <http://dx.doi.org/10.1016/j.chemosphere.2017.05.060>.
- Hjorth, R., Holden, P., Hansen, S.F., Colman, B., Grieger, K., Hendren, C.O., 2017b. The role of alternative testing strategies in environmental risk assessment of engineered nanomaterials. Environ. Sci. Nano. <http://dx.doi.org/10.1039/C6EN00443A>.
- Holden, P.A., Gardea-Torresdey, J.L., Klaessig, F., Turco, R.F., Mortimer, M., Hund-Rinke, K., Hubal, E.A.C., Avery, D., Barcelo, D., Behra, R., Cohen, Y., Deydier-Stephan, L., Ferguson, P.L., Fernandes, T.F., Harthorn, B.H., Henderson, W.M., Hoke, R.A., Hristozov, D., Johnston, J.M., Kane, A.B., Kapustka, L., Keller, A.A., Lenihan, H.S., Lovell, W., Murphy, C.J., Nisbet, R.M., Petersen, E.J., Salinas, E.R., Scheringer, M., Sharma, M., Speed, D.E., Sultan, Y., Westerhoff, P., White, J.C., Wiesner, M.R., Wong, E.M., Xing, B., Horan, M.S., Godwin, H.A., Nel, A.E., 2016. Considerations of environmentally relevant test conditions for improved evaluation of ecological hazards of engineered nanomaterials. Environ. Sci. Technol. 50, 6124–6145. <http://dx.doi.org/10.1021/acs.est.6b00608>.
- Hund-Rinke, K., Simon, M., 2006. Ecotoxic effect of photocatalytic active nanoparticles TiO₂ on algae and daphnids. Environ. Sci. Pollut. Res. 13, 225–232. <http://dx.doi.org/10.1065/espr2006.06.311>.
- Hund-Rinke, K., Herrchen, M., Schlich, K., Schwirn, K., Völker, D., 2015. Test strategy for assessing the risks of nanomaterials in the environment considering general regulatory procedures. Environ. Sci. Eur. 27, 1–12. <http://dx.doi.org/10.1186/s12302-015-0053-6>.
- Hund-Rinke, K., Baun, A., Cupi, D., Fernandes, T.F., Handy, R., Kinross, J.H., Navas, J.M., Peijnenburg, W., Schlich, K., Shaw, B.J., Scott-Fordsmand, J.J., 2016. Regulatory ecotoxicity testing of nanomaterials – proposed modifications of OECD test guidelines based on laboratory experience with silver and titanium dioxide nanoparticles. Nanotoxicology 10, 1442–1447. <http://dx.doi.org/10.1080/17435390.2016.1229517>.
- ISO, 2012. Water Quality - Fresh Water Algal Growth Inhibition Test With Unicellular Green Algae. (Geneve, Switzerland).
- Jensen, L.H.S., Skjolding, L.M., Thit, A., Sørensen, S.N., Købler, C., Mølhav, K., Baun, A., 2016. Not all that glitters is gold-electron microscopy study on uptake of gold nanoparticles in *daphnia magna* and related artifacts. Environ. Toxicol. Chem. 36 (6), 1503–1509. <http://dx.doi.org/10.1002/etc.3697>.
- Juganson, K., Ivask, A., Blinova, I., Mortimer, M., Kahru, A., 2015. NanoE-Tox: new and in-depth database concerning ecotoxicity of nanomaterials. BEILSTEIN J. Nanotechnol. 6, 1788–1804. <http://dx.doi.org/10.3762/bjnano.6.183>.
- Kalman, J., Paul, K.B., Khan, F.R., Stone, V., Fernandes, T.F., 2015. Characterisation of bioaccumulation dynamics of three differently coated silver nanoparticles and aqueous silver in a simple freshwater food chain. Environ. Chem. 12, 662–672. <http://dx.doi.org/10.1071/EN15035>.
- Kennedy, A.J., Gunter, J.C., Chappell, M.A., Goss, J.D., Hull, M.S., Kirgan, R.A., Steevens, J.A., 2009. Influence of nanotube preparation in aquatic bioassays. Environ. Toxicol. Chem. 28, 1930–1938.
- Klaine, S.J., Koelmans, A.A., Horne, N., Carley, S., Handy, R.D., Kapustka, L., Nowack, B., von der Kammer, F., 2012. Paradigms to assess the environmental impact of manufactured nanomaterials. Environ. Toxicol. Chem. 31 (1), 3–14.
- Klimisch, H.-J., Andreae, M., Tillmann, U., 1997. A systematic approach for evaluating the quality of experimental toxicological and ecotoxicological data. Regul. Toxicol. Pharmacol. 25 (25), 1–5. <http://dx.doi.org/10.1006/rtp.1996.1076>.
- Kühnel, D., Nickel, C., 2014. The OECD expert meeting on ecotoxicology and environmental fate-towards the development of improved OECD guidelines for the testing of nanomaterials. Sci. Total Environ. 472, 347–353.
- Lützhöft, H., Hartmann, N., Brinch, A., Kjølholt, J., Baun, A., 2015. Environmental Effects of Engineered Nanomaterials: Estimations of Predicted No-Effect Concentrations (PNECs). Copenhagen, Denmark.
- Lynch, I., 2016. Compendium of projects in the European NanoSafety Cluster, 2016 edition. <http://www.nanosafetycluster.eu/www.nanosafetycluster.eu/home/european-nanosafety-cluster-compendium.html>.
- Mackevica, A., Skjolding, L.M., Gergs, A., Palmqvist, A., Baun, A., 2015. Chronic toxicity of silver nanoparticles to *Daphnia magna* under different feeding conditions. Aquat. Toxicol. 161, 10–16. <http://dx.doi.org/10.1016/j.aquatox.2015.01.023>.
- Miao, L., Wang, C., Hou, J., Wang, P., Ao, Y., Li, Y., Lv, B., Yang, Y., You, G., Xu, Y., 2015. Enhanced stability and dissolution of CuO nanoparticles by extracellular polymeric substances in aqueous environment. J. Nanopart. Res. 17. <http://dx.doi.org/10.1007/s11051-015-3208-x>.
- Minetto, D., Volpi Ghirardini, A., Libralato, G., 2016. Saltwater ecotoxicology of Ag, Au, CuO, TiO₂, ZnO and C60 engineered nanoparticles: an overview. Environ. Int. 92–93, 189–201. <http://dx.doi.org/10.1016/j.envint.2016.03.041>.
- Noss, C., Dabrunz, A., Rosenfeldt, R.R., Lorke, A., Schulz, R., 2013. Three-dimensional analysis of the swimming behavior of *Daphnia magna* exposed to nanosized titanium dioxide. PLoS One 8. <http://dx.doi.org/10.1371/journal.pone.0080960>.
- Notter, D.A., Mitrano, D.M., Nowack, B., 2014. Are nanosized or dissolved metals more toxic in the environment? A meta-analysis. Environ. Toxicol. Chem. 33, 2733–2739. <http://dx.doi.org/10.1002/etc.2732>.
- Oberdörster, E., 2004. Manufactured nanomaterials (fullerenes, C(60)) induce oxidative stress in the brain of juvenile largemouth bass. Environ. Health Perspect. 112, 1058–1062. <http://dx.doi.org/10.1289/ehp.7021>.
- OECD, 2000. Guidance Document on Aquatic Toxicity Testing of Difficult Substances and Mixtures. ENV/JM/MONO (2000)6. Organisation for Economic Co-operation and Development, Paris, France.
- OECD, 2005. Guidance Document on the Validation and International Acceptance of New or Updated Test Methods for Hazard Assessment. ENV/JM/MONO(2005)14. Organisation for Economic Co-operation and Development, Paris, France.
- OECD, 2012. Guidance on Sample Preparation and Dosimetry for the Safety Testing of Manufactured Nanomaterials, Organisation for Economic Co-operation and Development, Paris, France.
- OECD, 2014. Ecotoxicity and Environmental Fate of Manufactured Nanomaterials: Test Guidelines. Expert Meeting Report. Series on the Safety of Manufactured Nanomaterials No. 40. ENV/JM/MONO(2014)1, Organisation for Economic Co-operation and Development, Paris, France.
- OECD, 2016. Physical-Chemical Properties of Nanomaterials: Evaluation of Methods Applied in the OECD-WPMN Testing Programme. Paris, France.
- OECD, 2017. Draft Guidance Document on Aquatic and Sediment Toxicological Testing of Nanomaterials. Organisation for Economic Co-operation and Development, Paris, France.
- Palmqvist, A., Baker, L., Forbes, V.E., Gergs, A., von der Kammer, F., Luoma, S., Lützhöft, H.C.H., Salinas, E., Sørensen, M., Steevens, J., 2015. Nanomaterial environmental

- risk assessment. *Integr. Environ. Assess. Manag.* 11 (4), 519–519.
- Petersen, E.J., Zhang, L., Mattison, N.T., O'Carroll, D.M., Whelton, A.J., Uddin, N., Nguyen, T., Huang, Q., Henry, T.B., Holbrook, R.D., Chen, K.L., 2011. Potential release pathways, environmental fate, and ecological risks of carbon nanotubes. *Environ. Sci. Technol.* 45, 9837–9856. <http://dx.doi.org/10.1021/es201579y>.
- Petersen, E.J., Henry, T.B., Zhao, J., Maccuspie, R.L., Kirschling, T.L., Dobrovolskaia, M.A., Hackley, V., Xing, B., White, J.C., 2014. Identification and avoidance of potential artifacts and misinterpretations in nanomaterial ecotoxicity measurements. *Environ. Sci. Technol.* 48, 4226–4246.
- Petersen, E.J., Diamond, S.A., Kennedy, A.J., Goss, G.G., Ho, K., Lead, J., Hanna, S.K., Hartmann, N.B., Hund-Rinke, K., Mader, B., Manier, N., Pandard, P., Salinas, E.R., Sayre, P., 2015. Adapting OECD aquatic toxicity tests for use with manufactured nanomaterials: key issues and consensus recommendations. *Environ. Sci. Technol.* 49, 9532–9547. <http://dx.doi.org/10.1021/acs.est.5b00997>.
- Rasmussen, K., González, M., Kearns, P., Sintes, J.R., Rossi, F., Sayre, P., 2016. Review of achievements of the OECD working party on manufactured Nanomaterials' testing and assessment Programme. From exploratory testing to test guidelines. *Regul. Toxicol. Pharmacol.* 74, 147–160. <http://dx.doi.org/10.1016/j.yrtph.2015.11.004>.
- Römer, I., White, T.A., Baalousha, M., Chipman, K., Viant, M.R., Lead, J.R., 2011. Aggregation and dispersion of silver nanoparticles in exposure media for aquatic toxicity tests. *J. Chromatogr. A* 1218, 4226–4233.
- Römer, I., Gavin, A.J., White, T.A., Merrifield, R.C., Chipman, J.K., Viant, M.R., Lead, J.R., 2013. The critical importance of defined media conditions in *Daphnia magna* nanotoxicity studies. *Toxicol. Lett.* 223, 103–108.
- Sakka, Y., Skjolding, L.M., Mackevica, A., Filser, J., Baun, A., 2016. Behavior and chronic toxicity of two differently stabilized silver nanoparticles to *Daphnia magna*. *Aquat. Toxicol.* 177, 526–535. <http://dx.doi.org/10.1016/j.aquatox.2016.06.025>.
- Schwab, F., Bucheli, T.D., Lukhele, L.P., Magrez, A., Nowack, B., Sigg, L., Knauer, K., 2011. Are carbon nanotube effects on green algae caused by shading and agglomeration? *Environ. Sci. Technol.* 45, 6136–6144. <http://dx.doi.org/10.1021/es200506b>.
- Sekine, R., Khurana, K., Vasilev, K., Lombi, E., Donner, E., 2015. Quantifying the adsorption of ionic silver and functionalized nanoparticles during ecotoxicity testing: test container effects and recommendations. *Nanotoxicology* 9, 1005–1012. <http://dx.doi.org/10.3109/17435390.2014.994570>.
- Selck, H., Handy, R.D., Fernandes, T.F., Klaine, S.J., Petersen, E.J., 2016. Nanomaterials in the aquatic environment: a European Union-United States perspective on the status of ecotoxicity testing, research priorities, and challenges ahead. *Environ. Toxicol. Chem.* 35, 1055–1067. <http://dx.doi.org/10.1002/etc.3385>.
- Shaw, B.J., Liddle, C.C., Windeatt, K.M., Handy, R.D., 2016. A critical evaluation of the fish early-life stage toxicity test for engineered nanomaterials: experimental modifications and recommendations. *Arch. Toxicol.* 90, 2077–2107. <http://dx.doi.org/10.1007/s00204-016-1734-7>.
- Skjolding, L.M., 2015. Bioaccumulation and Trophic Transfer of Engineered Nanoparticles in Aquatic Organisms. (PhD Thesis) Technical University of Denmark, Kgs. Lyngby, Denmark.
- Skjolding, L.M., Kern, K., Hjorth, R., Hartmann, N., Overgaard, S., Ma, G., Veinot, J.G.C., Baun, A., 2014. Uptake and depuration of gold nanoparticles in *Daphnia magna*. *Ecotoxicology* 23, 1172–1183. <http://dx.doi.org/10.1007/s10646-014-1259-x>.
- Skjolding, L.M., Sorensen, S.N., Hartmann, N.B., Hjorth, R., Hansen, S.F., Baun, A., 2016. A critical review of aquatic ecotoxicity testing of nanoparticles - the quest for disclosing nanoparticle effects. *Angew. Chem. Int. Ed. Engl.* <http://dx.doi.org/10.1002/anie.201604964>.
- Sørensen, S.N., 2016. Aquatic Toxicity Testing for Hazard Identification of Engineered Nanoparticles. Technical University of Denmark.
- Sørensen, S.N., Baun, A., 2015. Controlling silver nanoparticle exposure in algal toxicity testing a matter of timing. *Nanotoxicology* 9, 201–209. <http://dx.doi.org/10.3109/17435390.2014.913728>.
- Sørensen, S.N., Hjorth, R., Delgado, C.G., Hartmann, N.B., Baun, A., 2015. Nanoparticle ecotoxicity-physical and/or chemical effects? *Integr. Environ. Assess. Manag.* 11, 722–724. <http://dx.doi.org/10.1002/ieam.1683>.
- Sørensen, S.N., Engelbrekt, C., Lützhöft, H.-C.H., Jiménez-Lamana, J., Noori, J., Alatraktchi, F.A., Delgado, C.G., Slaveykova, V.I., Baun, A., Slaveykova, Baun, Anders, 2016. A multi-method approach for disclosing algal toxicity of platinum nanoparticles. *Environ. Sci. Technol.* (Submitted).
- Stone, V., Hankin, S., Aitken, R., Aschberger, K., Baun, A., Christensen, F., Fernandes, T., Hansen, S.F., Hartmann Isabella Bloch, N., Hutchinson, G., Johnston, H., Micheletti, C., Peters, S., Ross, B., Sokull-Kluettgen, B., Stark, D., Tran, L., 2010a. Engineered Nanoparticles: Review of Health and Environmental Safety (ENRHES) (RPRT), Engineered Nanoparticles. European Commission.
- Stone, V., Nowack, B., Baun, A., van den Brink, N., von der Kammer, F., Dusinska, M., Handy, R., Hankin, S., Hasselov, M., Joner, E., Fernandes, T.F., 2010b. Nanomaterials for environmental studies: classification, reference material issues, and strategies for physico-chemical characterisation. *Sci. Total Environ.* 408, 1745–1754. <http://dx.doi.org/10.1016/j.scitotenv.2009.10.035>.
- Syberg, K., Hansen, S.F., 2015. Environmental risk assessment of chemicals and nanomaterials - the best foundation for regulatory decision-making? *Sci. Total Environ.* 541, 784–794.
- Tangaa, S.R., Selck, H., Winther-nielsen, M., Khan, F.R., 2016. Trophic transfer of metal-based nanoparticles in aquatic environments: a review and recommendations for future research focus. *Environ. Sci. Nano* 3 (1), 966–981. <http://dx.doi.org/10.1039/C5EN00280J>.
- Tejamaya, M., Römer, I., Merrifield, R.C., Lead, J.R., 2012. Stability of citrate, PVP, and PEG coated silver nanoparticles in ecotoxicology media. *Environ. Sci. Technol.* 46, 7011–7017.
- Tella, M., Auffan, M., Brousset, L., Issartel, J., Kieffer, I., Pailles, C., Morel, E., Santaella, C., Angeletti, B., Artells, E., Rose, J., Thiery, A., Bottero, J.-Y., 2014. Transfer, transformation, and impacts of ceria nanomaterials in aquatic mesocosms simulating a pond ecosystem. *Environ. Sci. Technol.* 48, 9004–9013. <http://dx.doi.org/10.1021/es501641b>.
- Tella, M., Auffan, M., Brousset, L., Morel, E., Proux, O., Chaneac, C., Angeletti, B., Pailles, C., Artells, E., Santaella, C., Rose, J., Thiery, A., Bottero, J.-Y., 2015. Chronic dosing of a simulated pond ecosystem in indoor aquatic mesocosms: fate and transport of CeO₂ nanoparticles. *Environ. Sci. Technol.* 49, 653–663. <http://dx.doi.org/10.1039/c5en00092k>.
- Unrine, J.M., Shoults-Wilson, W.A., Zhurbich, O., Bertsch, P.M., Tsyusko, O.V., 2012. Trophic transfer of Au nanoparticles from soil along a simulated terrestrial food chain. *Environ. Sci. Technol.* 46, 9753–9760.
- Van Hoecke, K., De Schampelaere, K.A.C., der Meeren, P., Lucas, S., Janssen, C.R., 2008. Ecotoxicity of silica nanoparticles to the green alga *Pseudokirchneriella subcapitata*: importance of surface area. *Environ. Toxicol. Chem.* 27, 1948–1957. <http://dx.doi.org/10.1897/07-634.1>.
- Van Hoecke, K., De Schampelaere, K.A.C., Ali, Z., Zhang, F., Elsaesser, A., Rivera-Gil, P., Parak, W.J., Smaghe, G., Howard, C.V., Janssen, C.R., 2013. Ecotoxicity and uptake of polymer coated gold nanoparticles. *Nanotoxicology* 7, 37–47. <http://dx.doi.org/10.3109/17435390.2011.626566>.
- Wickson, F., Hartmann, N.B., Hjorth, R., Hansen, S.F., Wynne, B., Baun, A., 2014. Balancing scientific tensions. *Nat. Nanotechnol.* 9, 870. <http://dx.doi.org/10.1038/nnano.2014.237>.