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Aquatic Ecotoxicity Testing of Nanoparticles—The Quest To Disclose Nanoparticle Effects

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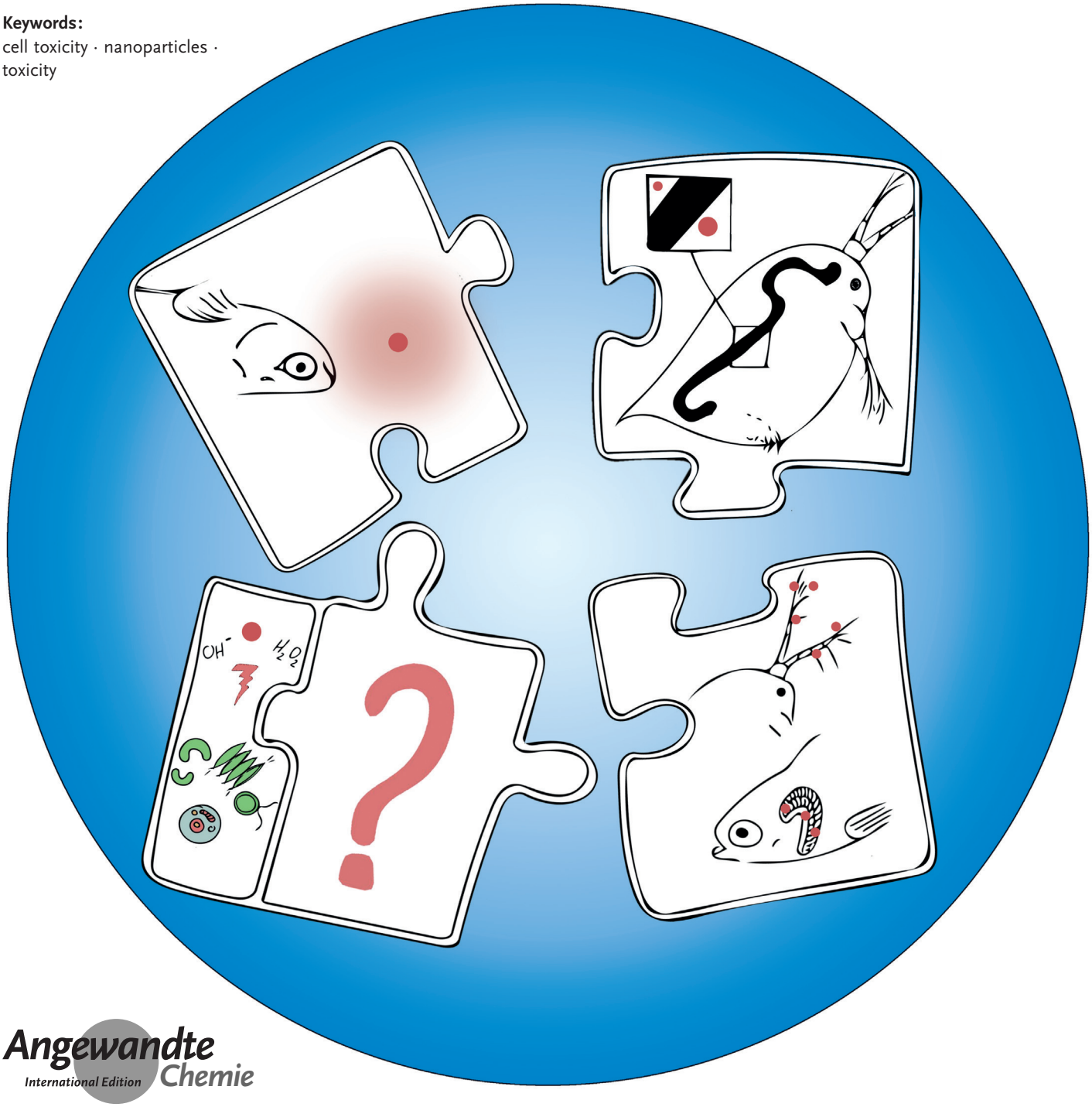

The number of products on the market containing engineered nanoparticles (ENPs) has increased significantly, and concerns have been raised regarding their ecotoxicological effects. Environmental safety assessments as well as relevant and reliable ecotoxicological data are required for the safe and sustainable use of ENPs. Although the number of publications on the ecotoxicological effects and uptake of ENPs is rapidly expanding, the applicability of the reported data for hazard assessment is questionable. A major knowledge gap is whether nanoparticle effects occur when test organisms are exposed to ENPs in aquatic test systems. Filling this gap is not straightforward, because of the broad range of ENPs and the different behavior of ENPs compared to "ordinary" (dissolved) chemicals in the ecotoxicity test systems. The risk of generating false negatives, and false positives, in the currently used tests is high, and in most cases difficult to assess. This Review outlines some of the pitfalls in the aquatic toxicity testing of ENPs which may lead to misinterpretation of test results. Response types are also proposed to reveal potential nanoparticle effects in the aquatic test organisms.

From the Contents

1. Introduction

During the last decade the use of engineered nanoparticles (ENPs) has rapidly increased and so has the number of consumer products claiming to contain ENPs.[1,2] A recent survey found that more than 2300 products containing nanoparticles are available to European consumers.[3] No exact production quantities of ENPs are currently publicly available. However, an estimation based on a company survey found the most produced ENPs to be TiO_2 (550–5500 t year^{-1}), SiO_2 (55–55 000 t year^{-1}), AlO_x (55–5000 t year^{-1}), ZnO (55–550 t year^{-1}), carbon nanotubes (CNTs; 55–550 t year^{-1}), FeO_x (5.5–5500 t year^{-1}), as well as CeO_x and Ag (both 5.5–550 t year^{-1}).[4] The use of ENPs in applications and products results in their release into the environment,[5–8] and any potential ecotoxicological effects of ENPs must, therefore, be evaluated. The first publication describing the detrimental effects of ENPs (fullerenes) on environmental organisms was published in 2004,[9] and while the effects observed in this study were later shown to be caused by solvent degradation products and not by the ENPs,[10] other early studies did find ENP effects in a range of organisms.[11–13] Since then, the field of nano-ecotoxicology has become well-established, with more than 750 papers published in total from 2006 to 2015.[14] The potential environmental exposure to ENPs along with emerging indications of ecotoxicity resulted in calls for frameworks and scientifically valid data suitable for environmental risk assessment.[15,16] In 2006, the OECD (Organisation for Economic Co-operation and Development) established the "Working Party on Manufactured Nanomaterials" (OECD WPMN) to "… *ensure that the approaches for hazard, exposure and risk assessment for manufactured nanomaterials are of a high quality, science-based and internationally harmonized*".[17] Since then, the amount of available information has rapidly expanded as a result of large national and international funding initiatives.[18] However, it soon became apparent that the knowledge on naturally occurring nanoparticles (1–100 nm) and colloids (1–1000 nm) was not sufficient to establish a detailed model of the fate and behavior of ENPs in the environment.[19] Without such a fundamental understanding of the fate and behavior of ENPs, risk assessment becomes an immensely challenging task.[20] Similarly, the assessment of the effects needed to complete the risk assessment of ENPs is hampered by knowledge gaps with regard to the causes of the biological responses observed when testing ENPs. The huge diversity of ENPs, in terms of chemical identity, particle size, and surface functionalization, makes it a difficult task to identify the inherent particle properties that determine the ecotoxicity of ENPs.[21,22] The lack of common descriptors, as well as the fundamental difference between ENPs and soluble compounds, raises serious concerns about the suitability of current guideline tests when applied to ENPs, and hence the

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relevance and reliability of the results obtained when it comes to risk assessment.[23] Unlike dissolved chemicals, the fate and behavior of ENPs in ecotoxicity tests, as well as in the environment, are not determined by partition coefficients.[24,25]

The major difference between ENPs and dissolved chemicals from a practical ecotoxicity testing point of view is that ENPs represent a solid phase with a confined physical shape similar to that of poorly soluble compounds. Thus, a clear boundary exists between the solid and liquid phase in ENPs in aqueous suspensions and the system will, hence, be affected to a greater degree by physical forces than molecular transformations.[27] A range of processes that can result in dynamic exposure concentration in tests with both dissolved chemical and ENPs are exemplified in Figure 1. However, ENPs in some cases exist as partly soluble entities that release ions (e.g. in the case of partly soluble metallic ENPs such as Ag, ZnO, and CuO), as illustrated in Figure 2. In colloidal science, a colloidal dispersion is traditionally defined as small particles that are stable in a liquid and undetectable under normal light conditions, but large enough to scatter an intensive beam of light.[28,29] Aqueous colloidal dispersions of ENPs can be synthesized by adding stabilizing agents (e.g. citrate) or by particle coating (e.g. polyethylene glycol (PEG) or polyvinylpyrrolidone (PVP)). In general, Ag ENPs and Au ENPs are stabilized in this way prior to ecotoxicity testing. Other ENPs are produced by milling to create dry powders (e.g. TiO_2 and ZnO ENPs), which then have to undergo a dispersion procedure to obtain a suspension suitable for ecotoxicity testing.[30] The overall stability of suspended ENPs is governed by the characteristics of both the particle and the media.[31,32] Thus, when used in aquatic ecotoxicity testing, the ENPs will vary in state and behavior, as illustrated in Figure 2, depending largely on the testing media.

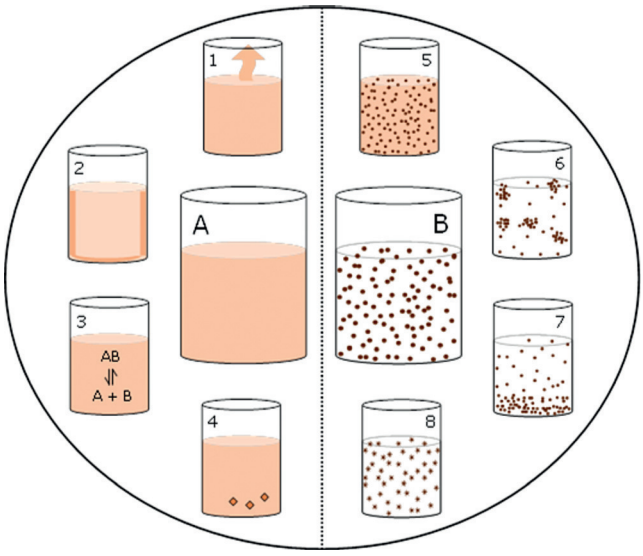

Figure 1. Different processes that can cause an unstable exposure concentration during exposure of a dissolved compound (A) and suspended ENPs (B). For a dissolved compound, these processes include 1) evaporation, 2) adsorption to test vessels, and 3) speciation reactions, including complexation and dissociation, and 4) precipitation of undissolved chemical and/or insoluble reaction products. For ENPs in suspension, the processes include 5) dissolution, 6) agglomeration/aggregation, 7) sedimentation, and 8) surface transformations and reactions, including catalytic effects, redox reactions, and changes to coatings/stabilizing agents (adapted from Ref. [26]).

Lars Michael Skjolding completed his PhD in 2015 at the Technical University of Denmark, in the Department for Environmental Engineering with Prof. Anders Baun, including a research stay at Gothenburg University (Sweden) in the group of Dr. Joachim Sturve. He remained as a postdoctoral researcher in the group of Prof. Anders Baun, focusing on ecotoxicological effects and environmental risk assessment of nanomaterials with a special focus on bioaccumulation, trophic transfer, imaging, and characterization of engineered nanoparticles in aquatic organisms.

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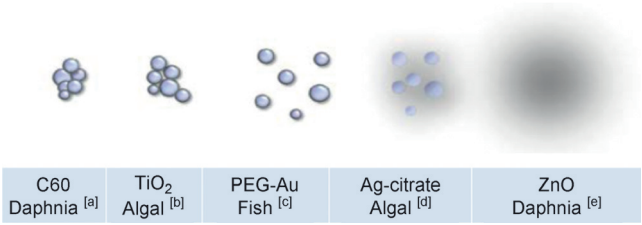

Figure 2. Different possible states of ENPs in an aquatic media together with examples of ENPs in different standardized testing media for ecotoxicity testing. From left to right: aggregation, agglomeration, single particles, partly dissolved, and fully dissolved. References: [a] Baun et al.,[11] [b] Hartmann et al.,[59] [c] Skjolding et al.,[94] [d] Sørensen et al.,[74] [e] Cupi et al.[145]

Although many reviews have been published that focus on, for example, 1) environmental realism of test conditions,[33] 2) modeling approaches,[34] 3) effects of organic matter on toxicity,[35] and 4) ecotoxicological effects of various ENPs,[36–39] there still seems to be a lack of reviews assessing nanoparticle effects in terms of test setups and effects observed. Handy et al.[40] and Petersen et al.[41] reviewed and presented a wide range of practical considerations to be considered when conducting ecotoxicological tests with ENPs. However, the practical considerations and the confounding factors of ENPs in test setups have to, our knowledge, not been reviewed in regard to assessing the effects of the nanoparticles. Isolating the nanoparticle effect caused by the novel intrinsic properties of the ENPs because of their size is not possible from the currently published reviews. Throughout this Review the implications of the properties of ENPs and their related behavior in test media before, during, and after ecotoxicity testing will be evaluated with a specific focus on the base set of aquatic organisms (fish, crustaceans, and algae) used for classification, labeling, and concentration–response assessment to determine the nanoparticle effect. For these organisms, standard toxicity tests and guidelines exist, and data are available for these tests as well as for scientific studies not necessarily following these guidelines.[14]

The general theme of this Review is presented in Figure 3, which illustrates three different types of responses that may influence or even dominate the aquatic toxicity of ENPs to a degree, and which make it difficult to determine nanoparticle effects. Herein nanoparticle effects are defined as effects related to the intrinsic properties of the ENPs as a result of the decrease in size compared to their bulk counterparts. The response types relate to the 1) physical attachment of ENPs to test organisms 2) dissolution of ENPs in the aqueous media, 3) discrete localization of ENPs within the test organisms. Furthermore, we also recommend that known effect mechanisms of ENPs, for example, the generation of reactive oxygen species (ROS), are included in ecotoxicity testing strategies. These effects will be evaluated in relation to aquatic ecotoxicological tests to address findings of excess toxicity beyond what can be explained by physical attachment, dissolution and discrete localization. This is what will be referred to as a nanoparticle effect.

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Anders Baun is Professor of risk assessment of nanomaterials and head of the Environmental Chemistry section at the Department of Environmental Engineering, Technical University of Denmark. He has an MSc in Environmental Engineering (DTU, 1994) and completed a PhD with Prof. Niels Nyholm at the Department of Environmental Engineering, Technical University of Denmark, on the application of biotests for the characterization of contaminated water samples. His postdoctoral research focused on risk assessment as well as chemical and biological aspects of groundwater pollution. His main research area is now the environmental risk assessment of nanomaterials.

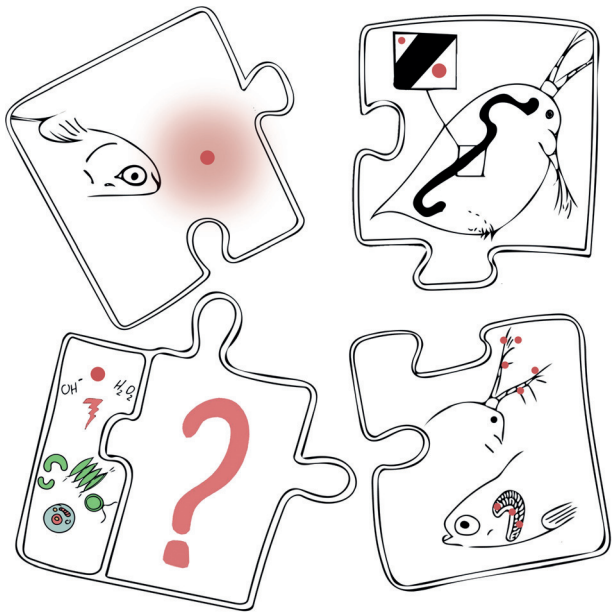

Figure 3. Three types of responses that might influence or dominate the aquatic toxicity of ENPs and mask the nanoparticle effect in aquatic organisms. The responses are related to: Effects related to the dissolved fraction (top left), effects of internalization and translocation of ENPs because of their small size (top right), physical effects of the nanoparticles (bottom right), and the nanoparticle effect with a proposed mode of action related to the generation of reactive oxygen species (bottom left).

2. Physical Effects of ENPs in Aquatic Toxicity Tests

Here we evaluate the results of ecotoxicity tests with the base set of organisms for risk assessment (fish, crustaceans, and algae) in terms of the physical effects of ENPs. Physical effects are in this context defined as mechanical effects not associated with chemical reactions caused by the ENPs or associated with dissolution of the ENPs to ionic species. Figure 4 illustrates test setups which can identify certain physical effects in tests with algae and daphnids (Figure 4 A,B).

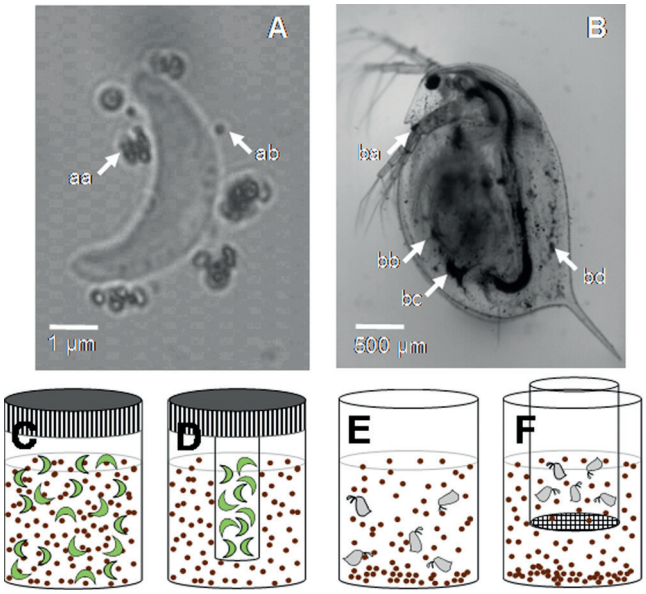

Figure 4. Top: Alga and daphnids exposed to C_{60} in aqueous suspension (adapted from Ref. [42]). Bottom: setups applied for the investigation of physical effects (adapted from Ref. [43]). A) The green alga *Pseudokirschneriella subcapitata* surrounded by C_{60} aggregates >1 µm (aa) and <200 nm (ab). B) The freshwater crustacean *Daphnia magna* after 48 h exposure to a suspension of 3 mg C_{60} L^{-1} in a bioaccumulation study with 49 µg L^{-1} phenanthrene. The black color of the digestive tract shows the uptake of C_{60}. Aggregates of C_{60} were also found on the antennae (ba), the thoracic legs (bb), the postabdominal claw (bc), and in the brood chamber (bd). The test setups applied for algae (C) and daphnia (E), where processes such as agglomeration, aggregation, sedimentation, and sorption to both test vessels and test organisms may interfere with the test outcome and cause physical effects. D) Physical shading effects in algal tests may be investigated by a double-vial setup, where algae are contained in a smaller inner vial, surrounded by the ENP suspension in a larger outer vial. F) Physical immobilization of daphnids arising from contact with larger aggregated/sedimented ENPs can be avoided by keeping the daphnids in a mesh-bottomed beaker inserted into larger beakers containing ENP suspensions (modified from Ref. [43]).

Reviews of the available ecotoxicity literature of ENPs show that attempts to reveal a nanoparticle effect in many cases have resulted in the use of high concentrations (>10 mg L^{-1}).[14] However, it has been shown that increased concentrations can change the behavior and corresponding fate of ENPs in water.[44] Likewise, high concentrations of ENPs can possibly cause effects which are not due to an actual toxic response, but instead caused by an overloading of ENPs in the test organisms. This may lead to physical effects such as shading in algal tests,[26,45] altered feeding behavior or impaired mobility of crustaceans,[46,47] and increased mucus production in fish.[48] It should be noted that the limited environmental realism in relation to the use of high concentrations have been extensively discussed and reviewed elsewhere,[34] thus the focus of this Review will be towards aquatic ecotoxicity testing within the current hazard identification paradigm and confounding factors that can arise from this approach.

Concentration, in terms of particles per volume, plays a crucial role in the potential for collisions between the ENPs and the organism. Therefore, it should be kept in mind that the total number of ENPs and their surface area is a function of the decreasing size of ENPs. For example, the total particle surface area for 100 nm ENPs is about four times as high as for 200 nm ENPs at the same mass-based concentration. There will be inherent collision interactions with any type of particles through surface charge interaction and Brownian mechanisms for particle collisions.[13] On a macroscale, the interaction between a larger moving organism (larger than the ENPs) and ENPs will mainly be governed by the shear gradient, which is correlated with the movement of the organism. The collision frequency will mainly depend on the velocity of the organism, that is, organisms with a higher swimming velocity encounter more collisions than organisms with a lower velocity.[47] Consequently, to what extent ENPs or agglomerates will encounter an organism will depend on the swimming velocities and the concentration of the ENPs (if the size of the ENPs is kept constant). Whether the collision will result in attachment to the organism is determined by the interacting surface charges on the organism and the ENPs.[49] This means that there will be an increase in ENP–organism interactions as the ENP concentration increases, independent of the mechanism of the toxic effect. Both electrostatic attractions and receptor–ligand interactions have been observed with a range of different algae and different types of ENPs,[50–52] but different functional groups can also affect the interactions[53] and possibly induce adsorption.

Physical effects have often been reported from tests of the inhibition of algal growth rate in the presence of ENPs. Inhibition of algal growth rate has been speculated to be influenced by pH changes in the vicinity of the algae, limited nutrient availability, or light availability (shading) as a result of physical effects.[26,45,54,55] Several studies have investigated the influence of ENP shading using setups that separate the algae from the ENP suspensions by the way that the light penetrates the ENP suspensions before reaching the algae (Figure 5). Such studies have both confirmed and rejected the influence of shading on growth inhibition.[54–56] However, although such tests are useful for investigating shading caused by dispersion turbidity they will not detect localized shading on a cellular level caused by the encapsulation of the cells by ENPs, which can, therefore, not be excluded.[54] Significant shading was identified for platinum ENPs of relatively low toxicity with an EC_{50} value at the higher end of the classification range of 10–100 mg L^{-1}.[26] This exemplifies the need to account for shading effects when testing for hazard

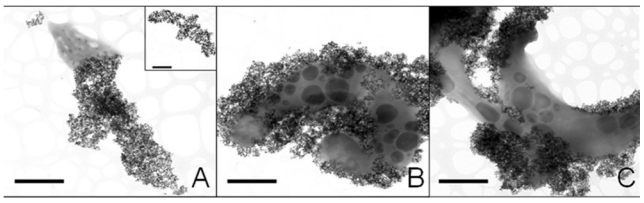

Figure 5. Algal cells with a high degree of attached TiO_2 ENPs after A) 24 h, B) 48 h, and C) 72 h exposure at 35 mg L^{-1} (scale bars: 2 μm; reprinted from Ref. [59] with permission).

identification purposes. Hjorth et al.[57] suggested a way to unveil such effects in algal tests by using relative changes in algal pigments, but this approach remains to be validated experimentally. Under the current standard testing scheme, physical effects are considered to be a confounding factor rather than part of the intrinsic toxicity of a chemical.[58]

Physical effects on *Daphnia magna* have also been observed after exposure to relatively high concentrations (10 mg L^{-1}) of CeO_2 ENPs, whereby aggregates sorbed to the carapace were observed.[60] The clinging of the CeO_2 ENP aggregates to the carapace did not significantly affect the toxicity over 96 h. However, in a long-term study over 21 days, the 10 mg L^{-1} CeO_2 ENPs caused 100% mortality after 7 days of exposure, while concentrations of 3 mg L^{-1} CeO_2 ENPs did not result in any mortality after 21 days. There were no apparent signs of impaired feeding after 96 h of exposure to 10 mg L^{-1} CeO_2 ENPs, as observed by lipid storage droplets surrounding the intestine as well as algae in the digestive tract (indicated by a green-colored digestive tract). However, growth was inhibited after 96 h, thus suggesting increased energy usage for, for example, feeding and depuration of non-nutritious CeO_2 ENPs taking up space in the gut. Furthermore, decreased molting was observed after exposure to smaller CeO_2 ENPs compared to larger micrometer-sized CeO_2 particles at high concentrations (10 mg L^{-1}).[60] It should be noted that no dissolution was observed in the experiment, thus the effect is most likely related to a physical effect of the ENPs. This may be through the attachment or through increased energy usage for depuration. Furthermore, *D. magna* is able to regulate its filtering activity based on food availability[61] and it has been suggested that if particles (or ENP agglomerates) are mistaken for food this can cause an increase in filtering activity.[62] Hence, the ingestion of non-nutritious ENP agglomerates combined with higher energy usage because of increased filtering activity could explain some of the observed effects.

A decreased swimming velocity of two daphnia species (*D. similis* and *D. pulex*) has also been observed after exposure to aggregated CeO_2 ENPs at a concentration of 1 mg L^{-1}.[46] The hopping frequency (frequency that daphnia beat their antennas to move) was not affected; correspondingly, the daphnia would cover less distance with the same energy input because of the lower swimming velocity indirectly influencing energetic metabolism. Conversely, Lovern et al. found a significant effect on the hopping frequency after exposure to 0.26 mg L^{-1} of C_{60} and a fullerene derivate ($C_{60}H_xC_{70}H_x$).[63] Daphnia generally feed by filtering water; however, as the energy usage for this process is high, daphnia tend to seek areas with high algae concentration for a maximum yield. Consequently, with a decreased swimming velocity, the energy associated with movement would increase, thus decreasing the energy for growth, as observed by Gaiser et al.,[60] and possibly reproduction, hence affecting population dynamics. Furthermore, the swimming velocity is also closely related to the respiration rate. While swimming, the daphnia generate a water current that facilitates the exchange of oxygen for respiration, but also for aeration of the brood pouch.[64,65]

The binding of ENPs to the exterior was also observed in the case of TiO_2 ENPs at a concentration of 2 mg L^{-1}.[47] TiO_2 ENP aggregates continuously adhered to the surface of *D. magna* during 96 h of exposure. The TiO_2 ENP aggregates were completely removed after the first molting, but reformed on the exoskeleton within 1 h and continuously grew to the end of the experiment (96 h). In a subsequent experiment it was shown that the first molting occurred similarly to controls within the first 36 h of exposure, whereas the second molting phase was significantly delayed in *D. magna* exposed to 2 mg L^{-1} TiO_2 ENPs. A lower molting success of only 10% was also observed, and this was despite a decreasing concentration of the water phase from 2 mg L^{-1} to 1.5 and 0.8 mg L^{-1} after 24 h and 72 h, respectively.[47] The Daphnia were not fed during the 96 h and, thus, starvation should be acknowledged as a confounding factor. Even so, sub-lethal effects of, for example, reduced molting occurred at concentrations < 1 mg L^{-1}. A similar attachment of Ag ENPs to the carapace was observed at concentrations as low as 0.01 mg L^{-1}, which also affected the swimming ability.[66] Although physical effects are identified for algae and crustaceans, the physical effects in fish are less frequently reported. In the early life stages, the adsorption of Ag ENPs to the chorion of fish embryos has been observed, similar to the adsorption on algae or crustaceans, and proposedly interferes with the pore channels.[67] In adult fish, ENPs have been shown to readily interact and adhere to gill surfaces.[68–71] Studies have found swelling and increased mucus production, which is a natural response to irritants at the gill sites.[48] Although this is not acutely problematic, the chronic effects could be pronounced.

Increased ventilation rates have been observed in a fish study with single-walled carbon nanotubes (SWCNTs); however, the mechanisms causing this effect were not documented in the study.[48] Aggressive behavior was also seen in the study, which could be caused by respiratory distress, although without further evidence it cannot be excluded that these effects are caused by the adhesion of ENPs to the gills. Although the adhesion of ENPs could be related to stress and potentially inhibit movement and predation, and therefore lead to changes in food web dynamics, there are still major knowledge gaps on the effects of the adhesion of ENPs and the subsequent interactions with biological interfaces.[72]

Clearly, ENPs cause sub-lethal effects associated with physical interaction not only at high concentrations (> 10 mg L^{-1}) but also at lower concentrations (< 1 mg L^{-1}). The sub-lethal effects at relatively low concentrations have most frequently been reported for crustaceans and were

related to energy deficits associated with, for example, reduced molting, feeding traits, and depuration of non-nutritious particles in the ingestible size range of crustaceans (e.g. *D. magna*). Therefore, physical effects need to be either accounted for or eliminated as far as possible. Alternatively, a change in testing procedures is necessary to acknowledge that ENPs may cause physical effects or contribute to the observed biological effects in other ways than those known for dissolved chemicals.

3. The Influence of the Dissolved Fraction on the Aquatic Toxicity of ENPs

Aqueous solubility plays a key role in the environmental fate, behavior, and effects of chemicals. Poorly soluble substances (organic and inorganic) receive special attention in guideline and standard ecotoxicity testing because of the link between solubility and the resulting biological effects. This issue has been addressed through specific guidelines and guidance documents.[58] In the traditional test for "conventional" chemicals, the dissolved fraction is considered to be responsible for biological effects. Hence, it is often specified in test guidance documents for aquatic ecotoxicity tests that the tested concentrations should not exceed the limit of the water solubility of the compound, as this would lead to erroneous results and the test guidelines are most easily applied to readily soluble substances. Hence, the aim of additional guidance documents, such as the "OECD Guidance Document on Aquatic Toxicity Testing of Difficult Substances and Mixtures",[58] is mainly to facilitate the dissolution of poorly soluble substances to create stock solutions which can be used to facilitate a stable exposure, for example, by using a solvent or by extended stirring, ultrasonication, and/or heating to increase dissolution. However, these methods have all been found to influence the ecotoxicity of ENPs and could, therefore, hamper interpretation of the results obtained.[22]

Dissolution becomes relevant for ENP ecotoxicity when ions or molecules are released from the ENP surface to the media. ENPs may be soluble to varying extents—from insoluble to poorly soluble, partly soluble, or completely soluble—depending on the ENP as well as on the testing conditions (e.g. the test medium), as illustrated in Figure 2. Understanding dissolution is fundamental to the interpretation of the ecotoxicity effects of ENPs. The dissolution kinetics (rate of solubility) and equilibrium solubility (amount of dissolved material) of ENPs will influence their biological effects in ecotoxicological tests. Ecotoxicity effects may be dominated by the particulate form of the ENPs and/or the presence of dissolved species,[41,73] which may contribute to toxicity through different modes of action. The overall effects will, therefore, be the combined effects of the dissolved and the particulate form of the material. This dynamic relationship of toxicity was clearly shown using an algal ^{14}C-assimilation test over 2 h using *P. subcapitata* (Figure 6A) and a 24 h acute toxicity test with *D. magna* (Figure 6B) exposed to Ag ENPs aged between 1 and 5 days, which resulted in significantly different EC_{50} values.[74]

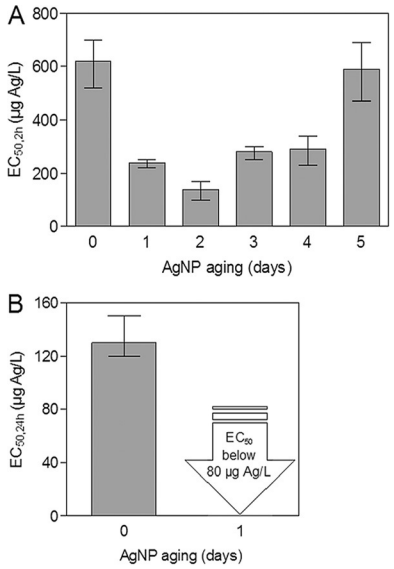

Figure 6. EC_{50} values as a function of Ag ENP aging using A) an algal ^{14}C-assimilation test for 2 h and B) a 24 h acute toxicity test with *D. magna* (note: aging Ag ENPs for 1 day resulted in 100% immobility at the lowest concentration of 80 µg Ag L^{-1}). The columns represent mean effect concentrations (EC_{50} values), and the bars 95% confidence intervals (adapted from Ref. [75]).

Despite a general trend of increasing dissolution with decreasing particle diameter, the relationship is not straightforward and is difficult to validate. For example, the presence of a particle coating can influence solubility and mask size-related changes in dissolution.[73] Bian et al.[76] studied the size dependency in the dissolution of ZnO ENPs by using three different particle sizes (4, 15, and 241 nm). The results demonstrated a qualitative trend of general enhanced dissolution for smaller particles (4 nm and 15 nm compared to 241 nm). However, when comparing the two smallest particle sizes, the 15 nm ZnO ENPs dissolved to a greater extent than the 4 nm particles, which is a deviation from the generally assumed size-dependent dissolution. Possible explanations include increased aggregation of the smaller particles or differences in surface tension hampering a quantitatively prediction of dissolution by classical thermodynamics.[76]

The tendency of aggregated ENPs to dissolve less could be explained by the decreased specific surface area of the aggregates compared to agglomerates or dispersed ENPs, and evidence to both support and contradict this theory are described in the literature. One study found the dissolution of Ag ENPs to be controlled by the initial Ag ENP size rather than by subsequent aggregation.[77] Conversely, another study found the dissolution of Ag ENPs to be slower at higher ionic strength, in which higher aggregation was observed, compared to in media with a lower ionic strength.[78] In support of these findings, a seemingly shape-dependent difference in the dissolution of spherical and rod-shaped CuO ENPs has been suggested to instead be an indirect result of shape-dependent differences in aggregation.[79] Furthermore, Baek and An[80] have observed that the ENP concentration influences the dissolution kinetics and equilibrium dissolution of Cu ENPs, whereby the dissolution rate decreased as the ENP concen-

tration increased. The concentration may in this case have an indirect effect on dissolution through a concentration-dependent increase in aggregation. Further investigations are needed to clarify the role of aggregation in ENP dissolution.

Although it is so far not well understood which physical and chemical parameters of the ENPs and the testing media control dissolution, it is widely agreed that Ag, ZnO, and CuO ENPs dissolve to a certain extent after addition to test media. Furthermore, it is well-established that the toxicity of these ENPs is partly—and in some cases fully—caused by the dissolved metal ions and complexes. As an example, Ag ENPs are known to release Ag^+ ions in the presence of water and oxygen,[81] and positive correlations have been found between the dissolution rate of Ag ENPs and their toxicity towards, for example, *D. magna*.[82,83] A similar trend was highlighted in a review on ZnO ENPs, and clearly indicates that the dissolved fraction caused the observed toxicity.[84] However, in some cases the toxicity of CuO ENPs and Ag ENPs could not be fully explained by the toxicity of the dissolved fraction. As an example, the expression profile of Ag ENPs differed from that of a dissolved control ($AgNO_3$) in *D. magna*, with Ag ENPs affecting protein metabolism and signal transduction while $AgNO_3$ affected developmental processes.[85] Similarly, the gene expression pattern in *D. rerio* was different after exposure to Ag ENPs compared to the ionic exposure.[86] However, the mechanisms driving those differences in toxicity are poorly understood. Nevertheless, it is recommended that already-established models for the speciation of trace metals, for example, the free ion activity model,[87] biotic ligand model,[88] and existing models for bioaccumulation, for example, biodynamic model[89] and chemo- and biodynamic models,[90] should be used to account for the effect of the dissolved fraction, thus facilitating interpretation of a possible nanoparticle effect.

Ecotoxicity testing of ENPs that may dissolve must take into account the complex relationship between ENP properties and media composition as well as keep a strong focus on dissolution kinetics and the non-equilibrium behavior of the dissolution of ENPs during toxicity testing. This will in most cases require additional (advanced) chemical analyses and some method development to be sure that the results reflect the actual in situ dissolution behavior of ENPs under the testing conditions.

4. Uptake, Internalization, and Translocation of Engineered Nanoparticles in Aquatic Organisms

Stone et al.[22] stated in a comprehensive review that "only few studies have dealt with bioaccumulation of metal ENPs". In 2013, Hou et al.[91] reviewed 65 papers on the biological accumulation of ENPs in water, soil, or sediment. They found that crustaceans ($n = 21$) and fish ($n = 27$) were the most tested organisms, and linear correlations were found between the concentration in the water phase and the concentration in the organism. However, this method inherently disregards the underlying mechanisms which would explain this linear correlation. In the reviewed literature, the uptake of ENPs by daphnia generally exceeded that observed in fish approximately a hundred times. However, these numbers should be taken with caution, as in many of the studies the observed body burden was mainly attributed to retention of ENPs in the gut section and thus not translocated in the organism. Furthermore, it should be noted that the aggregation of ENPs could render them in a size range for active filtration by *D. magna*, for example, which retains particles in the size range of 0.4 to 40 μm.[92,93]

A more recent review of the current literature (2013–2015) by Skjolding[94] considered 88 relevant scientific papers spread across aquatic (48 papers), sediment (12 papers), and terrestrial organisms (28 papers), with the most frequent aquatic species being daphnia (17 papers) and fish (14 papers) in the groups of invertebrates and protozoa, and aquatic vertebrates. ENPs have been shown to enter organisms at different trophic levels in unicellular algae compared to fish; however, it is still unclear if ENPs are internalized into cells and tissue and to what extent they are translocated in the organisms. Furthermore, it remains to be studied in detail whether the presence of ENPs in organs of aquatic organisms give rise to biological effects. Although the internalization of different ENPs in vitro has frequently been reported,[95] the literature on internalization mechanisms and evidence whereof in vivo is more scarce.

In the base set of ecotoxicity tests (algae, daphnia, and fish), algae are included as representatives of primary producers. For microalgae, the relatively thick and tough cell wall presents a barrier, commonly assumed to prevent the internalization of ENPs. However, cell walls have pores with diameters of 5–20 nm, and their permeability is also compromised during cell cycling.[96] Furthermore, internalization could be induced by, for example, cell wall pitting or membrane damage associated with dissolved metal species or generation of ROS, as discussed below. Various techniques have provided experimental evidence for the internalization of Ag, CuO, and TiO_2 nanoparticles in different algal species, although the uptake mechanisms and routes are not clear.[96] A study on the internalization of AgNPs in algae identified AgNPs inside cells regardless of whether they were exposed to AgNPs or ionic silver. Thus, AgNPs may not only be internalized as particles, but may also form inside the cell from assimilated silver ions,[97] thus complicating the identification of uptake mechanisms. Similar effects with regards to the formation or internalization of ENPs have more frequently been observed in plants (for a review, see Schwab et al.[98]).

Currently, there is limited knowledge on the effects following the internalization of ENPs in algae. A study indicated that the toxicity of CuO NPs was due to internalization and intracellular interactions, and that the primary mechanism was intracellular generation of ROS.[99] These conclusions were based on measured body burdens in washed algal cells, intracellular generation of ROS, and inhibition of photosystem II (PSII) activity of the alga *C. reinhardtii* when exposed to coated and bare CuO NPs and their dissolved fractions. Higher uptake was found in the presence of CuO NPs than on exposure to dissolved fractions only, and higher body burdens and toxicity was found for the coated CuO NPs compared to the bare NPs. Similarly, the internalization of Ag

NPs in the mixotrophic alga *Ochromonas danica* has been proposed as a pathway for algal toxicity.[100] This was based on the identification of intracellular Ag NPs by electron microscopy, and the finding that toxicity occurred even when free Ag^+ was eliminated from the medium through addition of glutathione.

The issue of internalization and translocation becomes more pronounced on moving up the trophic chain to crustaceans and fish. Although feeding traits of, for example, *D. magna* could promote the uptake of agglomerated or aggregated ENPs as mentioned in Section 2, the ingestion of primary ENPs is still possible through intake of water for digestion by Daphnia[101] or drinking of water by fish.[102] Although many studies have focused on aqueous exposure,[14,94] it is clear that dietary exposure as a result of the physical attachment of ENPs to food sources would enable access of ENPs to, for example, the gut region.[103]

Different methods have been used to elucidate the potential internalization of ENPs and the associated effects in, for example, Daphnia. TEM studies have demonstrated the presence of ENPs in the gut of *D. magna*.[104–106] Confocal microscopy images of whole organisms suggested translocation of, for example, polystyrene ENPs[107] and quantum dots.[108] Dark-field optical microscopy has also been used to show the ingestion of Ag rods in *D. magna*.[109] One of the most frequently used methods for studying the cellular interactions associated with the internalization and translocation of the ENPs is TEM. However, studies have shown that careful elemental analysis is required to avoid misinterpretation of high-density entities which could be mistakenly taken for ENPs.[110–112] Although theoretically feasible, the uptake of ENPs into epithelial gut cells has so far not been observed in vivo for, for example, *D. magna* (SWCNTs: Ref. [111], Au ENPs: Refs. [114,113,114], TiO_2 ENPs: Ref. [115]). In contrast to the above observations, some studies did observe uptake through the microvilli (ZnO ENPs: Ref. [116]; CuO ENPs: Ref. [105]; QDs: Ref. [108]). However, these studies have rarely been conducted with an ionic control or a bulk version of the same materials as the ENPs, and this prevents any firm conclusions with regards to the influence of nano-sized particles. In fish, the highest concentration of ENPs has also been found in the gut,[117–121] whereas elevated concentrations of different ENPs have also been found in, for example, gills, liver, and brain.[119–121] This shows a potential for translocation, even though the concentrations are lower than the concentrations observed in the gut. A systematic study by Osborne et al.[122] on the intestinal tissue of zebrafish can be seen as an example of the size-dependent difference in the translocation of ENPs (20 nm and 110 nm Ag ENPs) compared to an ionic control. The 20 nm Ag ENPs gained access to the basolateral membrane, whereas both 110 nm Ag ENPs and the ions were confined to the apical membrane.[122] Differences were also observed in gill tissue, where Ag ENPs were mainly located on the secondary filaments, whereas Ag was mostly present in the primary filaments of zebrafish exposed to the ionic control.[122] This highlights the need to account for differences in the internalization and translocation on the basis of the size of ENPs and also compared to a dissolved control. The underlying mechanisms causing these differences in internalization and translocation are, however, still not well understood.

It should also be mentioned that effects could possibly occur without internalization or translocation of the ENPs in the organism but result solely from the presence of ENPs in the gut. Protrusion of gut epithelia in fish was observed after exposure to TiO_2 ENPs, without evidence of internalization.[115] Reduction in food intake and energy inputs, changes in gut mobility, or effects on nerves or smooth muscle fibers have also been proposed as effects not necessarily associated with the internalization of ENPs.[123] Indeed, Mattsson et al.[103] observed effects on the feeding and shoaling behavior through a dietary pathway after long-term exposure to polystyrene ENPs.[103] Effects on rheotaxis behavior were also observed after exposure to Cu ENPs and Ag ENPs,[124] which could indicate interference with behavioral systems possibly related to translocation to the brain.[103] However, systematic studies of this type of effects are currently not well-established.

A fast initial depuration of ENPs has frequently been reported when moving test organisms, for example, daphnia, to clean media (Figure 7).[104,114,125] However, incomplete depuration of ENPs have been documented in several studies with fish[71,119,127,128] (Figure 8) and daphnia[113,114,126] (Figure 7). Interactions between the ENPs and gut epithelial sorption processes could play a pivotal role in this incomplete depuration. Retained ENPs could indirectly cause prolonged exposure to the ENPs. For example, a 1–3 h pulsed exposure of daphnids to CuO ENPs (0.2–3.2 mg $Cu\,L^{-1}$) followed by a post-exposure period in pure medium showed increased effects for the CuO ENPs compared to a dissolved control, with acute and chronic effects monitored after 48 h and 21 days.[129] The immobilization identified 48 h after the pulse was comparable to that of a continuous exposure for 24 h. In contrast, the 1–3 h pulses of CuO NPs were observed to affect both the time to first offspring as well as the production of offspring over 21 days. If exposure concentrations of CuO ENPs were based on the measured dissolved Cu fraction, the decrease in offspring production was greater for CuO ENPs than for $CuCl_2$. This could indicate effects associated with incomplete depuration leading to prolonged internal exposure of CuO ENPs compared to $CuCl_2$.

From the above it is clear that uptake, internalization, and translocation can possibly occur to different extents in the base set of test organisms, with effects most frequently reported at the higher trophic levels of daphnia and fish. It is also worth mentioning that currently there are no consistent and validated test guidelines considering behavioral effects or the presence of ENPs in, for example, the gut. Consequently, effects related to the exposure of ENPs could possibly be overlooked in acute toxicity tests.

5. Known Mechanisms of ENP Ecotoxicity—The Question of Particle Properties

From the previous sections it is clear that before a nanoparticle effect can be determined, one needs to account for already known effects (dissolved fraction, physical effects

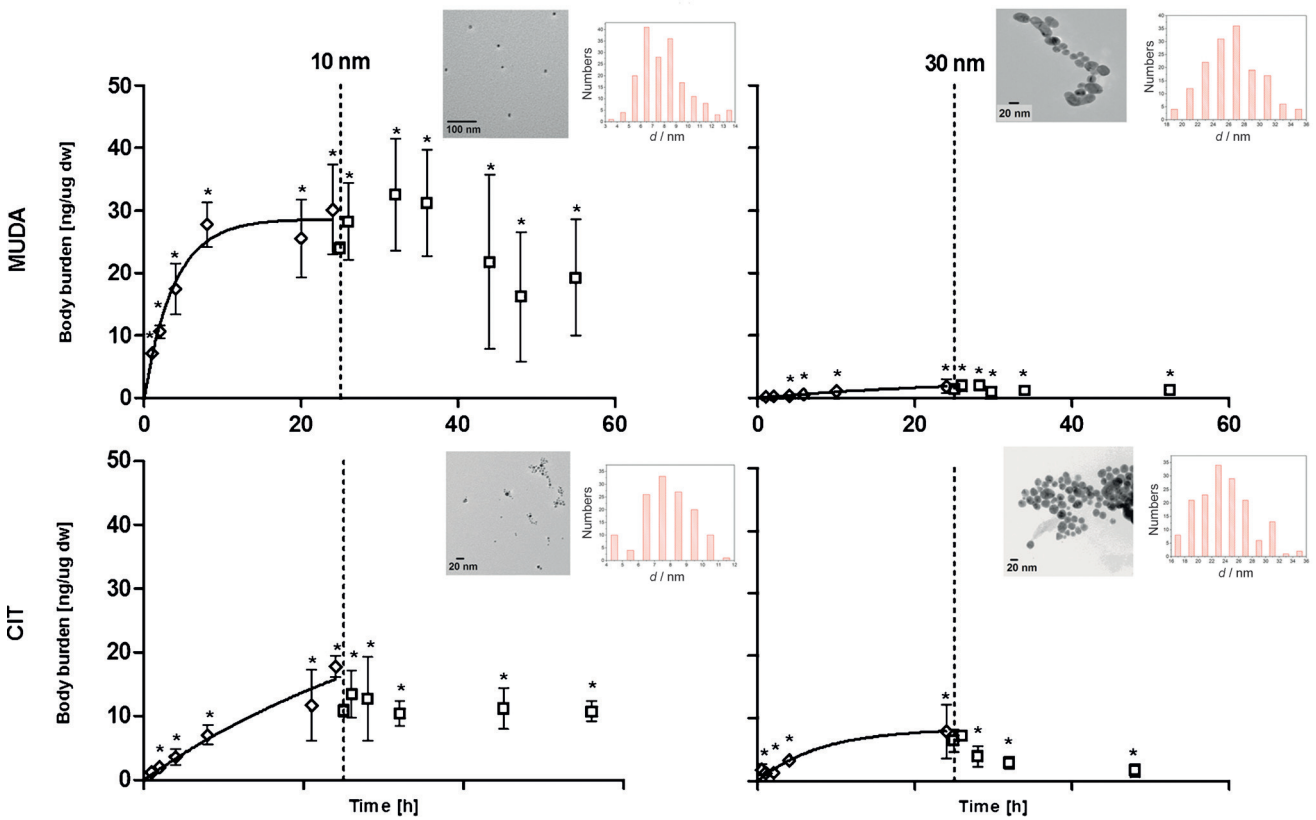

Figure 7. Uptake and depuration study with *D. magna* exposed to 0.5 mg Au L^{-1} for 24 h uptake (◇) and 24 h depuration (□) after the transfer of animals to clean media. Two different particle sizes (10 and 30 nm) and stabilizing agents (MUDA: mercaptoundecanoic acid, CIT: citrate) were used. Points denoted * are statistically significantly different from the control ($p < 0.05$). Insert: TEM images and number size distribution histograms of Au ENPs in MilliQ water from top left: MUDA 10 nm Au NPs ($d = (8.0 \pm 3)$ nm), MUDA 30 nm Au NPs ($d = (27.0 \pm 6)$ nm), CIT 10 nm Au NPs ($d = (7.5 \pm 3)$ nm), and CIT 30 nm Au NP ($d = (23.0 \pm 9)$ nm). Modified from Ref. [114].

etc.). Furthermore, identifying particle properties that are already known to cause a specific effect should be investigated before claiming or ruling out novel nanoparticle effects. The potential of ENPs to show environmental hazards has been scrutinized intensively in the last decade to elucidate such causation between particle properties and toxic responses. In human toxicology, there is a hypothesis that oxidative stress and the production of reactive oxygen species (ROS) is directly related to the increased total surface area of ENPs and that the generation of ROS should be considered the defining mode of action for ENPs.[130,131] Klaine et al.[132] similarly concluded for nano-ecotoxicology that the capability to generate ROS was indirectly the driving force behind a range of observed cellular responses to ENPs, including membrane and nucleic acid damage, protein destabilization, and lipid peroxidation; these are collectively termed oxidative stress, which can lead to genotoxicity and cytotoxicity.[133,134]

In recent years, several reviews have highlighted oxidative stress arising from the generation of ROS as the primary mode of action of ENPs towards aquatic organisms when effects cannot be related to the dissolution or physical interactions of ENPs.[14,135,136] However, the link between particle properties and ROS generation is still undetermined in ecotoxicology;[96,134] even whether size is actually correlated to toxicity is at times noted as being controversial,[134] and von Moss and Slaveykova[96] describe the relationship between particle properties and ROS generation as "*the most controversial issue and greatest challenge to nano-(eco)toxicology*". This claim is based on the notion that a correlation between the properties and effects does not imply causation between the two. A good example of this is a report by Angel et al.,[137] who studied the mechanism of CeO_2 toxicity towards microalgae. They found nanosized CeO_2 to be more toxic than micrometer-sized CeO_2, as well as a correlation between ROS generation and toxicity under normal light conditions. However, UV-filtered light reduced the amount of ROS generation but did not reduce the observed toxicity, thus indicating that ROS were not the governing mode of action of toxicity. The effect of dissolved cerium could also be disregarded because of negligible dissolution during the test. Instead, sorption of the ENPs to the algae was shown to be the most likely cause of the observed toxicity. There are many pathways that interlink ROS, oxidative stress, and cellular toxicity, thereby challenging the establishment of causality, as well as identifying ENP properties that govern these effects. The formation of extra- or intracellular ROS can trigger a cascade of cellular events, including oxidative stress and membrane damage that may ultimately lead to DNA damage and cytotoxicity.[96,133] The

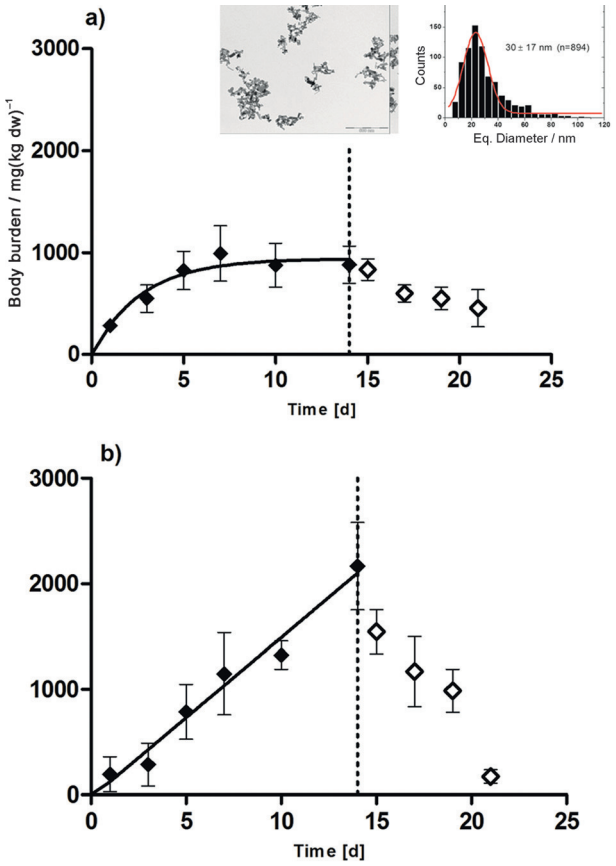

Figure 8. Transfer of ZnO ENPs to *D. rerio* fed on pre-exposed *D. magna* after 14 days of uptake (◆) and 7 days of depuration (◇). *D. magna* were exposed to 1 mg Zn L^{-} with a) ZnO ENPs or b) ZnO-octyl ENPs for 24 hours before feeding to *D. rerio*. A first order rate model fit is indicated by the solid line. Insert: TEM image of the ZnO ENPs in ultrapure water with a number size distribution histogram and an average size of (30 ± 17) nm (n = 894). Modified from Ref. [128].

reverse is also suggested, that is, ENPs may induce cellular toxicity by other mechanisms, such as DNA lesions, disruption of cellular homeostasis, and membrane damage that leads to cellular stress and accumulation of intracellular ROS.[138]

Numerous particle properties may be relevant for ROS generation. For example, Fu et al.[133] argued that the ENP properties that can affect ROS generation include: size, shape, particle surface, surface positive charges, surface groups, particle dissolution, metal-ion release from nanometals and nanometal oxides, activation by UV light, aggregation, mode of interaction with cells, inflammation, and pH value of the medium. von Moos and Slaveykova,[96] in contrast, stressed the importance of chemical composition and purity, particle size, shape, and the resulting relative large reactive surface area as well as surface chemistry. Properties that may theoretically be linked to ROS generation and oxidative stress are the catalytic or redox activity of ENPs, which enable Cu and Pt NPs to participate, for example, in electron-transfer/sharing/bonding processes with surrounding molecules. Indeed, CuO and Pt NPs are found to induce high levels of oxidative stress in microalgae.[26,139] The effect was not attributed to dissolved Pt in the case of Pt NPs, and the oxidative stress kinetics differed for CuO NPs and $CuCl_2$, also indicating a nanoparticle-specific toxic mode of action and/or an increase in the bioavailability of ions caused by the presence of the ENPs.

Ma et al.[84] conducted a review on the ecotoxicity of ZnO ENPs and concluded that "*There is not sufficient amount of studies toward a specific physico-chemical parameter (e.g., particle size) or a specific test species that allows for statistical analysis of correlation between physico-chemical properties and ecotoxicity*". However, Ma et al.[84] went on to highlight both solubility and photoreactivity as key properties for ZnO ecotoxicity. Theoretical band energy calculations to estimate the potential of oxide ENPs to interfere with the cellular redox equilibrium have shown that they, in general, cause oxidative stress in a predictable manner.[140] Band energies and the ionic index of the metal cation were found to be suitable descriptors in a structure–activity relationship (SAR) study of 24 metal oxide ENPs,[141] thus reaffirming the postulate of oxidative stress. However, this approach is simplistic and was not found to fully account for the metal oxide toxicity of all ENPs, for example, below a certain size.[96,142,143] In general, SAR, and especially quantitative SAR, studies are still in the early stages of development for ENPs and are, for example, "a long way off" before they can be considered a reliable tool in regulation.[144]

6. Implications of ENP Behavior in Guideline Tests for Assessment of Chemical Safety

For conventional chemicals, a range of OECD test guidelines are recommended for regulatory use, and quality measures of relevance and reliability are in place to ensure the adequacy of the test outcomes for regulatory decision making. The test guidelines are based on the assumption that the toxicity of a chemical to a given organism is dependent only on the chemical concentration, since all other potential influencing factors (e.g. test duration, media composition, pH, and temperature) have been defined. In test guidelines, suitable test organisms from the base set of aquatic ecotoxicity tests are also specified, thus enabling comparisons of values derived from concentration–response experiments. For the test results to be valid, a number of validity criteria need to be fulfilled. Among these, a constant and well-defined exposure concentration is crucial for the reliability of the test results. When these test guidelines are applied to ENPs, a number of technical obstacles, related to the nature and behavior of ENPs in the currently used aquatic toxicity tests, may prevent nanoparticle effects from being revealed. Many of these obstacles are related to difficulties in keeping exposure conditions stable throughout the incubation period in the toxicity tests.[41] These challenges are not unique for ENPs. For example, dissolution issues are known to influence the testing of highly lipophilic or sparingly soluble chemicals. Furthermore, the aquatic ecotoxicity testing of ENPs is more complex because of the gradual transformation of the ENP state during incubation (as illustrated in Figure 2). On the other hand, parallels may be drawn between ENPs and

conventional substances in terms of the phenomena that are encountered. This is especially relevant for the so-called "difficult substances", for which specific guidance for ecotoxicity testing is available.[58] Although it could be claimed that a stable ENP suspension is equivalent to a chemical in solution, there are major differences between the behavior of ENPs and dissolving chemicals in aquatic media as well as their interactions with biological systems. For (partly) soluble ENPs, experiences gained from conventional chemicals (mainly metals and metal salts) will contribute to uncovering potential excess toxicity found in the tests. However, for all ENPs (whether soluble or not), the presence of the particles in suspension presents a range of challenges for the interpretation and quantification of the effects observed. In this respect, the aggregation and agglomeration behavior of ENPs in aquatic media plays a major role and represents a challenge that is difficult to address.[30] Even for the same ENPs, for example, ZnO, differences in the preparation of stock suspensions (including timing) as well as test medium composition has shown to give results with orders of magnitude of differences in guideline tests.[145,146] These obstacles must be overcome to obtain reliable and comparable test results,[147] but this is difficult due to a number of factors that influence the stability of the test suspensions. Unstable suspensions lead to mixed exposure conditions in which the effective dose is not well-defined. This hinders evaluating the test results in terms of a correlation between the dose and response: if the dose is not defined, the quantification of effects needed for risk assessment is of course invalid. The dynamics of the processes occurring further complicate this picture, since unstable suspensions undergo a range of transformation processes during incubation if no precautions are taken.[44,145,148,74] The reason for this is dynamic—and often unpredictable—interactions with constituents of the testing medium (e.g. the presence of abundant divalent cations such as Ca^{2+} and Mg^{2+} in most standardized testing media), testing conditions (e.g. incubation time, pH, light, and temperature), as well as with test organisms (e.g. ingestion and biomodifications of ENPs in *D. magna*; algal exudates modifying the agglomeration behavior of NPs during incubation). Given this range of factors that can potentially influence the testing outcome and the fact that strong links between the inherent properties of the ENPs and the testing conditions remain to be discovered, it is at present very difficult to claim that a well-defined dose can be controlled when ENPs are examined using guideline tests.

Since it is difficult to strictly control the exposure during incubation, another approach is to describe and quantify the exposure by measurements over time. Measuring the concentration in the water phase will certainly provide useful information on the stability of ENPs in suspension. However, as described in this Review, it may not necessarily define actual exposure concentrations as a result of interference from dynamic phenomena such as particle adhesion to organisms, sedimentation, dissolution, and active uptake of ENPs. Finally, dilution of stock suspensions and test concentrations may affect the two processes assumed to be determinants for the toxicity of ENPs: dissolution and agglomeration behavior.[44,49,54] If attention is not paid to these influencing factors, artefacts may affect the outcome of standard toxicity tests to such an extent that the results are unreliable and even irrelevant.[149] As stated by Handy et al.,[150] control measures and best practices may help to overcome such problems within the defined tests; however, specific technical advice is still lacking.

To accommodate some of these concerns, a Guidance Manual for the Testing of Manufactured Nanomaterials was developed by the OECD WPMN in 2009 to ensure that the information collected from the OECD′s testing program on ENPs would yield reliable, accurate, and consistent results.[151] This was followed by a guidance document on Sample Preparation and Dosimetry, in which advice was given on how meaningful and reproducible test results could best be obtained by using the OECD test guidelines.[152] This guidance document includes a total of 15 issues to be documented for ecotoxicity studies performed using the current OECD guidelines. Among these are: method of suspension (different suspension methods may significantly change the ENP state or toxicity); quantification of media quality in both stock suspensions and testing media; and physical and chemical characterization of the ENPs in the test medium as a minimum (ideally the characterization should be carried out at multiple points during the test) before and after incubation (e.g. particle size and/or agglomerate size distribution and ENP concentration).[153] This "patch" to the existing test guidelines is a specific response to the technical problems of the test that—since it is difficult to fully control the exposure to ENPs during incubation—gives insight into the reproducibility, reliability, and relevance of the test results that can be gained by describing the exposure in detail.[23] Finally, it should be mentioned that the dose metric applied represents a challenge to the current procedures for the use of ecotoxicity test results in risk assessment. To obtain relevant and reliable results for dose-response assessments, the ecotoxicological studies must be expressed by an appropriate dose metric for the studied ENPs. Although particle number or specific surface area have been suggested as more appropriate dose metrics,[24,55,154,155] the number of studies that have applied these metrics is still too limited to draw firm conclusions on whether these novel metrics are better alternatives to expressing the effective concentrations than the traditional mass-based concentrations.

7. Concluding Remarks

This Review presents an approach to advance nano-ecotoxicity testing by defining three different types of confounding responses that may overshadow nanoparticle-specific effects. Not taking these into account will prevent a reliable evaluation of whether ENPs, in fact, represent novel and hitherto unknown hazards to the environment. We propose that all ecotoxicological studies of ENPs should address whether ENPs can physically attach to test organisms, undergo dissolution in the aqueous media, and internalize/discretely localize in/on the test organisms. Furthermore, we recommend that known effect mechanisms of ENPs, for example, the generation of reactive oxygen species (ROS),

should be included in ecotoxicity testing strategies. Based on the existing literature on the ecotoxicity of ENPs on the aquatic organisms used as the base set for risk assessment (i.e. fish, crustaceans, and algae), the following conclusions for each of the three response types can be drawn:

1) The dissolution of soluble ENPs can describe a large number of the observed effects in fish, crustaceans, and algae. However, effects not related solely to the dissolved fraction have been observed in some studies. To determine the contribution of the dissolved faction to the aquatic toxicity of ENPs it is pivotal to characterize the ENP dissolution behavior in the test media during testing.
2) Sub-lethal effects as a result of the physical attachment of ENPs to organisms have been documented for both high ($>10\,mg\,L^{-1}$) and low concentrations ($<1\,mg\,L^{-1}$). Whereas responses as a consequence of the physical attachment of ENPs to organisms have been documented for all three groups of organisms, sub-lethal effects at lower concentrations have most frequently been observed for crustaceans. In these cases, the effects may be related to energy deficits such as reduced molting rates, changes in feeding behavior, and depuration of non-nutritious particles in the ingestible size range of crustaceans. Therefore, physical effects need to be either accounted for or eliminated as far as possible, also at lower concentrations ($<1\,mg\,L^{-1}$), to prevent nanoparticle effects from being overshadowed.
3) ENPs were found to be internalized and accumulated in aquatic test organisms by mechanisms that are different to those that have traditionally been observed for dissolved chemicals. Furthermore, the transformation and reactivity of ENPs after internalization (e.g. release of metallic ions or ROS generation) have been shown to cause effects other than those of dissolved chemicals as a result of adherence to tissue and/or translocation in the organisms. However, the governing parameters for the internalization of ENPs and related toxic mechanisms in vivo in aquatic organisms are not well understood. The lack of reliable techniques to quantify and characterize ENPs in live organisms and tissue samples constitutes a limiting factor in this respect.

From a review of the current ecotoxicity literature on ENPs it was found that some of the most commonly used ENPs can be grouped according to the behavior of the ENPs during aquatic toxicity testing:

- Agglomeration is very important for all ENPs, especially for aquatic tests with TiO_2 and CeO.[54,60,145,154] In practice it is difficult to maintain a stable suspension of these particles in the media. Sedimentation of TiO_2 and CeO_2 ENPs are often reported, and physical effects on test organisms are likely to happen.
- Dissolution of Ag, ZnO, and CuO ENPs in the test medium and release of ionic metal species has often been found to explain the toxicity observed.[156] The dissolved metal ions will in most cases be more toxic than the corresponding ENPs; however, some exceptions have been found that point towards a nanoparticle effect. However, it is not trivial to quantify dissolution under actual test conditions and, without high analytical recoveries and complete mass balances, statements of a nanoparticle effect as a result of "more toxicity than what can be explained by the dissolved metal" should be carefully scrutinized. Furthermore, it is important to stress that the dissolution process is dynamic and on-going from the preparation of stock suspensions before testing as well as during the test period.[74]

Hence, the possibilities of revealing nanoparticle effects for these ENPs are directly linked to adequate quantification of their agglomeration and dissolution. In practice, a combination of the confounding response types is likely to occur simultaneously and, even when taking these responses into account, the identification of additional nanoparticle effects is not straightforward.

The interpretation of biological responses observed for ENPs in currently used test guidelines for risk assessment is challenged by difficulties in maintaining stable exposure conditions during testing. A number of technical challenges arise from the inherent differences between ENPs and the dissolved chemicals for which the tests were originally developed. The behavior of ENPs under testing conditions is very difficult to control and the reliability of test results depends on extensive characterization of ENPs and description of the observed biological responses in the test systems. We consider this the way forward to obtain data that, on the one hand, are adequate for regulatory purposes and, on the other hand, may disclose nanoparticle effects.

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