Aquatic toxicity testing for hazard identification of engineered nanoparticles

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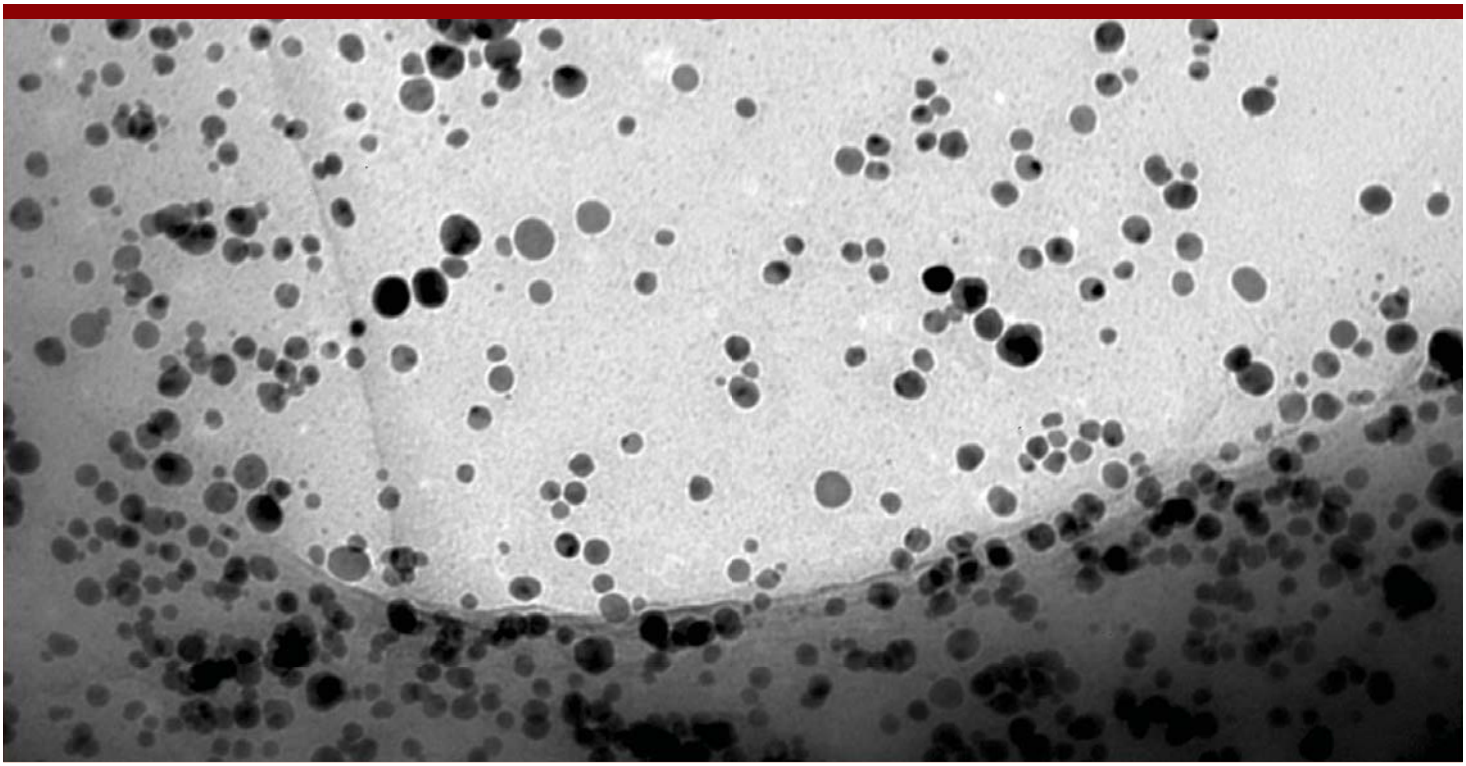

Sara Nørgaard Sørensen

PhD Thesis
June 2016

DTU Environment
Department of Environmental Engineering

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The synopsis part of this thesis is available as a pdf-file for download from the DTU research database ORBIT: http://www.orbit.dtu.dk

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Preface

This PhD thesis presents the scientific outcome of research conducted at the Department of Environmental Engineering, Technical University of Denmark (DTU) in the period April 2012 to April 2016 under supervision of Professor Anders Baun and co-supervision of Senior Researcher Hans-Christian Holten Lützhøft and Associate Professor Steffen Foss Hansen. Parts of the work were conducted during a four months stay at the University of Geneva, supervised by Professor Vera I. Slaveykova. The work was partly funded by the European Research Council (Grant no. 281579), as part of the project EnvNano (Environmental Effects and Risk Evaluation of Engineered Nanoparticles).

This thesis is organized in two parts: the first part puts into context the findings of the PhD in an introductive review; the second part consists of the papers listed below. These will be referred to in the text by their paper number written with the Roman numerals **I-V**.

I **Sørensen SN**, Baun A. 2015. Controlling silver nanoparticle exposure in algal toxicity testing – A matter of timing. *Nanotoxicology* 9(2):201-209.

II **Sørensen SN**, Engelbrekt C, Lützhøft HCH, Jiménez-Lamana J, Noori JS, Alatraktchi FA, Delgado CG, Slaveykova VI, Baun A. 2016. A multi-method approach for disclosing algal toxicity of platinum nanoparticles. (*Submitted*)

III **Sørensen SN**, Lützhøft HCH, Rasmussen R, Baun A. 2016. Acute and chronic effects from pulse exposure of *Daphnia magna* to silver and copper nanoparticles. (*Submitted*)

IV **Sørensen SN**, Hjorth R, Delgado CG, Hartmann NB, Baun A. 2015. Nanoparticle ecotoxicity – physical and/or chemical effects? *Integrated Environmental Assessment and Management* 11:722-724.

V Jensen LHS, Skjolding LM, Thit A, **Sørensen SN**, Købler C, Mølhave K, Baun A. 2016. Not all that glitters is gold – an electron microscopy study on uptake of gold nanoparticles in *Daphnia magna* and related artefacts. (*Submitted*)

In this online version of the thesis, the papers are not included but can be obtained from electronic article databases e.g. via www.orbit.dtu.dk or on request from DTU Environment, Technical University of Denmark, Bygningstorvet, Building 115, 2800 Kgs. Lyngby, Denmark, info@env.dtu.dk.

In addition, the following publications, not included in this thesis, were also concluded during this PhD study:

Cupi D, **Sørensen SN,** Skjolding LM, Baun A. 2015. Toxicity of engineered nanoparticles to aquatic invertebrates. In: Xing B, Vecitis C, Senesi N. Engineered nanoparticles and the environment: Physicochemical Processes and Toxicity. IUPAC Series on biophysicochemical Processes in Environmental Systems, Vol 4, Wiley-Interscience, Hoboken, NJ. (*In press*)

Hjorth R, **Sørensen SN**, Olsson ME, Baun A, Hartmann NB. 2016. A certain shade of green: Can algal pigments reveal shading effects of nanoparticles? *Integrated Environmental Assessment and Management* 12:200-202.

Skjolding LM, **Sørensen SN**, Hartmann NB, Hjorth R, Hansen SF, Baun A. 2016. A Critical Review of Aquatic Ecotoxicity Testing of Nanoparticles – The Quest for Disclosing the Nano-effect. (*Submitted*)

June 2016
Sara Nørgaard Sørensen

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My journey towards completing this PhD has been highly challenging both professionally and personally, but gratifying in terms of scientific insight, personal growth, friendships and adventures.

First and most importantly, I want to thank my supervisors: Anders Baun for giving me this opportunity in the first place, and for providing inspiration, problem-solving, and motivation along the way, Steffen Foss Hansen for valuable input and discussions, and Hans-Christian Holten Lützhøft for stepping in with clear guidance, advice and support.

Secondly, I need to thank the fantastic students, technical staff, PhDs and other employees at DTU Environment, whom in various ways contributed to my work and wellbeing. A special thanks to the nano-crew, I really value the enthusiasm, team-spirit and champagne we have shared.

Thanks to external collaborators and co-authors, especially Vera I. Slaveykova for the opportunity to expand my horizon at the University of Geneva, but also the amazing employees and students at the Institute F.A. Forel, who made my stay in Geneva so absolutely wonderful. In this regard, also thanks to Otto Mønsteds Fond and G.A. Hagemanns Mindefond for financial support enabling this stay.

Last but not least, much gratitude to my family and friends for their support and love, especially my parents for taking good care of Eddie when needed, and Vibeke for convincing me to pursue this PhD and always being at the other end of the line.

The essential lesson from my scientific work has ultimately infused my whole life over the past years: There is no absolute truth. The answers we search for, and find, rely on the questions we ask, as also stated by Werner Heisenberg in 1958:

"*What we observe is not nature itself, but nature exposed to our method of questioning*"

I hope for a future where we not only question the methods for toxicity testing of engineered nanoparticles, but also the necessity of producing and applying such synthetic materials and chemicals to the extent we do today.

Summary

Within the last few decades, major advances in the field of nanotechnology have enabled production of engineered nanoparticles (ENPs) for various applications and consumer products already available on the market. ENPs may exhibit unique and novel properties compared to their bulk counterparts, which is often related to a high surface-to-volume ratio. These properties have also caused concern amongst scientists and regulators, who have called for timely identification of the potential adverse effects of ENPs to human health and the environment.

Despite intensive research on the aquatic toxicity of ENPs, the applicability of the generated data for hazard identification purposes is generally impaired by poor reproducibility and reliability of data, and limited understanding of the underlying effect mechanisms. Consequently, it has been questioned whether the standardized aquatic toxicity tests, developed for testing soluble compounds, are equally applicable for ENPs. The preconditions for aquatic toxicity tests include aqueous solubility of the chemical test compound and stability during incubation. These criteria are not met for ENPs, as they are suspended rather than dissolved in aqueous media. Moreover, ENPs undergo time-dependent transformation processes of agglomeration, dissolution, sedimentation, and interactions with organisms and their exudates. Together, these processes challenge the establishment of traditional concentration-response data by affecting both the exposure and the response axes. The actual exposure experienced by organisms may not be reflected by the ENP-concentration in medium, commonly applied as the exposure metric, and the responses of organisms may result from various toxic and non-toxic mechanisms occurring simultaneously.

In this thesis, the challenges related to exposure control and response mechanisms in aquatic toxicity tests with ENPs are addressed through: 1) Exposure timing measures to minimize the transformation processes of ENPs during test incubation, and 2) Multi-dimensional approaches including investigations of other organisms responses than the traditionally applied, and determination of different exposure fractions such as the concentration of dissolved ions from ENPs and body burdens. Although these approaches are scientifically exploratory by nature, the aim is to generate data applicable for regulatory hazard identification of ENPs. The focus has been on the algal growth rate inhibition test and acute and chronic toxicity tests with crustaceans, all commonly applied in a regulatory context.

The exposure timing measures included aging of ENPs in test media prior to incubation, and/or shortened exposure duration. For algae, shorter exposure duration was obtained through the application of an acute 2h ^{14}C-assimilation test. For daphnids, a short-term (1-3h) pulse exposure was applied, followed by transfer of the organisms to pure medium, where acute and chronic effects were monitored according to standard guidelines during 48h and 21 days respectively. These approaches assisted to minimize the ENP transformation during incubation, but also influenced the toxicity. While aging of ENPs may both increase and decrease toxicity, the shortened exposure mainly appeared to involve a risk of underestimating, or in worst case overlooking chronic effects in algae and daphnids. Thus, more sensitive

endpoints may be relevant for algal tests with shortened exposure, such as oxidative stress, found to occur within few hours' exposure to certain ENPs.

The traditional endpoints of algal growth rate inhibition and daphnia immobility were found to be confounded by physical effects. As examples, the algal growth rate can be inhibited by ENPs physically obstructing the light available to the algae (shading), and the immobility of daphnids may partly result from ENPs adsorbed to these organisms' exterior. In addition to different effect mechanisms, several exposure fractions are available to interact with the organisms, including ENPs adsorbed to or internalized into the organisms/cells, suspended ENPs and ions dissolved from the ENPs. Together, these various exposure fractions and the multiple effect mechanisms complicate the establishment of traditional concentration-response relationships that are based on a single response and exposure dose-metric.

A multi-dimensional approach is therefore suggested for aquatic toxicity testing of ENPs for hazard identification purposes. This includes investigation of both physical and cellular effects in organisms in addition to the traditional endpoints. Also, the different exposure fractions of ENPs should be considered including the adsorbed and internalized fractions in organisms, as well as the dissolved and total concentrations in the medium. In practice it is neither unambiguous nor straightforward to determine the different exposure fractions and effect mechanisms, thus some consensus on the best available practices would be beneficial, as well as harmonization of testing approaches in a regulatory context.

Ultimately, a multi-dimensional approach may assist to identify which organism responses and exposure fractions are related and improve our understanding of the concentration-response data generated from aquatic toxicity tests with ENPs.

Dansk sammenfatning

De seneste årtiers store fremskridt indenfor nanoteknologi, har gjort det muligt at producere syntetiske nanopartikler (SNP) til diverse anvendelsesformål og til forbrugerprodukter, der allerede findes tilgængelige på markedet. SNP kan besidde unikke egenskaber der adskiller sig fra materialet i andre former, hvilket ofte relateres til et forøget overfladeareal ift. volumen for SNP. De særlige egenskaber har dog også givet anledning til bekymring hos videnskabsfolk og myndigheder, som har opfordret til rettidig identifikation af de potentielle skadevirkninger SNP kan forårsage hos mennesker og i miljøet.

Til trods for intensiv forskning i SNP's akvatiske toksicitet, så er anvendeligheden af de genererede data til fareidentifikationsformål generelt begrænset af en lav reproducerbarhed og pålidelighed af data, samt en begrænset forståelse af de underliggende effektmekanismer. Det er derfor spørgsmålet hvorvidt de nuværende standardiserede akvatiske toksicitetstests, hvilke oprindeligt er udviklet til at teste opløselige kemiske stoffer, ligeledes er brugbare for SNP. Disse tests forudsætter at det kemiske teststof opløses i testmediet og kan holdes stabilt i opløsning under inkubationen. Disse forudsætninger er ikke overholdt for SNP, eftersom de er suspenderet og ikke opløst i et vandigt medie. Desuden gennemgår SNP tidsafhængige transformationsprocesser, såsom agglomerering, opløsning, sedimentering samt interaktioner med organismer og deres udskillelsesprodukter. Tilsammen udfordrer disse processer etableringen af traditionelle koncentrations-respons data, ved at påvirke både eksponerings- og responsakserne. Den faktiske eksponering oplevet af organismerne afspejles ikke nødvendigvis af koncentrationen af SNP i mediet, der som regel er den anvendte eksponeringsenhed, og organismernes respons kan være resultat af diverse toksiske og ikke-toksiske mekanismer, der forløber simultant.

I denne afhandling adresseres udfordringerne med eksponeringskontrol og responsmekanismer i akvatiske toksicitetstests med SNP igennem: 1) Tiltag der vedrører eksponeringstiming, med hensigten at minimere de tidsafhængige transformationsprocesser af SNP under inkubationen, og 2) En flerdimensional tilgang der inkluderer undersøgelse af andre organismerespons end de traditionelt anvendte, samt bestemmelse af forskellige eksponeringsfraktioner, såsom koncentrationen af SNP på opløst form og i organismerne. Selvom disse tilgange er af en undersøgende videnskabelig karakter, så er formålet at generere data som er brugbare ved regulatorisk fareidentifikation af SNP. Der fokuseres på tests af algers vækstratehæmning samt akutte og kroniske test med krebsdyr, eftersom disse sædvanligvis bruges i regulatoriske sammenhænge.

Tiltagene vedrørende eksponeringstiming inkluder ældning af SNP i testmediet forud for inkubation, og/eller forkortelse af eksponeringsvarigheden. For alger blev en forkortet eksponeringsvarighed opnået ved brug af en akut 2t ^{14}C-assimilieringstest. For dafnier blev en pulseksponeringsmetode anvendt, hvor organismerne efter en 1-3t pulseksponering blev overført til rent medie og overvåget iht. vejledende retningslinjer for akutte og kroniske effekter i løbet af henholdsvis 48t og 21d. Disse tiltag medvirkede til at minimere transformationen af SNP under inkubationen, men påvirkede også toksiciteten. Mens ældning af SNP både kan øge og reducere toksiciteten, så virkede det til en forkortet eksponeringsvarighed hovedsageligt indebar

en risiko for at underestimere, eller i værste fald overse, kroniske effekter på alger og dafnier. Dermed kunne mere følsomme effektparametre være relevante for algetests med forkortet eksponeringsvarighed, eksempelvis oxidativt stress, der har vist sig at indtræffe ved få timers eksponering.

De traditionelle effektparametre algevækstratehæmning og dafnie immobilisering viste sig at være påvirkede af fysiske effekter. Algevækstraten kan for eksempel hæmmes ved at SNP fysisk blokerer lystilgængeligheden for algerne (skyggeeffekter), og immobilisering af dafnier kan til dels være resultat af SNP som er adsorberes til organismernes ydre. Foruden diverse effektmekanismer, så er der forskellige eksponeringsfraktioner tilgængelige for interaktioner med organismerne, heriblandt SNP adsorberet til eller optaget i organismerne/cellerne, suspenderede SNP og ioner der er frigivet ved opløsning af SNP. Tilsammen komplicerer disse forskellige eksponeringsfraktioner og effektmekanismer etableringen af traditionelle koncentrations-responsforhold baseret på en enkelt respons- og eksponeringsparameter.

En flerdimensional tilgang foreslås derfor ved akvatiske toksicitetstests til fareidentifikation af SNP. Denne inkluderer undersøgelse af både fysiske og cellulære effekter foruden de traditionelle effektparametre. Desuden bør de forskellige eksponeringsfraktioner for SNP tages i betragtning, inklusiv den adsorberede og optagede fraktion såvel som den opløste og totale koncentration i mediet. I praksis er det dog hverken entydigt eller ligetil at bestemme disse forskellige eksponeringsfraktioner og effektmekanismer, hvorfor en vis konsensus om de bedste tilgængelige teknikker vil være fordelagtig, såvel som en harmonisering af testprocedurer til regulatoriske formål.

I sidste ende kan en flerdimensional tilgang til toksicitetstestning bidrage til at identificere hvilke effekt- og eksponeringsparametre, der er forbundne og udvikle vores forståelse af de koncentrations-responsdata der genereres fra akvatiske toksicitetstests med SNP.

Table of contents

1 Background and aims

Within recent decades, major advances in the field of nanotechnology have enabled the production and use of engineered nanoparticles (ENPs) for various products and applications (Maynard et al., 2011). The amount of commercially available nano-enabled consumer products has increased markedly in recent years, covering a wide range of products from sports equipment to cosmetics (Hansen et al., 2016), and various applications are being investigated, including environmental remediation and targeted drug delivery (Gottschalk and Nowack, 2011). ENPs are utilized and desirable for various reasons; they generally have a high surface-to-volume ratio resulting in increased reactivity, and may also exhibit unique properties and/or behaviour in liquid media compared to soluble chemical compounds and larger-sized solid materials (Baalousha et al., 2014). Furthermore, ENPs may be tailored with regards to coating, size or shape, to target certain desirable characteristics, such as increased reactivity, and dispersion stability (Engelbrekt et al., 2010).

The unique properties of ENPs have also caused concern within scientific and regulatory communities, calling for timely risk assessment relating to human health and the environment. Consequently, the potential hazardous effects of ENPs to human health and the environment have been studied intensively over the last decade (Juganson et al., 2015; Oomen et al., 2014). The generated ecotoxicity data are however, generally impaired by poor reproducibility, reliability and altered concentration-response relationships for ENPs compared to soluble compounds (Hartmann et al., 2013; Petersen et al., 2014). Thus, despite intensive research and numerous tests conducted, the applicability of these data for hazard and risk assessment purposes is limited (Oomen et al., 2014), and aquatic toxicity testing of ENPs remains a complex task.

For environmental hazard identification in regulatory contexts, standardized aquatic toxicity tests (for example according to ISO standards and OECD guidelines) are applied for ENPs, although these tests were originally developed for soluble chemical compounds and do not take particle-related complexities into account (Palmqvist et al., 2015). The fact that ENPs are suspended and not dissolved in the test medium thereby challenges the validity of standard aquatic toxicity tests, as solubility of the chemical compound is a precondition to these tests. Furthermore, ENPs undergo various processes in aqueous suspension, which over time potentially modify the ENP stability and distribution in test containers during the exposure period. Thus, also the precondition of stable exposure conditions during the incubation is violated. Overall, the standard aquatic toxicity tests used for hazard identification of soluble compounds may not be appropriate for ENPs.

The challenges related to aquatic toxicity testing of ENPs are potentially influenced by numerous parameters and conditions of the test system, and may therefore be addressed by numerous approaches. These often include attempts to stabilize ENP suspensions through the adjustment of parameters such as pH, ionic strength, and natural organic matter (NOM) (for example Cupi et al., 2016, 2015; Römer et al., 2011) and dispersion protocols (Hartmann et al., 2015), or to characterize the ENPs as best and much as possible during the incubation of the test, as for example

described in work by the Working Party on Manufactured Nanomaterials (WPMN) of the Organisation for Economic Co-operation and Development (OECD, 2012a).

In this thesis, different approaches to address the testing challenges were explored. These included the use of exposure timing measures to increase ENP stability and test reproducibility, and the attempt to identify relevant endpoints and exposure parameters. The underlying reasoning was that ENPs are inherently different from soluble chemical compounds, and that standard aquatic toxicity tests needs to be reconsidered for ENPs, as also suggested by others (e.g. Kühnel and Nickel, 2014).

To keep some variables unchanged, it was decided to focus on guideline tests with daphnids (OECD, 2012b, 2004) and standardized algal tests (ISO, 2004), as the basis for investigations. This included use of commonly applied test organisms: The crustacean *Daphnia magna* and two species of unicellular green microalgae (*Pseudokirchneriella subcapitata* and *Chlamydomonas reinhardtii*). The ENPs applied for testing were chosen based on different criteria to fit the needs of the studies and include silver (AgNPs), copper oxide (CuONPs), gold (AuNPs) and platinum (PtNPs). Common for all of these, is their relevance in terms of use and environmental exposure, but also properties such as reactivity, density, toxicity and dissolution were key factors depending on the scope and analytical techniques applied in the different studies.

The overall aim of this thesis is to:

Address the challenges related to exposure control and response mechanisms in aquatic toxicity testing for ENP hazard identification purposes, through exposure timing and multiple endpoint approaches

In this context, the following research questions were addressed:

Influence of exposure timing on ENP aquatic toxicity testing

1. How does ENP aging in media prior to toxicity testing affect the outcome?
2. How does a shortening of exposure duration influence the testing outcome?

Appropriate endpoints for aquatic toxicity of ENPs

3. Are standard algal and daphnia toxicity test endpoints appropriate for ENPs?
4. What are the possible alternative toxicity endpoints relevant for ENPs?

Linking ENP exposure and effects in aquatic toxicity tests

5. How may the current concentration-response approach be adapted to become more appropriate for the hazard identification of ENPs?

The structure of this thesis involves initial background information on ENPs and challenges for aquatic toxicity testing of these (chapter 2). Then, the processes causing unstable exposure conditions for ENPs, and the use of exposure timing measures to address these challenges are covered by Chapter 3 and 4, respectively. Chapter 5 deals with the response-part of toxicity testing, including the mechanisms considered important for ENP-toxicity, and the challenges determining these. Finally, the implications of findings from all chapters and recommendations for aquatic toxicity testing with ENPs are discussed in chapter 6. Conclusions are in chapter 7.

2 Engineered nanoparticles

2.1 Definitions

There is no sole definition of a nanomaterial, as the definition may rely on the size, properties and intended applications (Baalousha et al., 2014). Generally though, the definitions commonly refer to a size range of 1-100 nm. For regulatory purposes, the European Commission has recommended the following definition of a nanomaterial (European Commission, 2011):

"A natural, incidental or manufactured material containing particles, in an unbound state or as an aggregate or as an agglomerate and where, for 50% or more of the particles in the number size distribution, one or more external dimensions is in the size range 1 nm to 100 nm."

Nanoscale materials may exhibit altered physical and/or chemical properties compared to larger sized bulk materials of the same chemical composition. This is often attributed to the larger surface-to-volume ratio at the nanometer scale (Klaine et al., 2012), causing more atoms to be exposed at the surface and altered properties of these surface atoms (Hassinger and Sellers, 2012). Attempts have been made to determine the size threshold governing nanospecific properties, and although selected physical chemical parameters was found to depend on the nanoscale particle size, current experimental data was deemed insufficient for defining an actual size threshold (Hassinger and Sellers, 2012). Generally, inorganic ENPs with diameters of 30 nm or less are reported to undergo dramatic changes in crystalline structure that enhance their interfacial reactivity (Auffan et al., 2009).

In the context of this thesis, the ENP size is not a pivotal parameter, although all tested ENPs had primary sizes (as synthesized) within the 1-100 nm range. More important is the physical entity of ENPs, distinguishing them from their water-soluble counterparts, often referred to as the ionic or dissolved form.

This physical dimension is included in the particle definition of the International Organization for Standardization (ISO): "A minute piece of matter with defined physical boundaries" (ISO, 2010). Moreover, the altered properties and behavior of ENPs in aquatic suspensions compared to their dissolved counterparts is considered crucial within this thesis, since solubility is a criterion for the standard toxicity tests applied for hazard identification of ENPs.

2.2 Production, use and environmental exposure

The production and use of ENPs has increased markedly during the last decade. The number of consumer products on the market claimed by the manufacturer to contain nanomaterials or make use of nanotechnology is recently reported by two product inventories for the European and global market, respectively as 2,231 (Hansen et al., 2016) and 1,814 (Vance et al., 2015).

These consumer products cover a wide range of use categories from personal care products, paints and cleaning agents to sports equipment, clothing and household appliances (Hansen et al., 2016; Vance et al., 2015). The ENPs are added to optimize the products in various ways, for example to provide protective coatings, or improve material strength, but most often ENPs are added for antibacterial protection (Vance et al., 2015). The most commonly declared nanomaterial in consumer products is by far AgNPs, accounting for 24% of the products and utilized mainly for its antibacterial properties (Vance et al., 2015). The nanomaterials with the greatest estimated annual production quantities are SiO_2 (55-55,000 t/year) and TiO_2 (550-5,500 t/year), whereas for Ag the amount is 5.5-550 t/year (Piccinno et al., 2012).

In addition, ENPs are applied in various industrial products and processes, including groundwater remediation, agrochemicals, pharmaceuticals (Gottschalk and Nowack, 2011). For examples, PtNPs are widely utilized in automotive catalysts (Ek et al., 2004) and iron ENPs for remediation (Gottschalk and Nowack, 2011).

The substantial production and use of ENPs will inevitably result in their release into the environment (Maurer-Jones et al., 2013). Experimental studies have presented evidence of ENPs being released from various products, such as textiles and food containers during use and washing, and paint on house facades exposed to rain and sun (Kaegi et al., 2008; Mackevica and Hansen, 2016). The wastewater system is shown to be a major pathway for environmental exposure of ENPs during production, use and disposal of ENPs and ENP-containing products (Nowack et al., 2012). However, direct exposure of the aquatic environment may also occur from ships, buildings, fields, and cars, where surfaces are treated with or composed of ENPs or ENP-containing products (Gottschalk and Nowack, 2011). Data on ENP concentrations in the environment are scarce, due to limitations of analytical methods for the detection and quantification of ENPs in natural matrices (Gottschalk and Nowack, 2011).

2.3 Aquatic toxicity testing – status and challenges

The same unique properties of ENPs considered beneficial for various applications also cause concern for potential adverse effects to human health and the environment (Klaine et al., 2012). The European Commission has up until 2012 funded 46 nano-safety projects amounting to investments of 130 million € (Oomen et al., 2014). The intense research in nano(eco)toxicology is also evident from the body of scientific literature published within the last decade. According to the ISI Web of Knowledge, the number of peer-reviewed articles from the search terms nano* AND ecotox* has increased from approximately 40 to 1100 in the period of 2006 to March 2016.

Despite these efforts to determine the ecotoxicity of ENPs, the data generated from standardized test methods are often reported as obscure, inaccurate or poorly reproducible (Hartmann et al., 2013; Klaine et al., 2012; Petersen et al., 2014). Thus, applicability of ecotoxicity data for hazard and risk assessment purposes remains

limited (Oomen et al., 2014). This has been attributed to missing information on: ENP characteristic, interaction with media and behavior during the test, as well as difficulties in dose-metrics and data interpretation (Potthoff et al., 2015). As published aquatic toxicity data for ENPs are generated for various different purposes, including regulatory compliance, environmental relevance and exploratory scientific research, different tests and setups are applied, hampering comparability (Wickson et al., 2014). It has been suggested that (eco)toxicity testing is conducted under either environmentally probable/relevant or worst case conditions, and that the test setup is designed accordingly (Potthoff et al., 2015). This thesis addresses the test-related challenges through more exploratory scientific research approaches, but the aim is to produce toxicity data relevant and appropriate for hazard identification, thus worst case scenarios need to be considered and as under controlled conditions as possible.

Generally, standardized aquatic toxicity tests provide information on the inherent capacity of chemical compounds to adversely affect aquatic organisms. The generated data are applied for hazard identification of the compounds, and thereby the foundation for further hazard assessment and communication, as well as risk assessment, management and regulatory compliance (Traas and van Leeuwen, 2007).

In Europe, especially two regulations are relevant in this regard: the CLP regulation on classification, labelling and packaging of substances and mixtures (European Parliament and Council, 2008), and the REACH legislation on the registration, evaluation, authorization and restriction of chemicals (European Parliament and Council, 2006). Neither of these includes specific provisions for ENPs, meaning the ENP "identity" is related strictly to their chemical composition and not their physical dimension. Discussions on adding special information requirements and assessment of the nanoparticle-form of a chemical compound under REACH are ongoing and new initiatives are awaited.

The first European legislation to implement requirements of a separate assessment for nanomaterials is the biocidal product regulation (European Parliament and Council, 2012), and entails that the approval of an active substance for biocidal products does not implicit cover the nano-form of the chemical compound in question (Brinch et al., 2016).

The regulations mentioned here all define which aquatic toxicity endpoints are appropriate for regulatory compliance, but do not provide specific requirements on *how to* conduct aquatic toxicity tests. Such guidance is provided by the European Chemicals Agency (ECHA, 2014), and also include specific guidance for ENPs, although very limited advise is related to aquatic toxicity testing, and mainly refers to characterization (ECHA, 2012a, 2012b). These guidance documents often refer to the use of internationally harmonized test guidelines prepared by organizations such as the Organisation for Economic Co-operation and Development (OECD). Much work is conducted to develop these guidelines to account for nanospecific issues, both within OECD and the scientific community in general.

For now, regulatory ecotoxicity testing of ENPs is conducted using the standardized test methods applied for "regular" soluble compounds, and include algae, crustaceans and fish, considered as surrogate of all aquatic organisms (European

Parliament and Council, 2008). Short-term regulatory toxicity endpoints include algal growth rate inhibition, immobility/lethality of daphnids, and lethality of fish.

The response is expressed as the mean effective concentration (EC_{50}) related to the endpoint within a defined exposure period, see Figure 1A. This paradigm is challenged for ENPs, as illustrated in Figure 1B. Firstly determining exposure concentrations (the X-axis) has proven to be difficult, as ENPs transform in aqueous suspension (Handy et al., 2012). Also, the transformation processes are often dynamic over time, introducing a third dimension of the concentration-response relationship. Finally, there are potentially multiple mechanisms influencing the detected responses of the organism (Y-axis), which are not yet fully understood (Juganson et al., 2015).

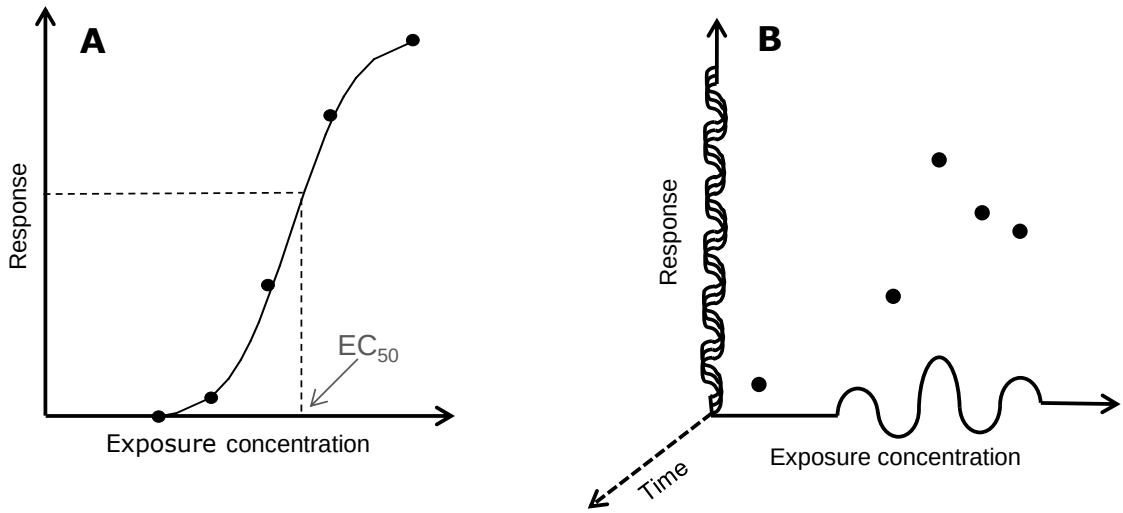

Figure 1. Illustration of typical concentration-response data for a regular, soluble compound (A), and the challenges related to obtaining similar data for engineered nanoparticles (B).

Traditionally, particles have not been considered in an ecotoxicological context (Klaine et al., 2012), therefore very limited experience exists to build upon in the recent efforts to (re)develop ecotoxicity testing setups and hazard identification approaches for ENPs.

Although ENPs are often mentioned as a type of xenobiotics, they constitute a diverse group and the environmental hazards will inevitably differ between ENPs, as it does between other chemical compounds. Since various ENPs are already on the market, hazard identification of these materials needs to be conducted rapidly. A challenge in this regard is the sheer number of ENPs to be tested. ENPs are not only defined by their chemical composition, but also other properties such as shape, size, functionalization, aggregation state, and dissolution kinetics. All these combined results in a vast number of ENPs to be tested. Consequently, much effort has gone into the development of high-throughput testing, screening tools and structure activity relationship (SAR) models (Juganson et al., 2015; Nel et al., 2013). This approach is however, challenged by the lack of knowledge on toxic mechanisms, as well as the determining exposure conditions and ENP properties.

3 Nanoparticle exposure dynamics in aquatic toxicity tests

When ENPs are added to an aqueous test medium, a suspension or dispersion is formed, whereas soluble compounds dissolve and form a solution. The processes dictating the spatial and temporal distribution of the test material, and thereby also the exposure conditions in aquatic toxicity tests, are very different in these two situations, as illustrated in Figure 2. The terms suspension and dispersion are often used interchangeably in the literature. Suspended particles are sufficiently large to settle usually >1 µm (Baalousha et al., 2009), whereas dispersed particles are smaller and in theory should remain dispersed. In practice, these are not easily distinguished and within this thesis the term suspension is used, unless describing theoretical phenomena relating strictly to dispersed ENPs.

While compounds in solutions overall are subjected to the laws of classical equilibrium based thermodynamics, other physical phenomena are important for ENPs. The processes described for regular, soluble compounds may however also apply to certain ENPs and/or the dissolved fraction of ENPs, for example ENP adsorption to the test vessel and chemical reactions on the surface. The ENP-specific processes, their governing forces and influencing factors are described in the following. In many cases, several of these processes occur simultaneously.

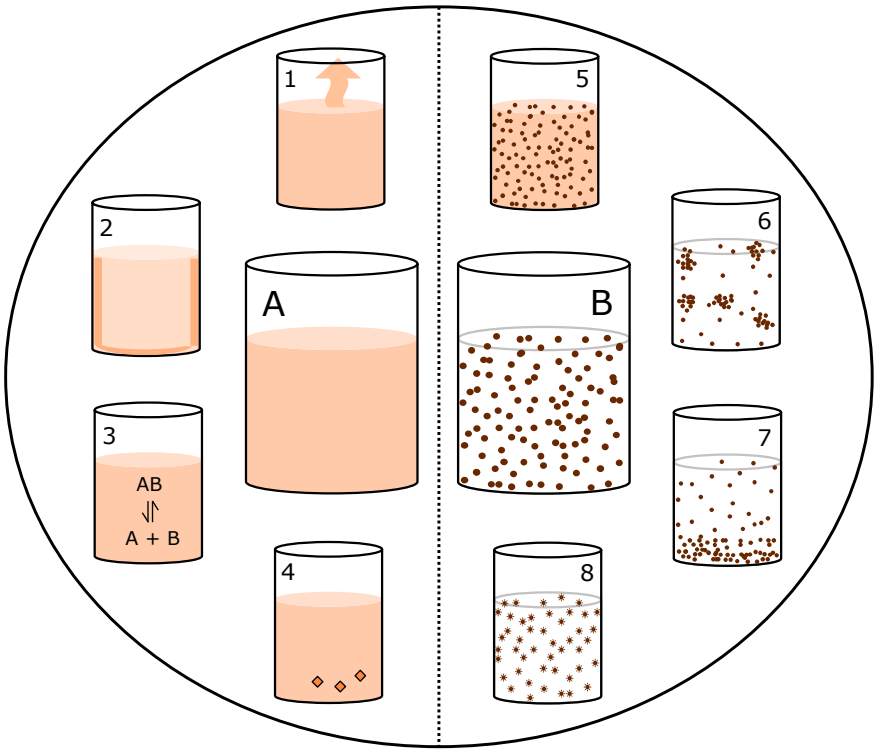

Figure 2. Illustration of processes that influence the distribution of a dissolved compound (A) and suspended ENPs (B). For a compound in solution these processes include 1) Evaporation, 2) Adsorption to test vessels, and 3) Speciation reactions including complexation, and dissociation, and 4) Precipitation 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 reaction and changes to coatings/stabilizing agents.

3.1 Agglomeration/aggregation and sedimentation

Theoretically, small particles that are dispersed (i.e. no gravitational settling) in a liquid will move according to Brownian motion. This means that particles are in constant random motion, caused by their collisions with molecules in the liquid. As the particles move around, they are also bound to collide with each other, thus Brownian motion has been suggested as a main driving force for agglomeration and aggregation (Petosa et al., 2010). Whether or not colliding ENPs form agglomerates or aggregates, is however also determined by the interparticular forces, including electrostatic, steric and van der Waal forces.

Electrostatic forces relate to the surface charge of ENPs. Between ENPs of similar charge, a repulsive force exists, whereas ENPs of opposite surface charge attract. Steric forces may also cause repulsion of ENPs, due to structural shielding. Van der Waals forces on the other hand cause attraction, due to electrical and magnetic polarization within two entities (Baalousha et al., 2009). Such forces are exploited in the efforts to keep ENPs dispersed and counteract aggregation, for example by coating ENPs with various polymers and organic molecules (Petosa et al., 2010).

Aggregation is generally defined as the process of ENPs being strongly bound, whereas agglomeration refer to individual or aggregated ENPs being weakly bound with the total surface area remaining unchanged (European Commission, 2011). Agglomeration is generally perceived to be reversible, whereas aggregation is not. In practice, it is however difficult to distinguish the two scenarios by the commonly applied characterization methods and most studies (including the work in this thesis) report changes to size distributions without knowing these details.

Several studies have shown how agglomeration/aggregation is influenced by ENP concentration and characteristics such as size, shape and coating, but also properties of the medium including pH, ionic strength, dissolved oxygen and NOM (Cupi et al., 2016, 2015; El Badawy et al., 2012; Liu et al., 2012; Zhang et al., 2011; Zhou and Keller, 2010). According to theory, Brownian motion causes smaller particles to move faster than larger ones, thereby being more prone to collide with other particles and agglomerate/aggregate. Also, an increasing ENP concentration will theoretically increase the collision rate and thus agglomeration and aggregation. This is also reported from experiments with silver and iron ENPs (Phenrat et al., 2007; Piccapietra et al., 2012).

Despite the many studies on the influence of various parameters such as pH, ionic strength and NOM, few studies have investigated the agglomeration/aggregation of ENPs in standard ecotoxicity test media as function of time only. For AgNPs with different stabilizing agents, substantial and rapid aggregation is reported immediately after suspension in OECD *D. magna* test medium and increasing after 24-72h, corresponding to the duration of an acute toxicity test with this organism (Römer et al., 2011; Tejamaya et al., 2012). Increasing sizes is also found for AuNPs (Skjolding et al., 2014) and AgNPs (Sørensen et al., 2016b Paper III) with different stabilizing agents during 24h in daphnia medium.

PtNPs are also found to undergo rapid (within 1h) and substantial agglomeration/aggregation in algal media over 48h (Sørensen et al., 2016a Paper II). The

number of aggregates detected by nanoparticle tracking analysis was 1000 fold higher in 4 × diluted tris-acetate-phosphate (TAP4) medium compared to the ISO medium, see Figure 3. In agreement with the studies on AgNPs, more agglomeration/aggregation of PtNPs occurred in the medium of highest ionic strength (TAP4).

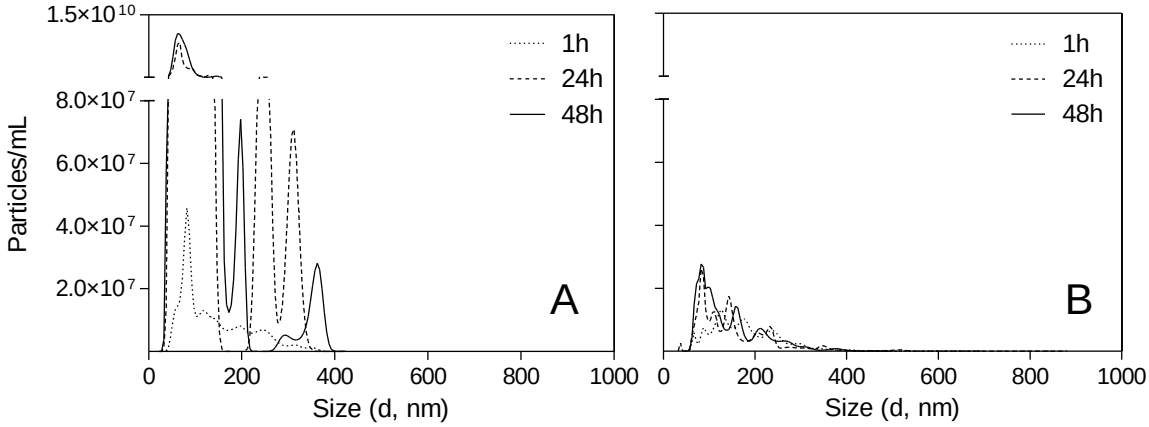

Figure 3. Number of agglomerates/aggregates for starch coated PtNPs (nominal size 5.8-6.0 nm including coating) suspended in TAP4 medium (A) and ISO medium (B) at 80 mg Pt/L, determined by Nanoparticle Tracking Analysis after 1-48h (Sørensen et al., 2016a Paper II).

Aggregation of suspended ENPs is often termed homo-aggregation, whereas aggregation of ENPs and other suspended particles including biota, organic and inorganic entities, sometimes is referred to as hetero-aggregation (Praetorius et al., 2014). Although standardized aquatic toxicity tests generally constitute relatively simple systems of synthetic freshwater (e.g. no sediment or natural particles), the biota and media alone may generate macromolecules/particles that may interact with ENPs.

An increasing particle size due to agglomeration/aggregation may potentially result in sedimentation of particles due to gravitational settling. According to Stoke's law, the velocity of the settling particles is related to the fluid viscosity and the particle density and radius (IUPAC, 2014). As agglomeration/aggregation and sedimentation are related, the factors influencing agglomeration will also impact sedimentation.

The sedimentation of ENPs may be examined by time-resolved UV-vis spectrophotometry (Sørensen et al., 2016a Paper II; Zhou and Keller, 2010), mass determination of aliquots sampled from the ENP suspension without stirring and subsequently sampled while stirring (Sørensen et al., 2016b Paper III). In some cases, ENP sedimentation is evident even from visual observations (Sørensen et al., 2016b Paper III; Tejamaya et al., 2012).

Sedimentation is a bigger issue for non-agitated tests, such as the *D. magna* immobilization test, compared to algal tests, where vials are shaken continuously, or tests conducted in a flow-through setup. For CuONPs, the water phase exposure concentration in *D. magna* immobilization tests can be reduced by 60% within few hours, due to sedimentation (Sørensen et al., 2016b Paper III), see Figure 4.

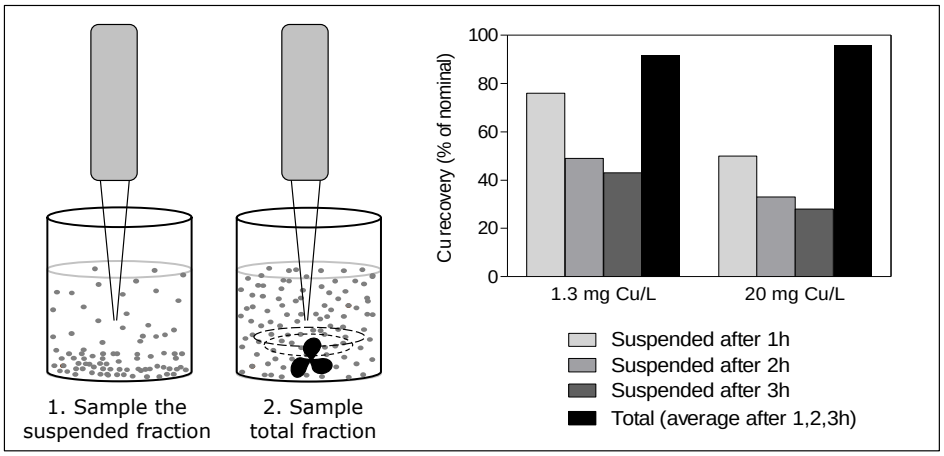

Figure 4. Sedimentation of CuONPs in modified M7 medium (1.3 and 20 mg Cu/) over 3h. The suspended Cu fraction was determined by ICP-MS in aliquots carefully sampled from the center of suspensions, and the total Cu was determined in another aliquot sampled from the same suspension while stirring (left illustrations). The suspended Cu fractions after1, 2 and 3h are provided as percent of the nominal concentration, and the total Cu as the average of 1, 2 and 3h (modified from Sørensen et al., 2016b Paper III).

3.2 Dissolution

ENPs composed of metals or metal oxides may undergo dissolution in test medium, and create an exposure scenario including both suspended ENPs and soluble ions. Dissolution occurs as molecules in the dissolving surface of the solid phase migrate through a diffusion layer, with the driving force being the thermodynamic solubility and the concentration gradient between the surface and the solution (Misra et al., 2012). The reverse of dissolution may also occur, i.e. that dissolved ions redeposit onto the surface of ENPs. Ostwald ripening is the phenomenon of dissolved species from smaller particles redepositing on the surface of larger particles (IUPAC, 2014), thus increasing the size of particles.

The importance of dissolution differs markedly for ENPs depending on the material composition. Whereas ENPs composed of materials such as TiO_2 undergo relatively limited dissolution in test media, others including those of silver Ag, CuO and ZnO are partly to highly soluble (The Danish Environmental Protection Agency, 2014).

Furthermore, both the rate of dissolution and the amount of dissolved material at equilibrium are influenced by different properties of the ENPs as well as the media. Amongst the physical and chemical properties of ENPs affecting dissolution, size is considered the most important, although parameters such as surface morphology, crystallinity, stabilization/capping agent also play a role (Misra et al., 2012). Therefore, aggregation (by affecting the size) and dissolution are interconnected, although this connection is not fully understood. Theoretically, decreased dissolution is expected with increasing ENP aggregation, due to a decreased surface area available for dissolution. However, the dissolution of AgNPs has been shown to depend more on their initial size than the aggregation (Kent and Vikesland, 2012).

The rate and extent of dissolution are also affected by the ENP concentration. This is for example shown for Ag and CuO ENPs, with dissolution correlating inversely with concentration (Baalousha et al., 2016; Baek and An, 2011; Jemec et al., 2016; S. N. Sørensen et al., 2016b Paper III), as also shown in Figure 5. This may be due to concentration-dependent aggregation, as described above.

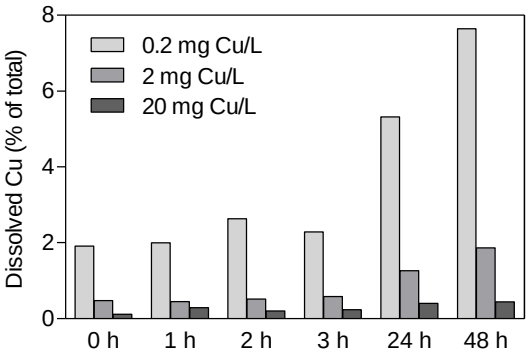

Figure 5. Dissolution of CuONPs suspended in modified M7 medium (0.2, 2 and 20 mg Cu/L) over 48h, as percent dissolved of the total Cu concentration determined by ICP-MS analysis of suspensions immediately after preparation, and in the supernatant after ultracentrifugation (Sørensen et al., 2016b Paper III).

As shown in Figure 5, substantial dissolution may occur during a 48h daphnia immobility test, and also vary with time and ENP concentration. Dissolution results in complex exposure scenario with the presence of both dissolved metals and ENPs. As the two fractions are subject to different transformation processes (as depicted in Figure 2), and possibly entail different bioavailability and toxicity mechanisms, it is crucial to differentiate the exposure and effects for these two fractions.

3.3 Other chemical and biological surface reactions

Some ENPs are reactive and undergo electron transfer with other chemical species. Redox reactions involve the processes of oxidation (a species losing an electron) and reduction (the gaining of an electron). Such reactions are also the basis for dissolution of inorganic ENPs, described in chapter 3.2. The redox potential is a measure for a chemical species' tendency to undergo oxidation or reduction (The Danish Environmental Protection Agency, 2014).

Other ENPs are not reactive themselves, but are catalysts for chemical reactions, including redox reactions. PtNPs are for example utilized in car catalysts to facilitate reduction and removal of contaminants including carbon monoxide (CO), hydrocarbons (HC) and nitrogen oxides (Palacios et al., 2000).

Regardless of their origin, oxidizing or reducing substances can interfere with the redox balance of cells, by decreasing the level of antioxidants or increase the production of reactive oxygen species (ROS). This may lead to inflammation and cytotoxicity and has been proposed as a toxic mechanism for ENPs (Nel et al., 2006). The ability of ENPs to generate abiotic ROS, i.e. without the interaction of living

cells, has been reported as an ENP characteristics in (eco)toxicological studies (Elder et al., 2007; Ivask et al., 2015; Rushton et al., 2010; Sørensen et al., 2016a Paper II). The catalytic activity of PtNPs increases with decreasing size, due to higher specific surface area (Engelbrekt et al., 2010). For these PtNPs, abiotic ROS was generated in algal media, but also in Milli-Q water, suggesting that the ROS generation may occur on the surface of PtNPs, and not by interactions with media components (Sørensen et al., 2016a Paper II).

Living organisms may transform ENPs via biodegradation and bio-modification. Although metal-based ENPs themselves are not biodegradable, biodegradation of organic coatings or stabilizing agents is possible and may compromise the ENP stability. Bio-modification of ENPs in the gut of *D. magna* has been reported for iron oxide ENPs and carbon nanotubes (CNTs), resulting in altered solubility of these (Kwon et al., 2014; Roberts et al., 2007). Also algae and other microorganisms have been shown to modify ENPs through exudates, affecting the aggregation and dissolution of CeO_2, Ag and CuO ENPs (Khan et al., 2011; Koukal et al., 2007; Kroll et al., 2014; Miao et al., 2015). Bio-modification and biodegradation processes are not specific for ENPs, although additional and/or different transformation processes are involved in the transformation of ENPs than for soluble chemical compounds, cf. Figure 2.

3.4 Adsorption to aquatic organisms and internalization

Several studies have reported significant adsorption or clustering of ENPs on the surface or around daphnids and algae, as illustrated for the freshwater alga *P. subcapitata* in Figure 6 (Angel et al., 2015; Aruoja et al., 2009; Baun et al., 2008; Booth et al., 2015; Campos et al., 2013; Dabrunz et al., 2011; Hartmann et al., 2013, 2010; Manier et al., 2013; Rodea-Palomares et al., 2011; Sørensen et al., 2015 Paper IV; Vallotton et al., 2015; van Hoecke et al., 2009; van Hoecke et al., 2008).

The same forces governing the interaction between ENPs, may also influence the adsorption of ENPs to cells and organism surfaces (The Danish Environmental Protection Agency, 2014). Biological factors however, also come into play when organisms are involved. The forces and mechanisms of major relevance to ENP-cell-interactions are related to specific features and functions of cells, as summarized by Ma and Lin (2013):

- The phospholipid bilayer of cells makes hydrophobic ENPs prone for adsorption via hydrophobic forces
- Cell surfaces of microorganisms generally have a net negative charge and may therefore attract positively charged ENPs through electrostatic forces
- Cell surface molecules and receptors enable receptor-ligand interaction and hydrogen bonding with surrounding ENPs
- Overall, the mechanisms, influencing factors, and kinetics of the adsorption processes are not fully understood, although the ENP concentration, and proper-

ties of ENPs and cells are important (von Moos et al., 2014). The ENP adsorption to test organisms may result in a locally increased exposure and cause both physical and toxicological effects, thus challenging the concentration-response paradigm.

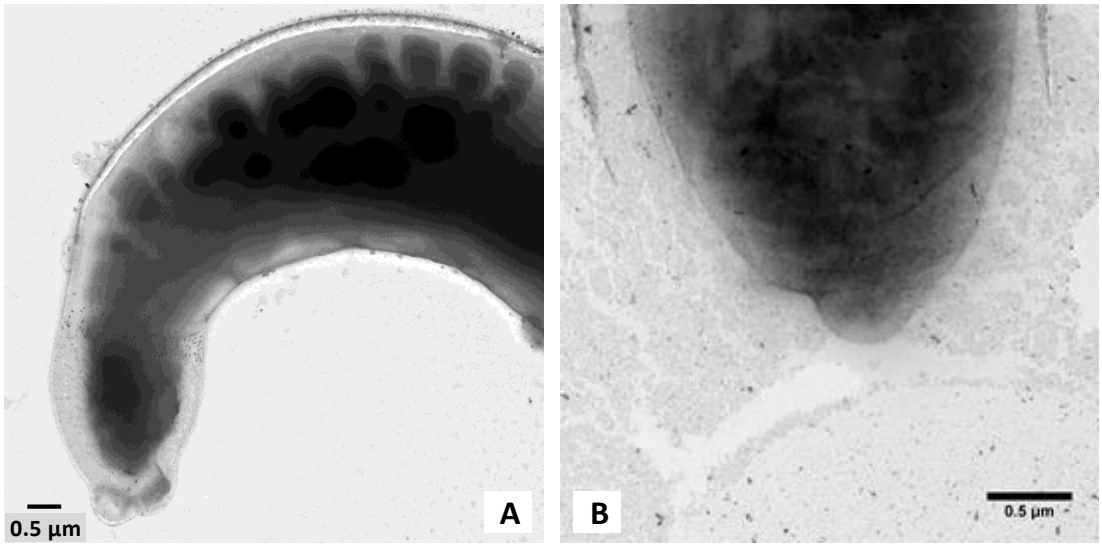

Figure 6. TEM images of the alga *P. subcapitata* upon 48-72h exposure to citrate stabilized AgNPs of nominal size 30 nm (A), and starch stabilized PtNPs of nominal size 1.7 nm (B) (unpublished images).

The potential internalization of ENPs into cells and tissues of living organisms is a concern due to the small size and unique properties of ENPs. Knowledge on internalization and distribution in cells and organisms would also assist to identify toxic mechanisms. Theoretical pathways and experimental evidence for their occurrence are described in literature (Ma and Lin, 2013; Schultz et al., 2015; von Moos et al., 2014). The major pathways are (also illustrated in Figure 7 below):

- *Dissolution* results in dissolved metal species and ions, which may be assimilated into cells via pathways for trace metals. An example is the internalization of silver ions through Na^+-channels in cells (Schultz et al., 2015)
- *Passive transport* of ENPs across intact cell membranes may be relevant for lipophilic ENPs in simple organisms lacking active transport mechanisms. Also, transport of ENPs into cells may occur across damaged membranes with increased permeability (Schultz et al., 2015)
- *Facilitated transport* occurs via carrier proteins and channels in the membrane. The pathway is generally considered highly specific and not relevant for most ENPs (Schultz et al., 2015)
- *Endocytosis* involves the folding of plasma membrane in a cavity-like structure that closes around the ENPs, forming a vesicle with the ENPs inside the cells cytoplasm. Various endocytosis mechanisms exist, depending on different carrier proteins and result in different sized vesicles (<100 nm to 5 µm) enabling up-

take of different sized ENPs. Eukaryotic cells like microalgae can have highly developed endocytic mechanisms (Schultz et al., 2015; von Moos et al., 2014).

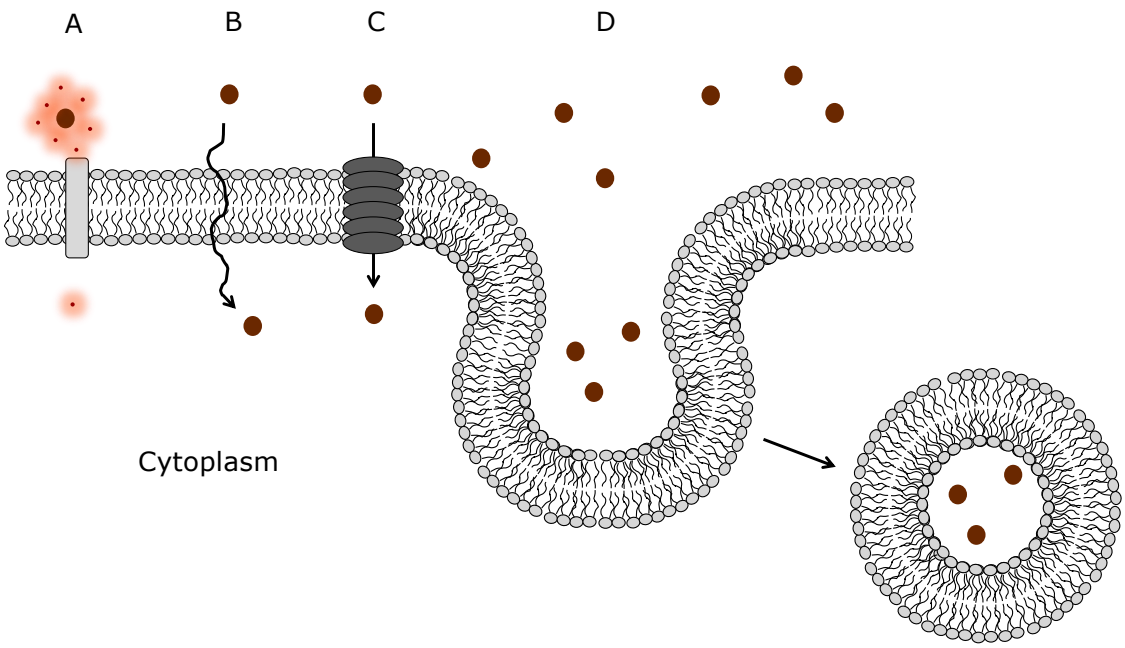

Figure 7. Illustration of cellular internalization pathways for ENPs, including uptake of dissolved ions through trace metal pathways (A), passive diffusion (B), facilitated diffusion (C) and an example of non-specified endocytic process (D).

For microalgae, the relatively thick and tough cell wall presents an additional barrier, commonly assumed to prevent ENP internalization. However, cell walls have pores with diameters of 5-20 nm, and their permeability is also compromised during cell cycling (von Moos et al., 2014). Experimental evidence for internalization of Ag, CuO and TiO_2 ENPs is reported for different algal species by various techniques, although the uptake mechanisms and routes are not clear (von Moos et al., 2014). A study on internalization of AgNPs in algae identified AgNPs inside cells regardless of being exposed to AgNPs or ionic silver. Thus, AgNPs may not only be internalized as particles, but also formed inside the cell from assimilated silver ions (Leclerc and Wilkinson, 2014).

For daphnids, the gut is considered the most important uptake pathway for ENPs (Ma and Lin, 2013). The filter feeding behavior of daphnids may result in filtration of up to 400 mL water/day, with the purpose of collecting food (mainly algae) in the size range of 0.4-40 µm (Geller and Müller, 1981; Gophen and Geller, 1984). This size range may overlap with ENP aggregates, and identified accumulation of various ENPs in the gut of daphnids is also frequently reported (Campos et al., 2013; Heinlaan et al., 2011; Jensen et al., 2016 Paper V; Khan et al., 2014; Lee et al., 2016). Consequently, *in vivo* cellular internalization of ENPs from the gut has been investigated, often by transmission electron microscopy (TEM) offering high resolution imaging of ENPs and cellular structures. Overall, available TEM studies report no or very limited internalization of ENPs via the daphnid gut, or they are inconclusive (Heinlaan et al., 2011; Jensen et al., 2016 Paper V; Khan et al., 2014; Lovern et al., 2008). The use of electron microscopy to investigate ENP internaliza-

tion at the cellular level presents various challenges. Extensive specimen preparation is needed, and imaging of many specimen sections is required to obtain representative information, or even confirm or reject cellular internalization. Also, there are artefacts related to the specimen preparation and interpretation of images, as structures can be formed inside cells and organelles closely resembling ENPs. Such false positive data on internalization are shown for AuNPs in *D. magna* (Jensen et al., 2016 Paper V), see Figure 8. Thus, element analysis in electron microscopy studies has been emphasized as crucial (Petersen et al., 2014; Jensen et al., 2016 Paper V). The use of cryo-techniques for imaging eliminate staining-related artefacts, and are likely preferable, although this remains to be investigated further.

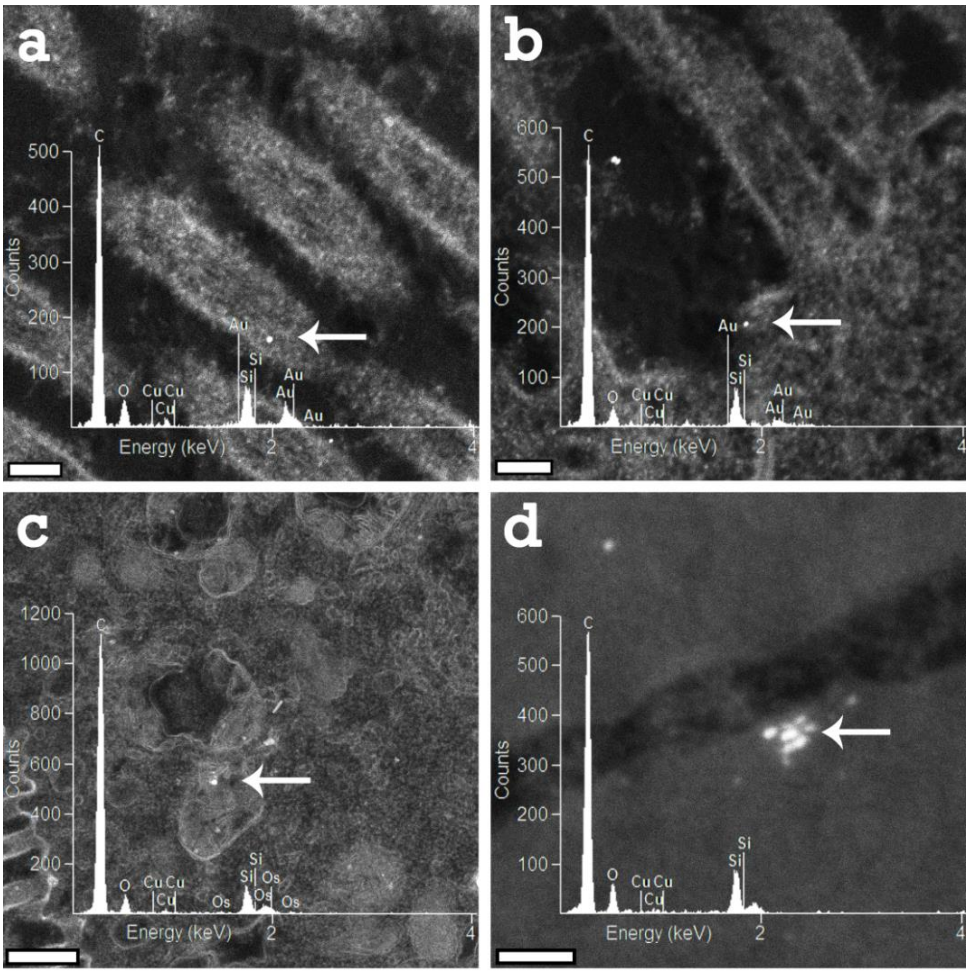

Figure 8. High-Angle Annular Dark-Field Scanning Transmission Electron Microscopy (HAADF STEM) images of gut epithelia from *D. magna* exposed to AuNPs (0.4 mg Au/L) for 24h. (a) AuNPs at microvilli, Scale bar = 100 nm, (b) AuNPs at crypt of microvilli, Scale bar = 100 nm, (c) Osmium-rich particle in mitochondrion, Scale bar = 0.5 µm, (d) Osmium-rich particle in lipid droplet, Scale bar = 50 nm (Modified from Jensen et al., 2016 Paper V).

4 Influence of exposure timing on testing outcome

The moment ENPs are suspended in aqueous media, various time-dependent transformation processes occurs as described in the preceding chapter. Over time, these processes will affect both the *magnitude* and *state* of the ENP exposure experienced by the organisms – and likely in a different way for each process.

Attempts to minimize this dynamic behavior of ENPs during aquatic toxicity testing are predominantly approached by adjusting the physical or chemical properties of the media (pH, ionic strength and NOM). While effective in certain cases, the approach also has limitations:

- Adjusting pH may reduce aggregation of electrostatically stabilized ENPs, but not necessarily sterically stabilized (Cupi et al., 2016).
- Addition of NOM may stabilize aggregation, but not for all ENPs, as shown for AgNPs (Cupi et al., 2015). Moreover, NOM can eliminate toxicity, (Angel et al., 2013; Cupi et al., 2015) which is very problematic for hazard identification.
- Reducing the ionic strength, for instance by using diluted medium, is reported to reduce ENP aggregation (Römer et al., 2011). This is however also suggested to affect the sensitivity of the test organism (Harmon et al., 2014).
- The media properties and conditions favourable to one transformation process may be unfavorable to another. For example, lowering pH is found to decrease ZnONP-agglomeration, but increase dissolution (Cupi et al., 2016). NOM suppresses aggregation of CuONPs, but increase dissolution (Miao et al., 2015).
- Perhaps most importantly; such approaches involving ENPs and test media only, do not take into account the transformation of ENPs that can occur from ENPs interaction with the test organisms and exudates. Thus, while stabilization through media adjustment may be a starting point, it does not address all the testing challenges related to ENP exposure dynamics. On the other hand, the presence of organisms may hamper the ENP characterization technique. This is for example the case when determining the particle size distribution of ENPs in samples containing algae or daphnia exudates.

All the transformation processes, regardless of governing forces and influencing factors, are time-dependent. Here, the effects of timing on aquatic toxicity testing outcome (exposure dynamics, toxicity and reproducibility) are addressed, looking into aging of ENP suspensions and exposure duration.

4.1 Aging nanoparticle suspensions before testing

The term “aging” is broadly applied in ENP-related literature, referring to ENP release from consumer products, environmental weathering or changes occurring during storage of the ENP as supplied by the manufacturer. Here, aging is defined as the time passed from the moment ENPs are suspended in the toxicity test medium and until ended toxicity testing, including all the transformation processes involved.

Aquatic toxicity testing with ENPs, as well as soluble compounds is predominantly conducted using freshly prepared stock and/or test suspensions in test medium. However, few studies have investigated the impact of aging in aquatic toxicity tests. These are summarized below, and the overall trends are discussed.

The ^{14}C-assimilation of algae *P. subcapitata* during 2h exposure to citrate stabilized AgNPs, is shown to vary significantly depending on aging (0-5d) in algal medium (Sørensen and Baun, 2015 Paper I), see Figure 9A. The greatest toxicity occurred after 48h aging, hereafter toxicity decreased with further aging. These changes were attributed to an initial rapid dissolution of ions, followed by interaction of these ions as well as AgNPs with media components, reducing bioavailability and toxicity. These AgNPs were also significantly more toxic to *D. magna* when aged (1d), than if freshly prepared (Rasmussen, 2014), Figure 9B. This was likely due to dissolution, as 5-10% of the total mass was dissolved within 24h aging and 24h exposure (Sørensen et al., 2016b Paper III).

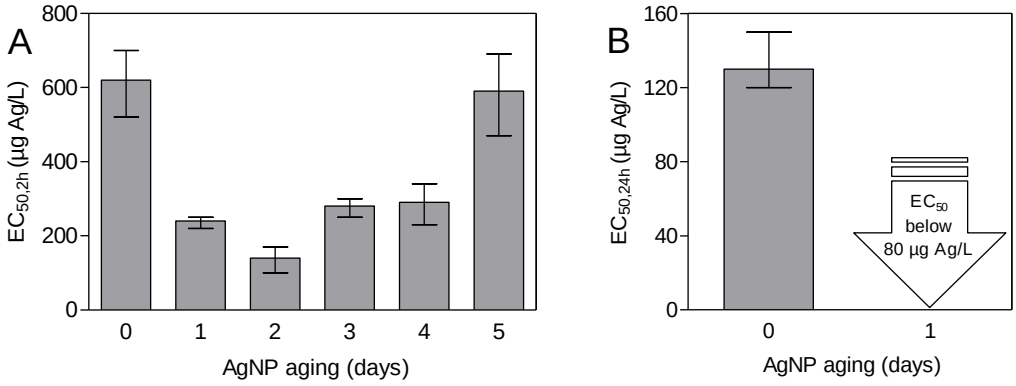

Figure 9. Toxicity of aged vs. freshly prepared AgNP suspensions in (A) 2h algal ^{14}C-assimilation tests (Sørensen and Baun, 2015 Paper I), and (B) 24h *D. magna* immobilization tests (note: 1d AgNP aging resulted in 100% immobility at lowest concentration of 80 µg Ag/L). Columns represent mean effective concentrations (EC_{50}-values), and the bars 95% confidence intervals (Rasmussen, 2014).

For algae, CNTs similarly inhibited the (96h) growth rate more when aged (3d) than if freshly prepared, with EC_{50}-values of 9 (8;10) and 24 (20;29) mg CNT/L, respectively (Schwab et al., 2011). This was correlated with increasing agglomeration of algal cells with CNTs over time, and a following increase in shading of these cells. On the other hand, aging of CeO_2NPs had no impact on the 72h algal growth rate inhibition, with EC_{50}-values of 5.6, 4.1 and 6.2 mg/L for suspensions aged 0, 3 and 30 days respectively (Manier et al., 2013). Although significant agglomeration of the CeO_2NPs occurred during aging, this obviously had no impact on toxicity. This was related to the overall surface area of ENPs available for algal interactions being unchanged for agglomerates (cf. also chapter 3.1), and concluded in line with other studies, that it is the primary size (or available surface area) that influence algal toxicity (Hartmann et al., 2010; Manier et al., 2013; van Hoecke et al., 2009).

For daphnids, 3 and 6 days of aging of TiO_2 decreased the 96h acute immobility roughly 2- and 4-fold, respectively. Also, the 21d chronic lethality was significantly less for 3d aged suspensions compared to when freshly made (Seitz et al., 2015). This decrease in toxicity was explained by increasing size with aging, causing visi-

ble sedimentation. Another study showed that 24-48h aging of representative AgNPs (NM-300K) had virtually no influence on the 48h immobility induced in *D. magna*, and EC_{50}-values were 33, 39 and 41 µg Ag/L for suspensions aged 0, 24 and 48 hours, respectively (Cupi et al., 2015). However, increased toxicity to *D. magna* is reported after 7d aging of AgNPs in US EPA media, compared to freshly prepared suspensions (Harmon et al., 2014). This was not related to aggregation, but rather dissolution of ions. For zinc oxide nanoparticles (ZnONPs), EC_{50}-values of 6.7, 1.7 and 4.7 mg/L are reported in *D. magna* for suspensions aged 0, 24 and 48 hours, respectively. Increased agglomeration occurred after 24h aging, and here the concentration-response pattern was not monotonous. However after 48h aging a clear dose-response relationship was obtained, likely due to increased dissolution (Cupi et al., 2015).

Indeed, the introduction of pre-testing aging as mean to increase stability during incubation, has been proposed and investigated in algal toxicity testing with AgNPs (Sørensen and Baun, 2015 Paper I). Using a 2h ^{14}C-assimilation test with *P. subcapitata* two consecutive test runs were conducted with freshly prepared citrate stabilized AgNPs from two different manufacturers (AgNP1 and AgNP2). The resulting concentration-response relationships were non-monotonous and poorly reproducible, see Figure 10A and C. However, when AgNPs were aged for 24h prior to testing, clear concentration-response relationships and a higher degree of reproducibility was obtained (Figure 10B and D).

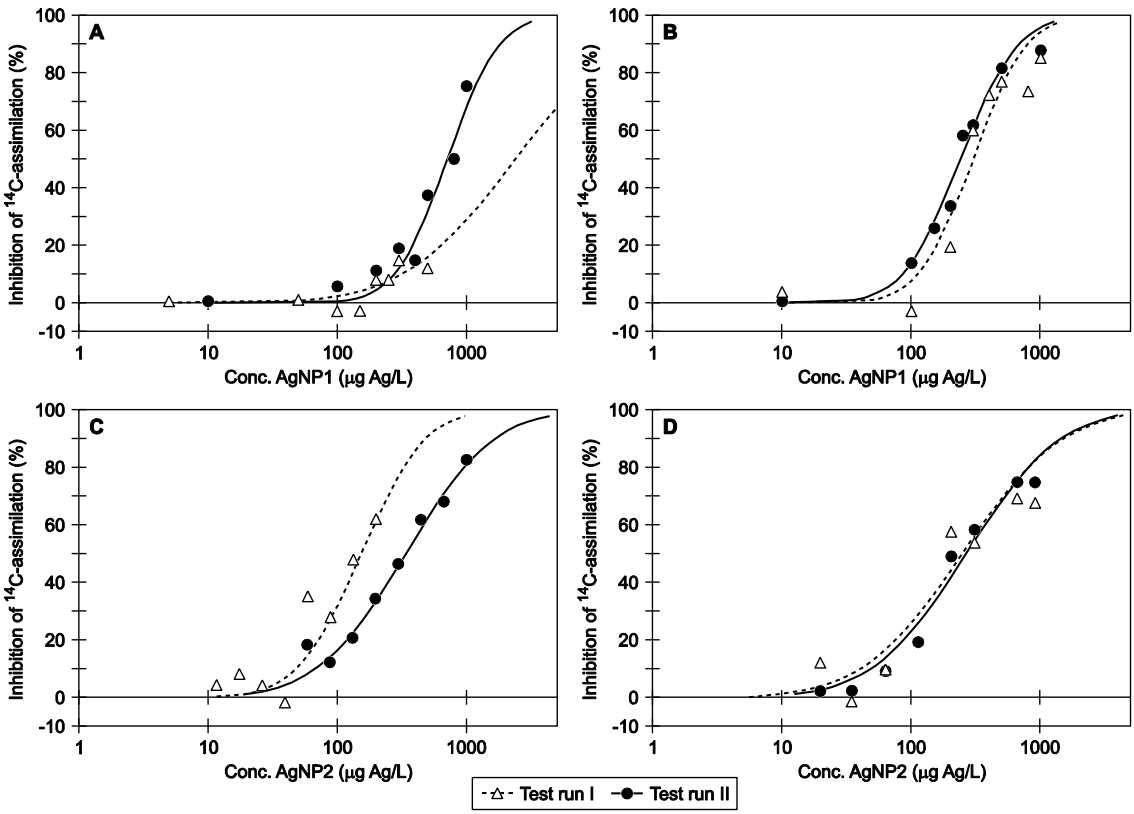

Figure 10. Concentration-response data and fitted curves by Log457 from 2h ^{14}C-assimilation algal tests with *P. subcapitata* exposed to AgNP1 (A and B) and AgNP2 (C and D) being freshly prepared (A and D) or aged for 24h in ISO algal medium in the dark (B and D). For each scenario (A, B, C and D) two individual test runs were conducted (Sørensen and Baun, 2015 Paper I).

Aging however, can also have the opposite effect and reduce stability. The acute 48h immobility of *D. magna* exposed to 24h aged CuONPs yielded such scattered data, and partly eliminated toxicity, that no concentration-response relationship could be established. A freshly prepared suspension in contrast gave monotonous response data (Sørensen et al., 2016b Paper III). The difference was attributed to the visible adsorption of CuONPs on the container walls during aging (occurred both in glass and plastic containers).

Taking all the above data into consideration, two key points are evident: 1) Very few studies have investigated the influence of suspension age on aquatic toxicity test outcome, especially in relation to test reproducibility, and 2) An aging step may both increase and decrease ENP toxicity as well as concentration-response pattern and reproducibility in aquatic toxicity tests. This may be trivial, considering that the transformation processes (outlined in chapter 3) also impact toxicity and stability in different ways, and that this complexity obviously is not eliminated simply by introducing an aging step. The key question therefore becomes *when* and *how* an aging step may be favorable to include for aquatic toxicity testing? This question is addressed in chapter 6.

4.2 Shortening the exposure duration

The exposure duration of aquatic toxicity tests for hazard identification is defined in the standard OECD and ISO guidelines, and is determined based on the generation time of the species and the endpoint in question. The exposure duration is known to be crucial for toxicity, and generally toxicity of soluble compounds increases with prolonged exposure. Therefore, the exposure duration is kept constant in tests applied for hazard identification, to enable comparison and ranking of chemicals according to their intrinsic toxicity (Traas and van Leeuwen, 2007). However, since ENPs often transform over time, in theory prolonged exposure may not necessarily increase toxicity.

Shortening the exposure duration has been proposed as a mean to minimize the extent of ENP transformation during incubation, and thus obtain more stable exposure conditions (Hartmann, 2011; Rosenkrantz, 2013; Sørensen et al., 2016b Paper III) and also a higher degree of reproducibility (Sørensen and Baun, 2015 Paper I). If successful, shorter test duration is also favorable due to less time and resources required for monitoring and characterizing ENP exposure conditions, which facilitates faster toxicity screening and hazard identification of ENPs. A study of stream mesocosms has found the fate of CeO_2NPs to differ for press and pulse exposures, and environmental risk assessment of ENPs was consequently recommended to address the implications of exposure duration (Baker et al., 2016).

4.2.1 Acute algal tests

A reduction of incubation time from the standard 72h to 48h has been validated for the relatively fast growing algae *P. subcapitata* (Arensberg et al., 1995). However, shortening incubation sufficiently to really reduce the impact of ENP transfor-

mation, e.g. to a few hours, is simply not feasible using growth rate inhibition as endpoint. An acute 2h ^{14}C assimilation algal test has been developed and evaluated for the testing of so-called "difficult" substances, for which exposure concentrations are not easily maintained (Rosenkrantz, 2013). For ENPs especially, a few hours incubation is theoretically favorable, as both the adsorption of ENPs to cells, and the production of exudates are minimized.

The applicability of this 2h test was evaluated for Ag and Pt ENPs, by comparing toxicity and reproducibility with those of 48h growth rate inhibition tests (Sørensen and Baun, 2015 Paper I; Sørensen et al., 2016a Paper II). For PtNPs, tests were conducted using two algal species (*P. subcapitata* and *C. reinhardtii*). The response of the 2h test was concluded to mainly rely on physical "shading" effects. The lack of effects in the 2h test was also evident for the dissolved Pt compound $PtCl_4$, as stimulation rather than inhibition occurred in *P. subcapitata*, see Table 1 (Sørensen et al., 2016a Paper II). As discussed in this study, 2h exposure is possibly too short for photosynthesis to be affected, and/or the algae may adapt photosynthesis to the "shaded" conditions in 48h, but not in 2h. Either way, a shortened incubation alleviated PtNP aggregation, but failed to disclose any "true" toxicity besides shading effects.

Table 1. Mean effective concentrations (EC_{50}) and 95% confidence intervals (95% CI) from 2h ^{14}C-assimilation and 48h growth rate inhibition tests with algae *P. subcapitata* and *C. reinhardtii* exposed to AgNPs, $AgNO_3$, PtNPs and $PtCl_4$ (data from Sørensen and Baun, 2015 Paper I; Sørensen et al., 2016a Paper II).

Test material	Algal species	2h ^{14}C-assimilation		48h growth rate	
		EC_{50}	95% CI	EC_{50}	95% CI
$AgNO_3$	*P. subcapitata*	6.0	5.1-7.1	4.9	3.9-6.2
NM-300K	*P. subcapitata*	50	46-56	140	120-160
AgNP1	*P. subcapitata*	710	620-830	310	270-360
AgNP2	*P. subcapitata*	150	130-190	75	63-90
$PtCl_4$	*P. subcapitata*	*		1.3	1.1-1.5
	C. reinhardtii	360	240-540	7.1	6.3-8.1
PtNPs	*P. subcapitata*	47	43-50	15	13-16
	C. reinhardtii	37	31-46	200	170-240

*Concentration-dependent stimulation was observed.

The algal uptake and toxicity of silver ions is known to be very fast, reported to occur within 1h (Navarro et al., 2008). Therefore, AgNPs are obvious candidates for the 2h test. However, while $AgNO_3$ yielded comparable EC_{50}-values for 48h growth rate inhibition and 2h ^{14}C assimilation, this was not the case for the three tested AgNPs, see Table 1 (Sørensen and Baun, 2015 Paper I). The hypothesis that shorter test duration alleviates variability in algal toxicity testing was not supported by the obtained concentration-response data. Two consecutive test runs conducted for two types of AgNPs, in both cases yielded non-comparable concentration-response data (cf. Figure 10A and C). The 2h test conducted in combination with aging of the AgNPs before the 2h test did however result in higher degree of reproducibility (as stated previously).

Rosenkrantz (2013) similarly concluded that the EC_{50}-values from the 2h ^{14}C-assimilation test were not comparable to those of 48h growth rate inhibition, for all the tested herbicides. The toxicity of sulfonylurea compounds was for example grossly underestimated, which was attributed to the toxic mode of action (inhibition of amino acid formation in cells) being too slow to yield a response in 2h. For herbicides, the toxic mode of action is generally well-known, whereas the toxic mode(s) of action for ENPs largely is unknown.

4.2.2 Post-exposure effects from pulse exposure of daphnids

Short-term exposures in *D. magna* as single or repeated pulse(s) have been applied for toxicity testing of soluble compounds, with the effects being monitored during a post-exposure period in pure medium (Andersen et al., 2006; Trac et al., 2015). The purpose of such pulse tests may be to decipher toxic mechanisms and simulate more environmentally realistic and relevant exposure scenarios for chemicals discharged into aquatic environments as pulses, e.g. from storm-water runoff, overflow of wastewater treatment plants, agrochemicals and veterinary pharmaceuticals (Handy, 1994; Hommen et al., 2010; Trac et al., 2015). Also, agrochemicals with a short half-life in the environment, even if not discharged as pulses, are suggested to exhibit pulse-like behavior (Reinert et al., 2002). Analogously, ENPs may also rapidly transform from their pristine form when released to the environment (Lowry et al., 2014) or added to test media (Petersen et al., 2014), as described in chapter 3. For ENPs, the use of more realistic release and exposure scenarios in toxicity testing has been highlighted as an important research need (Palmqvist et al., 2015).

Short-term (1-3h) pulse exposures of daphnids, followed by detection of acute and chronic effects during 48h and 21d post-exposure periods, has therefore been suggested relevant for ENPs, and the applicability evaluated for 24h aged Ag and freshly prepared CuONPs and soluble salts (Sørensen et al., 2016b Paper III). In this study, ENP transformation due to dissolution, agglomeration and sedimentation was less during 1-3 hours in modified M7 medium (corresponding to pulse exposures), than during 24 or 48 hours (corresponding to standard continuous exposure in acute tests and static renewal in chronic tests). The acute immobility of *D. magna* observed 48h post-exposure after pulses increased slightly with pulse duration for the test materials, except for AgNPs, causing similar immobility regardless of pulse duration, see Table 2. For all test materials, a 3h pulse yielded comparable EC_{50}–values (48h post-exposure) to those from 24h continuous exposure, according to overlapping 95% confidence intervals. For CuONPs, the 1-3h pulses appeared to cause slightly lower toxicity than continuous exposure, although not statistically significant, as the confidence intervals were quite broad (Sørensen et al., 2016b Paper III).

No chronic effects was determined in the 21d post-exposure period after 1-3h pulses of AgNPs (10-50 µg Ag/L) and $AgNO_3$ (0.2-2 µg Ag/L), in terms of the number of moltings, days to first live offspring and cumulated number of offspring. AgNPs even appeared to increase the number of offspring (3h pulse only). In contrast, pulses of CuONPs (0.2-3.2 mg Cu/L) and $CuCl_2$ (0.01-0.05 mg Cu/L) decreased the number of moltings and offspring, and CuONPs prolonged the time to first live off-

spring (Sørensen et al., 2016b Paper III). Generally, ENPs are believed to stay suspended only very shortly in natural aquatic environments upon exposure, due to transformations, adsorption and sedimentation processes removing them from suspension. Interestingly, a few hour exposures may, according to this study, be sufficient to induce adverse effects on daphnia reproduction.

Table 2. Mean effective concentrations (EC_{50}) and 95% confidence intervals in parentheses for acute immobilization of *D. magna* after 24h continuous exposure, and 48h post-exposure following 1, 2 and 3 hour pulses of AgNPs, $AgNO_3$, CuONPs and $CuCl_2$ (Sørensen et al., 2016b Paper III).

Exposure type	**AgNPs** µg Ag/L	**$AgNO_3$** µg Ag/L	**CuONPs** mg Cu/L	**$CuCl_2$** mg Cu/L
Continuous				
$EC_{50,24h}$	130 (120-150)	4.5 (4.0-5.2)	6.5 (3.7-46)	0.10 (0.080-0.13)
1h pulse				
$EC_{50,48h\ post\text{-}exposure}$	110 (88-140)	5.1 (4.3-6.5)	42 (15-89000)	0.22 (0.036-0.75)
2h pulse				
$EC_{50,48h\ post\text{-}exposure}$	110 (-)	4.6 (3.7-6.0)	19 (12-66)	0.14 (0.031-0.42)
3h pulse				
$EC_{50,48h\ post\text{-}exposure}$	110 (87-120)	4.3 (3.1-7.0)	17 (11-47)	0.12* (-)

(-) Estimation of confidence intervals is not possible from data.
*Determined from linear connection of data points (estimation by TOXCALC not possible).

Chronic tests with continuous exposure (static renewal) of *D. magna* exposed to the same type of CuONPs and $CuCl_2$ are reported to decrease the number of offspring at even lower concentration ranges: 0.037-1.4 mg Cu/L (CuONPs) and 0.004-0.060 mg Cu/L ($CuCl_2$) (Adam et al., 2015). Likewise for AgNPs, detrimental effects on survival, growth and/or reproduction are reported for *D. magna* continuously exposed to 10-50 µg Ag/L (Mackevica et al., 2015; Zhao and Wang, 2011). From these data is seems a short-term (1-3h) pulse of Ag and CuO ENPs does not adversely affect reproduction, molting, growth and lethality to the same extent and/or at similar low concentration levels, as 21d continuous exposure.

No other studies on the influence of a shortened exposure duration or pulse exposure for ENPs in tests with crustaceans have been identified. Rather, prolonged exposure (72-96h) in acute toxicity tests with TiO_2 is proposed (Clément et al., 2013; Dabrunz et al., 2011; Zhu et al., 2010). This revealed toxicity otherwise not observed, or at least found to be lower, from standard 48h exposure. Prolonging exposure may however compromise the general conditions of the organisms, as no feeding is provided. This may be why the control lethality exceeded the OECD validity criteria of 10% (OECD, 2004) in the study by Zhu et al. (2010). The generated EC_{50}-values were based on water phase concentrations (nominal or measured), although the exposure was reported to clearly change during exposure from aggregation and sedimentation. Thus, the dose "detected" by the organisms after 96h is likely much higher (due to previously mentioned adhesion and accumulation in the gut), than the (over time decreasing) water phase concentrations used for EC_{50}. This further makes the EC_{50} appear lower (and toxicity higher), than what may actually be the case. Thus, these studies prolonging exposure duration actually demonstrates the exact issues that are shown minimized during the shortened exposure duration.

5 Toxicity endpoints for aquatic toxicity testing of nanoparticles

Attempts to stabilize suspended ENPs during exposure, whether by timing or media parameter manipulations, will only address the stability issue. It does not change the inherent difference between ENP suspensions and chemical solutions. The test organisms will encounter nano-sized physical entities rather than (or in addition to) a soluble chemical fraction. This may change how the organisms respond to the test material, and how this response is interpreted. In the context of concentration-response data, the implication of toxicity endpoints thus becomes equally important to consider as the exposure part.

Despite the extensive research in nanoecotoxicology, the exact mechanisms of toxic action of ENPs are still not fully established. The mechanisms most frequently proposed to govern or influence ENP toxicity are illustrated in Figure 11 and include:

- Cellular effects relating to ROS and oxidative stress
- Effects of toxic, dissolved metal-ions released from ENPs
- Physical effects from interaction of ENPs and cells/organisms
- Effects relating to internalization and/or distribution of ENPs in cells/organisms
- Other nanoparticle-specific unknown effects

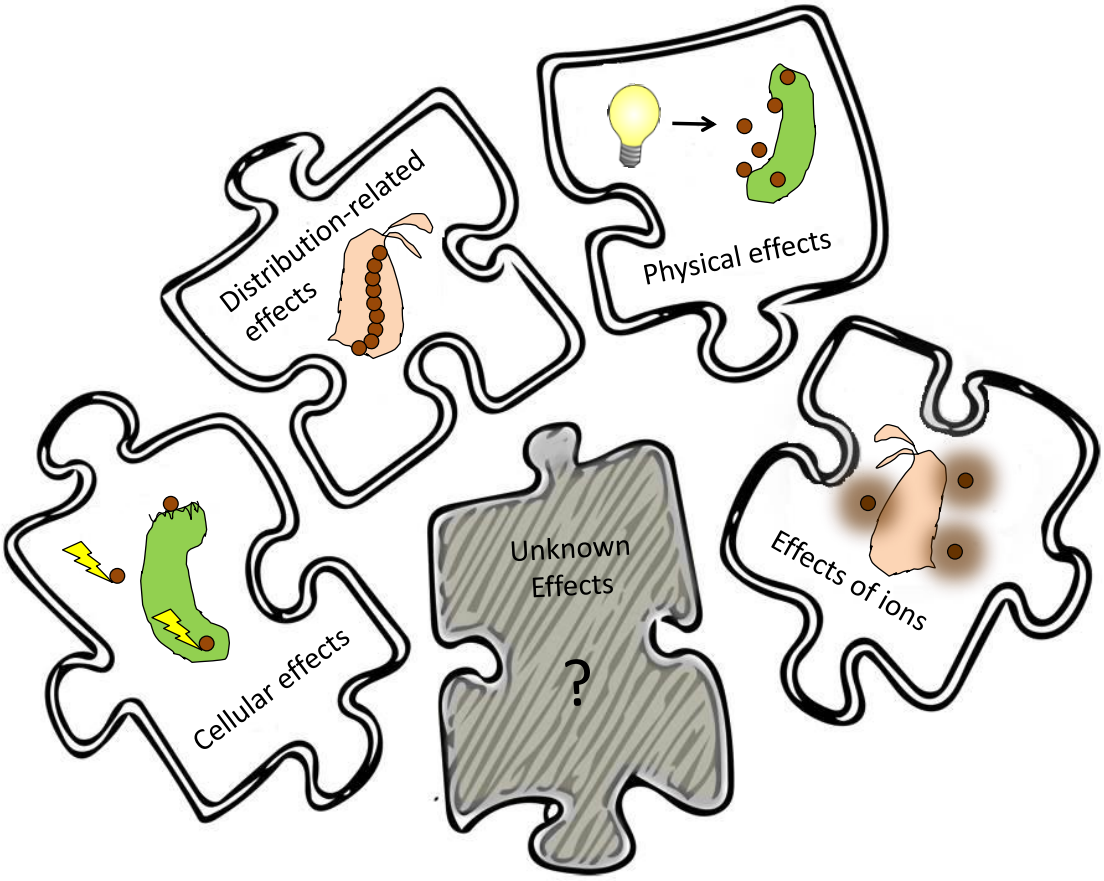

Figure 11. Illustration of the proposed nanoparticle-related effects in aquatic toxicity testing (modified from Skjolding et al., 2016).

5.1 Effects of dissolved ions

The most frequently reported potential toxic action of Ag, CuO and ZnO is the release of toxic ions (Juganson et al., 2015). There are however, several studies reporting toxicity beyond what can be explained by the measured dissolved fraction and/or possible nanoparticle-specific effects. These include exposure of algae to ENPs of Ag (Miao et al., 2010; Navarro et al., 2008; Oukarroum et al., 2012), CuO (Manusadžianas et al., 2012; von Moos et al., 2015), and Pt (Sørensen et al., 2016a Paper II) as well as daphnids to AgNPs (Ribeiro et al., 2014b; Stensberg et al., 2014) and CuONPs (Sørensen et al., 2016b Paper III).

Comparing these findings is complicated by the use of different endpoints, organisms/species and not least, the different approaches to differentiate toxic effects of the dissolved fraction from nanoparticle-specific effects. The simplest approach is comparing effects from exposures of ENPs with those of the dissolved reference, e.g. AgNPs and $AgNO_3$ as done by Ribeiro et al. (2014b). However, this provides no information on the underlying mechanism(s), and it is questionable whether a dissolved reference salt is fully comparable to the dissolved fraction of ENPs, in terms of chemical speciation, and exposure concentration kinetics.

Another approach is to expose organisms to the ENP suspension, and in a parallel setup, to the dissolved fraction of the ENP suspension, for instance through a dialysis membrane as in a study with algae and CuONPs by von Moos et al. (2015). This way, the exposure dynamics are accounted for, and toxicity (in this case oxidative stress) was shown to occur much faster in the presence of ENPs. This indicates that CuONPs exert a nanoparticle-specific toxic effect and/or modify the bioavailability of released ions, but the setup does not allow distinguishing these two mechanisms.

A third approach is to determine the ENP dissolution in the test medium, e.g. by ultracentrifugation, dialysis membrane or another method, and then compare the concentration-response data based on the total ENP mass, and the dissolved mass, respectively (Sørensen et al., 2016a Paper II and 2016b Paper III). Because ENP dissolution can change with time and concentration (cf. chapter 3.2 and Figure 5), dissolution should be determined over a time period equivalent to the exposure duration, and concentrations covering the exposure range. Otherwise, the concentration-response data based on the dissolved fraction may not be fully descriptive.

As the presence of test organisms may impact dissolution (Miao et al., 2015), it should ideally be quantified during the actual toxicity test, and not in a parallel setup, as often the case. Ligands can be added to the test system including test organisms, provided the ligand is not toxic, in order to neutralize the toxicity of released ions and thereby reveal potential nanoparticle-specific effects. The addition of citrate to algal tests with $AgNO_3$ and AgNPs, for example indicated that toxicity was mediated by Ag^+, but that AgNPs contributed to toxicity by serving as sources of these ions (Navarro et al., 2008).

The possibility of having locally increased dissolved concentrations around ENPs attached to or associated with the test organisms cannot easily be accounted for regardless of the procedure used.

5.2 Physical effects

Hazard identification toxicity tests are designed to reflect the direct toxic effects of a chemical compound on the test organism. However, ENPs are shown to affect the traditional endpoint applied for toxicity tests with algae (growth rate inhibition) and daphnids (immobilization) via seemingly non-toxicological mechanisms. Here, such non-toxicological effects are referred to as "physical effects".

During algal growth rate inhibition tests, the presence of ENPs may restrict light from reaching the algal cells and thereby inhibit the growth rate due to shading, rather than from inducing a toxic effect. Shading effects are reported for CNTs, Au and PtNPs (Schwab et al., 2011; Sørensen et al., 2016a Paper II; van Hoecke et al., 2013), while for ZnO, CuO and TiO_2 ENPs shading is found negligible (Aruoja et al., 2009; Hartmann et al., 2010; Hund-Rinke and Simon, 2006). In the tests with Au and Pt ENPs, shading could not fully explain the growth rate inhibition determined, indicating additional toxic and/or physical effects of these ENPs (Sørensen et al., 2016a Paper II; van Hoecke et al., 2013). Except for the study by Schwab et al., (2011) shading has been investigated using a setup where algae are separated from the ENPs suspensions and only exposed to light penetrating the ENPs suspension, see Figure 12A and B.

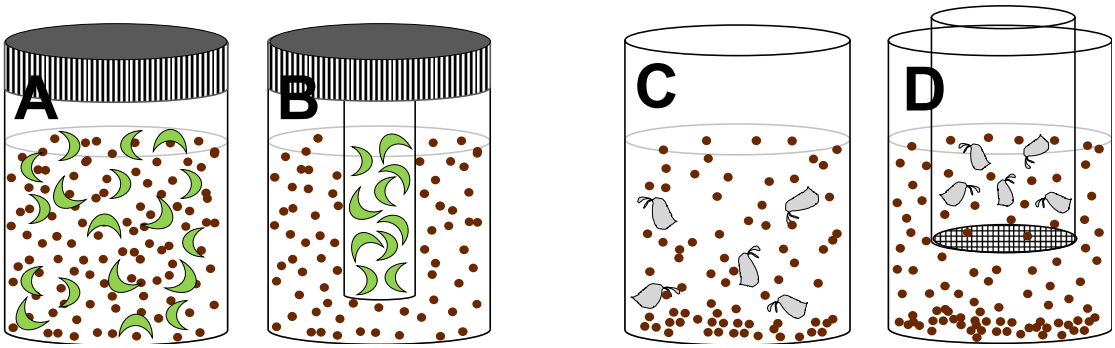

Figure 12. Illustration of regular setups applied for toxicity tests with algae and daphnids (A and C), and setups applied to distinguish physical and chemical effects of ENPs (B and D). Physical shading effects in algal tests may be investigated by a double-vial setup (B), where algal cells are contained in the smaller inner-vial, surrounded by the ENP suspension in the larger outer-vial. Physical immobilization of daphnids arising from contact with larger aggregated/settled ENPs can be avoided by keeping the daphnids in a mesh-bottomed beaker inserted into larger beakers containing ENP suspensions (D) (Sørensen et al., 2015 Paper IV).

While this approach reveals shading from ENPs located distant from algal cells, it does not reflect shading by ENPs in the close vicinity or adsorbed to/encapsulating the algal cells. This shading on a cellular level may contribute to the observed growth rate inhibition that is not identified from such a "separation setup". Another issue is that reduction of the algal growth rate from shading may mask toxicity, as slow growing algae may be less sensitive to toxicants (Cleuvers and Weyers, 2003).

Therefore, new testing endpoints or methods for identification of shading are essential for improve our understanding of the mechanisms involved in algal responses to ENPs. Analysis of the pigment composition in exposed algae compared to the control, has been suggested to provide a qualitative measure for "internal" shading, i.e.

as experienced by the algae (Hjorth et al., 2015). The approach rely on algal photo-acclimation, causing algae to rapidly adapt their pigment composition in response to changing light conditions: At excess light, the algae produce more xanthophyll pigments that protect against light stress, whereas low light conditions stimulate the chlorophyll production to optimize photosynthesis (Hjorth et al., 2015). This approach however, remains to be fully validated and tested using various ENPs.

Other physical effects have been suggested to result from the adsorption of ENPs to algal cells, including changes in pH, redox conditions, and reduced nutrient availability (Hartmann et al., 2013; van Hoecke et al., 2009).

The adsorption of ENPs to crustaceans also causes physical effects. Adsorption of CeO_2, PtNPs, and TiO_2 to the exoskeleton, cuticle and antenna of daphnids 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). Immobility is traditionally the toxicity endpoint applied in acute tests with daphnids, but when testing ENPs, it is problematic that physical and toxicological impairment of mobility cannot be distinguished.

Therefore, both the endpoints of immobility and lethality were recorded upon 48h exposure to PtNPs (Sørensen et al., 2015 Paper IV). The effective concentration for immobilization (IC_{50}) was lower than for lethality (LC_{50}), and organisms were seemingly immobilized from adsorbed PtNPs, but still alive. Daphnids are pelagic filter feeders, but will also search for food along bottom sediments. This behavior has also been observed in laboratory experiments, as daphnids may often be at the bottom of the test vessels. To reduce the adsorption of PtNPs to daphnids, the test was repeated using exposure beakers with inserted mesh-bottomed testing chambers as illustrated in Figure 12C and D (Sørensen et al., 2015 Paper IV). This allowed for exposure of daphnids to PtNPs in suspension, but hindered contact with larger aggregates and settled PtNPs at the bottom. Markedly less PtNPs on the exoskeleton of daphnids was observed. This setup resulted in similar IC_{50}- and LC_{50}-values, reflecting PtNP toxicity rather than physical restraints (Delgado, 2013).

5.3 Cellular effects

The ability of ENPs to generate ROS such as superoxide, hydroxyl, and hydrogen peroxide, and induce oxidative stress are considered the major mechanisms responsible for cellular effects of metal and metal oxide ENPs in aquatic organisms (Ivask et al., 2014; Juganson et al., 2015; von Moos and Slaveykova, 2014). 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 (Fu et al., 2014; von Moos and Slaveykova, 2014). The reverse is also suggested; that 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 (Kaweeteerawat et al., 2015).

The many pathways interlinking ROS, oxidative stress, and cellular toxicity challenge the establishment of causality, as well as the identification of ENP properties

governing these effects. As for other toxicity endpoints, the generation of ROS and oxidative stress are suggested to be influenced by various ENP properties. These include size, shape, chemical composition, surface charge, area and chemistry, dissolution, and aggregation (Fu et al., 2014; von Moos and Slaveykova, 2014).

The conduction band energy level of metal oxide ENPs is proposed as predictors of oxidative stress and cytotoxicity. An overlap of theoretical ENP conduction band energy with the cellular redox potential is hypothesized to allow electron transfer between ENPs and biomolecules, thereby creating free radicals and oxidative stress (Burello and Worth, 2011; Kaweeteerawat et al., 2015). Correlation between band energy and toxicity is found for 24 metal oxide ENPs in human cells and bacteria (Kaweeteerawat et al., 2015; Zhang et al., 2012). The approach however, was found to have limitations as the correlation was inaccurate for ENPs of TiO_2, CuO and ZnO (Kaweeteerawat et al., 2015; Zhang et al., 2012). Also, it may not apply tovery small ENPs for which energy band structures are considerably different (Burello and Worth, 2011).

Catalytic or redox active ENPs, such as Cu and Pt ENPs, which participate in electron transfer/sharing/bonding with surrounding molecules, are likely to generate ROS and oxidative stress. Indeed, ENPs of CuO and Pt have been found to induce high levels of oxidative stress in algae (Sørensen et al., 2016a Paper II; von Moos et al., 2015). For PtNPs, the effect was not attributed dissolved Pt, see Figure 13. For CuONPs and $CuCl_2$ the oxidative stress kinetics differed, also indicating a nanoparticle-specific toxic mode of action and/or vector-like mediated increase in bioavailability of ions (von Moos et al., 2015). Oxidative stress may therefore constitute an endpoint suitable for disclosing nanoparticle-specific toxicity, and is in line with the hypothesized toxic mechanism(s) of ENPs.

In larger organisms and humans, the relevance of *in vitro* toxicity tests is often questioned, as the exposure and toxic mode of actions may not reflect *in* scenarios. However, for unicellular organisms such as algae, cellular effects *are* organism effects, and there is no such discrepancy. The applicability of oxidative stress as toxicity indicator is challenged though, firstly by the lack of causality between the two, and secondly by the counteractive effect of cellular antioxidant systems. Generation and detoxification of ROS are constantly ongoing cellular processes, but toxicity only arises if the antioxidant capacity is exceeded. For PtNPs, negligible membrane damage was detected in algae, despite a high level of oxidative stress, indicating the antioxidant systems could cope with the oxidative stress and prevent its progression to membrane damage (Sørensen et al., 2016a Paper II).

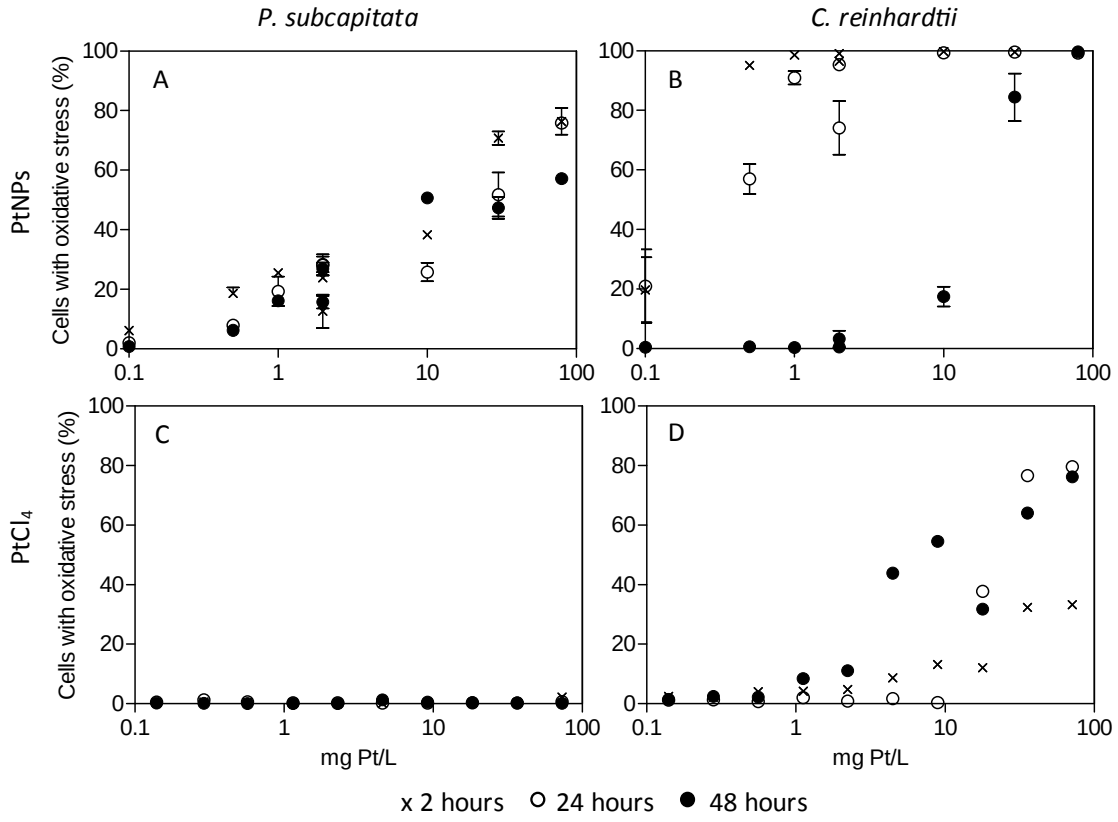

Figure 13. Oxidative stress in *P. subcapitata* and *C. reinhardtii* upon 2, 24 and 48 hours exposure to $PtCl_4$, in single concentrations (0.14-73 mg Pt/L) and PtNPs in two parallel tests with triplicate low concentrations (0.1-2 mg Pt/L) and high (2-80 mg Pt/L), respectively. Data for *C. reinhardtii* exposed to $PtCl_4$ for 2h are based on very low cells numbers (Sørensen et al., 2016a Paper II).

5.4 Effects related to nanoparticle internalization

Linking ENP toxicity to their internalization and/or distribution inside organisms/tissues/cells relies on applicable techniques to verify actual internalization. As mentioned in chapter 3.4, the application of electron microscopy techniques for this purpose presents numerous challenges, and routinely use for toxicity testing is hindered by the labor- and cost intensity of these methods.

The internalized ENP fraction may also be quantified by determining the mass of ENPs in the exposed organisms. However, such body burdens in organisms such as daphnids and fish often include ENPs accumulated in the gut (Skjolding et al., 2016). Although this fraction potentially contributes to toxicity, it is not internalized in cells or tissues. Some degree of internalization does seem plausible though, as uptake and depuration studies have reported incomplete depuration. For example, incomplete depuration is reported for AuNPs when exposed daphnids are transferred to pure medium (Khan et al., 2014; Skjolding et al., 2014). This indicates retention and/or internalization of these ENPs via the gut.

Although not necessarily internalized, ENPs retained in the gut may impact toxicity simply by prolonging and/or locally increasing exposure within the daphnia gut, compared to exposure of a soluble substance. This mechanism is likely responsible for the more pronounced reproductive effects of CuONPs (when expressing the concentration in terms of the dissolved Cu) compared to $CuCl_2$, in a 21d post-exposure period following 1-3h pulse exposures (Sørensen et al., 2016b Paper III), see Figure 14. Accumulation of CuONPs in the gut was suggested to prolong the exposure beyond the pulse duration, as the gut content is transferred with the organism to pure medium for the post-exposure period. In contrast, there is no such carry-over of accumulated $CuCl_2$ in the gut after ended pulse. Moreover, the accumulated CuONPs may have dissolved more readily in the gut, as suggested for AgNPs (Gaiser et al., 2011), thus locally increasing the dissolved fraction available for uptake.

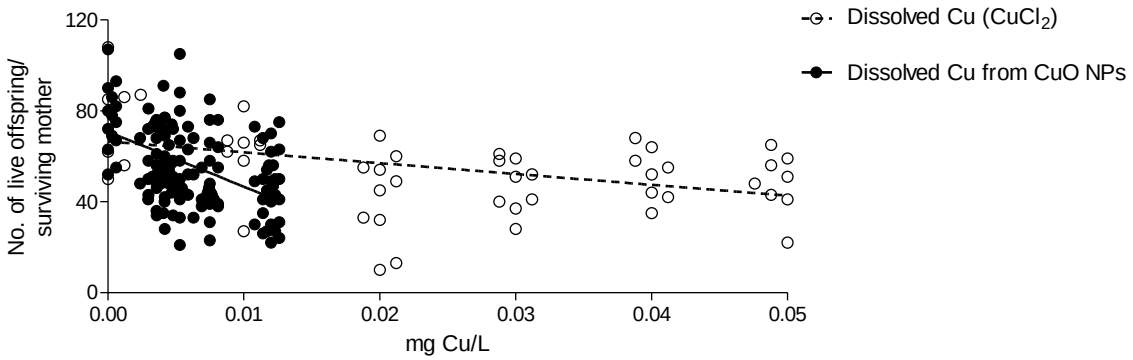

Figure 14. Number of live offspring produced by each *D. magna* mother animal in 21 days post-exposure period following 1-3h pulses of CuONPs (pooled data 1-3h pulses) and $CuCl_2$ (2h pulse). Response data and linear regressions are provided for $CuCl_2$ (circles and dotted line) and CuONPs, with the concentration expressed as the measured dissolved Cu fraction (black spheres and line) (Sørensen et al., 2016b Paper III).

For algae, a study with CuONPs indicated that the toxicity was due to internalization and intracellular interactions, and that the primary mechanism was intracellular ROS generation (Perreault et al., 2012). These conclusions were based on measured body burdens in washed algal cells, intracellular ROS generation and inhibition of photosystem II (PSII) activity of alga *C. reinhardtii* when exposed to coated and bare CuONPs and their dissolved fractions. Higher uptake was found in the presence of CuONPs compared to exposure of dissolved fractions only, and higher body burdens and toxicity was found for the coated CuONPs compared to non-coated.

Similarly, internalization of AgNPs in the mixotrophic species *Ochromonas Danica* has been proposed as pathway for algal toxicity (Miao et al., 2010). This was based on intracellular AgNPs identified by electron microscopy, and that toxicity occurred even when free Ag^+ was eliminated from the medium by addition of glutathione. In contrast, the uptake of AgNPs in the algae *Raphidocelis subcapitata* has been suggested to occur through dissolved ions, as no internalized AgNPs were identified by microscopy imaging (Ribeiro et al., 2014a).

For algae exposed to PtNPs, the Pt body burden (including strongly adsorbed PtNPs on the algal surface that are not removed by gentle cell washing) differed between two algal species. The body burden was higher in *P. subcapitata* than *C. reinhardtii*, possibly due to less PtNP-aggregation in the medium of *P. subcapitata* and/or a favored binding of Pt to their polysaccharide-rich cell wall compared to the glycoproteins of the *C. reinhardtii* cell wall (Sørensen et al., 2016a Paper II). Toxicity likewise differed in the two species: higher growth rate inhibition occurred in *P. subcapitata*, potentially as result of the higher body burden and/or adsorption to the cell surface and subsequent higher toxicity and/or shading. On the other hand, more oxidative stress occurred in *C. reinhardtii* with the lowest body burden (Figure 13A and B). Similarly, uptake of ZnONPs and following growth rate inhibition is reported for *C. reinhardtii*, in the absence of oxidative stress (Gunawan et al., 2013).

Overall, current data indicate that internalization and/or body burden of ENPs may relate to toxicity, although not in a simple way. The relation likely depends on the type of ENP, organism/species and toxic endpoint in question. Moreover, the terms internalization and body burdens are sometimes used without distinguishing actual internalization from the fraction accumulated in the gut and/or adsorbed to the surface of organisms.

5.5 Other nanoparticle-specific effects

ENP toxicity may possibly involve other, yet unidentified, nanoparticles-specific mechanisms. Many historic examples illustrate that novel technologies and chemicals may cause unpredicted effects not recognized until several decades after their introduction (Hansen and Gee, 2014).

ENP toxicity may also "simply" result from a combination of the outlined effects. This implies that ENP toxicity equals the sum of these effects, but the effects cannot be considered additive. They likely rely on different sites and modes of action, and effects may reinforce or counteract each other in synergistic and antagonistic ways. Moreover, the effects rely on different exposure fractions (e.g. the dissolved fraction, or the fraction of ENPs adsorbed to cells), which in turn are dependent on for example the medium composition, organism type, and ENP coating, size, and shape.

To account for the diverse effects of ENPs, multi-endpoint approaches to (aquatic) toxicity testing has been suggested (George et al., 2011; Oomen et al., 2014; Sørensen et al., 2016a Paper II). Other studies have applied multiple toxicity endpoints to investigate the underlying mechanisms of ENP toxicity, and attempted to relate these effects to ENP concentration, body burden, or the dissolved fraction.

The mechanisms governing toxicity of CeO_2NPs to the alga *P. subcapitata* has been studied by such a systematic approach (Angel et al., 2015; van Hoecke et al., 2009). Both studies disregarded effects of dissolved cerium due to negligible dissolution measured in the test medium. Angel et al. (2015) found ROS generation and growth rate inhibition correlated at normal light conditions, however diminishing ROS by using UV-filtered light, did not reduce growth rate inhibition, suggesting that ROS generation was not the (governing) toxic mode of action. Overall, sorption of CeO_2NPs to algae was shown to be the most likely cause of the observed toxicity (Angel et al., 2015). In line with this, internalization of CeO_2NPs and down-regulation of photosynthesis and carbon fixation in alga *C. reinhardtii* has been detected, without affecting growth (Taylor et al., 2016).

On the other hand, van Hoecke et al. (2009) did not find convincing evidence of internalization nor adsorption to cell walls. Also, hypotheses of shading and nutrient deficiency (caused by adsorption of nutrients to CeO_2NPs) as mechanisms involved in growth rate inhibition were rejected. However, clustering of CeO_2NPs around cells was observed and concluded to inhibit algal growth through localized shading, nutrient depletion and/or direct effects.

The discrepancies between these studies illustrate that a multiple endpoint approach may provide valuable knowledge within one study, but not necessarily ensure comparability between different studies. This may be a consequence of different methods and endpoints applied and hypotheses tested. Thus, some degree of consensus on how to conduct such tests in practise is necessary, and guidance on best practices/techniques would be beneficial in this regard.

Various algal toxicity endpoints has been investigated for PtNPs in two species (*P. subcapitata* and *C. reinhardtii*), including 48h growth rate inhibition, and 2h ^{14}C-assimilation, as well as the effect of dissolved fractions and shading in both these

tests. Also, the potential photosynthetic activity, oxidative stress, membrane damage and body burdens were determined (Sørensen et al., 2016a Paper II).

The 48h growth rate inhibition was found to be partly caused by shading, whereas the 2h ^{14}C-assimilation appeared to be completely governed by shading, see Figure 15. When exposures were based on the measured dissolved Pt fraction (determined by ultracentrifugation), the concentration-response curve aligned with that of $PtCl_4$ for *C. reinhardtii* indicating that the effects of PtNPs could be ascribed to the dissolved fraction of Pt in the medium (Figure 15A). For *P. subcapitata*, the same approach resulted in higher toxicity from the dissolved fraction of PtNPs than from the dissolved Pt reference salt, $PtCl_4$ (Figure 15C). This indicates an additional nanoparticle-specific effect. This could not be ascribed to membrane damage or oxidative stress (cf. Figure 13), as no membrane damage occurred from neither PtNPs nor $PtCl_4$. Also, the oxidative stress affected *C. reinhardtii* more than *P. subcapitata*, which is not in agreement with the growth rate inhibition. Instead, this nanoparticle-specific effect was related to higher body burdens in *P. subcapitata*, possibly as result of their cell wall composition and/or their lower growth rate than *C. reinhardtii*. A higher body burden (including intra-and extracellular Pt) would likely increase local shading from adsorbed PtNPs and/or other direct or indirect effects of PtNPs not accounted for by these endpoints (Sørensen et al., 2016a Paper II).

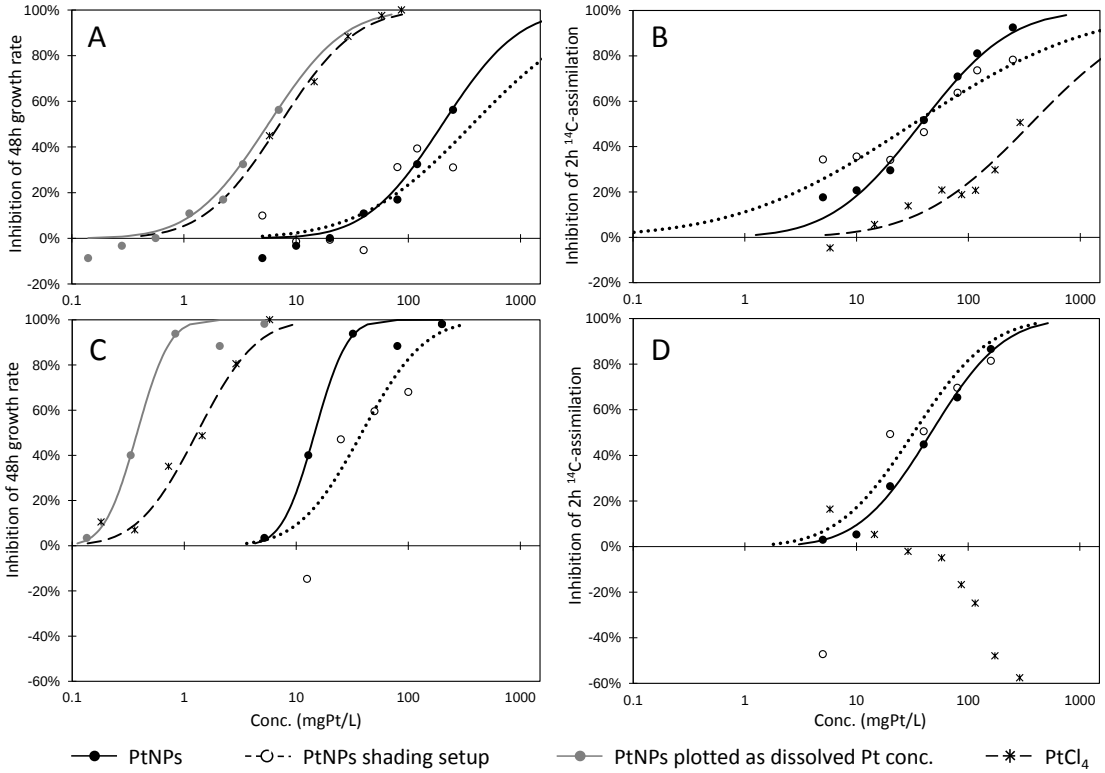

Figure 15. Concentration-response data and fitted curves from 48h growth rate inhibition tests (A and C) and 2h ^{14}C-assimilation tests (B and D) with *C. reinhardtii* (A and B) and *P. subcapitata* (C and D) exposed to PtNPs and $PtCl_4$. For PtNPs two setups were applied (as illustrated in Figure 12A and B): A regular setup and a double-vial setup for investigation of shading effects. Concentration-response data and curves for PtNPs was recalculated based on the dissolved Pt fraction, and plotted for A and B to reflect the toxicity of the dissolved Pt in the PtNP suspension (Sørensen et al., 2016a Paper II).

6 Implications of findings and testing recommendations

Currently, there are no ENP-specific testing strategies for the assessment of ENP toxicity to aquatic organisms in chemical regulations (Hund-Rinke et al., 2015). Regulatory testing within Europe is therefore conducted following the test guidelines referred to by the REACH and CLP regulations, including OECD and ISO protocols.

According to the OECD working party on manufactured nanomaterial (WPMN), the basic toxicological principles and test endpoints of the aquatic toxicity test guidelines are found adequate for the testing of ENPs (Kühnel and Nickel, 2014), although certain issues have been highlighted which need to be addressed. These include preparation of stock and exposure suspensions, but also the difficulties assessing actual exposures, and the artefacts related to the adsorption of ENPs to organisms, and shading which may complicate the interpretation of test results. Furthermore, improved knowledge on ENP toxicokinetics, ENP interference with test assays, and improved characterization during assays were highlighted as key research needs (Kühnel and Nickel, 2014). Except for stock suspension preparation, these issues have to some extent been addressed in this thesis, and the implications of findings in the context of toxicity testing for regulatory hazard identification will be discussed in this chapter.

6.1 Controlling exposure through timing considerations

The use of freshly prepared ENP suspensions in short-term algal toxicity testing has been shown to potentially cause an underestimation of toxicity (Sørensen and Baun, 2015). Thus, aging of ENPs in medium prior to testing may assist to disclose toxicity, otherwise overlooked or underestimated. In this regard, it is however, important to recognize the number of variables to account for during toxicity testing, and one may argue the addition of new ones to the list should perhaps be more substantiated, i.e. based on result from more studies. In line with this, the ecotoxicology of aged vs. pristine ENPs has been highlighted as an area needing more research, by the OECD WPMN (Kühnel and Nickel, 2014).

Additionally, an aging step is relevant for those ENPs where testing results in poor reproducibility and non-monotonous concentration-response data. Especially, this may be true for ENPs known to undergo dissolution, such as Ag, ZnO and CuO ENPs, as this dissolution is likely to influence toxicity substantially. The studies investigating aging effects, all find agglomeration/aggregation to increase with aging time, but the resulting impact on toxicity differs in various studies, making generalizations difficult. In toxicity tests without shaking or flow, such as the *D. magna* immobilization tests, sedimentation of ENPs may also be avoided by introducing an aging step. This however requires that the aging is conducted without shaking or stirring, and that only the suspended fraction is transferred from the aging container to the test vessels.

Also, defining the duration of the aging period is difficult. For some ENPs, it is possible to find data on dissolution kinetics, which can provide an idea of the appropriate aging duration in this regard. Another issue to consider when determining the duration of the aging step is that the quality of the media may be influenced by microbial contamination and/or chemical speciation of media components. To avoid excessive testing a single aging period may be applied, rather than a series, unless of course for strictly exploratory research purposes. The aging conditions applied in various studies differ in terms of conditions such as light, temperature, and shaking. It would be preferable to apply aging conditions, similar to the actual toxicity test conditions. This would make the transformations, otherwise ongoing through incubation, to occur during the aging step instead.

With regard to exposure duration, a shortened incubation was generally suggested as a mean to provide stable and uniform exposure conditions for ENPs. However, in algal testing, this approach revealed a risk of underestimating toxicity and it is therefore not suitable for hazard identification purposes. Also, the shortened 2h algal test did not necessarily facilitate exposure stability or reproducibility, as hypothesized. Shortened exposures does however have some eligibility in the combination with the pre-testing aging step, especially for exploratory research aiming to elucidate time-dependency of ENP toxicity, but also for regulatory algal testing to enhance reproducibility of the resulting concentration-response data.

To be broadly applicable, very short-term (few hours) acute algal tests must rely on a sensitive endpoint, preferably relevant to the toxic mechanisms hypothesized for ENPs. Such endpoint could be oxidative stress, proposed as a main mechanism governing toxicity ENP toxicity, and shown to occur very rapidly in algae; for instance within few hours exposure to PtNPs (Sørensen et al., 2016a Paper II), and CuONPs (Cheloni et al., 2016; von Moos et al., 2015).

For daphnids, shortening the exposure duration down to a few hours and observing the 48h post-exposure immobility of daphnids in pure medium, offered an acute toxicity test setup that reduced the transformation of ENPs during incubation, with comparable sensitivity to disclosing toxicity for Ag and CuONPs. The applicability for other ENPs remains to be investigated, but is likely similar for other soluble and/or toxic ENPs, such as ZnO, and CeO_2 ENPs.

Although 1-3h pulse exposure was found sufficient to induce adverse effects on daphnia reproduction, generally the effects observed were fewer and less pronounced compared to tests with continuous exposure for Ag and CuO ENPs, indicating the pulse testing was less sensitive. The pulse test setup, however allowed to identify nanoparticle-specific effects of CuONPs likely related to extended internal exposure beyond the pulse duration, than was the case for $CuCl_2$.

From other studies, prolonged exposure duration in acute immobility tests with daphnia has been proposed, as this caused effects not disclosed by a 48h test (Clément et al., 2013; Dabrunz et al., 2011; Zhu et al., 2010). On the other hand, these tests reported significant transformation of ENPs during the incubation. Evidently, there is a tradeoff between exposure stability and sensitivity from a shortened exposure.

Overall, attempts to control the exposure by minimizing the influence of transformation processes through approaches of aging, reduced exposure duration, media parameter adjustments, and dispersion protocols can be helpful to a certain extent. However, these changes may influence toxicity, and in worst case fail to disclose toxicity creating false negative data for hazard identification. Even when successful, exposure control measures do not eliminate the need for thorough exposure characterization.

6.2 Exposure characterization and dose-metrics

The transformation processes of ENPs in medium inevitably results in inhomogeneous distribution in the test system, and complicate determination of the exposure concentration. For soluble compounds, the concentration of the chemical compound in the test medium is in most cases uniformly represents the dose experienced by the organisms. For ENPs, the dose experienced by organisms may often not be well defined by the ENP-concentration in medium, because of the temporal and spatial fluctuations, and ENP-organisms-interactions as illustrated in Figure 16. In line with this, other authors have pinpointed the need for quantification of the ENP-distribution during exposure, including ENP sorption to organisms and abiotic surfaces, uptake by organisms, and sedimentation (Kroll et al., 2013).

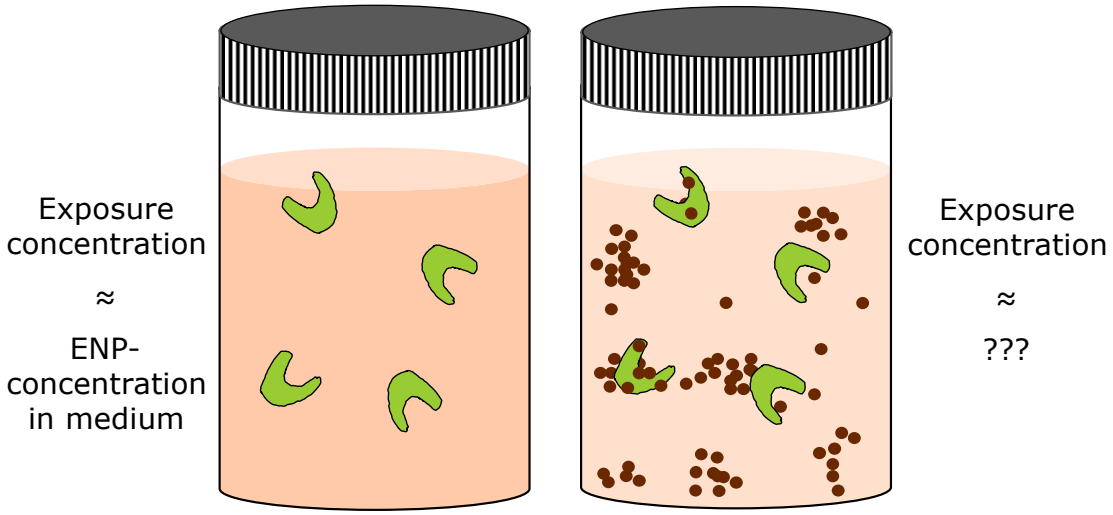

Figure 16. Illustration of the exposure scenarios in algal tests with a soluble compound being homogenously distributed (left) and suspended ENPs of inhomogeneous distribution (right).

It has been demonstrated by several studies that the measured ENP-concentration in medium may decrease over time, resulting in low EC_{50}-values, e.g. for the immobility of daphnids over 96h exposure to TiO_2 (Dabrunz et al., 2011). However, when ENPs are shown to be adsorbed to the exterior and accumulated in the gut of the organisms, an EC_{50} based on the ENP-concentration in medium is meaningless and should perhaps rather be related to the dose experienced by the organisms. Also for algae, it has been stated that speciation of e.g. AgNPs cannot assumed to be the same at the biological surface as in the solution overall (Leclerc and Wilkinson,

2014), and the attachment of AgNPs to algal cells is reported to increase bioavailability of particle-associated Ag ions (Miao et al., 2010; Navarro et al., 2008).

It has been suggested that the understanding of biochemical responses of organisms to ENPs, despite the escalating number of studies published, is hampered by insufficient physical and chemical characterization prior to the test (Vale et al., 2016). However, pre-testing characterization obviously does not facilitate improved understanding of the mechanisms occurring *during* incubation. To address this issue is has been suggested to thoroughly characterize the ENPs and their transformation *in situ* and cover the duration and ENP-concentrations of toxicity tests (Jemec et al., 2016; OECD, 2012a; Petersen et al., 2014; Pettitt and Lead, 2013).

While inarguably characterization is necessary, it remains questionable how to go about it in practice. Various methods and techniques are available for characterization of parameters such as ENP size, agglomeration, and dissolution, all with different advantages and limitations. Therefore multiple methods and analyses are often needed to ensure reliable information and data. Thus, as ENP characterization must be conducted over time, and cover the concentration range of the test, toxicity testing becomes very time and cost intensive. These consequences aside, and more importantly: Complete characterization of the exposure dose *as experienced by the organism* during a test, is not obtainable by any approach related to characterization of ENPs in medium. Consequently, other dose-metrics are needed which take into account the different exposure fractions of ENPs.

The key must therefore be to identify which of the exposure fractions that are related to which of the various effect-mechanisms. This raises the question of how to distinguish these relations during the toxicity test. Exposure characterization is obviously necessary, but the setup must be designed accordingly, and not be conducted blindly following a generic protocol with the risk of generating data without enabling any deeper understanding of these exposure-effect mechanisms.

This issue is also relevant for very lipophilic compounds, and attempts have been made to relate toxic effects to the internal concentration in exposed organism (body burdens), although further development and validation is needed for regulatory purposes (ECHA, 2014). For ENPs, the body burden has also been suggested as alternative dose-metric to the ENP-concentration in medium (Hartmann, 2011; Petersen et al., 2015), although this may be even more challenging than for lipophilic substances as adsorption to the organism is not strictly governed by chemical equilibrium processes, but influenced by various chemical interactions from the core and the coating/stabilizing agent as well as the physical particle forces.

From the few studies relating ENP-toxicity to body burdens, it is evident that this approach involves challenges. The question is whether body burdens should include the ENPs accumulated in the gut and adsorbed on the surface. These fractions may or may not contribute to toxicity, likely depending on the toxic endpoint monitored. Ideally, body burdens distinguishing the extra- and intracellular ENP fractions should be determined during toxicity tests, and compared with various toxicity endpoints. This allows for some elucidation of the ENP-cell/organism-interactions, toxic mechanisms and their correlation. As examples, algal growth rate inhibition and daphnia immobility are possibly related (at least partly) to the adsorption of

ENPs on the organism surface, while oxidative stress in algae may be affected both by internalized and externally bound ENPs, as ROS can be generated both intra- and extracellularly. How to determine these fractions is another issue, as these are difficult to differentiate in practice (Kühnel and Nickel, 2014). Although imaging techniques may allow identification of ENPs inside cells/tissue, it does not provide body burdens. On the other hand, determination of body burdens by mass analysis of organisms does not differentiate dissolved ions and ENPs (Kroll et al., 2013). In many cases it is also difficult to distinguish internalized and externally bound ENPs because complete removal of external ENPs (and dissolved ions) is not easily ensured. Also, body burden measurements are hampered by often low internal ENP concentrations in organisms, requiring large amounts of biomass for analyses. In algal tests, the use of body burden is further complicated by the exponential growth, possibly "diluting" the body burden over time. Overall, a combination of techniques and different considerations are needed to quantify body burdens.

The use of mass-based dose-metrics only has been identified as inadequate for quantifying the exposure of ENPs (ECHA, 2014). The total ENP-mass/medium volume is however, by far the most frequently applied dose-metric (Kroll et al., 2013). The specific surface area have been suggested as more appropriate dose-metrics (Auffan et al., 2009; van Hoecke et al., 2009), but very few studies have investigated the applicability of these metrics, and it remains unclear whether they represent better alternatives for the establishment of effective concentrations, than the mass-based concentration (OECD, 2012a). The surface area and particle number are not easily determined in practice though, due to the time- and concentration dependent processes of aggregation and dissolution. Also, the surface area per liter medium or mass ENP is still not representative of the interaction with the organisms. Thus, the surface area *in contact with the organism* is likely a better dose-metric, but practically difficult if not impossible to quantify experimentally. When novel dose-metrics are applied in aquatic toxicity tests, it may be beneficial to continue also reporting the traditional dose-metric of mass per liter to enable linking and comparison of results from future aquatic toxicity studies with past work, as also implied by OECD (OECD, 2012a).

6.3 Traditional toxicity endpoints and/or alternatives?

The endpoints for toxicity are clearly defined by regulations and test guidelines, including algal growth rate inhibition, acute immobility/lethality and chronic effects related to growth, survival and reproduction of crustaceans. Generally, endpoints are selected according to biological and practical considerations, but also their susceptibility to the hazardous agent (Traas and van Leeuwen, 2007). The later criterion may not apply to ENPs. Both the algal growth rate inhibition and daphnia immobility are shown to be influenced by physical effects that are not part of current hazard identification scheme. Also, these endpoints may not be the most susceptible and thus relevant endpoints for ENPs, in light of the toxic mechanisms reported as majorly important for ENPs.

Because EC_{50}–values in many cases can be generated from toxicity tests relying on the traditional endpoints, it may be assumed that the tests and paradigm are applicable for ENPs. For PtNPs, two studies even report similar $EC_{50,48h}$–values (15 and

17 mg/L) for growth rate inhibition in alga *P. subcapitata* determined in accordance with the ISO algal test standard (Ksiazyk et al., 2015; Sørensen et al., 2016a Paper II), which may lead to the conclusion that the algal test is not only applicable, but even reproducible for these ENPs.

However, the generation of (perhaps even reproducible) EC_{50}-values is not sufficient to conclude applicability of the test systems. The underlying mechanisms leading to the response must be considered as well. As the toxic mode of action is not traditionally considered for the hazard identification of chemical compounds, it may be questioned why the effect mechanisms should then be considered for ENPs. However, based on the inherent differences between ENP-suspensions and chemical solutions, as well as the potential for physical and unknown effects to occur, it seems that insight into effect mechanisms is pivotal in order to conduct aquatic toxicity testing and hazard identification of these materials.

In the current hazard identification scheme physical effects may be considered an artefact, or at least an essentially different response than chemically induced toxicity. Therefore, the use of EC_{50}–values as comparative parameters referring to a scale of "chemical toxicity", will not equally apply to ENPs. Consequently, the applicability of EC_{50}–values for hazard identification, ranking and classification of ENPs is questionable and further extrapolation of EC_{50}-values for hazard assessment and generation of PNEC values, even more so. As described in chapter 5, EC_{50}-values from ENP toxicity tests are evidently influenced by multiple mechanisms relating to both exposure and responses of ENPs. Thus, simply replacing current traditional endpoints with other, seemingly more appropriate ones, such as oxidative stress, still does not address this issue of several effect mechanisms.

6.1.1 A multi-dimensional testing strategy

At the current state of knowledge, a strategy for optimized toxicity testing with algae and daphnia, should as minimum involve the investigation of:

- Physical effects
- Effects of dissolved ions
- Traditional standardized toxicity endpoints
- Oxidative stress and membrane damage in algae

This multi-dimensional approach to aquatic toxicity testing of ENPs is illustrated graphically in Figure 17, and involves determination of different response and exposure parameters. This approach may assist to further elucidate the effect mechanisms and toxic mode of action for different ENPs, and generate substantial data, e.g. as foundation for the development of SARs and an improved aquatic toxicity testing scheme for ENPs. An ecotoxicity testing strategy including multiple toxicity endpoints as immuno- and genotoxicity, bioaccumulation, behavioral and multi-generation effects, has similarly been suggested for ENPs (Hund-Rinke et al., 2015). The argumentation is that additional data input may support assessments, and definitely be beneficial for research purposes.

Also, the risk of generating false positive or false negative results is minimized by multi-dimensional approach. On the downside, it is more time-consuming, costly and labor-intensive. Dependent on the point of view, this could also be considered an advantage, as extended testing requirements could limit the number and volume of ENPs produced, to the products and applications of largest economic benefits. Also, considering the huge sums of money used for the development of nanotechnologies in contrast to safety research (Hansen and Gee, 2014), elevated costs for the later does not seem so unreasonable.

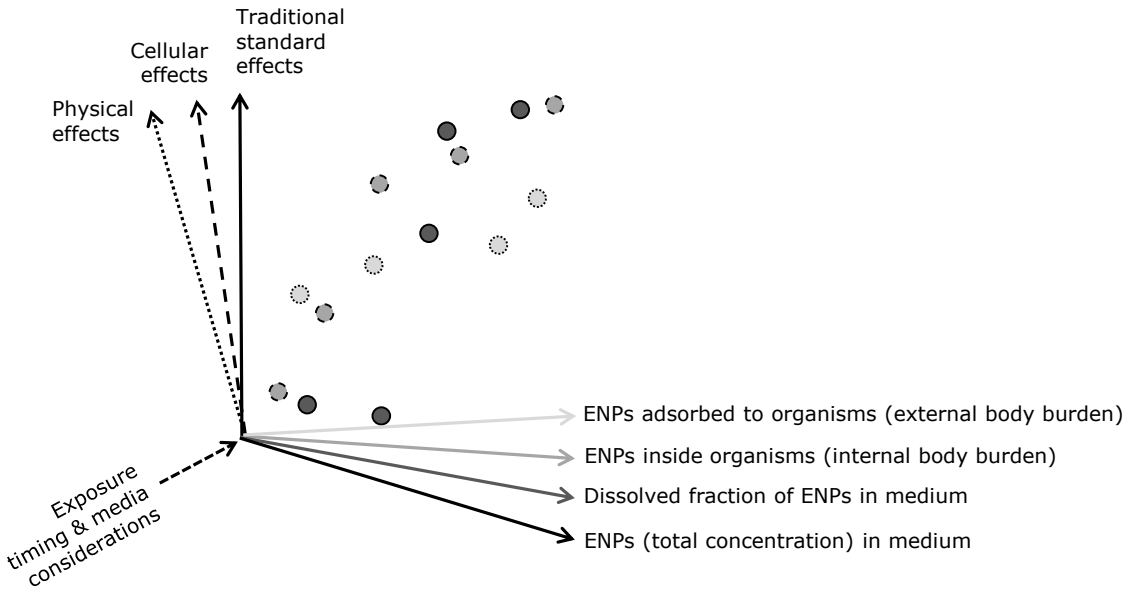

Figure 17. Illustration of a multi-dimensional strategy for aquatic toxicity testing of ENPs. The approach involves generation of several exposure-response relationships, combined from the different response parameters and exposure fractions, as illustrated by the various types of data points. Also, certain exposure timing (and/or media adjustment) approaches may minimize the influence of time on ENP transformation during incubation.

The addition of endpoints raises the question how these should be characterized and quantified. In contrast to the traditional endpoints, there are no guidelines or standardized procedures to follow for these additional endpoints. The variability between test protocols and result from different laboratories counteracts the comparability of result and also development of SARs (Ivask et al., 2014). Thus, some degree of harmonization is necessary for comparability, and some written guidance on the best available techniques/practices would be beneficial although this is not straightforward. Approaches to this issue are discussed below in the order of physical effects, effects of ions, and cellular effects.

Accounting for shading may be particularly important for ENPs that produce dark/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). Shading effects are most easily investigated through a separation setup, as previously described, although these disregard localized shading caused by ENPs adhering to algal cells. Despite of this, such setups are the most feasible setup available, requiring only special vials/plates, whereas the incubator

and analysis methods are unchanged. Ideally, shading as experienced by the alga should be determined, but such methods are not currently fully developed for standardized use. In daphnia, physical effects from ENPs may be overcome by simply separating the organisms from larger aggregates and settled ENPs through a mesh. Also, lethality should be reported in addition to immobility.

While physical effects may be more pronounced at higher ENP concentrations, they are also reported at lower concentration (~1 mg/L). Even when effects *are* occurring at high ENP concentrations, the questions remains how to deal with this, as regulatory toxicity testing must be able to generate EC_{50}-values up to 100 mg/L. Should these limits then be changed for ENPs? For soluble compounds however, these limits are not questioned when the effective concentration range is environmentally unrealistic. Also, disregarding effects of ENPs at higher concentrations ranges, simply because of their physical nature, could mean lowering the protection level for ENPs compared to soluble compounds. It may even be argued that the biological response is the key parameter, independent of its origin being physical or chemical. From this point of view, the endpoints influenced by both physical and chemical effects are actually the most suited for ENPs. This however, would require a shift in the underlying assumptions of classical toxicity testing and the use of EC_{50}-values in hazard identification. For both scientific and regulatory purposes, it is necessary to investigate and account for physical effects of ENPs.

Ideally, dissolution should be determined from the actual test, and not from a parallel setup of ENPs in test medium, as the interaction of organisms with ENPs may increase dissolution. The dissolved fraction could for example be determined at the beginning of, midways and after ended exposure by ultracentrifugation of an aliquot sampled from the test (including algae in algal tests) and following mass determination of the supernatant. This allows the response to be based on both the total and the dissolved fractions, and compared with the soluble metal salt. This highlights the importance of including a soluble salt control, as also suggested by others (Jemec et al., 2016) However, it is not trivial to quantify dissolution under actual test conditions and conclusions are often hampered by incomplete ENP mass recoveries. For certain ENPs, such as Ag and ZnO, the toxicity is mainly governed by very toxic dissolved ions. Consequently it may be argued that the data used for hazard identification of soluble Ag and Zn salts, also could apply the ENPs of these materials. Although this approach might be reasonable to ensure environmental protection from these ENPs, as also d it does not contribute to deeper understanding of the nanoparticles-specific effects also reported for these ENPs, or the development of a more appropriate testing scheme and SARs.

Investigations of oxidative stress and membrane damage in cells are relatively straight forward, compared to physical effects and effects of dissolved ions. In principle, this is simply an added toxicity endpoint, parallel to the traditional. Therefore the role of physical effects and effects of dissolved ions should also be accounted for in relation to cellular effects. Often the outcome is reported as percentage of cells with oxidative stress/membrane damage, as detected by use of fluorescent dyes. A bigger issue is that cellular effects are not clear-cut parameters for toxicity. The relation between oxidative stress and toxicity is, as mentioned previously, not well established, and occurrence of oxidative stress does not necessarily

evolve into toxicity. Generally, biomarker responses of individual organisms are not useful predictors of ecological or even whole-organism effects, but they may serve to explain the mechanistic bases of organisms effects following exposure to xenobiotics (Forbes et al., 2006). As oxidative stress appear to be an important mechanism for ENPs, the gathering of systematic data may however contribute to development of correlation between oxidative stress and toxicity, again for the benefit of future SARs or safe-by-design approaches. Also, the occurrence of oxidative stress may serve as a warning sign for potential toxicity when data are applied for regulatory purposes.

While standardization of aquatic toxicity test methods and testing approaches may increase interstudy comparability and regulatory relevance, it may also be counteractive to the discovery of mechanisms governing the exposure and effects mechanisms of ENPs. Also, it has been argued that the current state of knowledge does not offer a clear pattern for standardization of toxicity testing to be based upon (Wickson et al., 2014). The multi-dimensional approach addresses this issue by generating more knowledge on ENP toxicity, which may provide basis for development of future standard toxicity testing approaches for ENPs.

Also, the traditional standardized toxicity tests are biased towards known effects, therefore these do not provide further insight into underlying mechanisms, and also involve a risk of overlooking unforeseen effects (Wickson et al., 2014). In this regard, it is important to distinguish the purpose of regulatory and exploratory toxicity testing: According to current state of knowledge, toxicity testing for regulatory purposes could be harmonized to involve *at least* the four mentioned effects described here. However, this does not preclude exploratory toxicity testing for scientific purposes, as well as continuous development of the regulatory testing approach and paradigms according to increasing knowledge from research findings.

The four effect types suggested for hazard identification in this thesis could be conducted in a step-wise manner, triggered by certain criteria, e.g. the production volumes and hazard of the bulk counterpart. Such a tiered approach is suggested by Hund-Rinke et al. (2015), to include an initial toxicity screening using sensitive endpoints, short term duration and low work load testing, which may in certain cases be followed by more comprehensive testing. Testing the oxidative stress of algae may provide a foundation for such initial ENP screening and fulfil these criteria.

Currently, guidance on how to include results from non-standardized tests in environmental risk assessment is lacking (Palmqvist et al., 2015). In this regard, an obvious question related to a multiple endpoint testing is how to interpret and apply the generated data for hazard identification. Does the EC_{50} (relating to the traditional endpoint) remain the outcome used for hazard identification, but with additional insight from the other endpoints? The generation of EC_{50}-values relies on the paradigm that toxicity increases with increasing chemical concentration, which may not be true for ENPs. Alternatives include single concentration testing, or the use of criteria, such as for example the toxicity endpoints listed here, evidence for internalization, and quantification of the adsorption of ENPs to organisms. This however, would entail an inherent change in the evaluation of aquatic toxicity data for hazard identification in the context of existing chemical regulations such as REACH and CLP.

7 Conclusions

Based on the findings presented in this thesis it can be concluded that the current approach to aquatic toxicity testing for regulatory compliance, may fail to generate accurate, reproducible or meaningful data for the hazard identification of ENPs. A major reason for these issues may be that ENP concentration-response data covers several exposure fractions and effect mechanisms. ENPs namely undergo time-dependent transformation processes in medium such as agglomeration, dissolution, sedimentation and adsorption to test organisms, several ENP exposure fractions are present during incubation which may change with time and concentration. Also, ENPs may induce both physical and chemical effects, besides potential, unknown toxic mechanisms.

The issue of ENP instability during test incubation is generally mainly addressed through attempts to control and/or describe the exposure conditions during incubation. In this regard, the use of exposure timing measures, including aging of ENPs in medium prior to incubation, and/or shortened exposure duration may assist to stabilize the exposure during incubation, by allowing ENP transformation processes including agglomeration, dissolution and sedimentation of ENPs, to proceed prior to, rather than during, the test incubation. It is clear however, that such approaches may cause both increased and decreased toxicity of the tested ENPs.

Aging of ENPs may increase their toxicity, illustrating how the use of freshly prepared ENPs in medium may underestimate toxicity, but the opposite may also occur. If aging is to be implemented as tool in regulatory test guidelines, some harmonization of the aging conditions is needed for interstudy comparability. Shortened exposure durations may yield EC_{50}-values comparable to those of longer standardized exposure duration, but this is not always the case. If to be considered for regulatory testing regimes, shortened exposure duration algal tests must incorporate more sensitive endpoints to minimize the risk of false negatives. Such endpoints may include oxidative stress, which is not currently considered for hazard identification. It may serve as an initial screening tool, and/or assist to elucidate the toxic mechanisms related to ENPs.

The currently applied toxicity endpoints of standard hazard identification toxicity tests include algal growth rate inhibition and daphnia immobilization. These endpoints are shown to be confounded by physical effects and the challenges of distinguishing effects of dissolved ions and ENPs themselves. Thus, in addition to influencing the concentration-response data reproducibility and interpretability, this also hampers elucidation of potential nanoparticle-specific effects. As minimum, regulatory aquatic toxicity testing should account for the occurrence of physical effects, and the effects related to the dissolved ions.

Overall, a multi-dimensional approach for aquatic toxicity testing and hazard identification of ENPs is suggested to include these organism responses, and thorough quantification and characterization of exposure fractions including the adsorbed and internalized fractions in organisms, and the dissolved and total fractions of ENPs in medium. This approach may assist to: 1) Determine which effects and exposure fractions are related, 2) Improve the understanding of the generated concentration-response data, and 3) Identify potential nanoparticle-specific effect mechanisms.

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I **Sørensen SN**, Baun A. 2015. Controlling silver nanoparticle exposure in algal toxicity testing – A matter of timing. *Nanotoxicology* 9(2):201-209.

II **Sørensen SN**, Engelbrekt C, Lützhøft HCH, Jiménez-Lamana J, Noori JS, Alatraktchi FA, Delgado CG, Slaveykova VI, Baun A. 2016. A multi-method approach for disclosing algal toxicity of platinum nanoparticles. (*Submitted*)

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IV **Sørensen SN**, Hjorth R, Delgado CG, Hartmann NB, Baun A. 2015. Nanoparticle ecotoxicity – physical and/or chemical effects? *Integrated Environmental Assessment and Management* 11:722-724.

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