Engineered Nanoparticle (Eco)Toxicity

Towards Standardized Procedures for Hazard Identification

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– *Towards Standardized Procedures for Hazard Identification*

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PhD Thesis
May 2015

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Preface

This dissertation is submitted in partial fulfilment of the requirements for the Degree of Doctor of Philosophy at the Department of Environmental Engineering at the Technical University of Denmark (DTU). The research for the entitled thesis "Engineered Nanoparticle (Eco)toxicity – Towards Standardized Procedures for Hazard Identification" was conducted at the Department of Environmental Engineering at the Technical University of Denmark (DTU) in the period November 2011-February 2015, under the supervision of Professor Anders Baun and co-supervision of PostDoc Nanna Hartmann. The project was partially funded by FP7 Project MARINA – Managing Risks of Nanomaterials (Grant no. 263215). The content of the thesis is composed of two parts: the first part reveals the background and motivation behind the research conducted, gives an overview of the literature on the field in relation to the main findings of this research, and provides discussion and conclusion; the second part presents the peer-reviewed work that has resulted from the PhD study in the form of a book chapter and five papers. Throughout the thesis these will be referred to using the Roman numerals as Paper I-VI and will be cited [1-6].

I **Cupi D.**, Hartmann N.B., Baun A. (2015). The influence of natural organic matter and aging on suspension stability in guideline toxicity testing of silver, zinc oxide and titanium dioxide nanoparticles with *Daphnia magna*. *Environmental Toxicology and Chemistry* 34:497-506.

II **Cupi D.**, Hartmann N.B., Baun A. (2015). The influence of pH and media composition on suspension stability of silver, zinc oxide and titanium dioxide nanoparticles and immobilization of *Daphnia magna* under guideline testing conditions. *Submitted*.

III Hartmann N.B., Jensen K.A., Baun A., Rasmussen K., Rauscher H., Tantra R., **Cupi D.**, Gilliland D., Pianella F., Riego Sintes J.M. (2015). Techniques and protocols for dispersing nanoparticle powders in aqueous media – (what) are we ready to harmonize? *Submitted*.

IV **Cupi D.**, Sørensen S.N., Skjolding L.M., 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*

V **Cupi D.**, Baun A. (2015). Methodological considerations for using *umu* assay to assess photo-genotoxicity of engineered nanoparticles. *Submitted*.

VI **Cupi D.**, Baun A., Dreher K. (2015). Metal oxide nanomaterial alternative pulmonary toxicity assessment and mechanism of injury: Not all metal oxide nanomaterials are created equal. *Manuscript*.

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:

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Last but not least, I would like to thank my wonderful parents and two sisters for being my backbone and for encouraging me to continue higher education. Their kindness, patience and support have led to completion of my graduate studies.

Summary

In the past decade, the use of nanotechnology has led to a large variety of products in the market, and is projected to markedly increase in value in the years to come. The use of manufactured nanomaterials comprises various technological and economic benefits due to their novel physico-chemical characteristics. It is these unique physico-chemical properties that have raised concerns during the last decade regarding the potential risk nanomaterials pose towards human health and the environment. Similar to other chemicals, nanomaterials have to be tested and ranked in order to obtain information on hazard identification, which is an integrated part of risk assessment. The complex nature and behavior of nanomaterials in the different environmental compartments and test systems has made it difficult for the scientific community to conduct robust and reproducible tests, and consequently, for regulatory bodies to take action.

Standard test guidelines developed for conventional soluble chemicals, have been used to test nanomaterials. Concerns have been raised whether these test systems are adequate for addressing particle properties under different testing conditions and assessing toxicological outcomes. In fact, various international organizations (e.g. Organisation for Economic Cooperation and Development and International Organization for Standardization) have recognized the need to amend and refine the current standard tests in relation to nanomaterials. Methodological considerations to standard testing for the purpose of testing engineered nanoparticles (ENPs) in aquatic system are a central theme in this thesis. The research presented herein has included acute tests with freshwater cladoceran *Daphnia magna*, genotoxicity tests with bacteria *Salmonella typhimurium*, as well as acellular and *in vitro* assays.

An understanding of different physico-chemical properties and specific characteristics of various nanoparticles employed in this project has been attained by reviewing the literature in the field. Specific processes such as agglomeration in aquatic suspension, influence of environmental conditions on toxicity testing, dissolution, phototoxicity and inflammation were identified as important parameters and considered for further toxicity testing of Ag, ZnO, TiO_2 and CeO_2 ENPs; to investigate specific properties and improve test stability and reproducibility.

The issue of agglomeration of ENPs in aqueous suspensions was investigated by attempting to prepare stable stock and test suspensions of various nanoparticles. A step-wise approach was presented to develop tailored

dispersion protocols for (eco)toxicological testing of ENPs, based on the identification of critical issues and parameters for stock dispersion protocol development. This may serve as a basis for the development of a harmonized dispersion protocol for ENPs. Natural organic matter (NOM) and aging of suspensions prior to testing were employed in an attempt to stabilize aqueous suspensions of three different ENPs. While NOM helped in stabilizing ZnO ENPs suspensions, it caused agglomeration in TiO_2 ENP stock suspensions and an underestimation of toxicity for Ag ENPs. Likewise, aging only aided in the stability of ZnO ENPs. It was concluded that NOM can mitigate or eliminate toxicity of Ag ENPs and is not recommended for use. The ratio of NOM to ENP proved to be important in stabilizing non-capped ENPs. Another attempt towards stable suspensions involved adjusting different standard testing parameters, such as the pH and ionic strength of the test media. It was found that point of zero charge measurements should be conducted prior to ecotoxicological studies. Testing media of very low ionic strength at a pH where the ENPs have the lowest agglomerate size should be employed. This will control agglomeration and increase the stability and reproducibility of the test results. For ENPs such as TiO_2, toxicity of smaller agglomerates was significantly higher than larger agglomerates, highlighting the importance of size distribution in relation to toxicity. It was recognized that it is difficult to give general advice that is applicable for testing all nanomaterials, thus, a case-by-case evaluation should be conducted.

Another topic in this thesis was to evaluate the feasibility of current methods to screen and rank toxicity of ENPs in a high-throughput manner. Investigation of TiO_2 phototoxicity using the *umu* assay revealed that UV light caused damage to the bacteria and that high ENP concentrations had a shading effect, which were categorized as confounding factors. Similarly, an attempt to measure inflammation response caused by CeO_2 and TiO_2 ENPs, revealed that the high surface area of ENPs has a high affinity and binding capacity for protein molecules/assay reagents. These artifacts questioned the feasibility of these assays for testing ENPs. The influence of test parameters and confounding factors/artifacts should be taken into account and investigated prior to undertaking nano-toxicological studies. These results indicate that test guidelines need to be revised and tailored according to ENP properties, as test conditions affect toxicity. The information presented in this thesis may help the scientific community and regulators better understand test design and outcomes of nano-(eco)toxicological studies, which in turn may lead to a stronger scientific basis for regulation of nanomaterials.

Dansk sammenfatning

Igennem det seneste årti har brugen af nanoteknologi medført en lang række produkter på markedet, og en markant værdistigning af denne teknologi forventes i løbet af de kommende år. Brugen af syntetiske nanomaterialer afstedkommer forskellige teknologiske og økonomiske fordele grundet deres nye og særlige fysisk-kemiske egenskaber. Det er disse unikke fysisk-kemiske egenskaber, der har vakt bekymring i det seneste årti på grund af den potentielle risiko disse nanomaterialer kan have for menneskers sundhed og miljøet. I lighed med andre kemikalier, skal nanomaterialer testes og rangeres for at tilvejebringe oplysninger om fareidentifikation, hvilket udgør en integreret del af en risikovurdering. Nanomaterialers komplekse natur og opførsel i de forskellige delmiljøer og testsystemer, har gjort det vanskeligt for det videnskabelige samfund at gennemføre robuste og reproducerbare tests, og dermed er det vanskeligt for regulatoriske instanser at regulere og gribe ind på dette område.

De retningslinjer for standardtests, som oprindeligt er udviklet til konventionelle opløselige kemikalier, er blevet anvendt til at teste nanomaterialer. Der er blevet udtrykt bekymring for hvorvidt disse testsystemer tager højde for partikelegenskaber under forskellige forsøgsbetingelser og dermed er brugbare til at vurdere de toksikologiske effekter. Faktisk har forskellige internationale organisationer (f.eks. Organisationen for økonomisk samarbejde og udvikling, OECD, og den internationale standardiseringsorganisation, ISO) erkendt behovet for at ændre og forbedre de nuværende standardtests i forbindelse med nanomaterialer. Metodiske overvejelser omkring brugen af standard testmetoder med henblik på testning af syntetiske nanopartikler (NP'er) i akvatiske systemer er et centralt tema i denne afhandling. Forskningen præsenteret i nærværende afhandling har inkluderet akutte test med ferskvandskrebsdyret *Daphnia magna*, genotoksicitetstest med bakterien *Salmonella typhimurium*, samt acellulære og *in vitro* tests.

En forståelse af de forskellige fysisk-kemiske egenskaber og særlige karakteristika ved forskellige NP'er, anvendt i dette projekt, er opnået ved at gennemgå litteraturen på dette område. Specifikke processer såsom agglomerering i en vandig suspension, indflydelsen af diverse miljøforhold på toksicitetstestningen, opløselighed, og fototoksicitet blev identificeret som de vigtige parametre. Disse parametre blev derfor taget i betragtning ved efterfølgende toksicitetstestning af Ag, ZnO, TiO_2 og CeO_2 NP'er; i et forsøg

på at undersøge specifikke egenskaber og forbedre testenes stabilitet og reproducerbarhed.

Problemet med agglomerering af NP'er i vandige suspensioner blev undersøgt gennem forsøg på at fremstile stabile stam- og testsuspensioner af forskellige NP'er. En trinvis tilgang for dispersionsprotokoller tilpasset særligt til (øko)toksikologisk testning af NP'er blev fremlagt, baseret på identifikation af kritiske problemer og parametre ved fremstilling af stamsuspensioner; denne tilgang kan tjene som grundlag for udvikling af harmoniserede dispersionsprotokoller for NP'er. Naturligt organisk materiale (NOM) og "ældning" af suspensioner forud for toksicitetstestning blev anvendt i et forsøg på at stabilisere vandige suspensioner af tre forskellige NP'er. Mens NOM hjalp til at stabilisere visse suspensioner af ZnO NP'er, forårsagede det agglomerering af TiO_2 NP-stamsuspensioner samt en underestimering af giftigheden for Ag NP'er. Ligeledes, bidrog "ældning" af testsuspensioner til generering af mere monotone koncentrations-respons kurver for ZnO NP'er. Et andet forsøg på at stabilisere suspensionerne, indebar en justering af forskellige standard testparametre, såsom pH og ionstyrke af testmediet. Det blev konstateret, at målinger af det isoelektriske punkt ("point of zero charge") bør foretages forud for økotoksikologiske tests. Tests bør udføres i medie med en meget lav ionstyrke og ved den pH, hvor NP'erne har den mindste agglomeratstørrelse. Dette vil have en kontrollerende virkning på agglomerering og forøge stabiliteten og reproducerbarheden af testresultaterne. For NP'er såsom TiO_2, var mindre agglomerater mere toksiske end større agglomerater, hvilket understreger betydningen af størrelsesfordelingen an NP'er i forhold til deres toksicitet. Det blev erkendt, at det er vanskeligt at give generelle råd, anvendelige for tests med alle nanomaterialer, hvorfor der i stedet bør gennemføres ad hoc vurderinger.

Et andet emne i denne afhandling var evaluering af anvendeligheden af de nuværende metoder til at screene og rangordne giftigheden af NP'er ved brug af højkapacitets-screeningsmetoder. Fototoksiciteten af TiO_2 blev undersøgt ved hjælp af *umu* tests, hvor resultaterne viste at UV-lys forvoldte skade på bakterierne, og at høje NP-koncentrationer forårsagede skygning af lyset, hvilket var en konfunderende faktor for bestemmelsen af genotoksicitet. Tilsvarende viste forsøg med inflammationsmålinger forårsaget af CeO_2 og TiO_2 NP'er, at det høje overfladeareal af NP'erne har høj affinitet for proteinmolekyler/analysereagenser. De påviste konfunderende faktorer sætter spørgsmålstegn ved anvendeligheden af de anvendte metoder til testning af

NP'er. Betydningen af testparametre og konfunderende faktorer bør tages i betragtning og undersøges forud for nanotoksikologiske undersøgelser. Disse resultater viser, at testprotokoller bør revideres og tilpasses i overensstemmelse med NP-egenskaber, eftersom testbetingelserne påvirker de toksiske effekter. Den viden, der præsenteres i denne afhandling, kan hjælpe det videnskabelige samfund og lovgivende instanser til en bedre forståelse af testdesign og resultater fra nano(øko)toksikologiske tests, hvilket igen kan føre til et stærkere videnskabeligt grundlag for regulering af nanomaterialer.

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Abbreviations

CCC	Critical coagulation concentration
CLP	Classification, labelling, and packaging of substances
DOC	Dissolved organic carbon
DLS	Dynamic light scattering
DVLO	Derjaguin, Landau, Verwey, and Overbeek
ECHA	European Chemicals Agency
ELISA	Enzyme-linked immunosorbent assay
ENPs	Engineered nanoparticles
ENMs	Engineered nanomaterials
EPA	Environmental Protection Agency
FBS	Fetal bovine serum
IL-8	Interleukin-8
IR	Induction ratio
IS	Ionic strength
ISO	International Organization for Standardization
KGM	Keratinocyte growth medium
MARINA	Managing Risks of Nanomaterials
NNI	National Nanotechnology Initiative
NOM	Natural organic matter
SR-NOM	Suwanee River natural organic matter
OD	Optical Density
OECD	Organisation for Economic Cooperation and Development
PDI	Polydispersivity index
PZC	Point of zero charge
REACH	Registration, Evaluation, Authorization and Restriction of Chemicals
ROS	Reactive oxygen species
SCENIHR	Scientific Committee on Emerging and Newly Identified Health Risks
SEM	Scanning electron microscopy
TEM	Transmission electron microscopy
TG	Test guideline
WPMN	Working Party on Manufactured Nanomaterials

1 Background and aims

The increase in production, and the different types of nanomaterials found in consumer products has raised concerns during the last decade on their potential hazard to human health and the environment [7]. The nano scale dimension gives higher surface to volume ratio than their bulk counterparts, which makes some particles more reactive and exhibit unique properties [7-9]. As these novel materials need to be legislated, the hazard identification step in risk assessment is of great importance [10-12]. Currently, there is a lack of specific requirements for nanomaterials within regulation, and there are questions regarding the applicability of standardized methods (e.g. ISO and OECD guidelines) for testing nanomaterials [13]. Moreover, it is unsure if some of the currently available methods can be used or adapted to test specific properties of various nanomaterials in a high-throughput manner.

Aquatic toxicity testing is an important part of environmental testing, and is used in regulatory and nonregulatory applications. This includes testing of a variety of fresh and saltwater species, starting from unicellular species such as algae, several crustaceans (including *D. magna*), and species higher up in the food chain [14]. Human toxicological tests/assays also involve dissolving or suspending the chemical/particle of interest in a water-based media. For nanomaterial testing, it is crucial to consider properties and behavior in water and media in order to figure out the best testing practices that can lead to reproducible results. In the literature to date, this has proven to be a very challenging task due to the large variety of nanomaterials and their different behavior in aquatic suspension, which will be discussed in this thesis.

Keeping in mind these challenges, the aims of this thesis are to review and improve test guidelines and by giving recommendations on:

1) Stock suspension preparation and dosimetry, as well as characterization of nanomaterials before and during testing (Paper I, II, III);

2) Change of media composition or test conditions to improve nanoparticle test suspension stability and reproducibility (Paper I, II);

3) Methodological issues for specific test conditions in high-throughput assays using nanomaterials, in order to avoid confounding factors and artifacts, and improve testing procedure (Paper V, VI).

This was done by testing Ag, ZnO, TiO_2 and CeO_2 ENPs to illustrate how test conditions can govern test outcomes.

2 Nanotechnology and engineered nanoparticles

Nanotechnology is a field of technology that utilizes engineered nanomaterials (ENM) by incorporating them into processes and products that can improve their function by exploiting surface and quantum properties that are exhibited at the nano scale. The potential of this field was seen in the late 1950s by physicist and Nobel Laureate Richard Feynman, who had a vision for new technologies by manipulating atoms and molecules [15]. A great variety of ENM has been produced by a bottom-up or top-down manufacturing process. It has been projected that by 2020, the market value of products from nanotechnology will greatly increase [16].

To date, many organizations/institutions have tried to construct a definition for a nanomaterial/nanoparticle. This has been a challenging task that has involved several tiers, including nano definition of the primary and agglomerated size in different environmental components. The International Organization for Standardization (ISO) defines nanoscale as "size range from approximately 1 nm to 100 nm" and a nanomaterial as "material with any external dimensions in the nanoscale or having internal structure or surface structure in the nanoscale" [17]. In 2011, the European Commission gave the following recommendation: "Nanomaterial means 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-100 nm. In specific cases and where warranted by concerns for the environment, health, safety or competitiveness the number size distribution threshold of 50 % may be replaced by a threshold between 1 and 50 %". It also recommends that a number-based particle size distribution should be used [18]. The U.S. National Nanotechnology Initiative (NNI) states that "nanotechnology is the understanding and control of matter at dimensions between approximately 1 and 100 nanometers, where unique phenomena enable novel applications" and "Nanomaterials are all nanoscale materials or materials that contain nanoscale structures internally or on their surfaces" [19]. It is specifically this small scale that gives the ENPs their unique properties. Depending on the organization and region, many nano-definitions exist. However, most of these definitions agree on a size range of approximately 1-100 nm. In this thesis, ENPs will be referred to as particles of 1-100 nm in their primary particle form.

2.1 Nanoparticle uses and exposure

The work conducted in this thesis will focus on Ag, ZnO, TiO_2 and CeO_2 ENPs, which are used in many applications and consumer products. Moreover, they are selected as representative nanomaterials by the OECD Working Party on Manufactured Nanomaterials (WPMN) [20]. A search done using the recently compiled Nanodatabase [21], more than 1400 products have been listed as containing ENMs. A search conducted in this database (on March 3, 2015) revealed a total of 246 products from which, Ag was the most used ENP, and found in categories such as appliances, automotive, electronics and computers, food and beverages, children's goods, health and fitness and home and gardens. ZnO, TiO_2 were mainly found in products from the last two categories [21]. Previous literature has reported similar uses of these ENPs in products. Ag ENPs are very widespread due to their properties as antimicrobial agent, are found in various consumer products, food packaging and medical devices [22]. TiO_2 ENPs are used in photovoltaic devices, water treatment, and degradation of pollutants [23]. TiO_2 and ZnO ENPs are used in sunscreens and cosmetics [24]. CeO_2 are used in applications in coatings and fuel cells, as a polishing agent, catalyst and in automobile emissions control, and in coatings [25].

As described above, the application and use of ENPs are quite extensive. In order to understand their potential risk of ENPs to the environment and human health, it is important to have knowledge on exposure and transformation processes. It is reasonable to assume that ENPs will be discharged into the aquatic environment during the cycle of production, use and disposal [26]. Some processes involving release and exposure of ENPs to different environmental compartments and their respective species, as well as human exposure at a general population level and occupational exposure are shown in Figure 1. Manufacturing of pristine ENPs may lead to human occupational exposure during production and handling, and environmental exposure close to the manufacturing site. Likewise, incorporating these particles in various products can lead to human exposure. After use, these products go through waste handling processes, which may involve waste water treatment (WWT), incineration or depositing in landfills. This may lead to environmental exposure in the air, soil and water compartments, and the different biological species, especially in the aquatic compartment. This might later contribute to human exposure from contact with any of these compartments or species. However, limited data exist on the release and concentration of ENPs in the environment. This is majorly due to the lack of

instruments and techniques able to measure environmental concentrations [26], and the presence of complex matrices and biological matter [27].

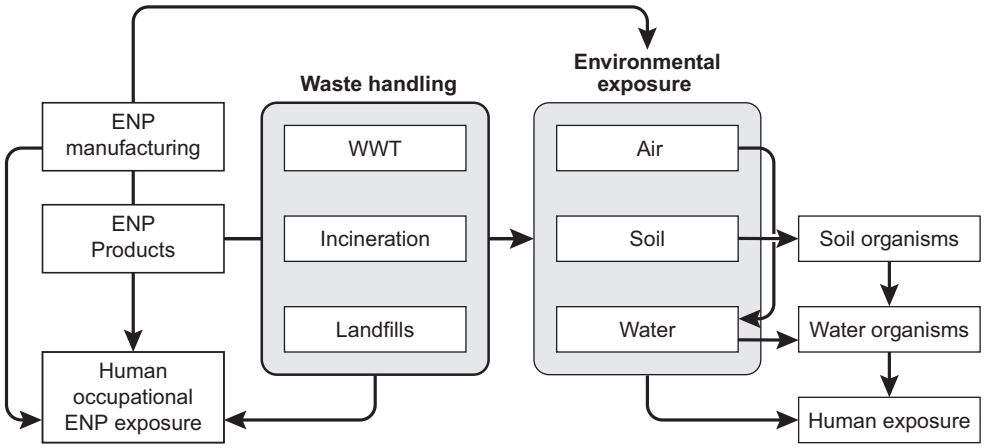

Figure 1. Diagram depicting release and transformation processes of ENPs, transfer into different environmental compartments and human exposure. Modified from Nowack et al. 2012 [26].

Due to transformation processes, it is less likely that the ENMs will be found in their original form in the environment [26]. Some of these processes may involve oxidation and reduction, including photo-catalytical processes, bio-transformation and degradation, dissolution, agglomeration, aggregation and precipitation, and adsorption and desorption [28-31]. Given the large amount of transformation processes and the lack of data on environmental concentrations of ENPs, such testing might not give a lot of information on the intrinsic toxic properties of pristine primary ENPs.

2.2 Current guidelines for safety assessment of nanoparticles

Understanding and assessing the environmental health and safety of ENPs is a challenging process that involves many tiers. The regular framework of risk assessment of chemicals is composed of hazard identification, hazard assessment/dose-response assessment, exposure assessment and risk characterization [11].

In 2009, the Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR) concluded that “A suitable framework for the assessment of all engineered nanomaterials requires exposure and hazard data on a wide range of products”. This includes robust methodology to be used in research and development stages to mitigate potential human health and environmental risks of nanomaterials [32].

Regarding legislation in Europe, the European Union (EU) Biocidal Products Regulation and the Cosmetic Products Regulation has specific provisions, requiring labeling and argumentation on the applicability of specific tests for nanomaterials. Registration, Evaluation, Authorization and Restriction of Chemicals (REACH) covers nanomaterials, but does not contain any nano-specific provisions [33]. For regular chemicals a setoff limit 100 mg/L has been based on the regulation on the classification, labelling, and packaging of substances and mixtures (CLP Regulation), [34], although no specific requirements on nanomaterials exist. However, the recommendation given by the European Commission in 2011 [18], should be followed by REACH and CLP [7]. In October 2012, the European Chemical Agency (ECHA) established a nanomaterials working group (ECHA-NMWG) to assist REACH and CLP processes and to give recommendations on technical and scientific issues [7]. In the US, nanoscale materials are covered under the Toxic Substances Control Act (TSCA). US EPA is conducting a comprehensive regulatory approach under TSCA including: notifications for new ENMs prior to manufacturing, and information gathering rule on new and existing ENMs where the information on production volume, release and exposure, and available health and safety data are provided [9].

The hazard identification process (which is also the first step in risk assessment), is mainly based on inherent physico-chemical, biological and toxicological properties of chemical or physical agents [11]. Standard testing methods were developed to screen and rank chemicals for hazard identification purposes. The most commonly used standard test protocols are those developed by international organizations such as Organisation for Economic Co-operation and Development (OECD) and International Organization for Standardization (ISO). The *OECD Guidelines for the Testing of Chemicals* serve as a tool for assessing the potential effects of chemicals on human health and the environment; and are used by various academic and government institutions, as well as the industry [35]. On the other hand, the ISO International Standards are strategic tools that ensure that products and services are safe, reliable and of good quality [36].

For testing of ENPs, the scientific community has to date used the OECD and ISO standard test guidelines that were originally developed for dissolving chemicals, such as OECD 202 [37] and ISO 6341 [38] for aquatic testing with crustaceans. Similarly, the U.S. Environmental Protection Agency (US EPA) has methods for measuring the acute toxicity of freshwater and marine

organisms [39]. However, ENPs exhibit particle/colloid properties, do not completely dissolve in aquatic suspensions, and go through processes of agglomeration/aggregation and settling, which make it difficult to test using current guidelines.

In order to serve as a subsidiary body to the OECD Chemicals Committee, the OECD Working Party on Manufactured Nanomaterials (WPMN) was established in September, 2006. It aims to ensure that the approaches to different steps risk assessment for manufactured nanomaterials contain high quality science and are internationally harmonized [40]. This would encourage international co-operation in health and environmental safety of manufactured nanomaterials. Indeed, since the establishment of the program, participating countries and organizations have given updates of current activities, at the national and international level.

In November 2007, OECD's (WPMN) launched a Sponsorship Programme to gather expertise and to fund the safety testing of 13 representative manufactured nanomaterials. The term "representative" means that the selected nanomaterials are available or soon to enter into commerce, for inclusion in a set of reference materials to support measurement, toxicology and risk assessment of nanomaterials [20]. Without any particular order, these materials include single-walled carbon nanotubes (SWCNTs), multi-walled carbon nanotubes (MWCNTs), silver nanoparticles, iron nanoparticles, titanium dioxide, aluminium oxide, cerium oxide, zinc oxide, silicon dioxide, dendrimers, nanoclays and gold nanoparticles. Other criteria for selecting these representative nanomaterials were production volume and the likely availability of such materials for testing. It was also mentioned that the relevance of this list of nanomaterials might change over time [20].

Based on the OECD Sponsorship Programme, information on the human health and environmental safety can be attained by testing the ENMs. This can be done in two phases: phase 1 is more exploratory in nature, involves the utilization of existing relevant information, generation of new information, as well as test guideline development; phase 2 will take into account the information from phase 1 and address additional endpoints that can shed light on the hazard potential of the representative nanomaterials which could feed into the risk assessment process, and may involve additional aspects such as life cycle assessment [41].

Various guidance documents have been published to guide and give recommendations in regards to suspension preparation and toxicity testing. One of the first documents, *Guidance Manual for the Testing of Manufactured Nanomaterials*, aims to give recommendations on endpoints needed for testing nanomaterials, including physical-chemical properties and material characterization, environmental fate, toxicological and ecotoxicological effects, and material safety [41]. The OECD *Guidance on Sample Preparation and Dosimetry for the Safety Testing of Manufactured Nanomaterials* covers more specific test-based issues in nano-(eco)toxicology. Considerations covered include appropriate dose-metrics, sample preparation and dosimetry, physical-chemical properties; samples of nanomaterial in exposure media for ecotoxicity studies, for degradation, and transformation and accumulation studies [42]. More specific to ecotoxicological testing, *Ecotoxicology and Environmental Fate of Manufactured Nanomaterials: Test Guidelines* states that testing should be conducted in several tiers where dissolution and dispersability, agglomeration state and dispersion stability, should be taken into account in order to investigate particle behavior over time. This guideline also states that there is a need for developing standard methods for stock suspension preparation and that the current regulatory practice for chemicals is not applicable to nanomaterials [43].

In 2012, ECHA updated their guidance documents by the addition of an Appendix R7-1, with applicable recommendations to Chapter R7a Endpoint nanomaterials [44]. This document was developed to provide advice to registrants when preparing dossiers for nanomaterials. It contains strategies for particle size distribution, surface area, and shape, as well as general advisory notes on toxicity assessment [44].

In 2014, ISO published a technical report on *in vitro* and *in vivo* methods for toxicological and ecotoxicological screening of nanomaterials. In this document it is stated that modifications that need to be applied to the test system will depend on the type of medium, test organism and the properties of the nanomaterials. Standard methods could however, be a good starting point, followed by careful consideration on nanomaterial suspension preparation [45]. Employing and validating standard guidelines will not only prove useful to scientific community and different organization, but also regulatory agencies and government bodies attempting to conduct risk assessment of nanomaterials.

3 Nanoparticle behavior in aquatic test systems

In order to understand the processes undergoing during testing when using standard test guidelines, it is important to analyze the behavior of ENPs in aquatic systems. This behavior will depend on various factors, but will be more affected by the physico-chemical properties of the specific nanoparticle and the chemical composition of the medium [22,46-49]. These properties will affect their stability, bioavailability, and consequently their toxicity towards aquatic organisms. ENPs can exhibit colloidal-like properties with some common governing processes that will be discussed below. Before discussing these processes, it is relevant to clarify certain terms that will be used herein. According to the definitions recommended by the European Commission [18]:

(a) ‘particle’ means a minute piece of matter with defined physical boundaries;

(b) ‘agglomerate’ means a collection of weakly bound particles or aggregates where the resulting external surface area is similar to the sum of the surface areas of the individual components;

(c) ‘aggregate’ means a particle comprising of strongly bound or fused particles.

3.1 Governing forces and processes

Since processes of agglomeration and aggregation will govern the behavior of ENPs in aquatic suspension, it is important to understand the conditions that could lead to these processes. Generally, ENPs will have a surface charge (positive or negative) based on the ions that are attracted to the surface due to chemical interactions. This comprises the first layer, and is commonly known as the Stern layer (Figure 2). Conventionally, the next layer would be what is called a slipping plane, which separates the Stern layer from the mobile fluid of the suspension/diffusive layer. The electric potential at this plane is measured as zeta potential. The zeta potential is measured due to electrophoresis, where a charged particle will move with a certain velocity in a voltage field. The second layer, called the diffusive layer is a loosely bound to the Stern layer via the Coulomb force (Figure 2). The double layer occurs in order to neutralize the charged surface, this way causing an electrokinetic potential between the surface and any point in the mass of the suspension.

When two ENPs are in close proximity in suspension, the major dominating interparticle forces will be the electric double layer (electrostatic), steric, and van der Waals forces [50]. The latter is an ever-present electrodynamic attraction as a result of dipole moments between two close bodies (or ENPs). In theory, particles suspended in a liquid are always in movement due to Brownian motion (resulting from the collision with the atoms or molecules present in the medium), and the smaller ones tend to move more quickly. Brownian diffusion has been considered as the main contributor to aggregation [51].

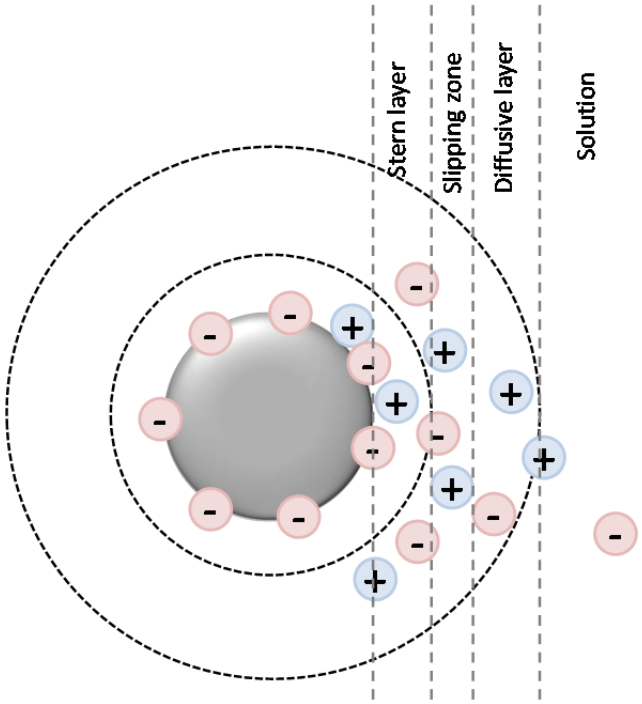

Figure 2. Schematic view of state of an ENP in aquatic suspension. A charged ENP will have a Stern layer and a slipping plane, where the zeta potential is measured. The surrounding suspension will be characterized by a diffuse layer and the electrical double layer [31].

Although nanomaterials exhibit different properties than colloids, it can serve as a fundamental basis to understand ENP interactions [52]. This thesis will focus on agglomeration on the aspect of homoagglomeration/aggregation, which occurs between two similar particles e.g. ENP-ENP interations [52]. In order to achieve stable ENP suspensions, the van der Waals attraction need to be overcome [50]. This is mostly explained by the Derjaguin, Landau, Verwey, and Overbeek (DLVO) theory of aggregation that states that van der Waals and electric double layer forces dominate these interactions as discussed in Hotze et al. (2010) [52].

However, the classical DLVO theory alone is not sufficient to explain/predict aggregation behavior, as it does not take into account steric forces from coatings or natural organic matter (NOM). Along with other short-range forces such as bridging, osmotic, hydrophobic Lewis acid-base and magnetic forces, steric forces are included in the extended DLVO (XDLVO) theory [52]. Moreover, the kinetics of ENP aggregation is governed by other factors such as size, chemical composition, shape, coatings and surfactants, and crystal structure. When the size of ENPs gets small enough, the surface curvature cannot be assumed to be flat. Moreover, surface charge behavior and reactivity can be altered. Chemical composition and crystal structure can alter Hamaker constant, which governs van der Waals attractions [52]. Also, non-spherical particles tend to have greater attraction than spherical particles when found at distances smaller than their mean diameter [53].

3.2 Factors affecting nanoparticle stability

Most of the surface properties of ENPs will depend on characteristics such as chemical composition, surface functionalization, crystallinity and porosity, hydrophobicity/philicity and shape [54-56]. Ionic strength and pH play an important role on ENP agglomeration/aggregation [47,52,57,58], (Paper II) [2]. These factors can promote aggregation through electrical double layer screening and surface charge titration [52]. The increase in ionic strength can lead to critical coagulation concentration [50], which should be avoided in both stock and test suspensions. The main charging mechanism for metal oxides in aquatic suspension is the dissociation of surface hydroxyl groups and is mainly dependent on pH of the suspension [50]:

$$M\text{–}OH_2^+ \overset{H_3O^+}{\rightleftharpoons} M\text{–}OH \overset{OH^-}{\rightleftharpoons} M\text{–}O^- \quad (1)$$

A representation of the forces playing an important role in agglomeration and aggregation, and that are investigated in studies conducted for this thesis are presented in Figure 3. Electrostatic stabilization can be achieved as a result of the charges from the electric double layer, which can be positive or negative, depending on the pH of the medium. Steric forces are normally achieved by coating ENPs with capping agents, or with thin repulsive coating of a polymer or surfactant, in this case NOM, this way avoiding agglomeration and keeping ENPs stable in suspension. In fact, particle aggregation and settling can occur as a result of high reactivity due to high surface area to volume ratio, unless capping agents are present [59]. However, when ENPs

are dispersed in NOM in the presence of metal ions (especially divalent) a bridging effect is observed [60,61].

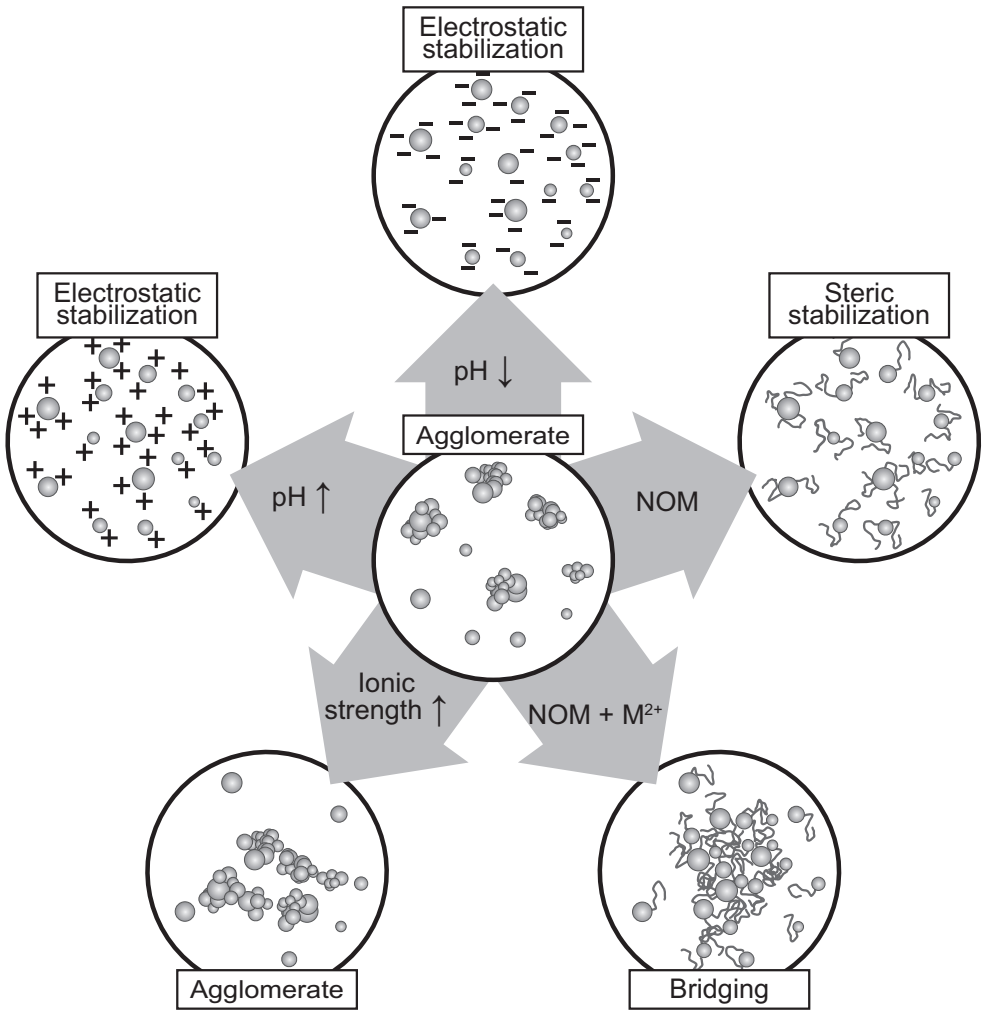

Figure 3. Illustration of the main mechanisms for stabilization of nanoparticle dispersions, including electrostatic stabilization due to pH, steric stabilization from presence of NOM and role of ionic strength in stability. Modified from Faure et al. (2013) [50].

One of the main mechanisms of adsorption of NOM to mineral surfaces (that could also apply to ENPs) is discussed in Yang et al. (2009) [62] and includes ligand exchange between mineral surfaces and carboxyl and hydroxyl groups of NOM. Additionally humic acid adsorption on metal oxide ENPs is highly dependent on pH and surface properties of the particles. Electrosteric stabilization (electrostatic + steric) can also be achieved in a suspension but not investigated in the work presented in this thesis. The ionic strength is one of the most important parameters as its increase can decrease the electrical double layer and cause agglomeration /aggregation. The minimal concentration of the counter ions that can destabilize suspensions is called the

critical coagulation concentration (CCC) [63]. As discussed in Baalousha et al (2013) [47], according to the Schulze–Hardy rule the CCC is inversely proportional to the valence of the counter ions. This means that higher valence ions will have lower CCC and cause more agglomeration. This has been seen in various studies, where the media containing divalent ions has contributed to higher ENP agglomeration than monovalent ions, and will be discussed in more detail later in Chapter 4 of this thesis.

In nano-(eco)toxicological studies zeta potential (zp) has provided a good numerical measure for the stability of suspensions. ENP suspensions are generally considered stable when -30 mV < zp > 30 mV. For values falling far outside this limit, the particles will not have enough repulsion and will agglomerate and aggregate, creating unstable suspensions (as shown in Figure 4). High zeta potential will be characterized with high interparticle repulsion that will keep the particles away from each other and hence, cause the suspensions to remain suspended. The most critical point will be the point of zero charge (pzc) or isoelectric point, which is the pH value where the net surface is zero, and the repulsion between the ENPs will be the lowest allowing agglomeration/aggregation to occur. The CCC will also be a function of pH, and it increases significantly as the pH is further away from pzc [64]. Polydispersivity index is often used to describe how disperse a sample is (distribution of a molecular mass in a given sample). The role of these parameters in suspension stability will be discussed further in Chapter 4.

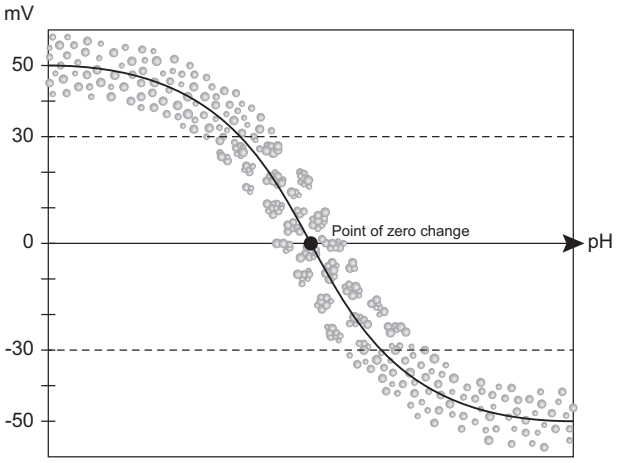

Figure 4: Stability of ENPs in suspensions at different pH. Generally stable suspensions are achieved when zeta potential is greater than 30 in absolute value. More agglomeration is seen at point of zero charge.

3.3 Nanoparticle dissolution

Another important process that metal and metal oxides are subjected to in aqueous suspensions is dissolution. Dissolution can be classified as a dynamic process where molecules of the dissolving solid migrate from the surface through a diffusion layer [65], and is controlled mainly by solubility [66]. Some of the main factors affecting dissolution are discussed in Misra et al. (2012) [66], and include size and its related parameters such as surface area and morphology, crystallinity and crystal structure; shape and agglomeration/aggregation, capping agents, presence of organic compounds (NOM, proteins, polysaccharides); and the characteristics of the surrounding media such as pH, ionic strength and storage conditions. Correlations between dissolution and physico-chemical properties of the ENPs are not very straightforward as a change in one of the properties can induce another change in another physico-chemical property [66].

ENP properties and media components can influence particle dissolution kinetics (rate of solubility) and equilibrium solubility (amount of dissolved material) [31]. Dissolved ions or molecules can interact with media components (hydrogen carbonate, sulphate, chloride and organic matter) and form complexes or precipitates. Dissolution and precipitation are controlled by the solubility product Ksp (the equilibrium constant for a solid substance dissolving in an aqueous solution), and can depend on inherent substance or ENP properties as well as media composition (ionic strength, ligands, pH, and temperature) [31]. The scenarios encountered in an ENP suspension can include: ENPs/ions, ions/complexes, suspended/agglomerate/ precipitates, and their bioavailability and toxicity [66].

TiO_2 ENPs are believed to be insoluble or have very low equilibrium solubility (as discussed in Schmidt and Vogelsberger (2006) [67]), whereas Ag and ZnO can partly dissolve in aquatic suspensions. Ag ENPs undergo dissolution through oxidation with dissolved oxygen and protonation [68]

$$4Ag_{(s)} + O_2 \leftrightarrow 2Ag_2O_{(s)} \quad (2)$$

$$Ag_2O_{(s)} + 2H^+ \leftrightarrow 2Ag^+_{(aq)} + H_2O_{(l)} \quad (3)$$

ZnO ENPs can undergo dissolution with protons and hydroxide ions under acidic and alkaline conditions respectively. As discussed in Bian et al. (2011) [69], the following reactions can lead to ZnO ENP dissolution:

$$ZnO_{(s)} + 2H^{+}_{(aq)} \leftrightarrow Zn^{2+}_{(aq)} + H_2O_{(l)} \quad (4)$$

$$ZnO_{(s)} + H^{+}_{(aq)} \leftrightarrow Zn(OH)^{+}_{(aq)} \quad (5)$$

$$ZnO_{(s)} + OH^{-}_{(aq)} + H_2O_{(l)} \leftrightarrow Zn(OH)_3^{-}{}_{(aq)} \quad (6)$$

$$ZnO_{(s)} + 2OH^{-}_{(aq)} + H_2O_{(l)} \leftrightarrow Zn(OH)_4^{2-}{}_{(aq)} \quad (7)$$

At acidic pH values (below 6), the ionic species in suspension will be Zn^{2+} and $Zn(OH)^{+}$; for pH values above 9 the hydroxide products will be $Zn(OH)_2^{-}{}_{(aq)}$, $Zn(OH)_3^{-}{}_{(aq)}$, $Zn(OH)_4^{-}{}_{(aq)}$; and pH values in between these, $Zn(OH)_2$ will precipitate (discussed in Bian et al. (2011) [69]).

Dissolved species in suspension can also affect the size distribution by undergoing Ostwald ripening where the ions redeposit on the surface of ENPs. For some ENPs, dissolution controls behavior in aquatic systems and can greatly affect toxicity. Various studies have concluded that the dissolved ions are responsible for the toxicity of ENPs in suspension [70-76]. On the contrary, some studies have suggested that the nano scale is responsible [77,78]; and others concluded that dissolution cannot account for all the toxic effects seen in and could be a combination of both factors [69,79-82]. For *Daphnia magna* silver can cause reduction of Na+ and inhibition of uptake, due to blockage of the Na+/K+-adenosine triphosphate channels [83]; and zinc can have a competitive effect on Ca uptake [84]. Elucidating on toxicity mechanism of ENP vs. metal ion is outside the scope of this thesis, however, dissolution measurements have been included in Papers I and II to investigate the effect of media parameters and suspension stability on dissolution and its effect on toxicity.

3.4 Catalytic and photocatalytic activity of ENPs

ENPs can exert their toxic effect either based on chemical composition or stress/stimuli caused by characteristics such as surface, size and/or shape, though it is difficult to differentiate between the two [85]. TiO_2 ENPs have a special mechanism of action in the presence of light, especially UV. Under UV radiation, TiO_2 acts as a photocatalyst by increasing the rate of chemical reaction without being consumed [86]. The most common crystalline structures of TiO_2 include rutile and anatase, with the latter displaying more photocatalytic effects [23], due to the high band gap (which is the energy difference between the top of the valence band and the bottom of the conduction band). UV is able to activate an electron on the surface of TiO_2 ENPs, which leaves a valence hole that can extract electrons from water or

oxygen ions and generate hydroxyl and oxygen radicals as known as reactive oxygen species (ROS). This process can be initiated by photons having equal or higher energy than its band gap (~3.2 eV) [87]. The mechanisms of formation of ROS from photoactivation of TiO_2 ENPs has been documented extensively [88,89] and is presented schematically in Figure 5.

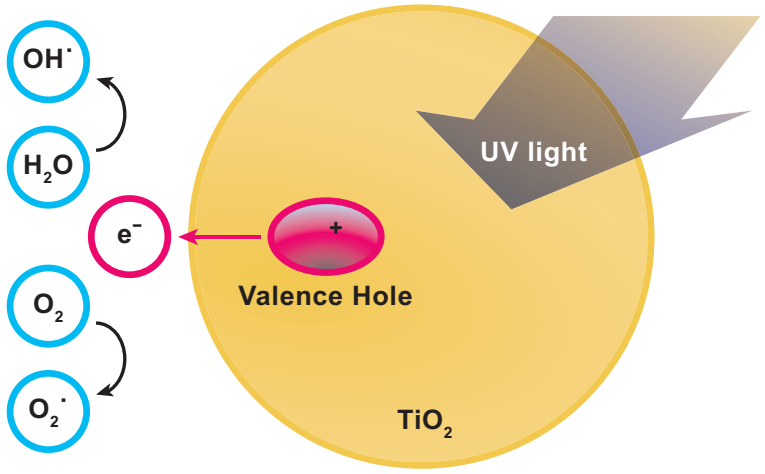

Figure 5. Photocatalytic activity of TiO_2 NPs. Once the particle is exposed to UV light, it absorbs a photon, this way exciting an electron which results in a valence hole. The electron released is able to interact with molecules present (H_2O and O_2) and create reactive oxygen species (Paper IV).

ROS molecules are damaging to DNA and can lead to strand breaks, abasic sites and base and sugar lesions [90]. DNA damage, along with point mutations and altered gene expression are commonly reported genotoxic responses [91]. Genotoxicity can be classified as direct from physical changes to DNA; or indirect, from reduced repair or ROS [91,92]. This makes the photocatalytic activity of TiO_2 ENPs an important mechanism of toxicity in both human and eco-toxicology (Paper IV) [4].

ENPs have significantly higher specific surface area than their larger counterparts, so the proportion of atoms on the surface is also greater, giving rise to higher surface reactivity. CeO_2 ENPs can donate and store oxygen. Due to its oxidative capacity, they act as a catalyst by assisting in the combustion of hydrocarbons and soot. CeO_2 ENPs can also strongly adsorb ultraviolet radiation [8]. CeO_2 ENPs have also showed to cause significant increases in the cellular ROS concentrations may exert toxicity through oxidative stress [93]. This is important as continuous oxidative stress can lead to chronic inflammation, which is associated with various diseases (as discussed in Reuter et al. (2010) [94].

4 Methodological challenges and improvement of current standard guidelines

In the last few years, the amount of studies on nano-(eco)toxicological testing has greatly increased [95,96]. In the vast array of these studies, various testing methods can be found, including nonstandard/customized using different exposure methods and endpoints, as well as standard OECD, ISO and US EPA methods [13]. As discussed previously, these methods were developed for traditional chemicals and some debate has followed on their feasibility, and generally on difficulties and challenges encountered during testing of NMs. Some of these challenges and generic issues in test methodology have been pointed out in the different OECD and ISO guidance documents, as well as critical reviews and nano-(eco)toxicological studies [13,42,43,45,97,98]. These relate to processes of ENP behavior in the test system, experimental design and stock and test suspension preparation, media components, size control, use of dispersing agents, artifacts and confounding factors; and will be discussed into more detail in the following chapters of this thesis. Stock suspensions will be referred to as ENPs in liquid media and test suspensions are prepared from aliquots of stock suspension in a test medium at desired ENP concentration.

4.1 Stock suspension preparation

Dispersion of ENMs in stable aquatic suspensions is the first step towards toxicological and ecotoxicological testing. Different methods of preparation of (stock) dispersions can lead to variation in ENM toxicity. Therefore, harmonization and standardization of dispersion methods applied in toxicity and ecotoxicity testing is necessary in order to ensure reproducibility and comparability and minimize test artifacts caused by modifications of the nanomaterials during dispersion preparation process (Paper III) [3]. The importance of stock suspension preparations has been highlighted in the early days of testing of ENMs as different preparation methods can yield different results (Paper II, III) [2,3].

ENPs in the form of powders are not easily dispersed in aqueous media and exhibit agglomeration tendencies. Dispersibility of a suspension can be improved by modifying test conditions such as pH and/or ionic strength or addition of compounds that act as dispersants followed by a de-agglomeration

energy using various procedures for ultrasonication, stirring, or shaking [99]. Stock dispersion methods should ideally minimize variations in dispersion procedures between the different test systems and laboratories, and fulfil the following criteria: 1) minimize artifacts from modifications of the nanoparticles, 2) enable a link between the observed effects and the physicochemical properties of the pristine ENPs, and 3) produce stable and homogenous stock suspensions that enable precise and representative sampling for test suspensions (Paper III) [3].

Several nanomaterial dispersion protocols such as NANOGENOTOX, PROSPECT, NANOMMUNE, ENPRA and NIST/CEINT have been proposed in the last few years as discussed in Paper III [3]. Due to their specificity, the applicability to other test systems and other types of ENPs is limited. However, key parameters identified from these protocols should be taken into consideration for stock dispersion preparation and include: nanomaterial properties, nanomaterial stock concentration, volume of dispersion medium, dispersion media/water quality, stabilizing/dispersing agents, pre-wetting of nanomaterial powders, dispersion procedure (mechanical and ultrasonication), temperature control, maintaining stability prior to dosing, and performance or quality assurance (Paper III) [3].

In regards to the ENM stock suspension concentration, a wide range of concentrations (ranging from 0.015 to 20 g L^{-1}) were chosen by the different dispersion protocols discussed in Paper III [3]. Ecotoxicological studies usually employ synthetic aquatic media and toxicological prepare dispersions in cell media for *in vitro* studies. The dose in both types of studies is very important as agglomeration and aggregation processes, which in turn can have an effect on toxicological outcomes [99,100], (Paper II) [2]. In the ecotoxicological studies conducted in Paper I and Paper II, the stock suspensions were prepared at 1000 mg L^{-1} taking into account the highest test concentration (100 mg L^{-1}) in order to avoid more than 10% of nutrient reduction in the medium.

Another point to consider is the quality of ultrapure water, which should have a resistivity of ≥18.2 MΩ·cm, and bacterial contamination, especially for toxicological studies. Bacteria such as endotoxins do not only have a potential for heteroaggregation of ENPs [52], but can affect the outcome of the test due to their toxicological mode of action. In ecotoxicology, stock suspensions in ultrapure water or MilliQ have been commonly used. Stock suspensions in aquatic media are generally not recommended as the ionic

strength can influence the agglomeration and aggregation in stock suspension (Paper II) [2], and unless undergoing a process of de-agglomeration, it can be transferred to aquatic media, this way contributing to a larger extent of agglomeration/aggregation.

Regarding ENM properties, composition, surface properties including hydrophilicity/phobility, water solubility and size distribution of the primary size should be taken into consideration. For hydrophobic ENPs, a pre-wetting step is included to facilitate dispersion in water. In the protocols reviewed in (Paper III) [3], a 0.5% (v/v) ethanol solution is normally employed by making a paste of the ENP powder. A solvent that is hydrophilic or has lower surface tension than water may be used; however, the final concentration should not be toxic to the test system/species and media controls should be used.

One of the processes that have the most impact on the deagglomeration of the ENPs in aquatic suspension is that of utrasonication, which applies sound energy with ultrasonic frequencies. This can involve water bath, probe, and cup horn sonicators. Other less vigorous methods include shaking, magnetic stirring and vortexing. In order to achieve reproducible results, the energy delivered to the sample is very important, along with sample volume, particle concentration, medium viscosity and temperature Paper III [3]. In preliminary studies conducted for this thesis, it was shown that the probe sonicator was more efficient in dispersing TiO_2 and CeO_2 ENPs creating a suspension with lower average size distribution (30-50 nm difference in zeta average), than the bath sonicator, which remained more stable over time (up to 48 hours). The probe sonication followed a similar soncation procedure to the NANOGENOTOX protocol [101]. The difference was the placement of the stock suspension beaker in a water bath instead of an ice-water bath [2] (Paper II). In the absence of the water bath, the temperature of the suspension increased by 10˚C above room temperature; and in the presence of the ice-water bath, the temperature of the suspension dropped to 4˚C. Changes in temperature could affect the agglomeration process, hence, water bath (where the temperature remained constant) is recommended.

Another mean of creating stable stock suspensions is the use of dispersants to stabilize for steric stabilization as shown in Figure 3. Dispersants can be natural (e.g. proteins and natural organic matter) or synthetic (e.g. surfactants and polaxamers). In toxicological studies, biological dispersants are highly preferred and generally include bovine serum albumin (BSA), fetal bovine serum (FBS) and dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) Paper III

[3]. The use of 0.1% DPPC + 10% FBS based on physiological relevance helped stabilize stock suspensions, and in their absence the ENPs precipitated to the bottom of the vial (Paper VI) [6]. Small amounts of protein in the media can improve dispersion and stability of particles in the solution [102,103]. Some substances such as tetrahydrofuran make very good dispersants for ENPs, however, are not compatible with biological systems [104]. As a good practice, dispersant controls should be included with all studies.

Parameters such as particle concentration, pre-wetting, dispersion media, sonication and dispersants are essential to produce a tailored ENP dispersion protocol that will be homogenous and stable. Appropriate controls need to be included if they are likely to entail modifications of the material properties or surface chemistry. However, other areas that need more investigation include effective sonication energy input, investigation relations between sonication procedures and biological effects, and ENP modifications in the presence of dispersants. As the issues covered here are identified from individual scientific studies and from large-scale research projects and international organisations, they may serve as a guide to researchers, companies, and regulators in nano-(eco)toxicological testing.

4.2 Influence of natural organic matter and aging on stock and test suspension stability

Natural organic matter in the form of humic and fulvic acids have been used as natural stabilizers in nano-ecotoxicology. These are high molecular substances containing hydrophilic and hydrophobic parts, aromatic rings and functional groups such as carboxylic and hydroxyl [105], which give NOM a good complexing capacity. Due to these properties, they can influence the mobility of contaminants through the process of adsorption, aggregation and sedimentation [106]. Various forms of NOM have lately been employed to stabilize and control dispersions of engineered nanoparticles. However, caution should be provided since dissolved organic carbon (DOC) can control metal bioavailability, slow dissolution and reduce toxicity; and any dispersant may change ENP dynamics and behavior (as discussed in Handy et al. (2012) [13]).

While various studies have investigated the effect of NOM on suspension stability [47,60,61,107-109], very few studies have considered its use in suspensions with relevance to crustacean ecotoxicological testing [110],

Paper I [1]. Moreover, no studies have considered the additional effect of aging to investigate the stability over the 48-hour duration in all test concentrations. In (Paper I) [1], all these parameters were investigated in three ENPs varying in physico-chemical characteristics; sterically stabilized Ag ENPs that were and expected to undergo only limited dissolution, pristine ZnO ENPs that can undergo dissolution, and highly agglomerating TiO_2 ENPs. The ubiquitous Suwannee River natural organic matter (SR-NOM) was used at concentration 20 mg L^{-1} (~10.5 mg C L^{-1}), which is in the range found in the environment [111] to investigate its stability on both stock and test suspensions. Stock I was prepared in MilliQ water and Stock II was prepared in MilliQ water in the presence of SR-NOM and 0 hour, 24-hour and 48-hour aging was performed (Figure 6).

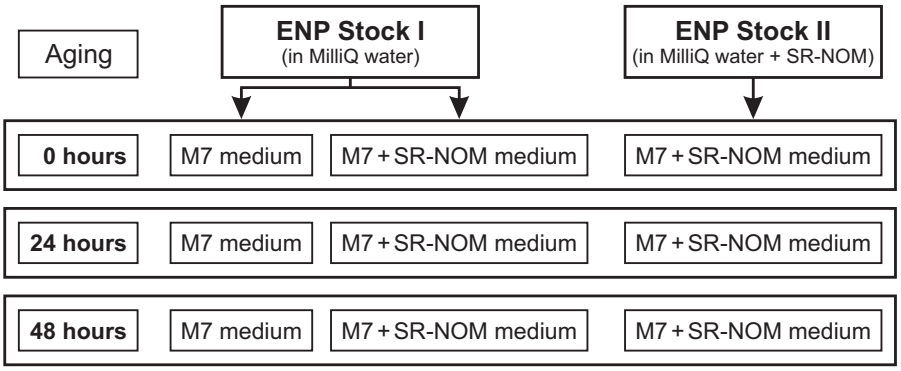

Figure 6. Experimental test set-up and preparation scheme of test suspensions of Ag, ZnO and TiO_2 ENPs for characterization and toxicity testing. ENP Stock I was prepared in MilliQ water at concentrations of 10 mg L^{-1} for Ag ENPs, 200 mg L^{-1} for ZnO ENPs, and 1000 mg L^{-1} for TiO_2 ENPs. ENP Stock II was prepared in MilliQ water containing 20 mg L^{-1} Suwannee River Natural Organic Matter (SR-NOM) in the same ENP concentration as ENP Stock I. The different test suspensions were prepared in triplicates, either in M7 medium, or M7 medium containing 20 mg L^{-1} SR-NOM. One set of test suspensions was tested freshly prepared (aged 0 hours), whereas the other two sets were tested aged 24 and 48 hours, respectively (Paper I) [1].

The zero hour aged Ag ENP Stock I and Stock II slightly agglomerated in suspension and aging did not considerably affect increase in z-average, PDI or zeta potential value. The addition of SR-NOM did not help the stabilization of Ag ENP stock suspensions. This could be due to the fact that these ENPs are already stabilized from the POE-Tween20 coating (Polyoxyethylene (POE) Glycerol Trioleate and Sorbitan mono-Laurate (Tween 20)).

Ag ENPs test suspensions prepared from Stock I and Stock II agglomerated both in M7 medium and M7+SR-NOM medium; and presence of SR-NOM

had no stabilizing effect, as observed by the zeta average or zeta potential values. Agglomeration and increase in PDI was observed for all test suspensions over time, up to 48 hours of aging. No difference was seen between agglomeration patterns between the different concentrations. Agglomeration in medium (M7), could be due to the high ionic strength medium as previously shown to cause aggregation of nanoparticles [47,107], where the divalent cations such as Ca^{2+} and Mg^{2+} will cause more aggregation than monovalent cations [47,60]. Despite NOM having shown to have a stabilizing effect on Ag ENP suspension [47,107], at higher ionic strength medium NOM does not help the stability of the system [112], most likely due to intermolecular bridging [60]. In fact, it has been shown that NOM can stabilize ENPs only for ionic strength values below the critical coagulation concentration (CCC) [47,113] and shifts the CCC to higher ionic strength [47]. The presence of SR-NOM under the conditions used in Paper I [1]does not seem to have a stabilizing effect in the case when ENPs are sterically stabilized by a coating.

NOM has also been reported to stabilize aqueous suspensions of ZnO ENPs and its effect is dependent on the concentration of NOM [64,108,114]. Similar results were seen in Paper I for stock and test suspensions [1]. ZnO ENPs in the test condition of 0 hour aging, the z-average and PDI were in the stable range for both Stock I and Stock II. Stock I prepared in MilliQ had a tendency to agglomerate over time, whereas the presence of SR-NOM in Stock II had a stabilizing effect. ZnO ENPs test suspensions prepared from Stock I agglomerated more in M7 medium than in M7+SR-NOM. The suspension in M7 greatly agglomerated over time, whereas the ENPs in M7+SR-NOM only agglomerated slightly. This difference is most likely due to the presence of ions in the medium and in particular the prevalence of divalent cations [114,115]. The same trend was seen for suspensions prepared from Stock II in M7+SR-NOM medium, where slight agglomeration was seen going from stock to test suspension, and the size distribution did not change with aging up to 48 hours. These results indicate a stabilizing effect of SR-NOM in both stock and test suspensions. However, careful consideration should be made when using NOM to increase the stability of ZnO ENPs, as addition of humic acid has shown to increase dissolution at high pH conditions [69]. Though not discussed in this thesis, another thing to keep in mind is the electrosteric stabilization as discussed in Omar et al. (2014). NOM presence promoted disaggregation, especially for pH values above point of zero charge [116].

In Paper I [1], TiO_2 ENPs in Stock I revealed to be stable. Preparation of TiO_2 ENP Stock II in SR-NOM revealed large agglomeration with z-average size > 2000 nm. Aging of the stock suspensions did not majorly affect the size distribution or zeta potential. The most stable stock suspension with limited agglomeration was TiO_2 ENP Stock I prepared in MilliQ water. High agglomeration was measured for Stock II despite various studies having reported a stabilizing effect of NOM on TiO_2 ENPs in aqueous suspensions [61,109,117]. However, at high cation concentrations aggregation was observed, most likely to Ca^{2+}–NOM bridging [61]. Since no ions were present in the stock prepared in MilliQ, aggregation might be effected by the amount of NOM or the charge of ENPs. TiO_2 ENPs in MilliQ were positively charged and since the NOM suspensions are negatively charged, agglomeration might be partly explained by the charge neutralization as seen before by iron oxide ENPs [118]. Moreover, stability of ENPs will depend on the quantitiy of NOM as only at high amounts could ENP stability be reached for iron oxide and ZnO ENPs [108,119].

As previously discussed in other studies [48,120] and similar to what was seen for Ag and ZnO NPs, TiO_2 ENPs agglomerated in M7 medium reaching values > 1 µm. Agglomeration has previously been reported for $CaCl_2$ concentrations over 0.1 mM, and pH above 5 [121], which are conditions met by the M7 medium. The suspensions prepared from Stock I in the presence of SR-NOM seemed to be more stable, and the size distribution only increased slightly over time. Test suspensions in SR-NOM from Stock II were also agglomerated, similar to the sizes found in the stock suspension. Based on these results, the use of NOM in stock suspensions should consider NOM: ENP ratio. In the case when the stock concentration is very high, either higher NOM concentrations should be used or other dispersion means should be used considering point of zero charge.

4.3 Influence of pH and ionic strength on stock and test suspension stability

As discussed previously in Figure 3, steric stabilization of ENPs is greatly affected by the pH and ionic strength of the media they are found in. With relation to pH, for every particle and medium used there exists a pH value where the ENP charge is zero, at which point the particle repulsion is the lowest (Figure 4). This is an important parameter since electrostatic repulsion and attraction are determinant factors in agglomeration [57,100]. This is more

relevant for non-functionalized (e.g. by coatings) ENPs, especially in the presence of ions in the media [57].

In order to investigate whether there is a certain set of parameters that could lead to stable aquatic suspensions, in this experimental study Ag, ZnO, and TiO_2 ENPs were dispersed in different media over a large scale of pH values. The aim was to achieve stable aquatic suspensions with lowest possible size of ENP agglomerates before and during testing under OECD guideline conditions. A variety synthetic freshwater media used for toxicity tests were employed: US EPA media ranging from Very Soft (VS) to Very Hard (VH), M4, M7 and ISO daphnia media with ionic strength ranging from 0.57 mM to 15.8 mM and hardness of 2.8 mg L^{-1} to 133.3 mg L^{-1} $CaCO_3$.

The zeta potential and size distribution were measured in the range of pH 2-12 for Ag, ZnO, and TiO_2 ENP stock suspensions dispersed in MilliQ water, VS, S, and M7 media (Figure 7). The zeta potential values of Ag ENPs in the different media were relatively unaffected by pH changes in the range relevant for toxicity testing (pH 6-9) (Figure 7, A2). However, since these ENPs are not electrostatically but rather sterically stabilized, the zeta potential will not necessarily reflect the suspension stability. Independent of pH and media composition, the z-average of Ag ENPs could be maintained around 100 nm (Figure 7, A2). This has also been seen previously, where pH and ionic strength did not have an impact on aggregation for sterically stabilized (PVP)-Ag ENPs but three other types of Ag ENPs (uncoated, electrostatically stabilized and electrosterically stabilized) were susceptible to the presence of Ca^{2+} (10 mM), which caused aggregation [58].

Dissolution of ZnO ENPs occurred for pH values lower than 6.5, so zeta potential and pzc of ZnO NPs could not be measured below this value. Dissolution of ZnO ENPs at pH values between pH 6.7 and 3.7 has also been observed earlier [116], and ZnO ENPs left in suspension at pH 6.1 were reported to dissolve completely [122]. ZnO ENPs in media indicated to be unstable based on the low zeta potential values (in absolute terms) (Figure 7, B1). High z-average and polydispersivity index increased at pH values above 7.5, especially for M7 medium (Figure 7, B2). Hydroxilation and precipitation can be a likely cause for the increase in size [69]. The point zero charge for the ZnO ENP suspensions was between 7.5 and 8.5 for MilliQ and VS EPA medium.

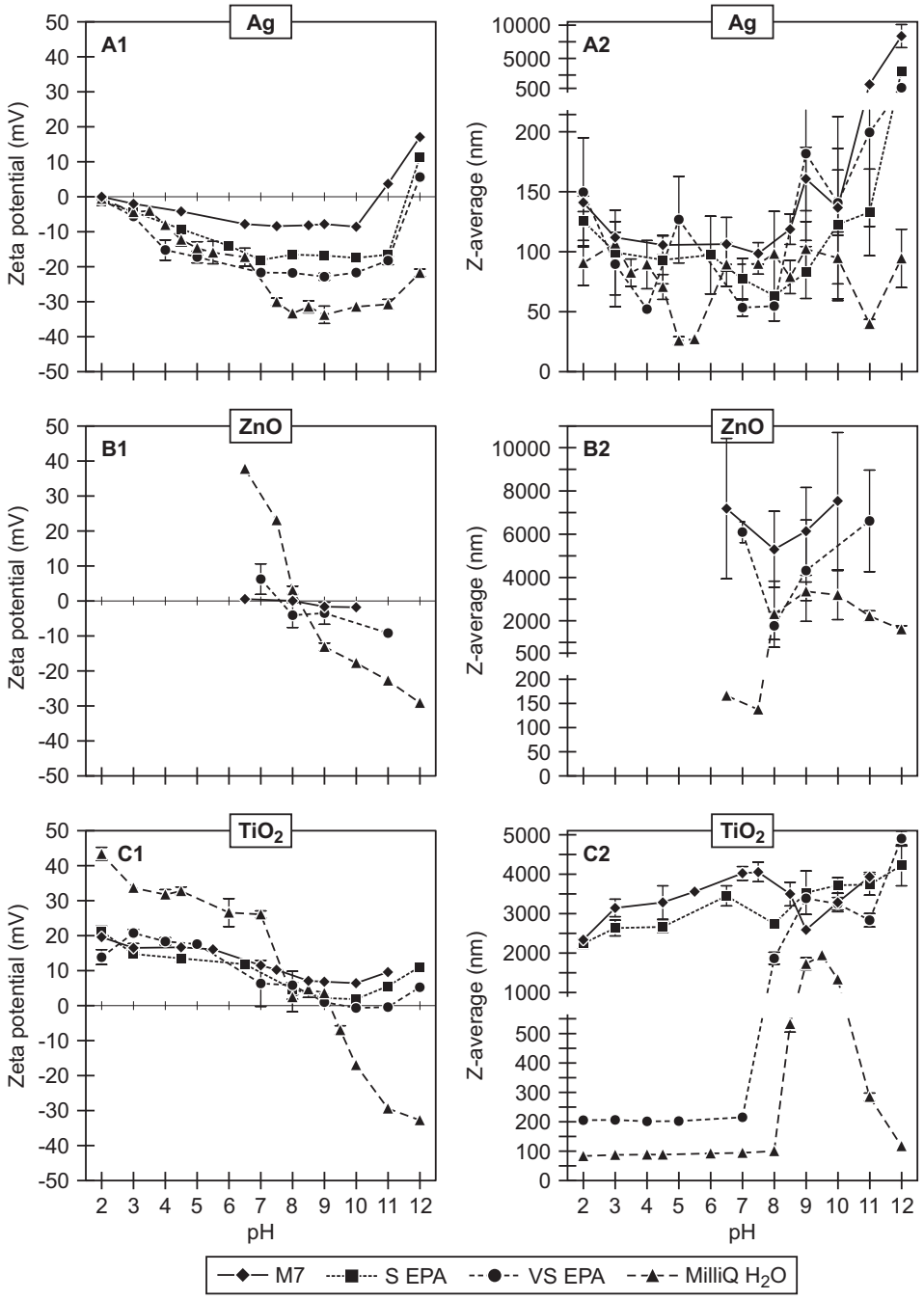

Figure 7. Zeta potential (left panel) and average size (right panel) of 10 mg L^{-1} Ag (A), 100 mg L^{-1} ZnO (B) and 100 mg L^{-1} TiO_2 ENPs (C) stock suspensions in MilliQ H_2O, M7, VS EPA, S EPA medium. Two identical stock suspensions were prepared and sonicated. First suspension was adjusted to acidic (HNO_3) and the other suspension to basic (NaOH). Please note the different scales on y-axis.

*ZnO ENPs in S EPA medium were highly unstable with sizes >8000nm, and high polydispersivity, and are therefore not shown in Figure 1B (Paper II) [2].

The ZnO ENPs in the most unstable conditions were those in M7 medium, and EPA medium where sizes >8000nm (Figure 7, B2). Due to the high instability of ZnO suspensions, the best option for preparing and testing stable suspensions was identified to only exist for VS EPA medium at pH 7 (see Figure 7, B2).

The discrepancy could be related to the fact that primary particle size and size distribution has been shown to affect pzc [48]. Point of zero charge for TiO_2 ENPs was observed around pH 9 for most media and MilliQ (Figure 7, C1). Anatase-rutile TiO_2 P25 ENPs revealed a pcz of approximately 7 [123]. Another study evaluated TiO_2 P25 ENPs dispersed in MilliQ water at pH 4, 6 and 8, and the isoelectric point was around 5. The most stable suspensions with regards to zeta average and zeta potential were those at the pH value of 8, which was the furthest away from the isoelectric point [124]. For the same particle, isoelectric point was around 6, when pH was adjusted to 3, 5.9, 7, 9 and 11. The smallest agglomerates were observed at pH 9 and 11 [125]. The zeta potential values correlated with the size distribution measurements (Figure 7, C2) where polydispersivity index and particle size increased drastically at pH>7 in VS EPA medium and pH 8 in MilliQ water, as the pH values approached the point zero charge. TiO_2 ENPs in S EPA medium and M7 medium were highly agglomerated at all pH values. As seen for other particles, an increase in ionic strength, and especially the presence of divalent cations in media induced agglomeration of TiO_2 ENPs at $CaCl_2$ concentration to 10 mM and 100 mM [126]. The lower ionic strength VS EPA medium at pH 6-7 was seen as the only "window of opportunity" where stable TiO_2 ENPs suspensions could be achieved (Figure 7, C2).

From the literature discussed above, there have only been a few studies investigating the stability of ENPs over a large pH range and pzc in test media that is physiologically relevant. This is important that pzc in all test media is measured as the CCC of ENPs has been found to be a function of pH [64]. In nano-ecotoxicological testing the set of parameters leading to stable suspensions should be investigated. In Paper II [2], this was achieved by using low ionic strength media that is physiologically relevant to the organism, and adjusting the media pH values to where the ENPs appeared to be most stable and low agglomerate sized were achieved. As a general rule, point of zero charge measurements should be necessary prior to ecotoxicity testing of ENMs.

5 Test condition implications in nano-ecotoxicology

Aquatic organisms such as algae, daphnia and fish are a base-set of organisms that are very commonly used in regulatory testing. Crustaceans, a very abundant and important group of invertebrates in marine and freshwater ecosystems are commonly used as a representative testing organism in aquatic and terrestrial systems [14,127]. This is due to their ecological importance, morphological and ecological diversity (Paper IV) [4], as well as small size, short life cycle, and ability to produce clones [128]. Daphnids are the link organism between primary producers such as algae, and secondary producers such as fish [14]. Moreover, they are among the most sensitive aquatic animals to chemicals and they are relatively easy to culture in the laboratory. As a result, *Daphnia magna* and *Ceriodaphnia dubia* are the most commonly used organisms in standardized tests for toxicity assessment of chemicals (and consequently nanomaterials) in OECD and ISO guidelines [14]. *D. magna* has been an important test species in the research presented in this thesis as it is used in OECD 202 guideline [37], which has been employed to test acute immobilization of daphnids in response to ENPs. The work presented in this thesis focused on standard regulatory testing for the purpose of screening and ranking the toxicity of the primary-sized ENPs taking into account CLP (classification, labelling, and packaging of substances) regulation [34]; and developing best practices for testing ENPs under these guidelines.

In nano-ecotoxicity testing, various parameters should be kept in mind including experimental design, reference materials and particle size controls [13]. In case of investigating a nano-size effect particle size controls should be included and metal salts where dissolution is being investigated [129]. In fact, it is recommended that particle size distribution and measurement of dissolution, as well as investigation of dispersion controls should be investigated [43]. As discussed in Petersen et al. (2012) [130], studies involving ENMs have additional uncertainties compared to regular chemicals that are related to dispersion and dynamic processes undergoing during testing. Artifacts can arise from interference with assay reagents or toxic byproducts from dispersion process [130]. When the test concentrations are high and the suspensions are not stable, ENPs tend to agglomerate and sediment to the bottom of the test vial. These precipitates could restrict the movements of invertebrates (e.g. *D. magna*) and cause false positive results

(as discussed in Handy et al. (2012) [97]). Agglomeration and processes it affects during testing can also be considered artifacts.

The acute toxicity of Ag, ZnO and TiO_2 ENPs to *Daphnia magna* has been investigated by a large number of studies. However, given the discrepancy of results encountered in nano-ecotoxicology it is difficult to hypothesize if the source is related to uncertainty, differences in experimental procedure, or artifacts [130]. A literature review was conducted for state-of-the-art knowledge on the current status of acute testing (48 hours) with crustacean *Daphnia magna*, to investigate whether these data are suitable for risk assessment. The search was conducted in ISI web of science using the keywords nano* and *Daphnia magna* and acute toxicity and Ag, or ZnO or TiO_2. The acute toxicity focused only on 48 hour exposures and one invertebrate to investigate patterns of toxicity. Studies using EPA or OECD 202 guidelines were selected. The data is presented in Appendix 1 and Fig. 8.

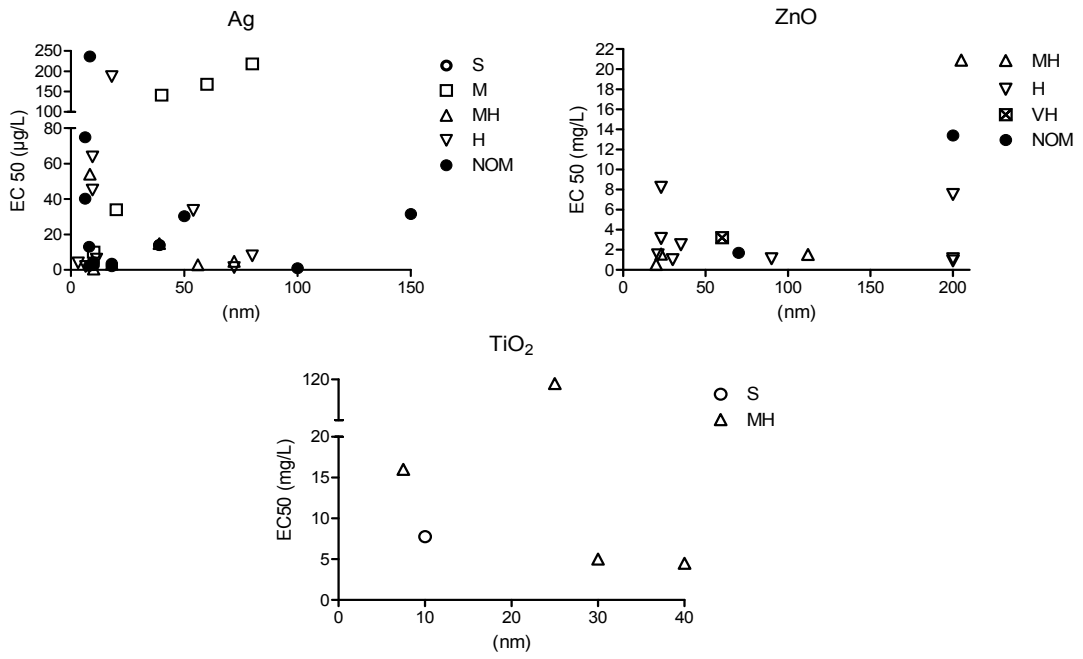

Figure 8. Literature review on the acute tocixity test of Daphnia magna to Ag, ZnO and TiO_2 ENPs. Data represents EC50 values vs. primary size of the particles with a specification of the medium strength. The classification into soft (S), moderately hard (MH), hard (H) and very hard (VH) was done according to EPA synthetic water criteria [39]. Data is also presented in Appendix 1.

The graphs in Figure 8 present EC50 values vs. primary size of the particles with a specification of the medium strength and presence of NOM. The

classification into soft (S), moderately hard (MH), hard (H) and very hard (VH) was done according to EPA synthetic water criteria [39]. The average size of the primary particle size was considered when plotting, which might not be the right parameter to take into account considering all the processes ENPs undergo in aquatic suspension. However, for most studies, no information was given on the size distribution in media. There were no clear trends between primary particle size, media composition and acute toxicity. Toxicity differed from a few (ZnO, TiO_2 ENPs), to several hundred orders of magnitude (Ag ENPs). The employed ENPs often differ in size, surface chemistry and coatings, as well as preparation methods. Discrepancies between these studies and the large agglomeration in test medium (where reported) raise question whether there is a risk of underestimating toxicity.

5.1 The influence of test suspension stability on ecotoxicity

Chapter 3 of this thesis covered the factors affecting stability of ENPs, and Chapter 4 presented cases on how NOM, aging, ionic strength and pH can influence the stock and test suspension stability. Since it has previously been shown that different suspension preparation methods can influence toxicity outcomes [131,132], it is important to investigate the effect of the suspension preparation (including aging, NOM, pH and ionic strength) as presented in Chapters 4.2 and 4.3 on toxicity towards the freshwater crustacean *Daphnia magna*.

5.1.1 The influence of aging and presence of SR-NOM

In Paper I, Ag ENPs test suspensions in M7 medium from ENP Stock I caused immobilization of daphnids within the first 24 hours and the response did not change up to 48 hour of exposure, showing a 48-hr EC50 of 32 µg L^{-1}. Aging of test concentrations in M7 medium up to 48 hours did not significantly affect toxicity. As discussed in Chapter 3.3, and as shown in previous literature, Ag ENPs are able to undergo dissolution in suspension [133,134]. In the present study, dissolution increased with aging (up to 24 hours) even in the presence of SR-NOM. This could be due to the fact that SR-NOM did not did not affect the size distribution of Ag ENPs, though the link between dissolution and physico-chemical properties of the ENPs are not very straightforward [66]. Increases in ion release during storage have also been reported earlier [135]. Exposure of *D. magna* to the freshly prepared and aged Ag ENP suspensions caused similar immobilization response.

Presence of SR-NOM under all conditions eliminated the acute toxicity of Ag ENPs at all concentrations tested. This is in accordance with earlier literature where the presence of NOM was able to mitigate toxicity [110,136] and this effect was more evident with increasing amounts of dissolved organic carbon (DOC) [135]. It was hypothesized that this response could be due to inhibition of ion release from adsorption of NOM onto Ag ENPs [133]; however, it is not plausible since dissolution increased after 24 hours. Elimination of toxicity from the presence of SR-NOM might be rather due to the formation of Ag^{+}-NOM complexes, which in themselves might not be toxic to the organisms. Based on current literature and results presented in Paper I [1], addition of NOM to Ag ENP suspensions can lead to mitigation of toxicity and is grounds for underestimation of toxicity.

ZnO ENPs test suspensions at zero hour aging in M7 medium from ENP Stock I caused immobilization of daphnids yielding 48-hr EC50 of 6.7 mg L^{-1}. No monotonous concentration-response curves could be achieved for ZnO ENPs in M7 + SR-NOM media prepared from ENP Stock I or ENP Stock II, although the overall immobilization was higher than in M7 medium (Figure 9). Depending on their concentration and chemical composition, presence of NOM can either enhance or reduce Zn^{2+} release from ZnO ENP, as NOM can protect ZnO ENPs from attack/dissolution [72]. Similar to this study, Blinova et al. [73] found that the presence of DOC did not decrease the toxicity of ZnO to freshwater crustaceans. NOM might have different effects on toxicity of different metals, as metals can bind to different moieties within the NOM [137]. Test suspensions from Stock I and Stock II that were aged for 24 hours, revealed a scattered response with similar 48 hour immobilization. However, after 48 hours of aging, concentration-response curves were attained for all testing scenarios with an overall increase in immobilization both 24 and 48 hour exposure, especially in the presence of SR-NOM (Figure 9). This might be due to an increase in dissolution over time since ZnO ENP toxicity is believed to be linked to Zn^{2+} ion release [70,73,76]. Kinetic studies have revealed that ZnO ENPs (at pH 7.5) can reach dissolution steady state in 24 hours [138]. In the present study (pH 8.2), stable testing conditions and concentration-response curves were only reached at 48 hour aging (Figure 9). Presence of SR-NOM and aging for 24 hours contributed to stabilization of the test suspensions and might lead to increased standard tests reproducibility.

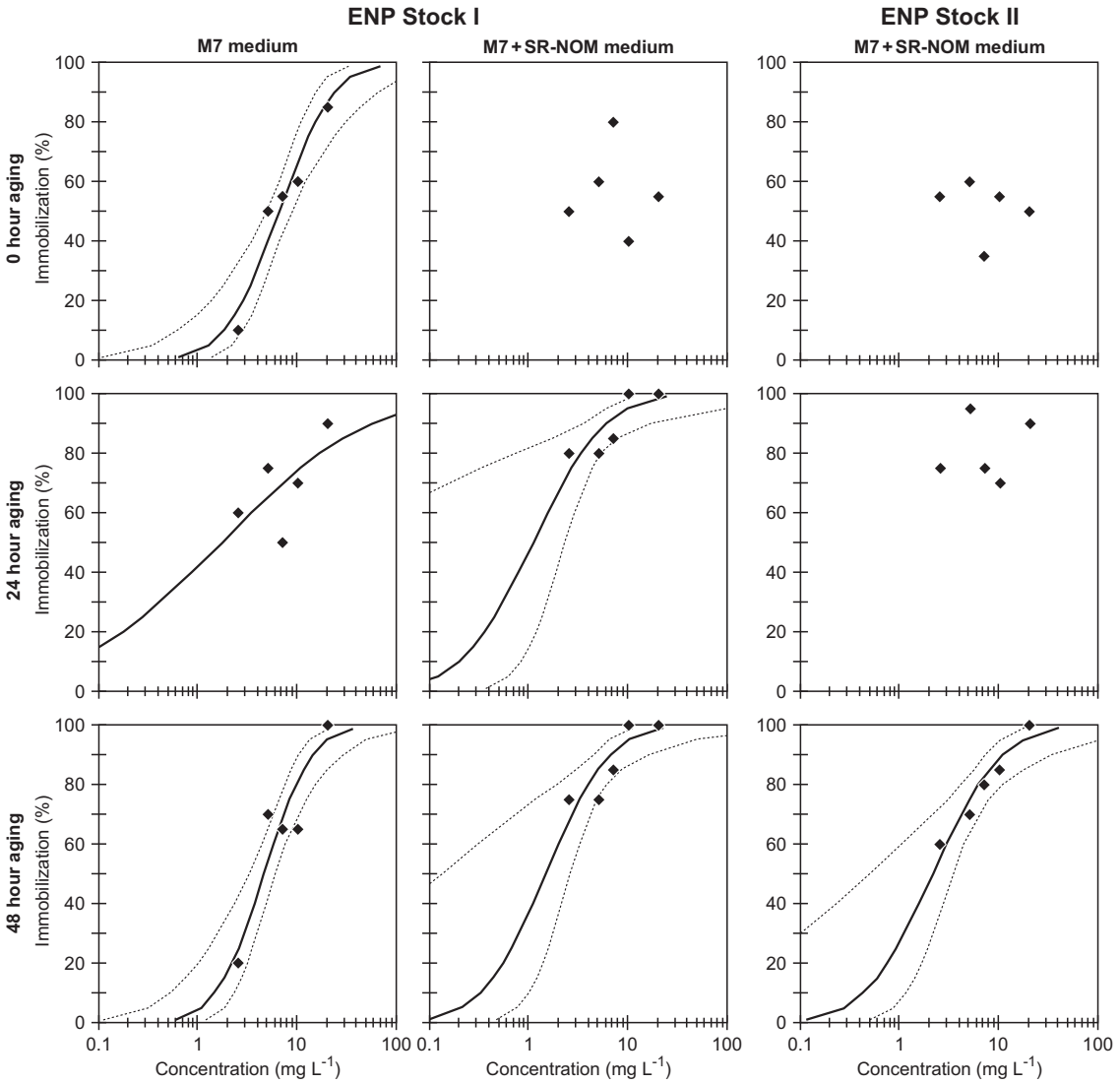

Figure 9. Immobilization of *Daphnia magna* neonates in response to 48-hour exposure to different concentrations of ZnO ENPs prepared from Stock I (MilliQ) and Stock II (MilliQ + SR-NOM) in the presence and absence of SR-NOM and with the additional effect of aging. Dotted lines represent 95% confidence intervals (Paper I) [1].

TiO_2 ENPs did not cause immobilization of *D. magna* in concentrations up to 100 mg L^{-1} under the different testing conditions, which is in agreement with previous literature [139,140]. In contrast, humic acid stabilized TiO_2 ENPs and a small increase was observed in zebrafish (*Danio rerio*) [141]. In Paper I [1], size distribution/stability did not have an effect on immobilization of the daphnids, but it might be rather the coating of NOM that can affect the toxicity of TiO_2 ENPs.

5.1.2 The influence of pH and ionic strength

As discussed in Figure 4 and Chapter 4.3, pH plays an important role in steric stabilization of ENPs in aquatic suspensions. Agglomeration may alter the stability of the suspensions, and hence the bioavailability and toxicity of ENPs, which in turn will affect the reproducibility of the test results. Colloidal stability is a significant parameter in bioactivity of ENPs that may account for different biological outcomes [142]. A few studies have investigated the stability of Ag ENPs, in different ionic strength media relevant for regulatory ecotoxicity tests [46,143,144]. The ISO medium for daphnia testing [38] was used in the original formulation, and in a diluted form of 2, 5 and 10 times in an earlier study [46]. The undiluted media revealed the most agglomeration, which influenced the nature of the exposed particles [46]. In a similar study by the same group [144], unmodified OECD M7 medium, a 10 times diluted M7 medium, and other medium modifications involving replacing of chloride with nitrate or sulfate were studied. The charge stabilized (citrate) Ag ENPs were more unstable than the sterically stabilized ENPs and replacement of chloride ions and use of high ionic strength media cannot be recommended [144]. Although these studies provide important information on the agglomeration behavior of Ag NPs in media relevant for regulatory testing, they only cover one type of ENP. Moreover, the modification of media raises questions regarding the adaptation of the animals to this media, despite the lack of mortality in the controls.

As discussed in Chapter 4.3, the study presented in Paper II [2] employed media that are physiologically relevant and that have been used in standard tests with crustaceans. The influence of suspension stability of Ag, ZnO, and TiO_2 ENPs was investigated towards acute toxicity of *D. magna* (Figure 10, 11, 12). The stable and unstable conditions to VS EPA medium with pH adjusted to 7 and M7 medium (unadjusted pH 8.6), respectively, selected from the stability study presented in Figure 7.

Despite being sterically stabilized, test suspensions of Ag NPs in VS EPA and M7 media were largely agglomerated, especially at lower concentrations (Figure 10, A1, B1). *D. magna* immobilization was higher in VS EPA media with a 48-h EC50 value of 51 µg L^{-1} than M7 medium with a 48-h EC50 value of 66 µg L^{-1}. This was due to an increase in dissolution in VS EPA medium, which could in itself be attributed to media composition and the lower pH. Here dissolution was related to concentration rather than size, where higher degree of dissolution was measured at higher concentrations than lower concentrations. Dissolution is a dynamic process that can be

influenced by surface chemistry (surface chemistry, crystallinity, and exposed plane), external factors (exposure media and storage conditions), size and surface area effects (agglomeration/ aggregation, shape, surface area and composition) [66]. An increase of Ag ENP dissolution has been reported with a decrease in pH [133], and sterically stabilized Ag ENPs dissolve more in acidic conditions than in water [145]. In the case of sterically stabilized ENPs, it seems that the dissolution, rather than the size distribution will govern the toxicity.

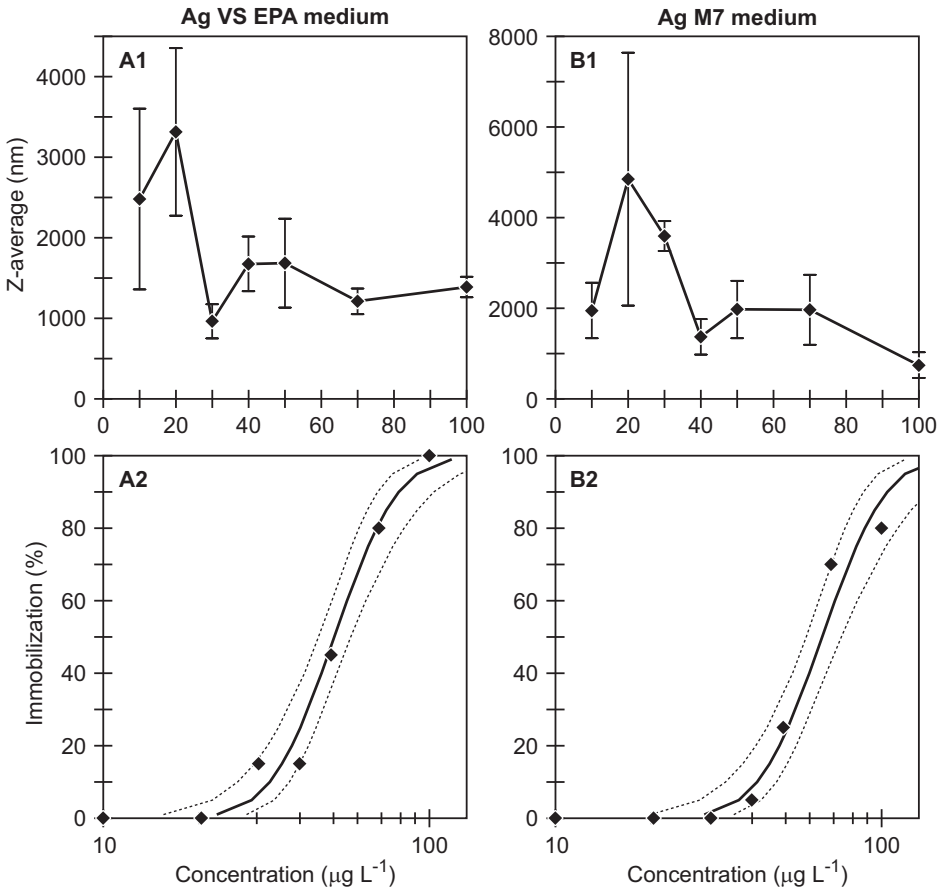

Figure 10. Zeta average size of Ag ENPs in VS EPA and M7 media as a function of test concentration (A1 and B1, respectively) and corresponding 48-h immobilization concentration-response curves for *Daphnia magna* neonates (A2 and B2, respectively). Please note the different scales on secondary axes (Paper II) [2].

For ZnO ENPs, a higher degree of agglomeration was seen at the highest and lowest concentrations, and high instability and PDI at concentrations ≤ 1 mg L^{-1} (Figure 11, A1, B1). This could be due to dissolution, which increased with a decrease in concentration, and was significantly higher in VS EPA medium. This higher amount of dissolution in VS EPA medium is in agreement with other studies where increase in pH caused decrease in

dissolution [146]. The increase in particle size distribution observed at the lower concentrations may be due to Ostwald ripening where the high amount of dissolved Zn^{2+} species are able to deposit back onto the surface of ENPs. The larger particle sizes seen at the highest concentration (20 mg L^{-1}), could be explained by particle interactions; due to increased probability of collision between the particles [147], thus decreasing the surface area and consequently dissolution [148].

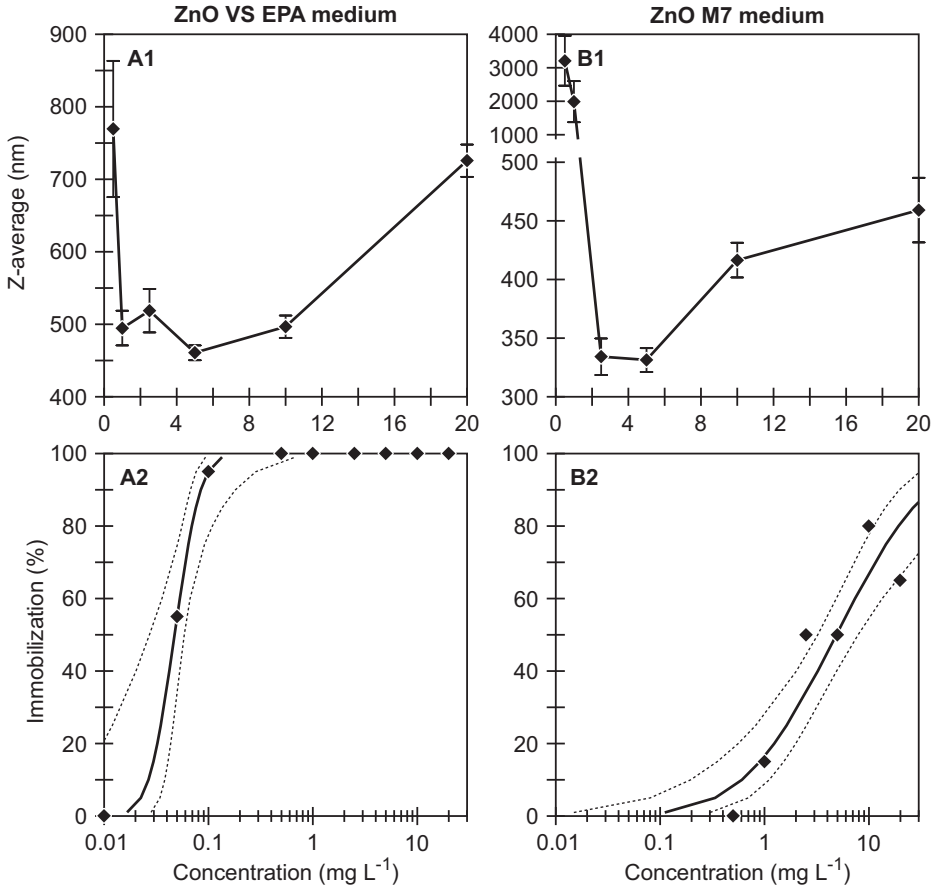

Figure 11. Zeta average size of ZnO ENPs in VS EPA and M7 media as a function of test concentration (A1 and B1, respectively) and corresponding 48-h immobilization concentration-response curves for *Daphnia magna* neonates (A2 and B2, respectively). Please note the different scales on secondary axes (Paper II) [2].

Due to the process of dissolution, significantly higher toxicity was observed for ZnO ENPs in VS EPA medium at pH 7, 48-h EC50 value of 0.047 mg L^{-1} than in M7 medium at pH 8.6, 48-h EC50 value of 4.9 mg L^{-1}; as toxicity of ZnO ENPs has been mainly attributed to the presence of zinc ions [70,73,149]. Low toxicity response was observed, despite the high dissolution at the lower concentrations in M7 medium. This response could be explained

by the high ionic strength, as increased Ca^{2+} concentrations (2.5 mM) has shown to have a protective effect on the uptake of Zn in *D. magna* [150].

Toxicity of ZnO ENPs on *D. magna* has earlier been tested using both synthetic media of different ionic strength and natural waters. A 48-h EC50 value around 1 mg Zn L^{-1} was reported, however, z-average of ENPs was >1000 nm [76]. ZnO ENPs tested in six different river waters had EC50 values in a range of 2.1 mg L^{-1} to 11.2 mg L^{-1} ZnO, (1.7 mg Zn L^{-1} to 9 mg Zn L^{-1}) [73]. High ionic strength media has shown different toxicity results varying from 0.67 mg L^{-1} ZnO (0.54 mg Zn L^{-1}) [151] to 22 ± 12.2 mg L^{-1} ZnO [79]. Toxicity of ZnO ENPs was investigated in different media compositions, but significant aggregation was reported due to high ionic strength (EPA medium hard water, EPA hard water, and ISO daphnia medium) [152]. The lowest toxicity was found in the EPA hard water medium, despite ISO medium having the highest ionic strength. This emphasizes the importance of considering not only the ionic strength but also the media composition when defining media that facilitates toxicity testing stable ENP suspensions. In Paper II [2], media composition did not affect the size distribution of ZnO ENPs significantly, but rather dissolution governed the toxicity outcome.

For TiO_2 ENPs, small agglomerate sizes (~200 nm) and stable suspensions were achieved in VS EPA medium, whereas in M7 medium large agglomerates (>1000 nm) were formed and settled to the bottom of the beaker during testing (Figure 12, A1, B1). Regardless of the pH, the increase in ionic strength has been shown to lead to higher aggregation [153]. TiO_2 NPs revealed a scattered concentration-response relationship in the *D. magna* acute immobilization test (Figure 12, A2), though the toxicity higher in VS EPA medium with 48-h EC50 value of 14 mg L^{-1} compared to 48-h EC50 >100 mg L^{-1} in M7 medium. This result has great importance as it shows that size distribution has a direct effect on toxicity while some studies have referred to TiO_2 as inert and non-toxic ENP [139,154]. Most studies have not tested TiO_2 NP suspensions in stable conditions where agglomerate sizes are kept in the “nano” range.

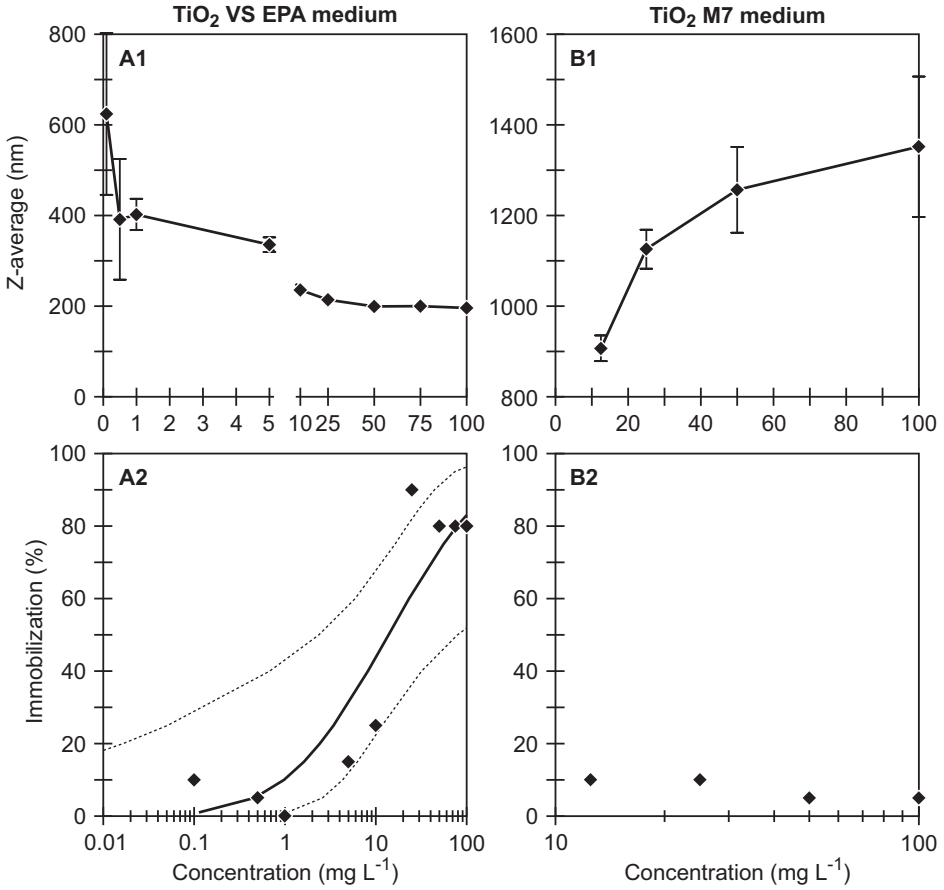

Figure 12. Zeta average size of TiO_2 ENPs in VS EPA and M7 media as a function of test concentration (A1 and B1, respectively) and corresponding 48-h immobilization concentration-response curves for *Daphnia magna* neonates (A2 and B2, respectively). Please note the different scales on secondary axes (Paper II) [2].

In the literature search conducted to date, TiO_2 ENPs have revealed different toxicity responses toward *D. magna* acute toxicity, depending on the type of particle and the test setup. 48-h EC50 values of 7.75 mg L^{-1} have been reported in COMBO media [155]; and 5 mg L^{-1} (ppm) in EPA in moderately hard reconstituted water (MHRW), where the TiO_2 ENP suspensions were filtered [156]. Increasing the exposure time to 72 and 96 hours increased toxicity towards *D. magna* [157-159], although this response might be linked to stress due to lack of nutrients, and amplified by the presence of TiO_2 ENPs, rather than the effect of ENPs. This discrepancy in EC50 values reported for TiO_2 might be due to testing of unstable suspensions and not due to large intra-species sensitivity as assumed by Gottschalck et al. [160].

6 Test condition implications in high-throughput testing

Increasing numbers of ENMs in production warrant testing for toxicological outcomes. Currently, there is a need for *in vitro* assays that screen and assess the (eco)toxicity of the growing number of ENMs. There are many advantages to *in vitro* toxicological testing and it includes the ethical considerations in reducing the amount of animals, lower cost and fast testing [161] and reproducible results [162]. Since there is a wide range of ENMs being produced and used, it is very challenging to perform toxicological testing on every specific material. According to the 'Toxicity Testing in the 21st Century: A Vision and a Strategy', screening methods should be developed to transition from descriptive animal testing, to quantitative and mechanistic toxicity testing using high-throughput approaches [163]. Another similar approach to this strategy has been presented by Nel et al. (2013) [164] to use *in vitro* high-throughput screening to make predictions on toxicology of ENMs *in vivo*. These alternative toxicity methods will give rise to rapid screening and ranking of nanomaterials based on toxicity, determine mechanisms and modes of action, assist in designing *in vivo* testing, and correlating nanoparticle properties with the respective effects [165].

Some of the most commonly studied toxicological endpoints to ENP exposure involve cytotoxicity, effects on growth and reproduction, and mortality. However, increasing amount of evidence indicates that ENPs can induce genotoxicity to various species [91]. A variety of endpoints are used to assess genotoxicity of substances, which apply to human and eco-genotoxicity. In the current tiered process of assessing the genotoxic effect of a substance/particle, the initial step is the *in vitro* screening for genotoxicity. If these tests provide a positive result, then *in vivo* testing (often the micronucleus and DNA repair assay) is conducted to investigate the full genotoxic potential of a substance [97].

Inflammation is another very commonly studied endpoint in nano-toxicology, though it is a complex mechanism that involves multiple cell types. While it is not feasible to measure such a response *in vitro*, it is possible to measure markers of proinflammatory signaling and gene expression that could lead to translation and production of proteins [161]. Similar to *in vivo* tests, *in vitro* testing also shares concerns about dosing, effects on cells, and feasibility and validity towards using them for ENPs [97].

6.1 Testing considerations on photo-genotoxicity high-throughput method

While TiO_2 are some of the most studied ENPs in toxicological studies, investigation of photo-genotoxic effects in a high-throughput manner is an area to be explored. From a scientific and regulatory standpoint, it is important to investigate if current assays are adapt to screen and detect potential genotoxicity of ENPs under different test conditions, such as presence of UV light. To date, there are several gaps in investigating genotoxic effects of ENPs including the lack of standardized methods, nominal dose considerations, and influence of co-exposed agents [91]. Several methods for detecting genotoxicity are used by the scientific community, including Ames test, comet assay, and *umu* assay. However, Ames test might not be feasible for testing NMs that cannot cross cell wall [97], and the comet assay is labor intensive. Moreover, interferences of ENMs with comet assay have been previously presented and discussed, which raise questions about the applicability in nano-genotoxicity [130]. Among these methods, the *umu* assay has the potential to be used for high-throughput screening of ENPs due to 96-well plate test design, standardized test procedure, and cost-efficiency. The *umu* genotoxicity assay measures the ability of chemicals/particles to induce *umu* gene expression in *Salmonella typhimurium* (TA1535/pSK1002). This strain contains *umuC-lacZ* fused gene, which is part of the SOS pathway, and gets induced in response to genotoxic compounds. Gene induction is estimated by β-galactosidase activity of the fusion gene. The kit used to test complied with ISO 13829 guideline on genotoxicity determination of water and wastewater [166].

High-throughput testing for measuring genotoxicity is relevant for both ecotoxicity and human health; however, this chapter will focus on the latter. Under UV illumination, TiO_2 NPs have shown increased toxic response towards freshwater organisms (daphnids) due to production of ROS [167,168]. Presence of ROS has been shown to play a role in genotoxic effects of particles, and its production may derive from various particle specific properties including surface type, shape and crystallinity [169]. Photocatalytic P25 exposed to either visible or UVA light was able to cause DNA lesions that were not present in the dark [170]. The question of organism exposure to UV rises when designing phototoxicity studies, as part of the toxic response could be contributed to light exposure (Paper IV)[4].

The genotoxic and photo-genotoxic effect of four different crystalline-structure TiO_2 ENPs was evaluated by using the *umu* assay. The degradation of methylene blue as a scavenger for ROS produced in the presence of illuminated TiO_2 ENPs under full spectrum UV light revealed a maximum response at 60 minutes. The maximum ROS production occurred anatase-rutile TiO_2 (NM-105) due to its structure. The initial genotoxicity tests were carried out using three TiO_2 ENPs of different crystalline structure (NM-105, NM-104, NM-103) with 60 min exposure to UV light. The induction ratio (IR), which is a measure for genotoxicity, was high (>1.5 fold induction). It could be hypothesized that ROS from photocatalytic activity could induce this response. However, the validity criterion for growth was not met due to damage from the UV to the control wells. Moreover, the two highest concentrations (333 µg mL^{-1} and 667 µg mL^{-1}) indicated cytotoxicity.

Based on these experiments and the fact that UV has DNA damaging properties [171], it became necessary to evaluate the damage of UV light alone on growth and genotoxicity of *S. typhimurium* used as a test species in the *umu* assay. As seen in Figure 13A, bacterial count number decreased with time, especially for exposures longer than 15 minutes. In order to evaluate the genotoxic effect of UV itself, and investigate if UV filters could lower this effect, *S. typhimurium* was exposed up to 60 minutes of UV in the absence of ENPs. The genotoxicity effect (Figure 13B) did not correspond with the count number/cytotoxicity data (Figure 13A). From Figure 13B it can be seen that UV exposures over 0.5 minutes caused a genotoxic effect on the cells. The use of mechanical filters WG-320 glass filter (blocking light <320 nm) and 50mM $CuSO_4$ generally lowered the UV damage/genotoxicity, however, the effect was still present at over 3 minutes of UV exposure.

Umu assay has previously been used to evaluate the effect of UV light on photo-genotoxicity of a mix of organic sunscreens and two crystalline-structure TiO_2 ENPs (anatase and anatase-rutile). At low concentrations, the presence of TiO_2 increased the induction ratio (IR). However, irradiation of the microplates induced β-galactosidase activity in the negative control wells of *S. typhimurium* strain [172]. Although IR was calculated by taking negative controls into account, any settings causing damage to these cells should be avoided as it compromises the validity of the test.

In the present study (Paper V) [5], light sources of full (UV) spectrum appear to cause genotoxic damage to the organism used in the umu assay, and are not recommended in future studies investigating photo-induced genotoxicity.

Similarly, the use of other light sources for this purpose should be followed by investigation of the effect of light on the bacterial strain *S. typhimurium*.

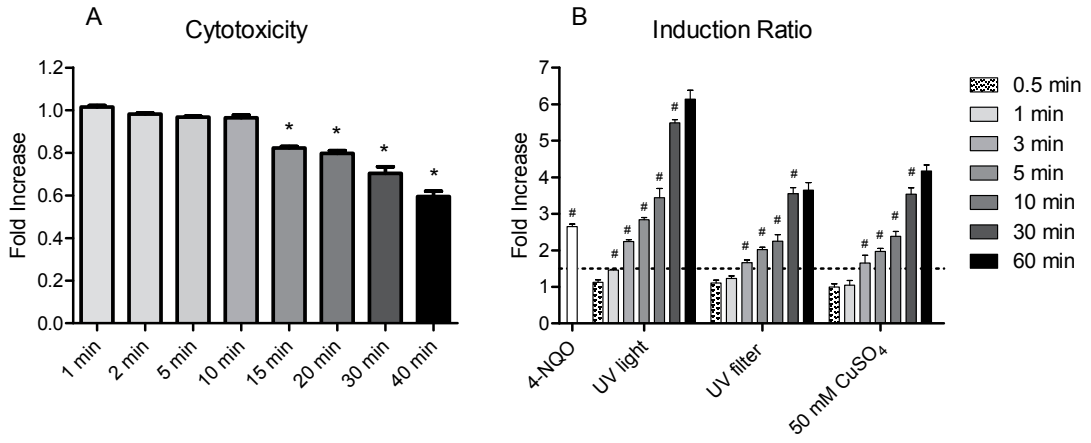

Figure 13. Effect of UV light on count number/cytotoxicity (A) and induction ratio (B) of *Salmonella typhimurium* TA1535/pSK1002 expressed as fold change over control. Graph A represents the decrease in bacterial density of Plate A after exposure to UV for 1, 2, 5, 10, 15, 20, 30, 40 minutes. Graph B represents a separate experiment as a response to UV light exposure in increments of 0.5, 1, 3, 5, 10, 30 and 60 minutes under UV irradiation, and with additional presence of WG-320 UV filter and 50 mM $CuSO_4$. N=4-8. Data was considered statistical significant when * $p \leq 0.05$ for the growth factor; and genotoxic when # $p < 0.05$, and the induction ratio ≥ 1.5 and growth factor ≥ 0.5 (Paper V) [5].

Other methodological issues in the *umu* assay included concentration considerations, not only due to the cytotoxicity previously seen at the two highest concentrations (333 µg mL^{-1} and 667 µg mL^{-1}), but also due to a shading effect from TiO_2 ENPs. This shading effect was seen to influence the growth factor calculations, and hence induction ratio and genotoxicity. This confounding factor was adjusted by taking into account the pre-incubatory readings of growth (Plate B).

The *umu* assay was used to test the genotoxicity of C_{60}, which showed a positive response [173]. However, this study used tetrahydrofuran (THF) in their dispersion method that has been subject to debate [104] (Paper III)[3] and did not account for the shading effect of these fullerenes, raising questions on the reliability of the method/study. Other similar assays using OD reading while employing ENPs should account for the shading effect, which in this case can be classified as a confounding factor. ENP interference with optical measurements has also been shown in other *in vitro* toxicity assays [174].

It has been pointed out that testing of genotoxicity of ENMs is a gray area where the best battery of tests for hazard identification have yet to be defined; and currently, the validity of OECD recognized genotoxicity assays is being questioned [175]. Since nano-toxicological studies need to identify and exclude potential artifacts in nano-genotoxicity, and critically validate and develop these test methods [161], the findings presented in Paper V [5] prove to be essential.

6.2 Testing considerations on inflammation high-throughput method

As discussed earlier in the specific properties of ENPs, metal oxides like TiO_2 and CeO_2 are able to cause ROS formation that can consequently lead to oxidative stress. Endpoints such as of oxidative stress and inflammation have been extensively used as toxicological endpoints for assessment of nanoparticles in *in vitro* studies [176-178]. Inflammation is usually measured by the presence or increase in the amount of major chemotactic factor Interleukin 8 (IL-8) [179] from release into the culture medium, and is more relevant for epithelial cells. The contents can be assessed by enzyme-linked immunosorbent assay (ELISA) [161].

Recent *in vitro* studies have shown that CeO_2 and TiO_2 ENPs can induce inflammation and oxidative stress in human bronchial epithelial cells [180,181]. Taking into consideration high-throughput screening, a 96-well (ELISA) was employed to screen and rank CeO_2 and TiO_2 ENPs for inflammation. ELISA uses antibodies and color change to identify a substance (in this case IL-8). In order to test the reliability of the assay and investigate any artifacts occurring from the presence of ENPs, an acellular study was conducted. Human recombinant IL-8 (150 pg/ml) was added to wells containing CeO_2 ENPs (8 nm) at concentrations 3.125-25 µg mL^{-1}. CeO_2 ENPs were suspended either in keratinocyte growth medium (KGM) or in KGM medium containing 0.1% DPPC + 10% FBS.

The amount of IL-8 cytokine was measured colorimetrically in both ENP dispersions (Figure 14). The IL-8 amount decreased in the presence of CeO_2 ENPs compared to IL-8 only in KGM media, which was the control. Though this decrease was not statistically significant due to high variation in the control group, the trend is very clear. This decrease was more pronounced for ENPs dissolved in KGM medium only at all concentrations. The wells containing ENPs dispersed in 0.1% DPPC + 10% FBS, measured higher

amounts of IL-8 in the supernatant at lower CeO_2 concentrations (6.25 and 3.125 μg mL^{-1}). Since no cells were present, the decrease in the amount of IL-8 in the supernatant can be attributed to the presence of ENPs, most likely due to the high surface area. This effect was greater for ENPs not coated in 0.1% DPPC + 10% FBS, indicating non-coated CeO_2 ENPs have higher affinity for IL-8.

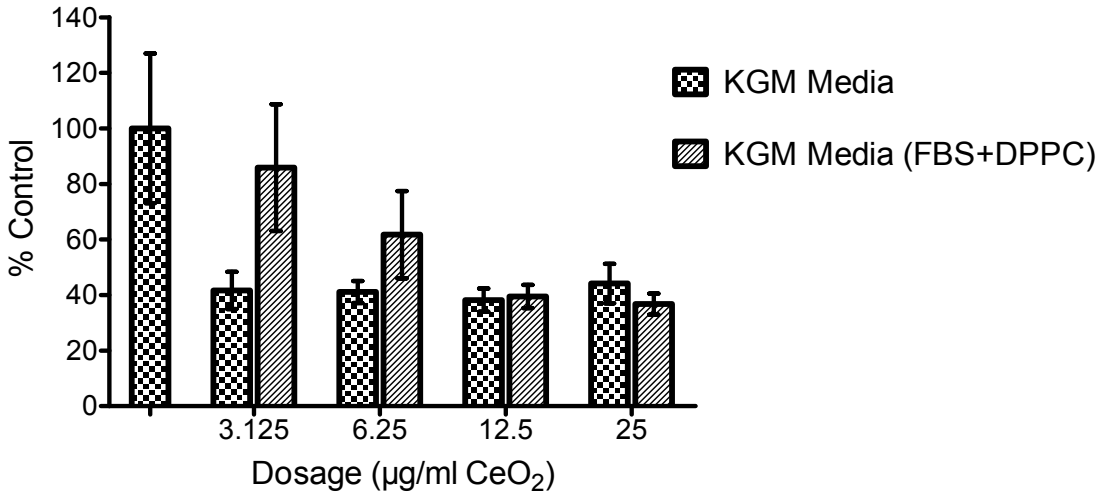

Figure 14. Interaction of NPs with IL-8 in a non-cellular assay. Various concentrations of CeO_2 Nano Amor (8 nm) NPs in the presence and absence of FBS + DPPC were added to KGM media containing human recombinant IL-8 at 150 pg/ml and incubated for 48 hours. After 48 hours the amount of free IL-8 was determined by an ELISA kit. Data represents means ± SEM for N=3 (Paper VI) [6].

To evaluate this response even further, lung epithelial cells (BEAS-2B) were exposed to four TiO_2 ENPs at concentrations of 6.35-100 μg/ml for a period of 4 hours. Since adsorption issue was raised from the acellular study, the first supernatant from the exposure was discarded and cells were washed and then incubated with fresh basal media for a period of 18 hours. The amount of IL-8 in the treated wells was lower than that in the controls (Figure 15). The IL-8 amount in the supernatant revealed a concentration gradient where higher amounts were measured for cells exposed to higher concentrations of TiO_2 NPs. However, the total amount of IL-8 was always significantly lower than the controls, indicating that cellular detection of IL-8 protein following nanoparticle exposure is compromised.

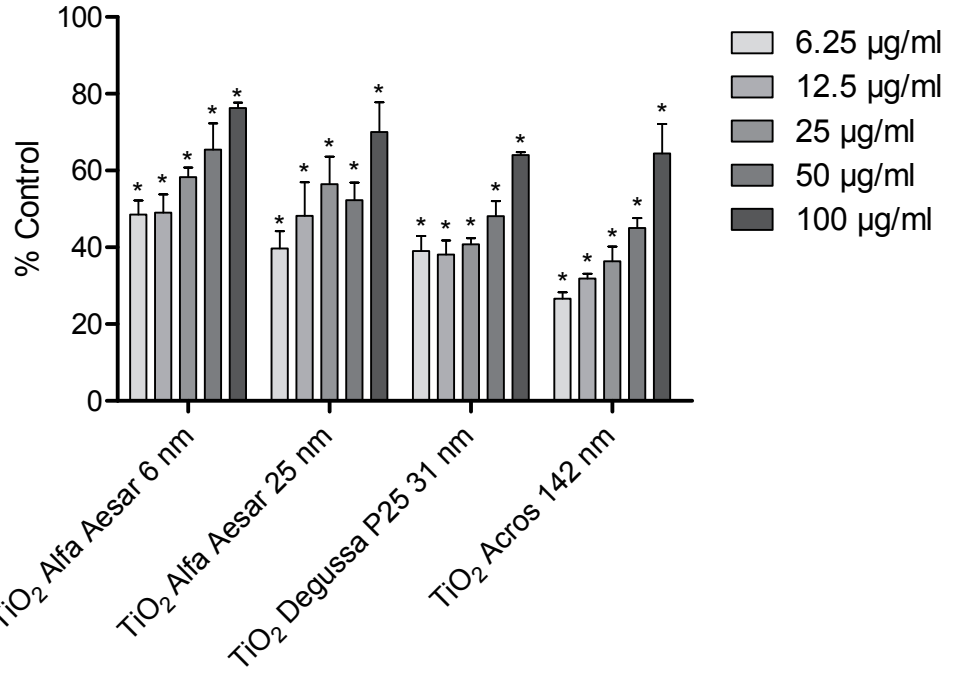

Figure 15. IL-8 protein expression of quiescent BEAS-2B cells after treatment with 4 TiO_2 nanoparticles at five different concentrations. Cells were grown to subconfluence before treatment. Cells were exposed for 4 hours and then the supernatant was discarded. Cells were washed with PBS, and fresh basal media was added to the cells for 18 hours prior to the start of the assay. An exposure time of 4 hours was chosen as RT-PCR data revealed a maximum gene induction around 6 hours. Data represents N=3 for treatments and N=6 for controls (Paper VI) [6].

Optical chemical and physical interferences have been reported for in vitro assays utilizing ENPs and discussed in Lee et al. (2015) [182]. Issues of adsorption of cytokines to ENPs have previously been discussed for metal oxide and carbon black ENPs [180,183,184]. Kroll et al. (2012) [174] investigated the adsorption capacity of 24 ENPs on IL-8 protein in a cell-free system. Only for one type of TiO_2 ENPs loss of IL-8 was significant. Similarly, TiO_2 and carbon black had high adsorption affinity, whereas SiO_2 and In_2O_3 ENPs exhibited low affinity [182]. This was not seen in the present study, as both CeO_2 and TiO_2 ENPs showed affinity for IL-8 protein. Similar to study in Paper VI [6], though to a larger extent, the adsorption affinity of ENPs decreased when ENPs were predispersed in FBS [182]. These discrepancies could be related to stock and test suspension preparation methods and the physico-chemical properties of the ENMs, as the adsorption was variable depending on the type of ENP. Generally, it can be concluded

that the tendency for proteins to adsorb to particle surfaces can lead to underestimation of cytokine production [98].

The results presented in Paper VI [6] indicate that the use of ELISA assays to measure cytokines such as IL-8, or any other endpoint protein could be influenced by artifacts caused by ENPs and is not recommended for use. This is a critical finding as it contributes to the development and improvement in vitro toxicity screening assays and indicates that measuring of expression at the protein level is not feasible for nano-toxicology studies. Gene expression methodologies such as RT-PCR would have to be employed to avoid test artifacts and measure endpoints such as inflammation and oxidative stress as successfully done in Paper VI [6].

7 Discussion

To date, many studies have investigated toxicity of ENPs using standard test guidelines. Since these guidelines were not developed specifically for ENPs, there are a lot of variables within the test system that can lead to discrepancies in toxicity results. As analyzed in Chapter 5, results from nano-ecotoxicological studies using the same chemical composition of a particle varied in toxicity from a few, to several hundred orders of magnitude. For the same particles, test conditions also seemed to affect the toxicity outcomes. These discrepancies raise questions whether these studies can lead to under- or overestimation of toxicity, and their applicability to the process of hazard identification.

One way to achieve reproducible data and harmonize test guidelines is to have stable test conditions. Keeping these factors in mind, *this thesis aimed to improve suspension stability and test reproducibility, as well as test 'nano-specific' properties*. As it has been recommended previously, when dealing with nanomaterials a case-by-case risk assessment should be considered [33], as there are many characteristics that can influence the potential hazard of ENPs [185]. Considering the large amount of ENPs, three ENPs were employed, varying in physico-chemical characteristics: sterically stabilized Ag ENPs and pristine ZnO ENPs that were expected to undergo only limited dissolution; and highly agglomerating TiO_2 ENPs.

More specifically, the aim of this thesis was achieved by giving recommendations on: 1) stock suspension preparation and dosimetry, as well as characterization of nanomaterials before and during testing; 2) change of media composition, or test conditions to improve nanoparticle test suspension stability and reproducibility; 3) provide methodological recommendations for specific test conditions and assays in nanotoxicology to avoid artifacts and improve testing procedure.

For nanomaterial testing, it is crucial to consider properties and behavior in water and media in order to determine the best testing practices that can lead to reproducible results. Some of the processes that affect the stability of ENPs were presented in Chapter 3. They include conditions that can affect the electric double layer, which governs the agglomeration and aggregation of ENPs. More specifically, electrostatic and steric forces, which can be modified by the presence of ions in the media, changes in pH values and presence of substances that create a layer around the ENPs, such as NOM.

In the most recent OECD expert meeting report, it is stated that the first step to such results underlies in preparing stable stock suspensions for comparability between test results and minimization of test artifacts [43]. The preparation of stock suspensions should have a high degree of monodispersity; though, when these stock suspensions are then transferred to testing media, the ionic strength and composition will influence agglomeration/aggregation [43]. Preparation of stable suspensions has been investigated in Papers I, II and III by developing tailored ENP dispersion protocols, based on:

- The identification of critical issues and parameters for stock dispersion preparation
- Stabilizing ENPs by development of a testing scheme that takes into account the influence of NOM and aging, not only in stock, but also in test suspensions
- Identifying a set of conditions by employing changes to ionic strength and pH that lead to stable suspensions for each of the employed ENPs.

While significance of the work presented in Papers I-VI in comparison to similar literature has been presented in Chapters 4-6, the discussion presented herein will focus more on the relevance of these findings in relation to OECD guidelines.

Stock suspension preparation

ENPs in a powdered form are not easily dispersed in aqueous media, as they exhibit agglomeration tendencies. Recommendations given by ECHA on nanomaterials, state that sample preparation is one of the most critical steps for successful (eco)toxicological testing of ENMs, which contains many variables [44]. These variables were examined in the five different protocols developed by EU and international projects. These often focussed on specific test types and/or certain ENPs, and their applicability to other ENP types is limited.

Paper III [3], presented more general recommendation for stock dispersion preparation that can be applied to many ENPs. It was concluded that the key parameters to be taken into consideration for include: nanomaterial properties, nanomaterial stock concentration, volume of dispersion medium, dispersion media/water quality, stabilizing/dispersing agents, pre-wetting of nanomaterial powders, temperature control, dispersion procedure (mechanical

and ultrasonication), maintaining stability prior to dosing, and performance or quality assurance. These parameters are essential in dispersion protocols for ENPs to produce stable and homogenous stock suspensions. As they are identified from individual scientific studies and from large-scale research projects and international organisations, they may serve as a guide to researchers, companies, and regulators in nano-(eco)toxicological testing.

The most recent OECD guideline on Ecotoxicity and Environmental Fate of Manufactured Nanomaterials [43] also suggests the investigation of particle behavior over time as an improvement to current test guidelines. It has previously been discussed that monitoring the mass concentration, particle size number and distribution needs to be monitored in order to better interpret the results [13]. Various considerations have been given to characterization requirements for stock suspensions, but less thought has been given to measuring the particle size distribution in the test suspensions [13]. Paper I [1] and Paper II [2] are one of the few studies that systematically investigated the stability of each individual ENP in stock and test suspensions, with an additional effect of aging, which shed light on the processes occurring during the test duration. This had implications in evaluating if the test conditions used are feasible for maintaining stable suspensions throughout the duration of the test.

Influence of SR-NOM

OECD 202 guideline recommends that the amount of total organic carbon in dilution water should be < 2 mg/l [37]. This amount has been proposed keeping in mind metal toxicity and might not necessarily be in quantities that allow successful stabilization of ENPs, especially at high concentrations. In Paper I [1], the presence of 20 mg L^{-1} SR-NOM in suspension revealed different results for Ag, ZnO and TiO_2 ENPs. SR-NOM did not have a stabilizing effect for ENPs that are sterically stabilized by a coating, as is the case with Ag, but stabilized ZnO in both stock and test suspensions. Agglomeration was seen in TiO_2 stock suspension II in the presence of SR-NOM in MilliQ water. This was due to the fact that the stock suspension was prepared at a high concentration (10x more than the highest test concentration). Addition of NOM in stock suspensions should consider NOM to ENP ratio to achieve enough steric repulsion to avoid agglomeration and/or aggregation. As seen before NOM stabilization effect is dependent on the concentration of NOM [64,108,114]. Moreover, addition of NOM in media will only stabilize suspensions when the ionic strength is below the CCC. This has also been seen previously, where NOM can stabilize ENPs

only for ionic strength values below CCC [47,113] and shifted the CCC to higher ionic strength [47]. Given these results, recommendations can be applied to other similar ENPs, where ENPs stabilized by capping agents will not be affected by NOM, so its use is not recommended in stock or test suspensions. In the case of highly agglomerating ENPs, appropriate amounts of NOM should be used to achieve stabilization, and addition of NOM is not recommended for media close to CCC. Additionally, the influence of aging on suspension stability should be evaluated in a case-by-case approach. Various studies have used NOM to improve the stability of ENPs test suspensions in ecotoxicology [47,60,61,107-109]. However, these have included mainly physico-chemical observations, and have not been fully relevant for ecotoxicological testing.

In relation to the stability effect on toxicity, similar toxicological responses were seen for Ag ENPs in freshly prepared and aged suspensions. The presence of NOM did not increase the stability of test suspension and caused an underestimation of toxicity which was seen both in Paper I [1] and in other literature [110,136]; which gives evidence for NOM not to be used in stabilizing and testing Ag ENPs. On the other hand, presence of SR-NOM contributed to stabilization of ZnO ENP test suspensions and might lead to an increase in standard test reproducibility.

Influence of ionic strength and pH

In the OECD *Guidance Manual for the Testing of Engineered Nanomaterials* it is also emphasized that conditions such as pH and ionic strength should be considered in testing nanomaterials, as they may affect both solubility and dispersibility; and where necessary, pH adjustments should be undertaken [41]. During acute toxicity testing in this thesis, questions arose as to whether specific conditions stated in standard guideline tests are adaptable for ENPs. The OECD 202 guideline on immobilization of *D. magna* states that pH conditions can be between 6-9 and that varying composition of dilution water can be used, but that an optimal water hardness is 140-250 mg/L $CaCO_3$ [37]. The ISO 6341 method for immobilization of *D. magna* provides more strict testing conditions: "The dilution water thus prepared shall have a pH of 7.8 ± 0.5, a hardness of (225 ± 50) mg/l (expressed as $CaCO_3$), a molar Ca/Mg ratio close to 4:1 and a dissolved oxygen concentration above 7 mg/l" [38]. When dispersed under such conditions (using M7 medium), the powdered ENPs (ZnO and TiO_2) agglomerated and precipitated to the bottom of the test vial during the 48-hour duration of the test (Paper I [1], Paper II [2]). Therefore, the regulatory relevance and reliability of results for ENPs (especially TiO_2)

in current guideline tests with *D. magna* would be very low. Taking all these parameters into considerations, it is particularly difficult to obtain stable suspensions and maintain compliance with OECD test guidelines, where both Ca^{2+} and Mg^{2+} are required in high amounts.

The later *Guidance on Sample Preparation and Dosimetry for the Safety Testing of Manufactured Nanomaterials* [42] states that test media parameters that can affect agglomeration/aggregation and should be taken into account include ionic strength, calcium concentration and hardness, DOC, alkalinity and dispersing agents. This guidance also points out that characterization has normally been limited to stock dispersions, rather than test dispersions in media; and that few studies have measured particle and agglomerate size distribution across a dilution series during exposure [42].

To investigate this further, media of different ionic strengths were employed to examine the size distribution and stability over a range of pH (Paper II) [2]. The optimal testing conditions, where the lowest possible agglomerate sizes could be achieved were investigated for the three ENPs. The optimal conditions revealed to be VS EPA media (which had the lowest ionic strength) at pH 7. This was due to the low media hardness, since it has been shown that divalent ions can cause more agglomeration than monovalent ions [47,60]. From these experiments, it was seen that dissolution, rather than size, governed the toxicity of sterically stabilized Ag ENPs, and of ZnO ENPs. Dissolution is an important mechanism that should be accounted for and measured in nano-ecotoxicology and ENPs.

Regarding ecotoxicity testing of ENPs, the most recent OECD expert meeting report [43] concluded that the most important information to be identified in the characterization of tested materials include dissolution and dispersability, agglomeration, and dispersion stability. This guideline also introduced the need for tests with aged ENMs, which was taken into account and implemented in Paper I [1]. Additionally, in this guideline it was also discussed that a standardization of medium used is recommended given the high variability of results with the current recommended media, and that the suspensions should be as monodisperse as possible. Dispersability and dissolution were pointed out to be key factors affecting test performance. Given the high diversity of ENMs, a material-by-material guidance was recommended [43]. These requirements were fulfilled in Paper I [1] and more so in Paper II [2], where smaller sized agglomerates were achieved in

suspension which revealed "nano-specific" properties, along with standardization of the VS EPA medium.

Stable suspensions of low-agglomerate sizes were achieved by taking into consideration the point of zero charge of Ag, ZnO and TiO_2 ENPs in media of different ionic strengths. A very crucial finding was that for TiO_2 ENPs, these smaller agglomerates were found to be more toxic than their larger agglomerates, despite some previous studies categorizing TiO_2 as non-toxic [139,154]. This shows that toxicity will depend on size distribution of ENPs. Therefore, it is recommended that measuring the point of zero charge in relevant test media prior to toxicity testing and identifying the optimal parameters such as pH, media composition, and ionic strength should be employed in standard guideline testing of ENPs.

Besides incorporating some of the recommendations given in the different OECD and ISO guidance documents, studies presented in Papers I, II, and III [1-3] presented work that provided additional information to the scientific community regarding the behavior and stability of ENPs in aquatic suspensions. This was done by identifying the influence of NOM addition, aging, media content and point of zero charge on suspension stability prior to testing. This could lead to more appropriate test conditions, with regards to reproducibility and reliability of results. While various peer-reviewed studies have investigated the effect of NOM, pH, and ionic strength on suspension stability, the studies presented here use ENPs of different chemical composition, structure and coating to systematically investigate ENP behavior and stability over time, while keeping in mind physiological relevance and coupling with ecotoxicological studies.

High-throughput screening

The third aim of this thesis was to provide methodological recommendations for specific test conditions and assays in nanotoxicology, to avoid artifacts and improve testing procedures. Similar to guidelines in ecotoxicology, assays used in toxicology have a set procedure/protocol. Processes affecting the behavior of ENPs in aquatic suspensions, also apply to nano-toxicology, although here, different test media are used. With the number of ENMs on the rise, and since nano-toxicological studies are time consuming, very costly, and might involve the use of animals, *in vitro* high-throughput methods have gained popularity among scientists and regulators. Since there are various advantages to *in vitro* testing, one of them being reproducible results [162],

such methods were employed to investigate the photo-genotoxicity and inflammation response of ENPs.

As discussed in Chapter 3.4, TiO_2 and CeO_2 ENPs possess photocatalytic and catalytic properties, respectively. These properties can lead to generation of ROS, which plays a role in genotoxic effects of particles [169], and is a precursor for oxidative stress, which when continuous, can lead to chronic inflammation [94]. Although the *umu* assay [166] has been used to assess the genotoxicity of chemicals in water and wastewater, only a few studies have used it to test ENMs [172,173]. However, these studies did not take any special provisions on the physico-chemical characteristics of ENPs, which can lead to confounding factors. In Paper V [5], the issues that were encountered included: the full spectrum UV light source caused cytotoxic and genotoxic damage to *S typhimurium* and this effect was not lowered by the use of UV filters; a shading effect from the presence of ENPs influenced the growth factor and induction ratio calculation, and hence, the genotoxicity outcome. These can be classified as confounding factors and make a very important finding, given that they were not considered by previous studies using the *umu* assay to identify genotoxicity of ENPs. In the case of use of other light sources, the *umu* assay needs to be accompanied with preliminary testing of effects of the light on the negative controls; and the shading effect of ENPs that could influence OD readings. Based on these considerations and the short lifetime of ROS, the employment of *umu* assay might not be sensitive enough to quantify the photo-genotoxicity of ENMs, therefore, other genotoxicity methods should be used.

The high-throughput method presented in Paper VI [6], also revealed that ENPs have specific properties which need to be accounted for during testing in order to minimize test artifacts. Using the IL-8 ELISA method for measuring the inflammation response of ENPs revealed high affinity for the large surface area ENPs for the IL-8 protein. Binding of IL-8 to ENPs occurred in both acellular and cellular assays, and was characterized as a confounding factor. Similar responses are expected for assays that require the measurement of proteins in the supernatant. A few previous studies have also indicated adsorption of cytokines to ENPs for metal oxide and carbon black ENPs [180,183,184,186]. However, these results were not consistent, and no recommendations for use of other methods were given. In Paper VI [6], recommendations given by ECHA were followed [44], where other toxicity tests (e.g. RT-PCR) accompanied the initial screening of in vitro ELISA assay. Papers V and VI [5,6] also highlighted the importance of testing

conditions on confounding factors and artifacts, and their influence on toxicity outcome.

While the work conducted as part of this PhD study may not fully provide the whole platform for testing ENPs, the presented results and recommendations provide: a foundation for understanding ENP behavior in aquatic suspensions, how to prepare stable stock and test suspensions and how test conditions, including confounding factors and artifacts, can affect toxicity. The research presented herein contributes to the environmental, and to a lesser extent, human health hazard identification of ENPs, by using harmonized and standardized reference methods. Achieving stable suspensions and reproducible test results through the hazard identification step, fulfils the criteria presented by the OECD WPMN where the different steps risk assessment of ENMs need to be internationally harmonized.

8 Conclusions and recommendations

In order to improve the feasibility of test guidelines towards ENPs, this thesis has critically analyzed the processes that ENPs undergo in aquatic suspension and methods to prepare stable test systems. It was concluded that processes like agglomeration/aggregation affect the stability and toxicity of ENPs. These can be controlled by modifications in media composition and pH, and addition of natural organic matter. NOM eliminated the toxicity of Ag ENPs and did not aid in their stability, however, increased the stability of ZnO ENPs. Monitoring of ENP behavior in media over time and through the process of aging, showed that stable suspensions are difficult to be maintained throughout the test period. Despite being a difficult process, it was possible to find a specific test media at a pH value where ENPs could be maintained at low-agglomerate sizes. This corresponded to VS EPA medium at pH 7, although it was ENP specific. TiO_2 ENPs revealed to be more toxic in low-agglomerate sizes. The process of dissolution revealed to be an important parameter that greatly affected toxicity, especially for ZnO ENPs, and was dependent on media composition and pH. Testing of ENPs under high-throughput *in vitro* methods revealed that certain test conditions can act as confounding factors, and influence test results. From the *umu* assay, it was seen that UV light caused genotoxic damage and presence of ENPs caused interference with readings. For the IL-8 ELISA, it was seen that ENPs had a binding affinity to test reagents in the absence and presence of cells.

One of the main findings from this thesis is that test conditions can affect the toxicity outcomes. In order to minimize variability and increase reproducibility, standard guideline tests used to test toxicity of ENPs should aim for stable stock and test suspensions. Test suspensions should be low in ionic strength, at the same time be physiologically relevant for the species used. Measurement of point of zero charge in media should be the first step investigating the pH value(s) where ENPs are most stable. Here, the lowest size distribution should be achieved so nano-sized effects can be investigated. If such conditions are not optimal, NOM should be added for steric stabilization. Use of NOM should consider the ENP to NOM ratio, and is not recommended when media reaches the CCC.

The results presented so far indicate that nano-(eco)toxicity testing is not so straight-forward. A case-by-case approach is recommended when ENPs have different chemical composition, coating, crystal structure and primary size.

However, some general methodological recommendations can be adopted for other ENPs under the OECD 202 guideline, and are presented in Figure 16.

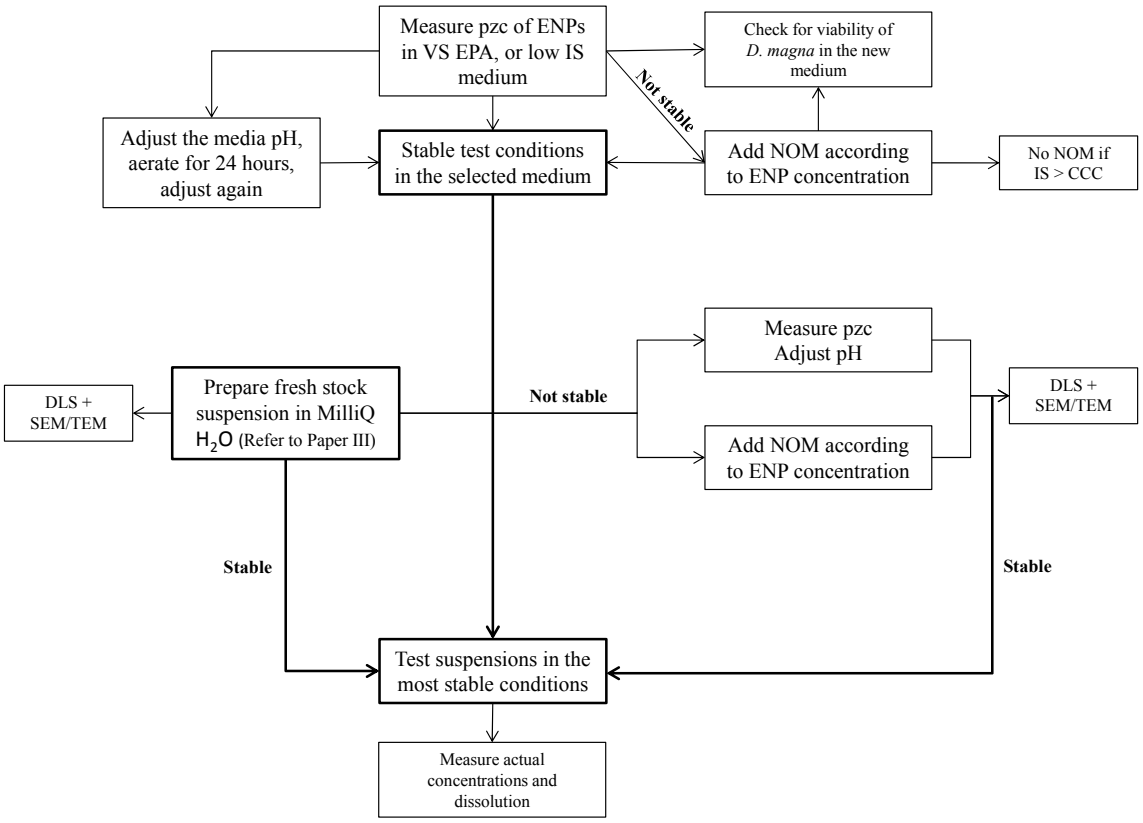

Figure 16. A stepwise approach to test ENPs under the OECD 202 guideline for acute immobilization of aquatic invertebrates. The conditions follow the guideline.

Despite these recommendations, more investigations are needed to elucidate on dynamics of ENM behavior in the different test media. ENMs that are difficult to disperse (e.g. those having hydrophobic properties) should receive special consideration. The ambiguity of the sources and types of NOM, and the different test media available, could pose an additional challenge in the process of harmonization. Therefore, employing test conditions presented in this thesis will provide additional data, which might lead to specifications on NOM type and test media in standard guidelines for acute toxicity testing.

Given the infancy of employing high-throughput *in vitro* testing for ENMs, there is a need for these assays that reflect on the physico-chemical properties and behavior of ENMs. Due to the potential interference of ENPs with the reagents/components of the assays, results should be validated, irrespective of the method requirements. Additionally, these assays should be combined with a battery of other assays or tests, to assess a specific endpoint. Lastly, it should be kept in mind that not all nanomaterials are created equal.

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10 Papers

I **Cupi D.**, Hartmann N.B., Baun A. (2015). The influence of natural organic matter and aging on suspension stability in guideline toxicity testing of silver, zinc oxide and titanium dioxide nanoparticles with *Daphnia magna*. *Environmental Toxicology and Chemistry* 34:497-506.

II **Cupi D.**, Hartmann N.B., Baun A. (2015). The influence of pH and media composition on suspension stability of silver, zinc oxide and titanium dioxide nanoparticles and immobilization of *Daphnia magna* under guideline testing conditions. *Submitted*.

III Hartmann N.B., Jensen K.A., Baun A., Rasmussen K., Rauscher H., Tantra R., **Cupi D.**, Gilliland D., Pianella F., Riego Sintes J.M. (2015). Techniques and protocols for dispersing nanoparticle powders in aqueous media – (what) are we ready to harmonize? *Submitted*.

IV **Cupi D.**, Sørensen S.N., Skjolding L.M., 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*

V **Cupi D.**, Baun A. (2015). Methodological considerations for using *umu* assay to assess photo-genotoxicity of engineered nanoparticles. *Submitted*.

VI **Cupi D.**, Baun A., Dreher K. (2015). Metal oxide nanomaterial alternative pulmonary toxicity assessment and mechanism of injury: Not all metal oxide nanomaterials are created equal. *Manuscript*.

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.

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