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**Skjolding, Lars Michael**

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Lars Michael Skjolding

DTU Environment
Department of Environmental Engineering

PhD Thesis
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Address: DTU Environment
Department of Environmental Engineering
Technical University of Denmark
Miljoevej, building 113
2800 Kgs. Lyngby
Denmark

Phone reception: +45 4525 1600
Fax: +45 4593 2850

Homepage: http://www.env.dtu.dk
E-mail: info@env.dtu.dk

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# Preface

This PhD thesis presents research in environmental effect and risk evaluation of engineered nanoparticles with a special focus on bioaccumulation. The study was undertaken between September 2012 and September 2015 at the Department of Environmental Engineering, Technical University of Denmark (DTU) under supervision of Professor Anders Baun. Co-supervison of the project was carried out by Associate Professor Henriette Selck at Department for Environmental, Social and Spatial Change (ENSPAC), Roskilde University (RUC).

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

**I** **Skjolding, L. M.**, Kern, K., Hjorth, R., Hartmann, N., Overgaard, S., Ma, G., Veinot, J.G.C., Baun, A., 2014a. Uptake and depuration of gold nanoparticles in *Daphnia magna*. Ecotoxicology 23, 1172-1183

**II** **Skjolding, L. M.**, Winther-Nielsen, M., Baun, A., 2014b. Trophic transfer of functionalized zinc oxide nanoparticles from crustaceans (*Daphnia magna*) to zebrafish (*Danio rerio*). Aquatic Toxicology 157, 101-108.

**III** Mackevica, A., **Skjolding, L. M.**, Gergs, A., Palmqvist, A., Baun, A., 2015. Chronic toxicity of silver nanoparticles to *Daphnia magna* under different feeding conditions. Aquatic Toxicology 161, 10-16

**IV** **Skjolding, L. M.**, Ašmonaitė, G., Jølck, R. I., Baun, A., Sturve, J., 2015, Uptake and localization of fluorescent labelled nanoparticles in living zebrafish (*Danio rerio*) using light sheet microscopy. (*Submitted*)

**V** Jensen, L. H. S., **Skjolding, L. M.**, Thit, A., Sørensen S. N., Købler, C., Mølhave, K., Baun, A., 2015, Not all that glitters is gold – an electron microscopy study on uptake of gold nanoparticles in *Daphnia magna*. (*Submitted*)

**VI** Thit, A., **Skjolding, L. M.**, Selck, H., Sturve, J., 2015, *In vitro* and *in vivo* effects of copper oxide nanoparticles and copper ions to Zebrafish (*Danio rerio*): Effects on Cells, Embryos and Fry. (*Manuscript*)

In this online version of the thesis, the papers **I-VI** are not included but can be obtained from electronic article databases e.g. via www.orbit.dtu.dk or on request from DTU Environment, Technical University of Denmark, Miljøvej, Building 113, 2800 Kgs. Lyngby, Denmark, info@env.dtu.dk.

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

Cupi, D., Sørensen, 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 Biopsysicochemical Processes in Environmental Systems, Vol. 4, Wiley-Interscience, Hoboken, NJ. (*In press*)

Hartmann, N. B., **Skjolding, L. M.**, Hansen, S. F., Kjølholt, J., Gottschalk, F., Baun, A., 2014, Environmental fate and behaviour of nanomaterials, new knowledge on important transformation processes, Environmental Project No. 1594, Danish Environmental Protection Agency, Copenhagen, Denmark

# Acknowledgements

A three years long journey has come to an end. On this journey I've met numerous of wonderful people who helped me with challenges I've faced along the way.

First I would like to thank my supervisor Professor Anders Baun, who always supported and inspired me even in the most troubled times.

Much gratitude goes to my co-supervisor Associate Professor Henriette Selck for inspiring scientific discussions and feedback.

Thanks to my fantastic colleagues here at DTU (Aiga, Biase, Lauge, Laura, Katrine, Nanna, Rune, Sara, Signe, Steffen, Stine, Susanne and many more) and collaborators at RUC (Amalie, Ronja and Stine). The times we shared at various conferences, courses and workshops made this journey an exciting and unforgettable time of my life.

I would like to thank Docent Joachim Sturve for giving me the opportunity to work in his laboratory during my external stay at University of Gothenburg. In this regard I would also thank Idella and Otto Mønsted Fonden for financial support during my research stay.

Finally, much gratitude goes to friends, Slænget, family and my love for their understanding and patience for my physical and mental absence at times.

"*I can feel I'm getting stronger, the longer, I'm pushed to the limit – said I'd do it someday, some day is now!*" - Scorpions, 2004

# Summary

Use of engineered nanoparticles (ENPs) (particles with a diameter of 1 to 100nm) is increasing. Engineered NPs are used in a wide variety of consumer product, industrial uses and remediation of pollutants. The increasing use is due to novel physical and chemical properties varying from that of their bulk forms.

With release of ENPs to the environment a need for evaluation of the potential risk of ENPs is necessary. Potential risks are assessed through a chemical safety assessment. Test guidelines (TGs) to evaluate the risk of compounds for the chemical safety assessment were developed for soluble chemicals. However, with fundamentally different chemical and physical properties of ENPs compared to soluble chemicals current TGs could be inadequate and possibly lead to wrong interpretation of results obtained.

One of the key issues is the dual action of ENPs consisting both of a chemical identity and a physical identity. For soluble chemicals the chemical identity has been the parameter controlling ecotoxicological endpoints (e.g. toxicity and bioaccumulation). However, with ENPs consisting of a wide range of particle sizes, coatings and functionalizations influencing the performance and result of test carried out the intrinsic properties of the ENP becomes critical in relation to endpoints assessed. Consequently, a central theme in this thesis is to increase the understanding of the intrinsic properties of ENP and how it influence bioaccumulation. Different particle sizes, coatings and functionalizations with different aquatic organisms were investigated. Furthermore, multiple microscopy methods were used to assess internationalization in the aquatic organisms. Finally, different exposure routes were used to determine if it could affect localization in the aquatic organisms.

The influence of different particle sizes, coatings and functionalizations were investigated using model ENPs (Au ENPs) with two different sizes (10 and 30nm) and coatings (citrate and mercaptoundecanoic acid (MUDA)) and a standardized test setup with a standardized test organism (*Daphnia magna*). It was found that while MUDA coated ENPs showed a clear trend of smaller ENPs taken up faster than larger ENPs contradictory findings was observed for the citrate coated ENPs showing similar uptake for both sizes. Consequently, both coating and size was found to affect bioaccumulation. Using differently functionalized ZnO ENPs (-OH and -Octyl functionalization) it

was found that large micron sized aggregates was also available for uptake in *D. magna* showing high uptake, possibly also associated with the carapace of the test organism. Functionalization with -Octyl increased the uptake compared to pristine ZnO ENPs while ZnO-OH ENPs showed no significant uptake compared to control. These results showed that larger size aggregates and functionalization could influence bioaccumulation potential. It should be highlighted that this type of interactions associated with the physical properties of ENPs and their influence on bioaccumulation is not accounted for in TGs.

Internalization of ENPs in the tested aquatic organisms was not identified through any of the microscopy techniques used. However, it was highlighted for proper interpretation of results multiple methods have to be used, and especially the need for element analysis was highlighted to identify artefacts and avoid misinterpretation of results. Furthermore, a general lack of understanding of internalization processes of ENPs after *in vivo* exposure was identified in the literature in regards to intrinsic properties of ENPs (e.g. particle sizes, coatings and functionalizations).

Exposure pathways were found to influence the localization of ENPs using light sheet microscopy. In zebrafish (*Danio rerio*) aqueous exposure to ENPs showed ENPs associated with gill, head region and gut whereas after dietary exposure ENPs were only found associated with the gut region. Consequently, ecotoxicological tests should be carried out for different exposure routes so possible effects are not overlooked due to the exposure route employed. However, it is not clear which pathway would be most relevant for testing with ENPs or if different pathways should be employed for different physical and chemical properties of ENPs.

Finally, it should be stressed that successful interpretation of all ecotoxicological tests with ENPs will ultimately rely on comprehensive characterization of the ENPs used, especially in relevant test media. This also underlines the immediate need for implementation of some level of physical characterization in TGs when testing ENPs.

# Dansk sammenfatning

Brugen af manipulerede nanopartikler (MNP'er) (partikeler med en diameter på 1-100 nm) er stigende i en bred vifte af forbruger produkter, til industriel brug, og til oprensning af forureninger. Den øgede brug er grundet forskellige fysiske og kemiske egenskaber sammenholdt med egenskaberne for bulk materialet.

I forbindelse med brugen af MNP'er vil udledninger til miljøet forekomme. Udledning af MNP'er vil nødvendigvis kræve en vurdering af risikoen for skadelige effekter på miljøet. En evaluering af sådanne skadelige effekter udføres med etablerede forsøgsretningslinjer (FRL'er) som er udviklet for opløste kemikalier, men ikke uopløste stoffer som MNP'er. De fundamentalt forskellige kemiske og fysiske egenskaber som MNP'er besidder i forhold til opløste kemikalier, kan medføre usikkerhed om hvorvidt de etablerede FRL'er er tilstrækkelige, og om hvorvidt de fundne resultater er retvisende for de skadelige effekter.

En af hovedårsagerne til denne usikkerhed ligger i den dobbelte virkning af MNP'er som både besidder en fysisk-og kemisk identitet. For opløste kemikalier har den kemiske identitet været styrende for resultaterne af økotoksikologiske forsøg (f.eks. toksicitet og bioakkumulering). De mange forskellige partikelstørrelser, overfladebehandlinger og funktionaliseringer af MNP'er kan have kritisk indflydelse på udfaldet og brugbarheden af økotoksikologiske forsøg. Derfor er kendskabet og forståelse af egenskaberne i MNP'er og deres indvirkning på bioakkumulering et centralt emne i denne afhandling. I denne afhandlingen er forskellige partikel størrelser, overfladebehandlinger og funktionaliseringer undersøgt for deres potentiale for at bioakkumulere i forskellige akvatiske organismer.

Derudover er der brugt forskellige mikroskopi teknikker til at kortlægge det cellulære optag af MNP'er i akvatiske organismer. Slutteligt er der anvendt forskellige eksponerings veje for at bestemme hvorvidt det kan påvirke placeringen af MNP'er i akvatiske organismer.

For at undersøge påvirkningen af forskellige partikelstørrelser, overfladebehandlinger og funktionaliseringer på bioakkumulering blev guld MNP'er med to forskellige størrelser (10 og 30 nm) og to overfladebehandlinger (citrat og mercaptoundecanoic syre (MUDA)) brugt i et standardiseret forsøgssystem

med standardiserede forsøgsorganismer (*Daphnia magna*). Resultatet var at små MUDA overfladebehandlede MNP'er blev optaget hurtigere end store MUDA overfladebehandlede MNP'er, modsat var resultaterne for citrat overfladebehandlede MNP'er som viste samme optag for begge størrelser. Altså blev det fundet at både overfladebehandling og størrelse havde en indflydelse på bioakkumuleringen. I et forsøg med forskellige funktionaliseringer af ZnO MNP'er (-OH og -Octyl funktionalisering) blev det fundet at aggregater i mikro-størrelse kunne resultere i et højt optag i *D. magna*, måske som følge af sorption til den ydre skald. Funktionalisering med -Octyl viste et øget optag i forhold til ubehandlede ZnO MNP'er, mens -OH funktionaliseringen ikke viste noget markant optag i forhold til kontrol organismerne. Resultaterne viser at også optag af større aggregater er muligt, samt at funktionaliseringen kan have en indvirkning på bioakkumulering. Med udgangspunkt i ovenstående skal det understreges at de fysiske egenskaber af MNP'er kan have indvirkning på bioakkumulering og at der i øjeblikket ikke er taget højde for dette i FRL'erne.

Der blev ikke observeret cellulært optag af MNP'er i forsøgene med akvatiske organismer og forskellige mikroskopi metoder. Det blev fundet at grundstof bestemmelse i forbindelse med mikroskopi observationer vil være nødvendige for at undgå fejlfortolkning. I litteraturen er der en generel mangelende forståelse af hvilke egenskaber for MNP'er (f.eks. partikelstørrelser, overfladebehandlinger og funktionaliseringer) der påvirker optagelsesmekanismer som følge af eksponering med MNP'er i levende organismer.

Med lys flade mikroskopi blev det vist at lokaliseringen af MNP'er i fisk var påvirket af eksponeringsvejen. Eksponering af zebrafisk (*Danio rerio*) via vandfasen viste MNP'er ved gæller, hoved-og tarm region hvor der ved eksponering gennem fødeindtag kun blev fundet MNP'er i tarm regionen. Følgeligt blev det sluttet at økotoksikologiske forsøg skal benytte forskellige eksponeringsveje, da forskellige effekter kan være forbundet med forskellige eksponeringsveje. Det er endnu ikke klarlagt hvilke eksponeringsveje der bør anvendes for forskellige fysiske eller kemiske egenskaber af MNP'er.

Slutteligt, blev det understreget at enhver fortolkning af økotoksikologiske forsøg vil kræve en fyldestgørende karakteriseringen af de benyttede MNP'er, specielt i relevante forsøgsmedier. Det fremhæves derved også at det er bydende nødvendigt at implementere en form for fysisk karakterisering i FRL'er når der udføres forsøg med MNP'er.

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# 1 Background and aims

Engineered Nanoparticles (ENPs) are generally defined as structures with all three dimensions in the range of 1-100 nm (for overview of definitions see Baalousha et al., 2014). With the decreasing size ENPs exhibit different chemical and physical properties than that of their bulk counterparts (Nel et al., 2006). Due to these properties ENPs are being used in an increasing number of products. This has raised concern for exposure to the environment following detrimental effects on both the environment but also human health (Nel et al., 2006; Klaine et al., 2008; Stone et al., 2010). In order to promote a sustainable product development a chemical safety assessment is necessary. For such assessment one must understand the fundamental properties that make ENPs different from soluble compounds for which current test guidelines for chemical safety assessment are valid (Peijnenburg et al., 2015).

A key issue for ecotoxicological tests is the huge diversity of ENPs which can consist of not only different core chemical composition but also a wide range of sizes, coatings and functionalizations which all influences the performance and result of the test carried out (Nel et al., 2006; Peijnenburg et al., 2015). Especially, when performing long term tests with the introduction of additional test parameters e.g. feeding and the challenge of keeping constant exposure concentration. Consequently, there is need for a basic understanding of the intrinsic properties of ENPs influencing their behavior in a given test setup and how these influences the endpoint assessed (Handy et al., 2012a; Handy et al., 2012b).

An endpoint which is proposed to have pivotal importance for chemical safety assessment of ENPs is bioaccumulation (SCENIHR, 2006; Baun et al., 2008; Handy et al., 2008a). While the concentration of ENPs in the environment is not expected to reach levels causing acute toxic effects for most ENPs (Boxall et al., 2007; Müller and Nowack, 2008; Blaser et al., 2008) concern have been raised about chronic toxicity (Nel et al., 2006), uptake and trophic transfer through the food chain (Holbrook et al., 2008; Zhu et al., 2010). Different functionalizations and coatings could enhance uptake due to properties which are already recognized to increase cellular uptake e.g. surface charge (Nel et al., 2006).

These findings have led to the following aim of this thesis:

To determine the influence of intrinsic properties of ENPs (size, shape, coating and functionalization) on the potential bioaccumulation in aquatic organisms.

In accordance the following research questions have been identified:

1) Do particle sizes, coatings and functionalizations affect uptake and depuration in aquatic organisms? (Paper I, II, III, VI)

2) Do the aquatic organisms internalize ENPs open exposure? (Paper IV, V)

3) Do exposure pathways affect the localization of ENPs in aquatic organisms? (Paper IV, V)

Given that intrinsic properties of ENPs (e.g. size, coating and functionalization) dynamically interact with biological endpoints (e.g. toxicity and bioaccumulation) addressing the above research questions becomes a complex challenge. Consequently, a need to minimize the variables is necessary. In this thesis gold nanoparticles (Au ENPs) was used as model ENPs for multiple reasons:

- Low toxicity enabling use for longer term biological studies.
- Chemically inert thus eliminating confounding factors of dissolution.
- Ease of synthesis of particles with multiple controllable sizes with identical coating, while also having the option for different coatings enabling testing of multiple variables independently.
- Low background concentrations thus limiting interference from natural and synthetic matrices.
- Ease of detection and low detection limit with e.g. Transmission Electron Microscopy (TEM) and chemical analysis.
- Possibility for attachment of fluorescent probes enabling microscopy techniques such as light sheet microscopy.

While the main focus of the thesis is on Au ENPs (paper I, VI, V), other ENPs (Paper II, III, VI) were also employed to exemplify some of the limitations and challenges of testing ENPs.

# 2 Nanotechnology and engineered nanoparticles

Nanotechnology is the tailoring and mastering of entities in the nanometer range. Generally, ENPs is defined as matter in the range of 1-100 nm which exhibit physical and chemical properties different from that of the bulk (for overview of definitions see Baalousha et al., 2014). These novel chemical and physical properties of ENPs are today used for tailoring of ENPs for specific purposes. In the following section some of the uses and releases to the environment will be presented. This is followed by a presentation of current guidelines available for chemical safety assessment and some of the challenges for those in relation to ENPs.

## 2.1 Use and environmental release of engineered nanoparticles in consumer products

Use of ENPs is increasing. In 2005 the Woodrow Wilson International Center for Scholars initiated a database to map the amount of consumer products on the market. Currently, 1824 entries are listed in the Woodrow Wilson database describing category, origin and nanomaterial (Woodrow Wilson International Center for Scholars, 2015). Additionally, a Danish database supported by the Ecological Council, DTU Environment and Danish Consumer Council, was launched in 2012 (The Nanodatabase, 2015). Both databases are based on producers claiming the use of nanotechnology in their product. The Danish database consists of 1459 entries, and has the advantage of an analysis section. The analysis section reveals that for the majority of the products (959) the identity of the ENPs used is not known. A similar trend is revealed by a categorization framework for characterization of the nature of ENPs in the product presented in the Woodrow Wilson database. In the categorization framework five categories are used;

- Category 1 describes products where ENPs have been described by e.g. research studies, patents or reports.
- Category 2 describes products where supporting information have been reported, but to lesser extent than category 1.
- Category 3 describes characterization done by the manufacturer, but without any official patent description of the product.

- Category 4 describes products where the manufacturer claim that nanotechnology is used in the product, but provides no characterization of description of the ENPs used.
- Category 5 describes products where sources, other than the manufacturer claim that the product contain nanotechnology.

Figure 1 shows the distribution of the different categories. The vast majority of products (71%) falls into category 4 (unsupported claim by manufacturer). Similar, in the Danish nanodatabase (66%) of the products enlisted contains an undefined source of ENPs.

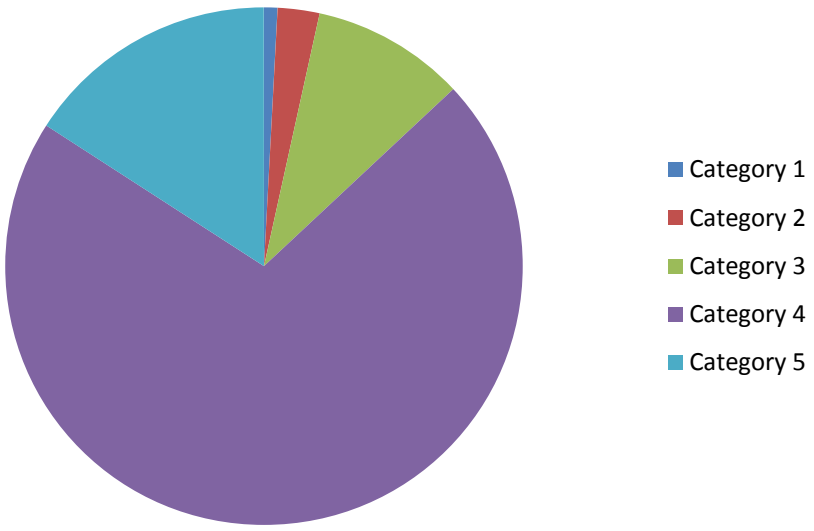


**Figure 1:** Distribution (%) of consumer products by category from the Woodrow Wilson database. Category 1 describes products where ENPs have been described by e.g. research studies, patents or reports. Category 2 describes products where supporting information have been reported, but to lesser extent than category 1. Category 3 describes characterization done by the manufacturer, but without any official patent description of the product. Category 4 describes products where the manufacturer claims that nanotechnology is used in the product, but provides no characterization of description of the ENPs used. Category 5 describes products where sources, other than the manufacturer claim that the product contain nanotechnology (Woodrow Wilson International Center for Scholars, 2015).

Considering the number of consumer products for which the identity of the ENPs employed is known the majority contain Silver (Ag) (42.8%), followed by CNTs (8.6%), phosphate (8.4%), $TiO_2$ (5.6%) and Au (5.4%). The remaining entries are limited to <5% per inventory.

According to the Danish nanodatabase the majority of the products containing Ag ENPs falls in to the category of Health and Fitness (56.1%), and

Home and Garden (18.7%). Within these categories the Ag ENPs is generally used due to its antimicrobial properties (Sharma et al., 2008; Sotiriou and Pratsinis, 2010; Hajipour et al., 2012). The Ag ENPs are e.g. attached or woven into fabric for clothing, applied as a film to products, or used in disinfection sprays (The Nanodatabase, 2015).

Studies on measurements of ENPs in the environment are almost non-existent possibly due to complex matrices, relative low release and lack of standardized robust methods for characterization. One of the few studies available is on release of $TiO_2$ nanoparticles from sunscreen into surface waters (Gondikas et al., 2014). An increase in Ti elemental ratios was observed during the summer period compared to spring. The increase was attributed to an increased amount of swimmers wearing sunscreen. Presence of clustered $TiO_2$ NPs in the suspended matter of the lake was confirmed using electron microscopy. The study underlines the possible direct release of ENPs to the environment. Another source of release to the environment could be through consumer products where the number of products containing ENPs, especially Ag ENPs, is increasing as outline previously. Multiple studies have assessed the potential release of Ag from fabrics which constitutes a large fraction of the category Health and Fitness presented earlier (Benn and Westerhoff, 2008; Geranio et al., 2009; Impellitteri et al., 2009; Benn et al., 2010; Kulthong et al., 2010). A great variation in terms of amount released and the form of release was observed. Benn and Westerhoff (2008) found that the release was primarily in the form of Ag ions. Geranio et al. (2009) observed a high degree of particles (>450 nm) when applying relevant washing conditions. Generally, it is expected that transformation processes will occur throughout the use and release phase. Impellitteri et al. (2009) showed that the use of bleach while washing would influence the speciation of the Ag present in textiles. Consequently, the release to environment would not only be in the form of the pristine ENPs but rather a mixture of different species and sizes.

ENPs are not only expected to be released to the environment from consumer products. ENPs have shown promising results in water treatment and degradation of pollutants thus possibly being intentionally discharged directly in the environment (Klimkova et al., 2010; Liga et al., 2011; Fei et al., 2008; Ilisz et al., 2003; Zhong et al., 2007; Crane and Scott, 2012). The most promising ENPs for remediation is nano-Zero Valent Iron (nZVI). nZVI have been proven highly effective for degradation/removal of e.g. azo dyes (Fan et al., 2009), chlorinated solvents (Lien and Zhang, 1999), polybrominated diphenyl

ethers (Li et al 2007), different transition and post transition metals (Klimkova et al., 2011; Li and Zhang, 2007). However, a fundamental understanding of the role and impacts of nZVI mobility, reactivity, fate and ecological effects is still to be fully established.

The above outlines the many uses, and the possibilities for ENPs to be a released to the environment. However, due to technical challenges to characterize and measure ENPs in the environment the expected release is not well documented. Due to these challenges probabilistic modelling have been used to estimate releases to evaluate possible scenarios for the environmental emissions of ENPs (Gottschalk et al., 2009; Gottschalk et al., 2010; Sun et al., 2014). In general, the estimations suggest that the concentrations will not reach acute toxic levels. However, there is still a risk for long term effects originating from bioaccumulation of ENPs or unforeseen endpoints e.g. behavioral (McNeil et al., 2014; Skjolding et al., 2015 – Paper IV; Thit et al., 2015 – Paper VI) or population level effects (Mackevica et al., 2015 – Paper III).

In summary, use and release to the environment have been identified both through intended and unintended release thus a need for chemical safety assessment of different ENPs is necessary.

In the following section will be presented some of the challenges associated with conducting chemical safety assessment of ENPs.

## 2.2 Current guidelines for testing of engineered nanoparticles

In this thesis focus will be on guidelines proposed by the Organization for Economic Cooperation and Development (OECD) which has a set of internationally-accepted test guidelines (TGs) for describing the release, fate, transport, transformation, exposure, and toxicity of chemical substances under controlled laboratory conditions. For the scope of this thesis the subset of TGs focusing on bioaccumulation in aquatic organisms is used. Assessing the potential bioaccumulation is an important part of chemical safety assessment and thus crucial for the sustainable development of ENPs.

TGs presently available for assessing bioaccumulation in aquatic organisms are TG305 Bioaccumulation in Fish: Aqueous and Dietary Exposure (OECD, 2012) and TG315 Bioaccumulation in Sediment-dwelling Benthic Oligochaetes (OECD, 2008). The main focus in this thesis is on pelagic aquatic

organisms, thus TG305 is the most relevant of the currently available TGs. It should be stressed that the range of difficulties presented below are not solely in relation to bioaccumulation testing, but rather in relation to testing ENPs in an aquatic setup.

In January 2013 the OECD Working Party on Manufactured Nanomaterials hosted an expert meeting on the applicability of OECD TGs to ENPs (findings summarized in Kühnel and Nickel, 2014). While it was recognized that the majority of the TGs could be used for testing of ENPs there was raised concern in regards to e.g. sample preparation, dispersion, analysis, dosimetry and characterization. Several other publications have also suggested regulatory testing assays to be "generally applicable to ENPs" (Handy et al., 2012a; Handy et al., 2012b; Kühnel and Nickel, 2014; OECD, 2014). However, additional concern has been raised due to the exposure of test organisms to particles rather than solely dissolved chemicals which were the intended use of the OECD TGs.

ENPs can undergo a range of processes, including changes in size, chemical composition, or state as described earlier. These processes depend on the intrinsic properties of the ENPs, coating, functionalization and media composition. Such dynamic changes results in key challenges for exposure-response estimations. Alternative dose metrics have been suggested e.g. surface area, particle number, or body burden (Petersen et al., 2015). However, this type of metrics is not considered in the current chemical safety assessment framework. Other factors like dissolution during exposure are expected for partly soluble compounds e.g. Ag or Zn ENPs (Liu and Hurt 2010; Ma et al., 2013), thus resulting in a combined exposure of ENPs and ions. Such combined exposure calls for a need to define both the dissolved fraction and the particulate fraction to determine which part exerts the observed effect (Palmqvist et al., 2015).

Bioaccumulation of soluble organic compounds and metal ions is fairly well understood and modelled through partition coefficients, chemical speciation and bioavailability modelling. These models are often associated with an "equilibrium" between multiple phases. Care should be taken that bioaccumulation of ENPs is not confused with the existing definition for hydrophobic organic contaminants (HOC) or dissolved metals as described by previous models (Handy et al., 2012b). Bioaccumulation of HOC is related to the passage of biological membranes through passive diffusion, or active uptake through ion channels or carrier mediated transport (Sijm et al., 2007). While

these transport mechanisms would correctly describe the uptake of the ionized fraction, studies show that this type of transport is rarely observed for the uptake of ENPs (Petersen et al., 2015).

A more dynamic behavior and interaction with the test system is to be expected for ENPs compared to soluble compounds thus hampering reproducibility and accuracy of OECD TGs. Media with high ionic strength will cause agglomeration and sedimentation of ENPs (Klaine et al., 2008). In many TGs (including 305) it is stated that:" *The concentration of the test substance in the chambers is maintained within ± 20% of the main of the measured values during the uptake phase*" (OECD, 2012). With processes including dissolution, sedimentation, adsorption and uptake by the test organism this requirement will be difficult to comply with for ENPs. A concentration gradient would be established through sedimentation yielding higher concentration at the bottom of the test vessel compared to the upper layers. This gradient can cause increased exposure of organisms situated at the bottom of the test vessel rather than the above water column.

The above interactions show how the guidelines face a range of challenges resulting from the instability of ENPs. In the following section will be described some of the processes governing this instability and how those, result in changes in the potential bioaccumulation of ENPs.

# 3 Behavior of engineered nanoparticles in aquatic test systems

Behavior of nanoparticles in the environment, but also in test systems is crucial for determining the fate, transport, uptake and toxic properties of ENPs. In the previous section it was identified that transformation in terms of dissolution, particle size, coating and functionalization could influence the behavior and fate of ENPs. In following section some of the key governing processes controlling the behavior of ENPs will be presented.

## 3.1 Processes affecting nanoparticle stability

Size is an inherent property for ENPs, and also crucial in the definition of those. Consequently, it is clear that determination of size and how size changes with time is paramount.

ENPs will generally move according to Brownian motion, which refers to the random movement caused by collision with atoms or molecules in the matrix surrounding them e.g. liquid or gas. The movement is affected by shear and gravitational forces and generally by the laws of diffusion (Handy et al., 2008c).

ENPs inherently have a charge based on the ions attracted to the outer surface as a result of chemical interactions. The charge is generally described by the Electric Double Layer (EDL) theory. The EDL theory describes two layers; The Stern layer and the diffuse layer. The Stern layer is present closest to the ENP, it consist of electrostatically bound ions. At the end of the Stern layer is situated the diffuse layer consisting of mobile ions repulsed by the Stern layer enabling a diffusive interchange of ions with the bulk solution. The EDL will thus create a repulsive force between two surfaces with the same charge (Baalousha et al., 2009). Another force which can cause repulsion is steric forces from e.g. coatings or natural organic matter (NOM). Furthermore, other forces such as bridging, osmotic, hydrophobic Lewis acid-base and magnetic forces could also cause repulsion dependent on the given environment. Opposed to the repulsive forces of the EDL are the attractive van der Waals forces. The van der Waals forces are caused by the electrodynamic attraction originating from the dipole moments between two entities (e.g. ENPs) (Baalousha et al., 2009). The van der Waals forces generally only occur at a relative short distance (<10 nm) compared to that of the EDL. The stability of a suspension is thus defined as the net result of the attractive van der Waals

forces and the repulsive EDL forces and steric forces (Baalousha et al., 2009).

A parameter which can be used to measure the stability of a suspension is the zeta-potential. The zeta-potential is measured at the point between the Stern layer and the diffuse layer, called the slipping plane (Handy et al., 2008c). A stable dispersion is generally defined as a dispersion having a zeta-potential with a numerical value of >30 mV. The range of -30 mV to +30 mV is thus described as an unstable dispersion with gradually further instability when approaching 0 mV. Such unstable dispersion will aggregate or agglomerate due to the decreasing repulsion and increasing attraction by the van der Waals forces (Handy et al., 2008c).

Aggregation is generally defined as the fusion of ENPs and not possible to be separated whereas agglomeration is the formation of loosely bound formations of ENPs which can be separated. While it can be difficult to distinguish the two in practice the terms are often used interchangeably. Throughout this thesis aggregation will be used to describe both processes unless the description specifically relates to agglomeration.

The aggregation kinetics is mainly explained by the Derjaguin, Landau, Verwey and Overbeek (DLVO) theory describing the interactions between the attractive van der Waals forces and repulsive EDL forces. While DLVO theory does not account for interactions with steric forces, the extended DLVO theory includes these (Hotze et al., 2013). However, there is still a range of parameters that affect aggregation kinetics which are generally not covered by DLVO theory such as size, shape, coatings and functionalization (Hotze et al., 2013).

## 3.2 Nanoparticle stability determines the dose

Quantifying the administered dose of a compound is crucial in understanding the outcome of ecotoxicological tests. Given the dynamic behavior of ENPs it is clear that maintaining the administered concentration can be difficult (Petersen et al., 2015). Even monitoring the dose has proven to be difficult when testing ENPs, especially at environmentally relevant conditions. Some of the difficulties reside on the issue of determining the different fractions administered e.g. dissolved or particulates (Louie et al., 2014). Furthermore, the high surface area to volume and rapid Brownian motion attributed to the small size makes ENPs very susceptible to attachment to surfaces. Those surfaces could be either collision with similar ENPs termed homoaggregation or collision

with other particles termed heteroaggregation (Baalousha et al., 2009). Both processes will eventually increase the size of the ENPs thus affecting sedimentation, uptake by organisms and toxicity. It is clear that sedimentation of the ENPs would naturally interfere with the concentration of the test setup as stated in section 2.3 “Current guidelines for testing of nanoparticles”.

Previously, it was highlighted that the exposure of ENPs and the dissolution of those could be problematic. While $TiO_2$ ENPs appear to be almost insoluble, ENPs of Ag, ZnO and Cu have been shown to undergo dissolution (Bian et al., 2011; Mudunkotuwa et al., 2012, Kent and Vikesland, 2012, Li et al., 2012; Thit et al., 2015 – Paper IV). However, since the scope of this thesis is on the importance of size, shape, coating and functionalization of a model particle in regards to bioaccumulation, the toxicity of ENPs vs metal ions and dissolution of ENPs will not be discussed further herein.

Since the unique properties of ENPs are exerted at the nano-scale, stable ENPs are preferable for the commercial use of ENPs. Such stability can be achieved through steric or electrostatic stabilization increasing the repulsive forces of the ENPs as described in previous section. Consequently, most ENPs used in consumer products are designed with a coating. The coating can consist of a great variety of organic compounds which provides an electrostatic or steric stabilization. Examples of low molecular weight organic molecules used for stabilization are the citrate coating of Ag NPs (Mackevica et al., 2014 - Paper III) or the MUDA coating of Au NPs (Skjolding et al., 2014a - Paper I). These types of coatings can create and maintain uniform ENPs with a narrow size distribution in MilliQ as shown in figure 2 for citrate and MUDA coated Au NPs (Skjolding et al., 2014a – Paper I). However, when introducing the ENPs into a high ionic strength test medium the stability greatly decreases due to the suppression of the EDL (Lee and Ranville, 2012; Park et al., 2014; Park et al., 2015). Table 1 shows how nominal sized Au NPs of respectively 10 and 30 nm with two different coatings increase in size over 24 hours in Elendt M7 media (Skjolding et al., 2014a – Paper I). At time 0 two peaks are observed for 10 nm citrate and MUDA coated Au NPs, one having a hydrodynamic diameter of 14±4 nm and 20±5 nm respectively corresponding approximately to the nominal size observed using TEM (Figure 2). However, a second peak of 112±47 nm for 10 nm citrate coated and 142±53 nm for 10 nm MUDA coated Au NPs was also observed. This clearly illustrates the rapid aggregation that can occur after addition to test media even with a coating. After 24 hours no second peak was observed for 10 nm MUDA coated Au NPs, while a fraction of smaller Au NPs was still observed

in the 10 nm citrate coated Au NPs. The increasing size was also in accordance with the measured zeta-potential that in all cases showed a numerical value below 30 mV (Table 1) (Skjolding et al., 2014a – Paper I).

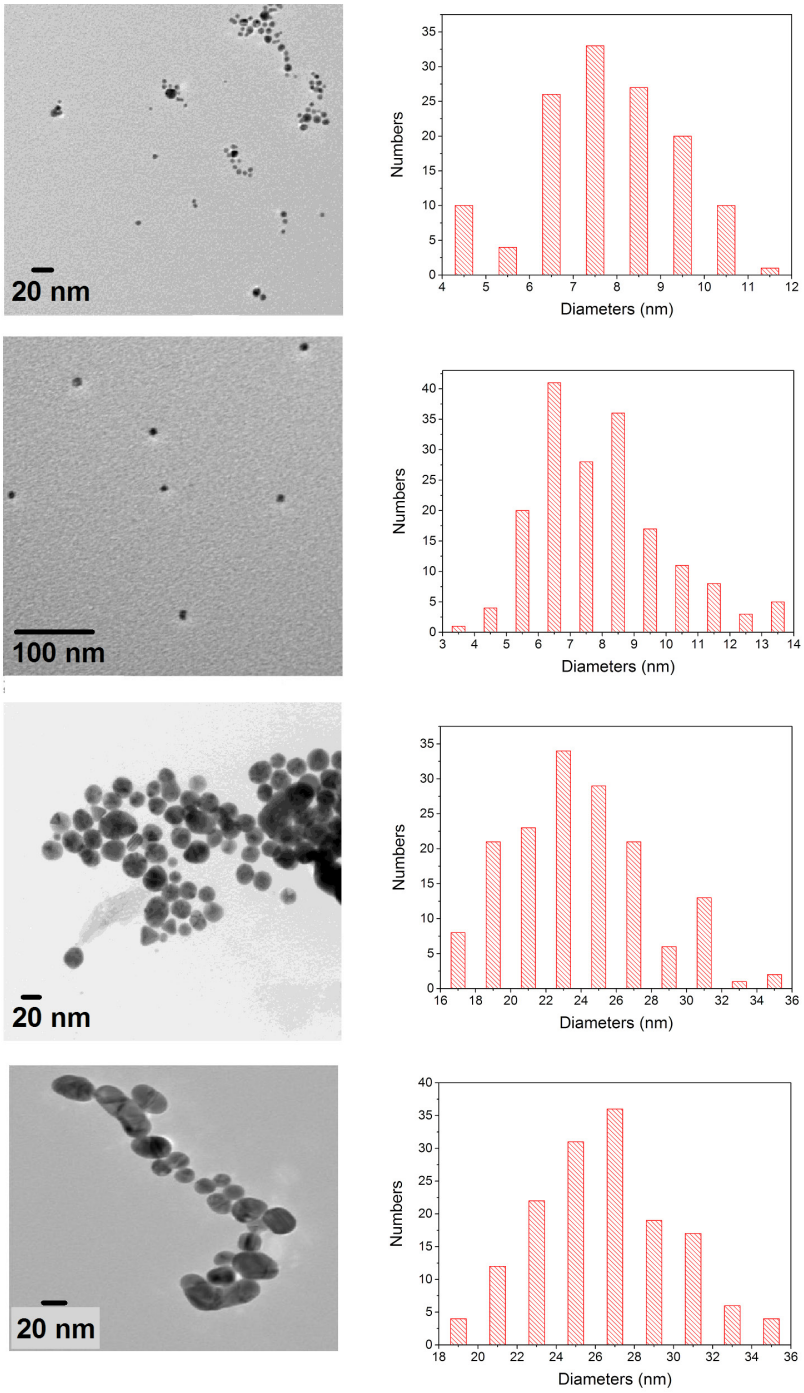


**Figure 2:** TEM images and statistical size distribution of Au NP in MilliQ water from top CIT 10 nm Au NP (d = 7.5±3 nm), MUDA 10 nm Au NP (d = 8.0±3 nm), CIT 30 nm Au NP (d = 23.0±9 nm), and MUDA 30 nm Au NP (d = 27.0±6 nm) (MUDA: mercaptoundecanoic acid, CIT: citrate) (Skjolding et al., 2014a – Paper I).

**Table 1:** Size peaks recorded (Percentage of particles in this range) and zeta-potential of Au NP in Elendt M7 after 0 and 24 hours measured by dynamic light scattering and transformation to volume-based distribution (mean ± standard deviation; n=3) (Skjolding et al., 2014a – Paper I).

| Test compound | Size Peak 1 [nm] | | Size Peak 2 [nm] | | Zeta-potential [mV] | |
|---|---|---|---|---|---|---|
| | t = 0 | t = 24h | t = 0 | t = 24h | t = 0 | t = 24h |
| MUDA 10 nm Au NP | 20±5 (71%) | 229±60 (100%) | 142±53 (29%) | N/A | -14±7 | -16±5 |
| MUDA 30 nm Au NP | 109±42 (82%) | 279±53 (100%) | 23±5 (18%) | N/A | -15±9 | -13±6 |
| Citrate 10 nm Au NP | 14±4 (91%) | 188±48 (60%) | 112±47 (9%) | 20±4 (40%) | -14±8 | -14±6 |
| Citrate 30 nm Au NP | 225±61 (100%) | 328±61 (100%) | N/A | N/A | -14±9 | -16±6 |

*mercaptoundecanoic acid. N/A: No applicable data.

Similar instability of citrate coated Au NP after addition to different types of synthetic media and natural waters has been reported (Lee and Ranville, 2012; Liu et al., 2012; Park et al., 2014; Park et al., 2015). In cases where synthetic media was used the increase in size was accounted to the suppression of the EDL due to an increase in ionic strength compared to MilliQ water. Park et al. (2014) observed a decrease in particle number concentration in three out of four tested synthetic media over 24h, only the lowest ionic strength media did not show a decrease in particle number concentration (Park et al., 2014). Lee and Ranville (2012) also observed a decrease in Au concentration by 45% to 75% correlating with different initial concentration after 24h of incubation in hard water (0.02 nM to 0.08 nM, lowest initial concentration corresponded with the largest decrease) (Lee and Ranville, 2012). Due to the method used, it was not possible to determine the governing process of the decreasing concentration. In a study by Skjolding et al. (2014a – Paper I) using a similar test system, a marked decrease in water phase concentration of four different Au NPs over 72 hours in Elendt M7 (figure 3) was found (Skjolding et al., 2014a – Paper I).

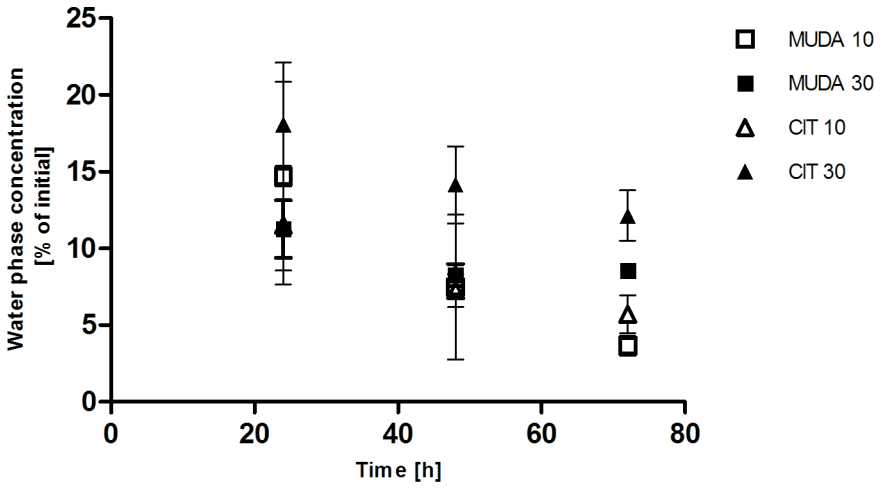


**Figure 3:** Water phase concentration of four different Au NPs (Initial concentration 0.4 mg Au/L) after 72h of incubation in Elendt M7 media. The number annotation after the abbreviation is the nominal size in nm. MUDA is Au NPs coated with mercaptoundecanoic acid, CIT is Au NPs coated with citrate (Skjolding et al., 2014a – Paper I).

For 30 nm citrate coated Au ENPs a more careful investigation of the fate of the Au ENPs were carried out. The mass was split into four fractions: sorption to test organisms, uptake by test organism (*D. magna*), adsorbed to test vessel and remaining in the water phase. It was found that sorption to the test organisms were negligible. The remaining fractions contained 38±2.4% in the test organisms, 32±2.9% adsorbed to the test vessel and 30±4.7% remaining in the water phase (Skjolding et al., 2014a – Paper I). Consequently, more processes than just sedimentation mediated by aggregation will partition in lowering the concentration in the aqueous phase.

While coatings with small macromolecules yielded stable suspensions in MilliQ water and media with relative low ionic strength, aggregation was still marked when introduced to synthetic media like Elendt M7. Larger synthetic macromolecules can be used to create highly stable ENPs e.g. Poly(ethylene glycol) (PEG) coated Au NPs (Skjolding et al., 2015 – Paper IV). These PEG coated Au NPs have been shown to be stable with very limited aggregation even in high ionic strength media. Furthermore, PEG coated Au NPs can be functionalized to exert certain behavior which possibly could increase their use (Farokhzad et al., 2004; Jolck et al., 2015). However, functionalization does not always correspond with stability. Different functionalization of ZnO ENPs showed marked aggregation resulting in micron-sized aggregates and

visible sedimentation after 24h of incubation with Elendt M7 (Skjolding et al., 2014b – Paper II).

The above section shows how different size, coating and functionalization can affect the behavior of ENPs in relation to both dynamic sizes changes related to aggregation but also changes in the nominal concentrations thus ultimately playing a crucial role for interpretation of findings.

# 4 Bioaccumulation of engineered nanoparticles in aquatic organisms

Uptake of ENPs into a wide range of different organisms has been reported in the literature (see appendix). Furthermore, it has been proposed that the drivers of uptake among others could be related to:

- Concentration of ENPs. Generally, a higher concentration of ENPs could drive a higher uptake and resulting increase in body burden.
- Exposure matrix. Reactions (e.g. dissolution) and transformations (e.g. binding of protein or humic acid) influence the form, fate and potential for mechanisms of uptake of ENPs but also exposure route.
- The properties of the ENPs. These include size, coating, functionalization, chemical composition and surface charge.
- The test organism. For example biological traits (e.g. feeding behavior) and physiology.

In the following sections specific focus will be on *D. magna* (4.3 and 4.4) and *D. rerio* (4.5 and 4.6) and the impact of the above mentioned drivers on uptake.

## 4.1 Bioaccumulation of engineered nanoparticles – a brief overview

In 2010 Stone et al. (2010) published a comprehensive review of literature dealing with environmental effects of ENPs (Stone et al., 2010). The review concluded that "only few studies have dealt with bioaccumulation of metal nanoparticles". Since then the body of available literature on bioaccumulation of ENPs has increased markedly. In 2013 Hou et al. (2013) reviewed 65 papers on the biological accumulation of ENPs consisting of studies conducted in water, soil or sediment. The most frequently used test species in water were identified as daphnia (n = 21) and fish (n = 27). A linear correlation was fitted to the datasets for daphnia and fish respectively, based on the concentration in the organisms and the concentration in the water phase. A good fit was generally found for both daphnia and fish ($R^2_{daphnia} = 0.99$, $R^2_{fish} = 0.85$). While the fit is good, the underlying mechanisms and the general nature of the data are not well presented by this method. As stated earlier there is currently no standardized method on reporting end points for ENPs e.g. wet or

dry body burden, or for example a measure like Bioconcentration Factor (BCF) as commonly used for HOCs. Furthermore, it was emphasized in the review that for the ease of comparison only nominal concentrations were used which due to processes of sedimentation, adsorption or uptake by organism would grossly overestimate the concentration of the aqueous phase exemplified in figure 3 (Skjolding et al., 2014a – Paper I; Thit et al., 2015 – Paper VI) and also illustrated in several entries in the overview table of the literature from 2013-2015 (see appendix, column "ENPs & Properties"). Hou et al. (2013) also underlined this drawback and highlighted it as a key parameter which should be considered in future studies.

The body of literature reviewed by Hou et al. (2013) clearly showed that daphnia had a greater potential for uptake compared to that of e.g. fish by ~2 orders of magnitude. The effect of higher uptake by daphnia was generally attributed to the unique feeding behavior by filtration of the surrounding water. Furthermore, it should be recognized that e.g. *D. magna* retains particles in the size range of 0.4 - 40 µm (Gophen and Geller, 1984, Geller and Müller, 1981), thus actively retaining larger aggregates of ENPs e.g. micron sized aggregates of ZnO ENPs (Skjolding et al., 2014b – Paper II). Another issue that was not addressed in the review was what parameters were driving the differences in uptake. While the review tried to use a modified version of the BCF for comparison it also stated that the premises for BCF was generally not fulfilled, as also described earlier in this thesis. Finally, the review concluded that the dataset presented generally lacked consistency in data, and that more data would be necessary to develop nano-QSARs (Quantitative Structure-Activity Relationship) in order to predict bioaccumulation of ENPs. More recent studies have focused on including the limitations of the early studies (<2012) with regards to characterization of the ENPs and monitoring the behavioral changes in terms of sedimentation, dissolution, aggregation, adsorption.

To create an overview of the more recent literature (2013-2015) regarding bioaccumulation a search in Web of Science was performed using the search phrase: (topic) bioaccumulation AND (topic) nano* AND (year published) 2013-2015, yielding 279 hits. The search was manually refined to 88 relevant scientific papers spread across aquatic (48 papers), sediment (12 papers) and terrestrial organisms (28 papers). For the scope of this thesis the 48 papers from aquatic organisms were split into three subcategories aquatic invertebrates and protozoa (34 papers), aquatic vertebrates (16 papers) and algae (5 papers), the accumulated number of papers exceeds the total number of pa-

pers found in the literature search since more organisms or ENPs were tested in some papers. The subcategory concerning uptake in algae was not included in this part. A complete overview of the literature and main findings was summarized in the appendix.

In general a similar trend as observed by Hou et al. (2013) was found in the literature from 2013-2015. Most studied species were still daphnia (17 papers) and fish (14 papers) for aquatic invertebrates and protozoa, and aquatic vertebrates respectively. With regards to endpoints reported body burden was directly reported or possible to extrapolate in the majority (90%) of the studies reviewed. However, the discrepancy between wet and dry weight reported still remains an issue as also highlighted in the review by Hou et al. (2013). In regards to characterization of the used ENPs the majority (81%) of the studies reviewed reported some information on state of aggregation, dissolution, or decrease in test concentration over time but rarely in a comprehensive manner (see appendix, column "ENPs & Properties").

In section 3.2 it was shown how critical comprehensive characterization both initially and during test is for right interpretation. However, the above overview illustrates that while some characterization parameters are reported in order to elucidate the confounding factors present in studies they are still not present in a comprehensive form in all studies which hampers general comparison.

## 4.2 Internalization of engineered nanoparticles

For internalization of ENPs a number of potential mechanisms exist. In the case of metal ions the transport is through carrier-mediated metal ions transporters (Petris, 2004; Hogstrand, 2011). However, for the scope of this thesis the focus is on internalization of non-dissolving ENPs. The main focus will be on Au ENPs as those are very electron dense and have a very low background concentration in nature thus uptake would solely be through added ENPs. Apart from the carrier mediated metal ion transporters there are various other specific and unspecific uptake mechanisms where uptake of ENPs is possible. Endocytosis describes an invagination of the plasma membrane surrounding a cell to form a vesicle (Ivanov, 2008). The vesicle is filled according to different mechanisms and allows different sizes of particles and material to enter the cell. Three major types of endocytosis have been proposed for internalization of ENPs; Clathrin-mediated endocytosis, caveloar-mediated endocytosis and micropinocytosis (Ivanov, 2008).

- Clathrin-mediated endocytosis describes a formation of a protein (clathrin) coated pit lined with receptors and the internalization occurs following a specific ligand-receptor interaction. Furthermore, the pathway is also governed by a size dependent uptake of ~120 nm particles (Petros and DeSimone 2010). Consequently, this type of endocytosis is rather specific both in mode of activation and size dependency. However, this type of endocytosis has been proposed for internalization of ENPs e.g. lysosomal uptake of Ag ENPs in oyster (*Crassostrea virginica*) (Ringwood et al., 2010).
- Caveloar-mediated endocytosis is often associated with the uptake of glycolypid signaling proteins or cholesterol trafficking (Vassilieva et al., 2008). The uptake is through interactions with caveloae creating invaginations with a size specificity of ~60 nm (Petros and DeSimone, 2010).
- Macropinocytosis or "cell drinking" is a nonspecific pathway with a size specificity of <1 μm. Consequnetly, also larger aggregates of ENPs could be proposed to be taken up through this pathway. Furthermore, the content of the vesicle is directly discharged in the cytosol (Ivanov, 2008) thus whole ENPs could reach sites normally not associated with this type of entities leading to possible toxic mechanisms induced by the ENPs (Moore, 2006)

Internalization *in vitro* has been described in literature and the above pathways are proposed to be associated with this type of uptake. Rapid uptake of surface modified Au ENPs (primary size 18 nm) in part by clathrin-mediated endocytosis was observed in human leukemia cells (Connor et al., 2005) and cancer cell lines (Chithrani et al., 2006). Furthermore, the use of Au ENPs for drug delivery have been widely recognized and used to study cellular uptake mechanisms (Johnston et al., 2010b).

## 4.3 Influence of size, coating and functionalization on the uptake and depuration of engineered nanoparticles by filter feeders

Filter feeders are important species in the aquatic food web compromising the role of a primary consumer feeding on algae and bacteria and as food source for fish. The literature overview showed that one species of filter feeders was more frequently used namely *D. magna* (see appendix). *D. magna* is considered to be a relevant test organism due to feeding traits, general behavioral habits and placement in the food chain (Baun et al., 2008). As mentioned earlier *D. magna* filters water to catch particles (mainly algae) in the size range 0.4–40 µm (Geller and Müller, 1981; Gophen and Geller, 1984). Due to agglomeration of ENPs in freshwater it is therefore likely that ENPs agglomerates will be ingested. This is in accordance with a number of studies with Daphnia spp. and different types of ENPs e.g. Lovern et al. (2008), Petersen et al. (2009a), Zhu et al. (2010) and Hu et al. (2012) (For overview of uptake of ENPs in recent literature (2013-2015) consult appendix). However, smaller ENPs than those falling in the range of 0.4-40 µm can possibly be taken up through the intake of water for e.g. digestion (Gillis et al., 2005) or aeration of eggs in the brood pouch (Rosenkranz et al., 2009). Additionally, adult *D. magna* with a size around 2-3 mm filtrates ~400 mL water each day, thus contact with the surrounding water is huge compared to the size of the organism (McMahon and Rigler, 1965). Uptake through diet has also been observed suggesting uptake via multiple exposure routes (McTeer et al., 2014; Pakrashi et al., 2014; Lee and An, 2015; Lee et al., 2015).

While studies using dietary exposure have been carried out as shown above the vast majority of studies on daphnia have included exposure through the aqueous phase. While effects on uptake was identified for different sizes and shapes (Chitrani et al., 2006), and surface chemistry (Hauck et al., 2007; Alkilany et al., 2009) *in vitro*, few *in vivo* studies have described those effects in aquatic organisms (Holbrook et al., 2008; Rosenkranz et al., 2009; Lewinski et al., 2010; Zhu et al., 2010; Hull et al., 2011; Glenn et al., 2012). Also, the effect of both size and coating has rarely been combined in a systematic study.

The effect on uptake of two sizes and different coatings of Au ENPs in *D. magna* showed difference in uptake rates and maximum body burden after incubation at 0.5 mg Au/L for 24h (figure 4) (Skjolding et al., 2014a – Paper I). No food was administered during the course of the experiment to eliminate

the confounding factor of adhesion of Au ENPs to algae (Hartmann et al., 2012) which would enable dietary uptake. A rapid uptake was observed during the first hours of the exposure for all Au ENPs, which was reflected by the modelled uptake rates (table 2). The order of uptake was 10 nm MUDA coated Au ENPs > 10 and 30 nm citrate coated Au ENPS > 30 nm MUDA coated Au ENPs. Consequently, the influence of size was not straight forward as both citrate coated Au ENPs were taken up at similar rates, whereas uptake of MUDA coated Au ENPs was higher for the smaller sized Au ENPs. Similar findings were reported for citrate coated Au ENPs with nominal sizes ranging from 6-30 nm in *D. magna* (Ranville et al., 2012; Khan et al., 2014; Wray and Klaine, 2015). In accordance with findings by Skjolding et al (2014a – Paper I), the smallest Au ENPs were taken up faster than the larger Au ENPs. No significant difference in uptake mechanisms of 10 and 30 nm Au ENPs could be due to fast initial aggregation rendering them in the same size range (table 2). The uptake rates found by Wray and Klaine (2015) was in the order: 6 nm Au ENPs (5.14±0.39 L/g organism/d) > 20 and 30 nm spheres (2.77±0.25 and 2.68±0.12 L/g organism/d, respectively). The uptake rate for the 20-30 nm spheres are of the same order of magnitude as found in the study by Skjolding et al. (2014a – Paper I) for citrate coated Au ENPs (table 2).

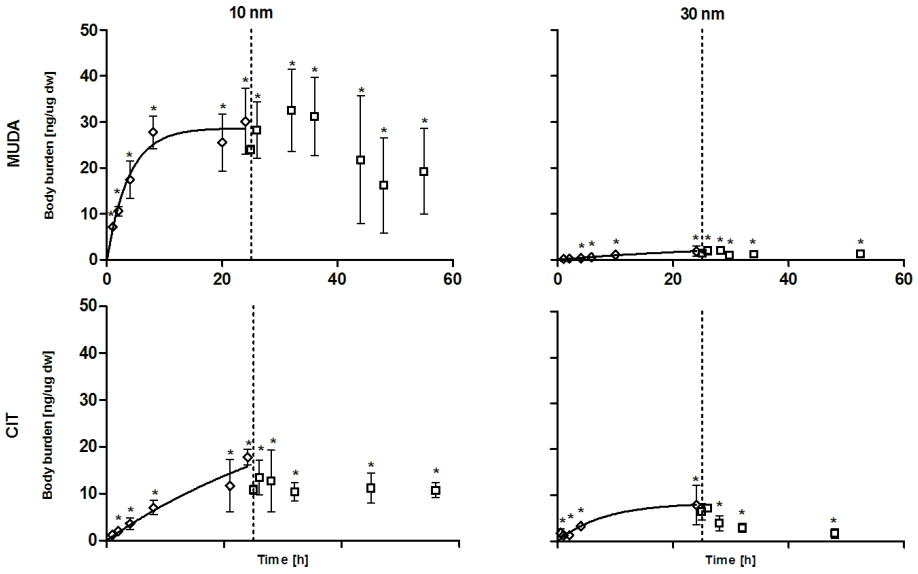


**Figure 4:** 24 hours of uptake (diamonds) and depuration (squares) in neonate *D. magna* during exposure to 0.5 mg Au/L in the uptake phase. The different size and stabilizing agent of the nanoparticles is indicated by the matrix (MUDA: mercaptoundecanoic acid). Points denoted * are statistical significantly different from the control ($p<0.05$) (Skjolding et al., 2014a – Paper I).

**Table 2:** Nominal size of particles and stabilizing agent along with modelled uptake and depuration rates, with corresponding $R^2$ and the remaining residual body burden of Au at the end of a 24 hours depuration period in clean Elendt M7 media. The values in the parentheses denote the 95% confidence interval with upper and lower boundary (Skjolding et al., 2014a – Paper I).

| Nominal size [nm] | Stabilizing agent | Uptake rate[a] [L $kg^{-1}$ dw $h^{-1}$] | Depuration rate [$h^{-1}$] | $R^2$ | Residual mass [ng Au/µg dw organism] |
|---|---|---|---|---|---|
| 10 | MUDA* | 4112-27720 | 0.26 (0.15; 0.37) | 0.81 | 16.1±10.3 |
| 30 | MUDA* | 35-306 | 0.03 (0; 0.11) | 0.68 | 1.2±0.76 |
| 10 | Citrate | 339-2911 | 0.02 (0; 0.09) | 0.84 | 11.2±3.2 |
| 30 | Citrate | 409-2275 | 0.10 (0; 0.25) | 0.65 | 1.7±1.0 |

[a] The range for the uptake rates were derived from kinetic modelling with the initial water phase concentration (lowest value) and the final water phase concentration (highest value) as input parameters. This was done to accommodate for changes in water concentration during the course of the experiment. *mercaptoundecanoic acid

While similar results and model fitting have been successful for predicting uptake of different sizes of Au ENPs (Wray and Klaine, 2015) discrepancies exist when trying to expand the findings to other types of ENPs. In the studies with Au ENPs a trend for smaller sized Au ENPs (6-30nm) to be taken up faster than larger sizes was reported (Wray and Klaine, 2015). Contrary, one study found that the uptake of $TiO_2$ ENPs correlated with the hydrodynamic size thus larger (aggregated) $TiO_2$ ENPs were taken up faster than smaller (well dispersed) $TiO_2$ ENPs in the size range 100-1000 nm (Kwon et al., 2014). It could be proposed that different uptake mechanisms are dominant within the size ranges studied. Natively, *D. magna* has an average mesh filter size of approximately 400 nm (Geller and Muller, 1981). Thus, larger particles would be retained by the filter mesh while uptake of the small Au ENPs would be through gravitational deposition, inertial impaction, motile-particle deposition and/or electrostatic interactions (Rubenstein and Koehl, 1977). Based on the intrinsic size related properties of ENPs, it is most likely that either diffusive particle deposition or electrostatic interaction is the main drivers for uptake of ENPs in the small size range (6-30 nm). Throughout the test rapid aggregation of the Au ENPs could increase their size thus rendering them available within the filter mesh size range (Skjolding et al., 2014a - Paper I). The stability of citrate and MUDA coated Au ENPs changed markedly over 24h (table 1), but uptake of Au ENPs is rapidly occurring within the first

few hours of exposure (figure 4) (Skjolding et al., 2014a – Paper I) in accordance with literature (Lovern et al., 2008; Khan et al., 2014). While aggregation did not seem to be the driving factor for the uptake of Au ENPs, it could be proposed as a confounding factor in the uptake of $TiO_2$ ENPs thus describing the difference in size related uptake patterns (Kwon et al., 2014). Consequently, when comparing size it would be crucial to consider such differences in mechanistic uptake pattern related to the organism used which ultimately can influence the correlation between size and uptake. Indeed a higher uptake was observed for highly aggregating ZnO ENPs compared to Au ENPs (Skjolding et al., 2014b – Paper II). A bulk ZnO powder (<5 µm) was also used to simulate a primary micron sized particle. The study found that uncoated ZnO ENPs were taken up to lesser extent than those of the bulk ZnO particles, even with aggregated sizes of the ZnO ENPs in the micron range (2020±120 nm). A functionalization with hydroxyl groups to the ZnO ENPs increased the uptake in *D. magna* however the size was also increased to large aggregates (6490±1660 nm) (table 3) (Skjolding et al., 2014b – Paper II). It should be noted that adsorption of ZnO ENPs to the exterior of the *D. magna* was not assessed in this study, which could overestimate the fraction taken up.

A two part regime could be proposed for *D. magna* to consist of ENPs larger than the mesh filter size to behave significantly different in regards to size correlated uptake than those of sizes smaller than the mesh filter size (Kwon et al., 2014).

**Table 3:** Results from uptake studies using *D. magna* exposed for 24 hours to $ZnCl_2$, ZnO bulk, ZnO NP and ZnO-octyl NP (1 mg Zn/L) followed by a depuration period for 24 hours. Uptake and depuration rates were modelled using a first-order rate model. Numbers in parentheses are 95% confidence intervals of parameter estimates. All values are corrected for background content of Zn measured in clean animals. N/A: no data (Modified from Skjolding et al., 2014b – Paper II).

| Parameters | $ZnCl_2$ | ZnO bulk | ZnO NP | ZnO-octyl NP | ZnO-OH NP |
|---|---|---|---|---|---|
| Body burden[a] [mg Zn/kg dw] | 1660 ±1020 | 17500±4300 | 7690±3580 | 37230±2460 | 287±91 |
| Uptake rate [L $kg^{-1}$ dw $h^{-1}$] | N/A | N/A | 24500 (5410; 43700) | 38200 (27300; 49200) | N/A |
| Depuration rate [$h^{-1}$] | N/A | N/A | 3800 (570; 7020) | 1100 (690; 1410) | N/A |

[a]Average body burdens (including ENPs adsorped to the exterior of the *D. magna*) are shown as ±standard deviations.

Another factor which could explain the discrepancies in trend for uptake of different ENPs could be related to differences in adsorption behavior of the different ENPs to the exterior. As mentioned earlier citrate coated Au ENPs had a low tendency to sorb to the carapace while $TiO_2$ have been found to stick to the carapace of *D. magna* (Dabrunz et al., 2011). Such sorption to the outer body wall would vastly increase the ENPs associated with the test organism thus impacting the overall body burden measure. Associated physical effects of sorption to the carapace may include difficulties in molting (Dabrunz et al., 2011), filtration efficiency and decreased mobility (Lovern et al., 2007).

The potential to cause chronic effects would be dependent on the potential for test organisms to depurate the ENPs.

Fast initial depuration of Au ENPs have been reported in literature (Lovern et al., 2008; Ranville et al., 2012; Khan et al., 2014; Skjolding et al., 2014a – Paper I). However, for other types of ENPs and test organism depuration have been found to be slow (Rosenkranz et al., 2009; Lewinski et al., 2010; Zhao and Wang, 2010; Zhu et al., 2010; Feswick et al., 2013). During starvation *D. magna* have been found to preserve food in the gut. Thus, the lack of food during the depuration period may have caused retention of ENPs in the gut. The gut retention time in *D. magna* is between 2-55 min, trending towards low values with food present (Rigler, 1961, Schindler, 1968, McMahon, 1970, Gliwicz and Sieniawska, 1986). Consequently, it is expected that the presence of food will affect the depuration rate of ENPs. Accordingly, an increased depuration rate was observed after addition of food during depuration (figure 5) (Zhu et al., 2010; Skjolding et al., 2014a – Paper I). The residual body burden of Au ENPs was however not affected by the addition of food during the depuration period (figure 5) (Skjolding et al., 2014a – Paper I). It could be proposed that the corresponding increase in depuration was insufficient to significantly reduce the body burden due to the variation in data.

The residual body burden could be proposed to be internalized ENPs thus not expected to be removed during relative short time span (<24h). A higher residual body burden was observed for smaller MUDA coated Au ENPs (10 vs 30 nm) which correspond with increased interaction potential of small ENPs and possible increased sorption to microvilli which would reduce depuration (Skjolding et al., 2014a – Paper I).

To investigate this type of effects the most visual and direct way is through microscopy techniques which will be presented in the following section.

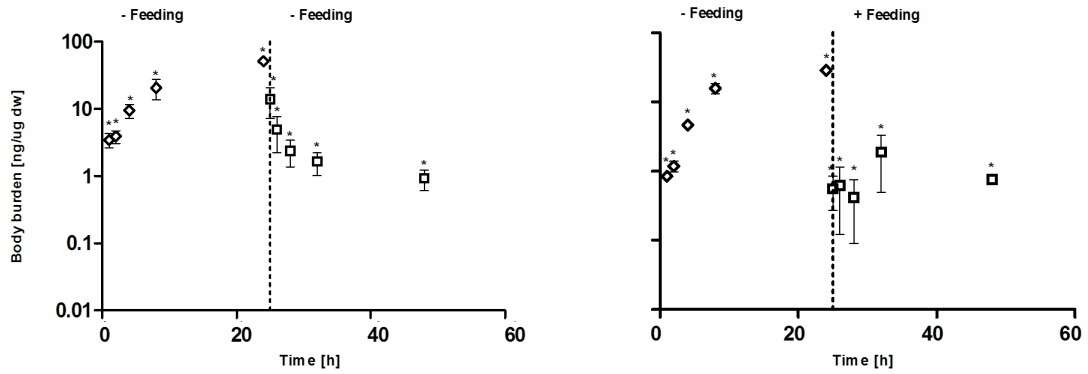


**Figure 5:** Relation between body burden in *D. magna* and time during 24 hours of uptake (diamonds) and depuration (squares), respectively. *D. magna* was exposed to 0.4 mg 10 nm CIT coated Au ENPs/L in systems without food (both during uptake and depuration) and with food (only during depuration). Points denoted * are statistical significantly different from the control ($p<0.05$) (Modified from Skjolding et al., 2014a).

## 4.4 Microscopic investigation of engineered nanoparticles uptake in filter feeders

For microscopic investigation of ENPs electron microscopes are usually preferred utilizing high resolution to provide direct evidence of internalization and visualize individual electron dense ENPs.

In *in vivo* experiments the results reported in literature using electron microscopy are diverse and not straight forward. General trends in terms of uptake mechanism or physical-chemical parameters of the ENPs controlling uptake is often difficult to obtain. Even if internalization is indicated the interpretation can be tricky, an example could be Ag ENPs where dissolution following formation of AgS granules is a known method for detoxification (Luoma and Rainbow, 2008). Furthermore, artefacts induced by regular staining procedures have caused concern with regards to interpretation of *in vivo* results from electron microscopy studies (Edgington et al., 2014; Jensen et al., 2015 – Paper V). Consequently, when conducting this type of studies a range of controls or multiple detection methods has to be used in order to quantify internalization. For example using a combination of TEM and element analysis citrate capped Ag ENPs were indeed found in epithelial cells of ragworms (*Nereis diversicolor*) and observations of endocytotic pits gave direct evi-

dence of the uptake pathway (Garcíca-Alonso et al., 2011). However, such direct evidence is not predominant in the literature.

Multiple studies have assessed uptake of different ENPs in *D. magna* with various microscopy techniques (TEM e.g. Lovern et al., 2008; Heinlaan et al., 2011; Zhao and Wang, 2012; Confocal microscopy e.g. Rosenkranz et al., 2009; Lewinski et al., 2010; Feswick et al. 2013; Dark-field optical microscopy e.g. Scanlan et al., 2013). While TEM surpass the other techniques in terms of image resolution a clear disadvantage is the extensive sample preparation and time consuming analysis of specimen to get qualitative datasets compared to some of the other techniques presented (for further discussion see Goodhead et al., 2015).

A promising technique to overcome some of those shortcomings of TEM includes Light Sheet Microscopy (LSM) (Skjolding et al., 2015 – Paper IV, Jensen et al., 2015 – Paper V). LSM has rarely been used in ecotoxicological studies of NP. However, it offers some advantages that could be used complementary with the widely used electron microscopy. Light SM possess the major advantages that whole living organisms can be studied. Furthermore, the sample preparation is quick and easy, the method has a low level of invasiveness for embedding and imaging, good penetration depth and low photobleaching compared to other types of microscopy. But most importantly LSM retains the integrity of the living organism, which in electron microscopy would undoubtedly be lost. However, a drawback is the image resolution and the need for light emitting properties of the investigated ENPs (either through attached fluorescent probes or inherently due to the nano-size). LSM was used as a first line of evidence in studying the uptake of Au ENPs (Jensen et al., 2015 – Paper V). Utilizing a fluorescent probed Au ENP at 0.4 mg Au/L a clear fluorescent signal was observed in the living *D. magna* after 2h of uptake (figure 6). Increasing fluorescence from the gut tract was observed up until 24h of uptake where the whole gut tract was fluorescent (figure 6) (Jensen et al., 2015 – Paper V).

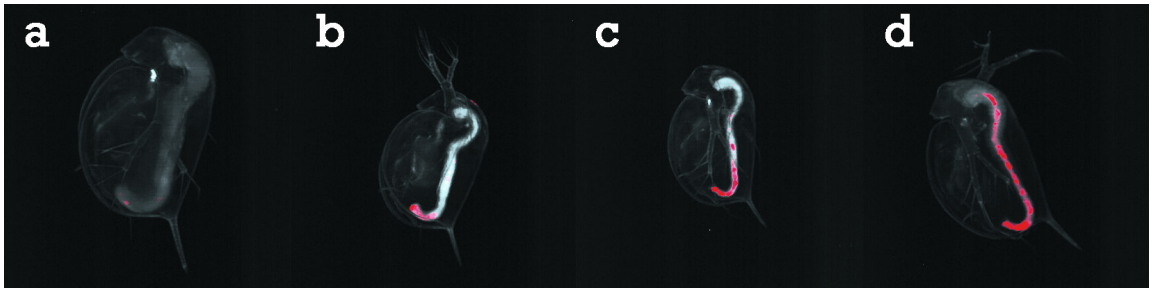


**Figure 6:** *D. magna* after (a) 1h, (b) 2h, (c) 4h and (d) 24h exposure to Au ENPs (0.4 mg Au/L) using a 20x magnification water immersed objective (Jensen et al., 2015 – Paper V).

No fluorescent signal was observed outside the gut section of the daphnia or on the carapace. Au ENPs initially accumulated in the hindgut as expected for inorganic particles with no nutritional value (Hardy and McDougall, 1893). The pattern of uptake also corresponds to findings by Skjolding et al. (2014a – Paper I) showing a rapid increase in body burden during the first hours, and plateauing after approximately 8h (figure 4) (Skjolding et al., 2014a – Paper I). No uptake past the gut tract was observed during the exposure period (24h) (figure 6). However, using fluorescently probed ENPs raises the question of limit of detection. While electron microscopy can visualize individual electron dense ENPs this is so far not feasible using fluorescent probes. Thus, only clusters of Au ENPs will be visible through this method. However, LSM could be relevant as a screening tool or in chronic tests in order to determine sites for possible accumulation.

Focused Ion Beam – Scanning Electron Microscopy (FIB-SEM) is another microscopy technique that has not been frequently used in regards to ecotoxicological studies of ENPs. Using a focused ion beam, milling of a sample is possible thus making it possible to achieve 3D like images which could possible distinguish adsorbed ENPs from internalized ENPs (Jensen et al., 2015 – Paper V). After exposure to citrate coated 10 nm Au ENPs at a concentration of 0.4 mg Au/L a strong back-.scattered electron signal was registered from the gut lumen, indicating regions with high concentration of Au ENPs and thus ingestion of Au ENPs into the gut tract (figure 7a, arrow) (Jensen et al., 2015 – Paper V). The peritrophic membrane (PTM) appeared to retain the Au ENPs in the gut lumen with only few bright signals (proposedly Au ENPs) near the microvilli, and seemingly no internalization in the cell structures (figure 7b, arrow) (Jensen et al., 2015 – Paper V). No disruption of cell structures or cell membrane was reported following the exposure. Contrary, protrusion of the midgut lumen was observed for CuO ENPs following expo-

sure to 4.0 mg/L for 18h and could be related to oxidative stress (Heinlaan et al., 2011). Cellular morphological changes and protrusion was also observed using $TiO_2$ ENPs with a size of 23±7 nm and a mixed crystalline structure (Kwon et al., 2015). However, similar to findings by Jensen et al. (2015 – Paper V) no internalization was found in intact epithelial cells in either of the studies. While morphological changes was not found using citrate coated Au ENPs (Jensen et al., 2015 – Paper V) Reactive Oxygen Species (ROS) generation have been observed in the gut of *D. magna* using Au ENPs with different coatings (mercaptopropionic acid, polyallylamine hydrochloride (PAH), cetyltrimethylammonium bromide (CTAB)) and a primary particle size of 4 nm which could possibly could lead to cellular damage (Dominguez et al., 2015). Furthermore, two positively charged Au ENPs with different coating (PAH and CTAB) were found to exert significantly different levels of toxicity (Bozich et al., 2014). This underlines that different cellular effects could induce a potential for different uptake dependent on size, surface coating and functionalization as also indicated previously in this section.

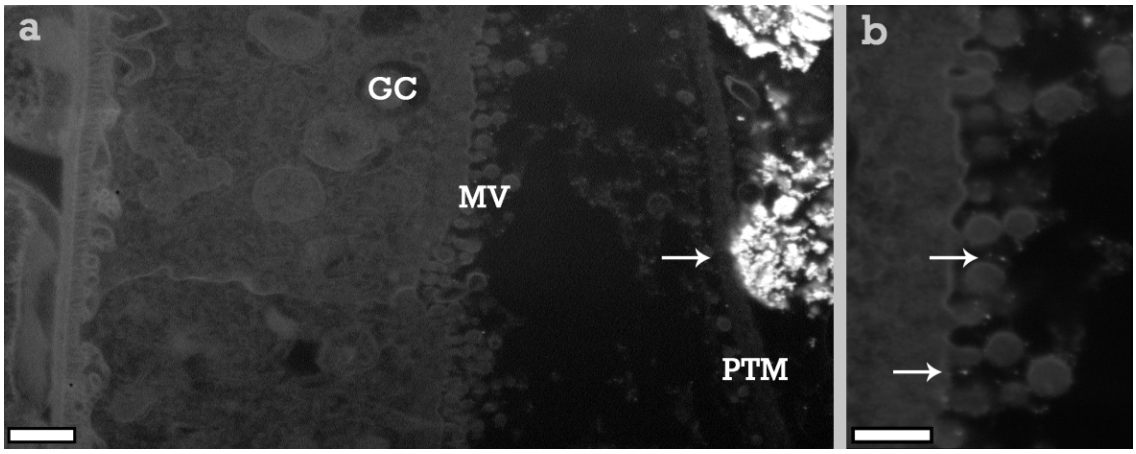


**Figure 7:** FIB-SEM images of D. magna gut epithelia after 24h exposure to Au NPs (0.4 mg Au/L). Electron dense structures appear white. Au NP-like objects are marked with arrows. (a) Scale bar = 1 µm, (b) Scalebar = 0.5 µm. GC = gut cells, LU = Lumen, MV = Microvilli, PTM = Peritrophic membrane (Jensen et al., 2015 – Paper V).

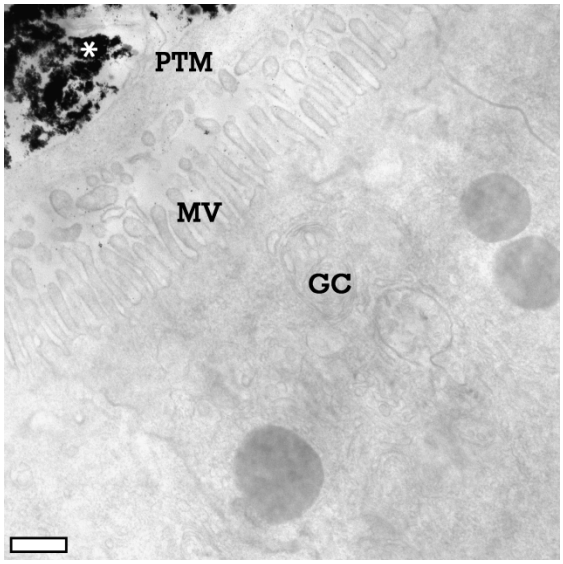


**Figure 8:** D. magna gut epithelia after 24h exposure to Au NPs (0.4 mg Au/L). Overview of gut lumen and microvilli, scale bar = 0.5µm. *= Au NP- like objects. GC = gut cells, MV = Microvilli, PTM = Peritrophic membrane (modified from Jensen et al., 2015 – Paper V).

In order to more carefully assess the individual cellular structures and their morphology and/or possible uptake of individual ENPs TEM was used (Jensen et al., 2015 – Paper V). In accordance with previous findings in the literature (Lovern et al., 2008; Khan et al., 2014; Santo et al., 2014) uptake of Au ENPs was observed in the gut lumen after 24h exposure (figure 8) (Jensen et al., 2015 – Paper V). Contrary to previous published results using Au ENPs (Lovern et al., 2008; Khan et al., 2014) electron dense structures were also found in considerate numbers past the PTM and associated with the microvilli (figure 9a) and few within the cells (figure 9b) (Jensen et al. 2015 – Paper V). In accordance with findings using FIB-SEM the majority of the Au ENPs were retained in the gut lumen by the PTM (figure 7).

As mentioned earlier artefacts due to specimen staining or embedding is of concern in regards to interpretation of TEM images. Consequently, two methodological controls (a staining control, C1 and embedding control, C2) were examined by TEM (Jensen et al., 2015 – Paper V). For C1 Au NP suspension was added with uranyl acetate during en bloc staining. For C2 Au ENP suspension in acetone was added instead of propylene oxide during embedding. No confirmed Au ENPs (by Scanning Transmission Electron Microscopy Electron-Dispersive X-ray (STEM EDX)) were identified inside the C1 and C2 control organisms, indicating that any possible Au NPs observed

inside *D. magna* cells in the subsequent STEM EDX analysis would not result from the staining or embedding procedure (Jensen et al., 2015 – Paper V).

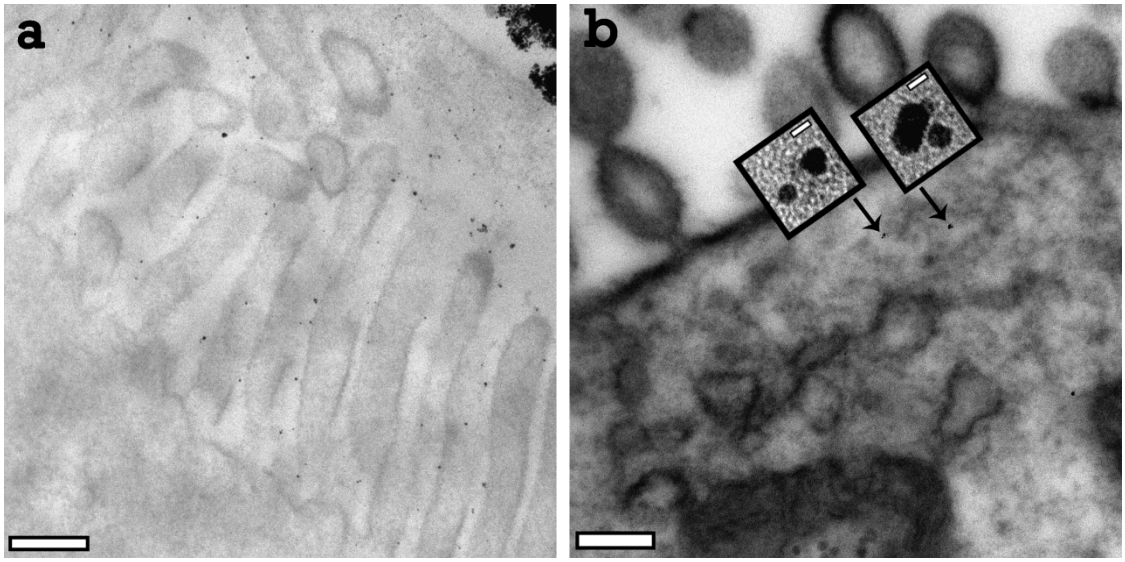


**Figure 9:** TEM images of *Daphnia magna* gut epithelia exposed to Au ENPs (0.4 mg Au/L) for 24h showing intracelluar structures and objects which resemble ENPs. (inserts in figure 4b). (a) Scale bar = 200 nm and (b) Scale bar = 200 nm, inserts = 5 nm (Jensen et al., 2015 – Paper IV).

While the methodological controls confirmed that the internalization of the ENPs was not due to physical movement of ENPs into the cells during embedding or staining it did not confirm the origin and chemical structure of the internalized ENPs (figure 9b). To confirm the chemical composition of the internalized ENPs elemental analysis with EDX was used (Jensen et al., 2015 – Paper V). The results revealed that the identification of ENPs solely on observations (i.e. contrast, size and morphology) can be deceiving, especially for NPs inside the cellular matrix where both nano- sized cellular structures and precipitates can exhibit similar contrast and size ranges. The large Au NP aggregate-like structures retained by the PTM inside the gut lumen were confirmed to be Au by EDX (Jensen et al., 2015 – Paper V). In order to confirm the internalization of Au ENPs a large number of high contrast objects resembling the added Au ENPs was analyzed with EDX (examples are shown in figure 10). High contrast particles attached to surface (figure 10a) and in the crypts (figure 10b) of microvilli were confirmed to contain Au, thus most likely Au ENPs (Jensen et al., 2015 – Paper V). Elemental analysis of high contrast material in mitochondria (figure 10c) and lipid droplets (figure 10d) inside intact cells were not confirmed to contain Au but were found to con-

tain osmium rich material, most likely precipitates from the staining procedure (Jensen et al., 2015 – Paper V).

As described earlier internalization of NPs can take multiple endocytotic routes depending on nanoparticle size, shape, surface charge and surface coating (Malgorzata et al., 2009; Cho et al., 2009; García-Alonso et al., 2011). With regards to the size, larger ENPs (~60 nm - 1 µm) are mainly taken up actively by endocytosis whereas smaller ENPs (<60 nm) will possibly aggregate on the cell surface before being taken up through endocytosis or through passive transport (Treuel et al., 2013). *In vitro* small particles have been shown to passively pass through cell membranes as shown in e.g. red blood cells which lack endocytotic uptake routes. Passive uptake might be a significant route of internalization in long-term exposure to low numbers of ENPs where threshold densities of ENPs on the cell membrane will be insufficient to activate endocytosis (Treuel et al., 2013). Relating this to the amount of ENPs that were crossing the PTM it is possible that there were simply not enough ENPs interacting with the cell surface and exposure time was too short or specific size was too large for passive transport (Jensen et al., 2015 – Paper V).

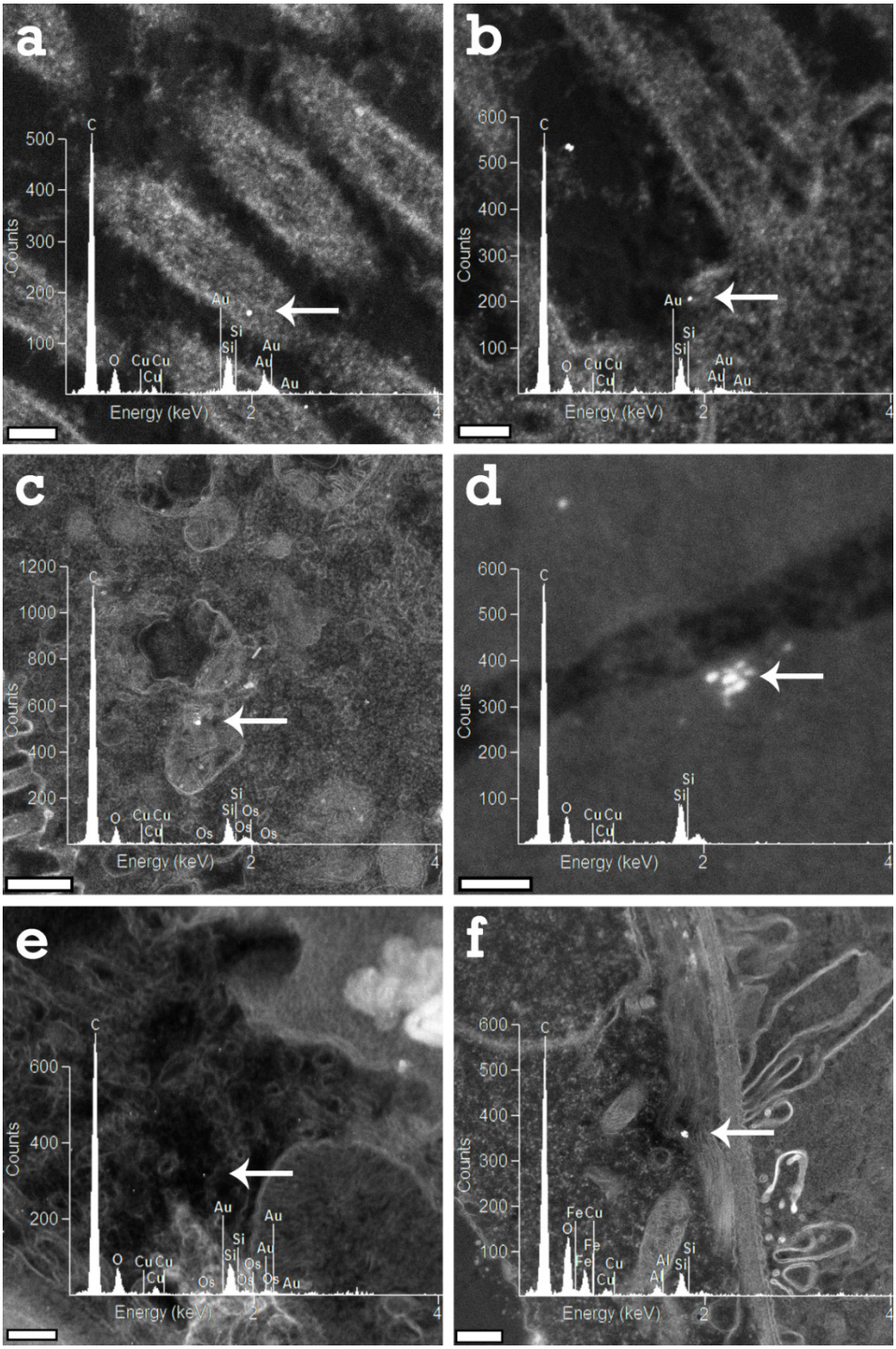


**Figure 10:** HAADF STEM images of *Daphnia magna* gut epithelia exposed to Au NPs (0.4 mg Au/L) for 24h showing intracelluar structures and possible NPs. (a) Au NP at microvilli, Scale bar = 100 nm, (b) Au NP at crypt of microvilli, Scale bar = 100 nm, (c) Os-rich particle in mitochondrion, Scale bar = 0.5 µm, (d) Os-rich particle in lipid droplet, Scale bar = 50 nm, (e) Au NP in a disintegrating cell, Scale bar = 200 nm and (f) Fe-rich particle below the basal lamina, Scale bar = 0.5 µm (Jensen et al., 2015 – Paper V).

Recently, it was reported that *D. magna* did neither internalize ~20 nm Au NPs in its gut cells nor were the NPs associated with the microvilli of the gut epithelia (Khan et al., 2014). The daphnia were exposed for 5 hours and allowed to depurate before analysis. Lovern et al. (2008) reported ~20 nm Au NPs close to microvilli and suggested the possible uptake of one single NP attached to microvilli. In this case daphnia were exposed for 12 hours without depuration (Lovern et al., 2008). In contrast, Jensen et al. (2015 – Paper V) found considerable amount of Au ENPs associated directly with the microvilli (figure 9a) (Jensen et al., 2015 – Paper V). It is possible that the discrepancy is due to the difference in size of Au ENPs and/or to the length of exposure. In regards to size, an uptake study of negatively charged fluorescent polystyrene beads (20 nm and 1 μm) in the gut of *D. magna* showed that both particle sizes translocated from the gut to lipid storage bodies distant from the gut (Rosenkranz et al., 2009). In the study a short uptake period of 30-60 min was used thus highlighting that for some ENPs the uptake and translocation could be fast. 1 μm particles were also detected by TEM inside the lipid storage bodies. Some 20 nm particles were also observed by TEM, but in both exposed daphnia and controls which points to the possibility that these images could in fact be artefacts from the heavy metal stains used in the preparation or post-staining which again underlines the need for element analysis to strengthen the interpretation of such findings. It is therefore currently not evident if single ENPs were able to enter the gut epithelial cells of *D. magna* in such short time points.

Another study using ZnO ENPs found both uptake inside microvilli and gut cells at various locations, as well as in gut muscles indicating crossing of the basal lamina (Santo et al., 2014). The ENPs used had a median size of 21 and 35 nm respectively, thus being similar to the previously mentioned studies. However, a marked increased toxicity of the ENPs was observed compared to Au ENPs. Thus, uptake could be related to toxic effects as proposed by Heinlaan et al. (2011) for CuO ENPs. Furthermore, an uptake period of 48h was used, which could also affect the uptake as highlighted earlier. However, no element analysis was carried out for observed ENPs. One study successfully confirmed internalization of differently functionalized ($NH_2$, PEG and COOH-PEG) Quantum Dots (QDs) in *D. magna* using a combination of confocal and electron microscopy coupled with element analysis and concluded that particle functionalization affected uptake more than surface charge (Feswick et al., 2013).

In the literature it is becoming increasingly recognized that TEM images of various nanomaterials in biological samples can be misinterpreted (Brandenberger et al., 2010; Edgington et al., 2014; Kobler et al., 2014). For example, the use of Electron Energy Loss Spectroscopy (EELS) showed QDs in macrophage-like cells. However, only one out of six areas that were originally believed to contain QDs did in fact contain a corresponding spectrum. The particle-like structures found in the other areas were probably either precipitates from the post staining or osmophilic structures within the sample (Brandenberger et al., 2010). Again, it is highlighted that there is a need for elemental analysis in the localization of intracellular ENPs using TEM. An additional source of artefacts in performing TEM of NP uptake can be imaging and analysis of particles inside dying cells. In this case, analysis is not a guarantee against false positives. The gut of *D. magna* undergo rapid turnover and renewal of cells in which the cell content is packed into vesicles and released by disruption of the cell wall into the gut lumen. Jensen et al. (2015 – Paper V) observed an example of such location of Au ENPs within these cells, and confirmed the presence of Au (figure 10e) (Jensen et al., 2015 – Paper V). This is not surprising considering the cell was not intact, however early in the turnover process the state of the cell can be difficult to identify (Jensen et al., 2015 – Paper V). This might lead to incorrect conclusions regarding uptake of ENPs even when performing elemental analysis.

The above underline the differences in internalization for different ENPs with regards to size, functionalization, time of exposure and chemical composition. Additionally, it highlights the pivotal role of applying multiple approaches to ensure the right interpretation of images obtained through microscopy, especially TEM.

## 4.5 Influence of size, shape and functionalization on effects and uptake in fish

Uptake of ENPs in fish is important as it represents a higher trophic level being a secondary consumer. Hence, being a likely target for substances found to accumulate in the food web through trophic transfer (transfer from e.g. daphnia to fish).

The literature overview (2013-2015, presented in section 3.3) identified that multiple fish species have been tested however one species was found to be more frequently used, namely zebrafish (*D. rerio*). Zebrafish is superior for testing due to its small size and ease of culturing. Furthermore, it is widely

used as a model organism and can be tested on different biological organizations e.g. cells in culture, embryos, fry and adult fish (Thit et al. 2015 - Paper VI). Two of these biological organizations have been used in OECD guidelines; OECD guideline 236 (Fish Embryo Acute Toxicity (FET) Test) and 305 (Bioaccumulation in Fish: Aqueous and Dietary exposure) (OECD, 2012; OECD, 2013).

Multiple routes of uptake of ENPs have been proposed for fish e.g. uptake through gill surface, through water due to stress induced drinking, through skin due to inflammation, or through diet (for extended discussion of uptake routes see e.g. Handy et al., 2008b). While the possible uptake routes have been identified they have been harder to experimental quantify *in vivo*. Numerous studies reported uptake of various ENPs resulting in elevated concentration of the given compound. $TiO_2$ ENPs with a primary particle size of 21 nm (Degussa P25) showed elevated uptake in carp (*Cyprinus carpio*) with elevated content in gill, liver, muscle and intestine using 10 mg/L of $TiO_2$ for 20-25d of aqueous exposure (Sun et al., 2007; Zhang et al., 2007; Sun et al., 2009). Contrary, using similar $TiO_2$ ENPs showed no significant accumulation in gill, liver, muscle or brain of rainbow trout *Oncorhynchus mykiss*) using concentrations of 0.1-5 mg/L for 14d of aqueous exposure (Federici et al., 2007, Johnston et al., 2010a). However, few aggregates of $TiO_2$ ENPs were found adhering to the gill membrane of some fish though gill tissue levels of $TiO_2$ was not significantly different from control after exposure to 5 mg/L (Johnston et al., 2010a). The contrary findings could be related to the difference in concentrations and time of exposure. One study reported $TiO_2$ ENPs (Degussa P25) aggregates of approximately 2500 nm resulting in sedimentation (Zhu et al. 2010). Similarly, Sun et al. (2007) also reported aggregation thus renewing the test medium daily to ensure a concentration of 10 mg/L of $TiO_2$ ENPs. Thus, the renewal of the test media daily would result in a dynamic process of newly aggregating $TiO_2$ ENPs. The resulting water phase concentration would most likely be higher in the test setup with daily renewal compared to a static test. Indeed, studies have shown a concentration dependent uptake of different ENPs in fish (Salari Joo et al., 2013; Chen et al., 2014; Jayaseelan et al., 2014).

Another issue could be the changes in ENPs size corresponding with different potential for dissolution. While this probably played a minor role for differences in uptake of for example $TiO_2$ ENPs it could influence the results for more soluble ENPs e.g. Ag and ZnO ENPs (Ates et al., 2014; Hao et al., 2013; Auffan et al., 2014). However, a more comprehensive description of

dissolved ionic species compared to ENPs is beyond the scope of this thesis (For a comprehensive discussion of the topic see e.g. Shaw and Handy, 2011).

Furthermore, it is important to remember that dynamic processes are governed by time. Consequently, direct comparisons without comprehensive characterization of the ENPs both at the beginning and during the test to identify changes as a function of time will be difficult if not impossible. Common for the many studies carried out are that parameters such as state of aggregation, water phase concentration during the test, dissolution etc. is rarely reported in a comprehensive manner thus limiting basis for comparison.

## 4.6 Influence of exposure pathway on uptake and localization in fish

While aqueous exposure is a likely scenario it might not constitute the most dominant route of uptake at higher trophic levels. Dietary exposure has been proposed as an important route of uptake for ENPs in for example fish (Handy et al., 2008b).

Previously, it was shown that daphnia had a high uptake of ENPs from the aqueous phase (section 4.2). It was concluded that for the majority of the ENPs tested uptake was primarily in the gut tract with limited or no internalization dependent on properties of the ENPs. While the ENPs situated in the gut tract of daphnia seemingly had little or no toxic effect on daphnia it would still be available for trophic transfer to e.g. fish.

One of the first studies showing direct evidence on trophic transfer of ENPs exposed QDs to ciliates (*Tetrahymena pyriformis*) as food source for rotifers (*Brachionus calyciflorus*) in a simplified food chain (Holbrook et al., 2008). Internalization was also observed using confocal microscopy however the extent of the trophic transfer was concluded to be minimal and that the transferred QDs were efficiently depurated by the rotifer (Holbrook et al., 2008). With trophic transfer occurring at lower levels of the food chain more adverse effects could be observed at higher level consumers (Schwarzenbach et al., 2005).

Trophic transfer was observed from crustaceans (*D. magna*) to zebrafish (*D. rerio*) in a simplified freshwater food chain pre-exposing *D. magna* to two different concentrations of $TiO_2$ ENPs (0.1 and 1.0 mg/L) (Zhu et al., 2010). Significant difference in resulting body burden after 14d of exposure was ob-

served for the two concentrations. Similar findings were observed for pristine ZnO ENPs and ZnO ENPs functionalized with octyl groups using 24h pre-exposed *D. magna* as food source for *D. rerio* (figure 11) (Skjolding et al., 2014b – Paper II). The difference in body burdens achieved in zebrafish could result from higher body burden in the feed. For the ZnO ENPs the different functionalization resulted in approximately 5 times larger body burden compared to pristine ZnO ENPs (exemplified in figure 11 and table 4) while the resulting body burden increase in the fish was 2.4. Similarly, the *D. magna* pre-exposed to $TiO_2$ ENPs contained approximately 13.5 times higher body burden at concentrations of 1.0 mg/L $TiO_2$ ENPs compared to that of 0.1 mg/L $TiO_2$ ENPs while the resulting body burden in the zebrafish exposed to the high concentration (1.0 mg/L $TiO_2$ ENPs) was approximately 4.3 times that of the low concentration (0.1 mg/L $TiO_2$ ENPs). However, in both cases the body burden reached was higher than that of the corresponding aqueous exposure. In both of the above studies the content was carried out as whole body burden, thus the issue of localization was not assessed. Thus, insights in target organs could not be quantified.

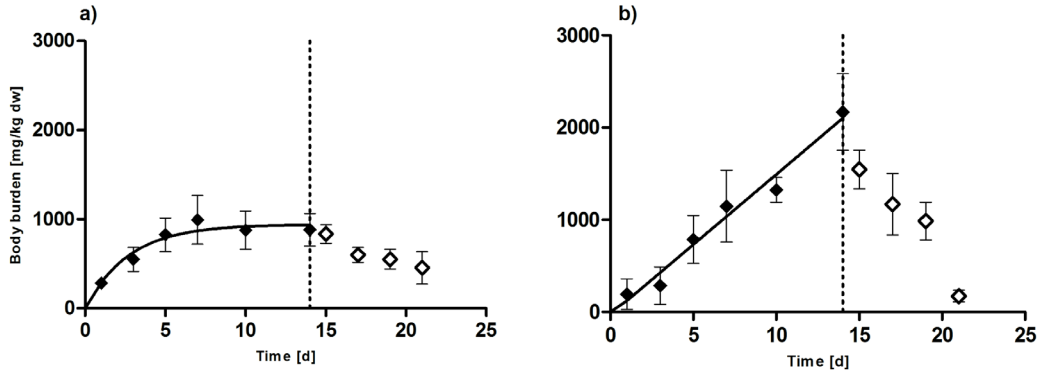


**Figure 11:** 14 days of uptake (filled diamonds) and 7 days of depuration (clear diamonds) with *D. rerio* feeding on pre-exposed *D. magna* (1 mg Zn/L with ZnO NP and ZnO-octyl for 24 hours) in the uptake phase and clean *D. magna* in the depuration phase (mean ± standard deviation; n=3). A first order rate model fit is indicated by the solid line (see table 4 for model parameters) (Skjolding et al., 2014b – Paper II).

Using neutron activation of Ag ENPs a significantly higher body burden was observed for zebrafish (*D. rerio*) fed contaminated food, compared to aqueous exposure (Asztemborska et al., 2014). However, it was also found that the majority of the content was located in the gut, region and was easily depurated after moving to clean media (Asztemborska et al., 2014). The level of

Ag in the rest of the fish remained similar for exposure through water and diet. Contrary, using dietary exposure of goldfish (*Carassius auratus*) to CuO ENPs and ZnO ENPs a lower body burden was observed compared to aqueous exposure at similar concentrations (Ates et al., 2014). However, the dissolution of the ENPs was highly dependent on the route of exposure. Thus, CuO ENPs and ZnO ENPs showed respectively 98% and 55% dissolution in the aqueous exposure compared to <5% in the dietary exposure for both ENPs. Consequently, the exposure was largely in the form of ions rather than ENPs for the aqueous exposure.

**Table 4:** Results from uptake studies for *D. rerio* after 14d uptake feeding on *D. magna* pre-exposed for 24h to 1 mg Zn/L of ZnO NP and ZnO-octyl NP, respectively. Depuration periods were 7d for D. rerio. Uptake and depuration rates were modelled using a first-order rate model. The $R^2$ is the correlation coefficient for the model fit. Numbers in parentheses are 95% confidence intervals of parameter estimates. All values are corrected for background content of Zn measured in clean animals (Skjolding et al., 2014b – Paper II).

| Parameters | ZnO NP | ZnO-octyl NP[a] |
|---|---|---|
| Body burden[b] [mg Zn/kg dw] | 890±180 | 2170±410 |
| Uptake rate [L $kg^{-1}$ dw $h^{-1}$] | 13 (7.1;21) | 6.3 (5;7.5) |
| Depuration rate [$h^{-1}$] | 15 (6.1;24) | 5.8 (1;11) |
| $R^2$ | 0.67 | 0.87 |

[a]A linear model was fitted to the data due to no plateau reached during uptake. Unit for uptake is [$h^{-1}$].

[b]Average body burdens are shown as ±standard deviations.

In order to limit the confounding factor of ENPs dissolution one study exposed juvenile zebrafish (*D. rerio*) to Au ENPs (Skjolding et al., 2015 – Paper IV). The results showed a significant difference in total body burden between the two exposures after two days (figure 12). To quantify the localization which is inherently associated with conducting total body burden Au ENPs used in the study was synthesized with a fluorescent probe, thus enabling use of a quantitative (Inductively Coupled Plasma – Mass Spectrometry (ICP-MS)) and qualitative (LSM) estimation method for the uptake and localization of the two different exposure pathways. Fluorescently labelled polystyrene ENPs were used as fluorescent reference for the uptake and localization of the fluorescently probed Au ENPs (Skjolding et al., 2015 – Paper IV).

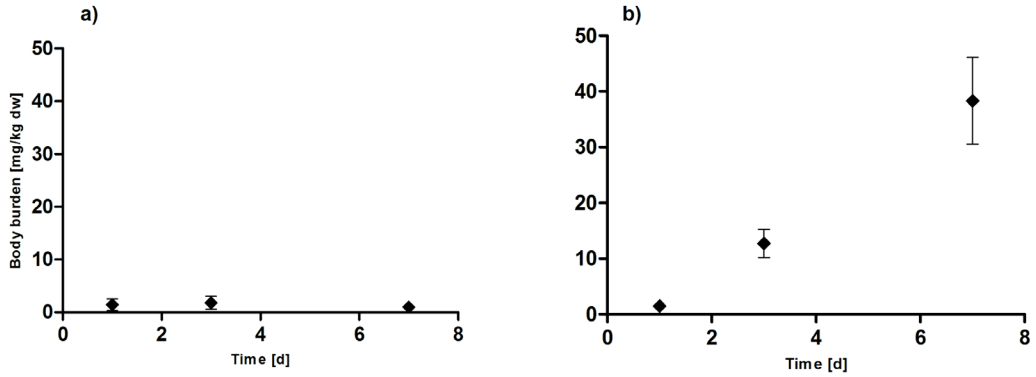


**Figure 12:** Mass quantification of Au NP uptake in zebrafish during seven days through aqueous phase (a) at 1 mg Au/L and dietary exposure (b) with *A. salina* pre-exposed to 1 mg Au/L for 24h before feeding to *D. rerio* (Skjolding et al., 2015 – Paper IV).

Uptake of Au ENPs from the two treatments was confirmed by ICP-MS (figure 12) (Skjolding et al., 2015 – Paper IV). Dietary exposure of Fluorescent Polystyrene Beads (FPBs) and Au ENPs resulted in fluorescent signal from the stomach and intestines of zebrafish. Aqueous exposure to FPBs resulted in increased fluorescent signal in gills, intestines and olfactory tract. Contrary, the Au ENPs were mainly associated with the intestine and the stomach (Skjolding et al., 2015 – Paper IV). It was concluded that the localization of FBP exposed through the aqueous phase was distinguishably different from that of the dietary exposure as also reported in literature (Aztemborska et al., 2014; Ates et al., 2014; Lee and An, 2015; Dedeh et al., 2015). Furthermore, it was noted that the limited fluorescence observed from the probed Au ENPs could be due to overlap of background fluorescence of the zebrafish excited at same wavelengths as the Au ENPs (Skjolding et al., 2015 – Paper IV). Additionally, the uptake could be in concentrations below the detection limit of the applied method thus only signal from clusters of ENPs would emit sufficient signal to be recorded. Clusters of approximately 150 fluorescently probed Au ENPs were suggested as the lower boundary in cell medium (Rothen-Rutishauser et al., 2014). A systematic evaluation of the threshold for the method was not employed by Skjolding et al. (2015 – Paper IV), however well dispersed ENPs in low melting agarose was not detectable (Skjolding et al., 2015 – Paper IV). Thus, it is possible that even though no clear signal was observed for Au NP in this study diffuse uptake across the gut lumen could be too low for detection with the present technique. Similar to study by Skjolding et al. (2015 – Paper IV) latex beads with a size of 39.4 nm was visualized in different compartments (brain, gills, liver, kidney,

gallbladder and intestine) of transparent medaka (*Oryzias latipes*) after 7d of aqeuous exposure (Kashiwada, 2006). The seemingly higher uptake of FPBs compared to Au ENP (figure 13 and 14) could be due to perturbation of lipid membranes caused by the polystyrene beads. This effect was observed in an *in vitro* model system using hydrophobic polystyrene chains aggregated to up to 7 nm (Rossi et al., 2013). It should be noted that the polystyrene ENPs used in Rossi et al., (2013) was markedly smaller than FPBs used in the study by Skjolding et (al., 2015 – Paper IV).

It should be noted that while the above methods could propose a possible qualitative assessment of distribution and excretion through microscopy techniques combined with ICP-MS measurements the development of suitable labels and methods to ensure that the label remains attached to the ENPs remains a future challenge.

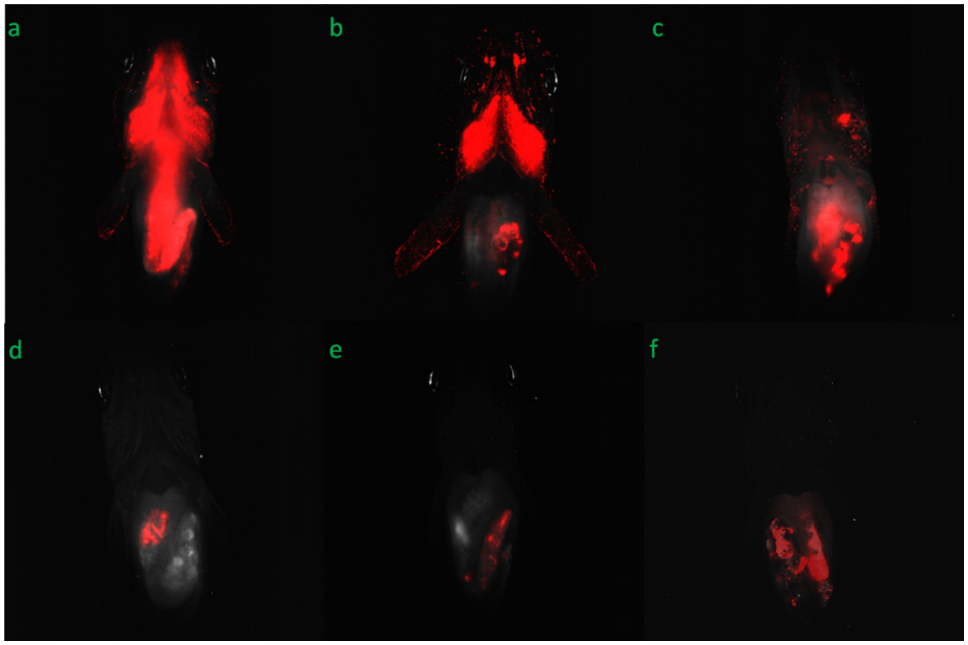


**Figure 13:** Max intensity light sheet microscopy images of zebrafish exposed to fluorescent polystyrene beads for 1, 3 and 7 days through aqueous phase (a, b and c) and pre-exposed *A. salina* (d, e and f) as diet respectively. Red signal corresponds to fluorescent polystyrene beads and grey signal corresponds with autofluorescence of the zebrafish (Skjolding et al., 2015 – Paper IV).

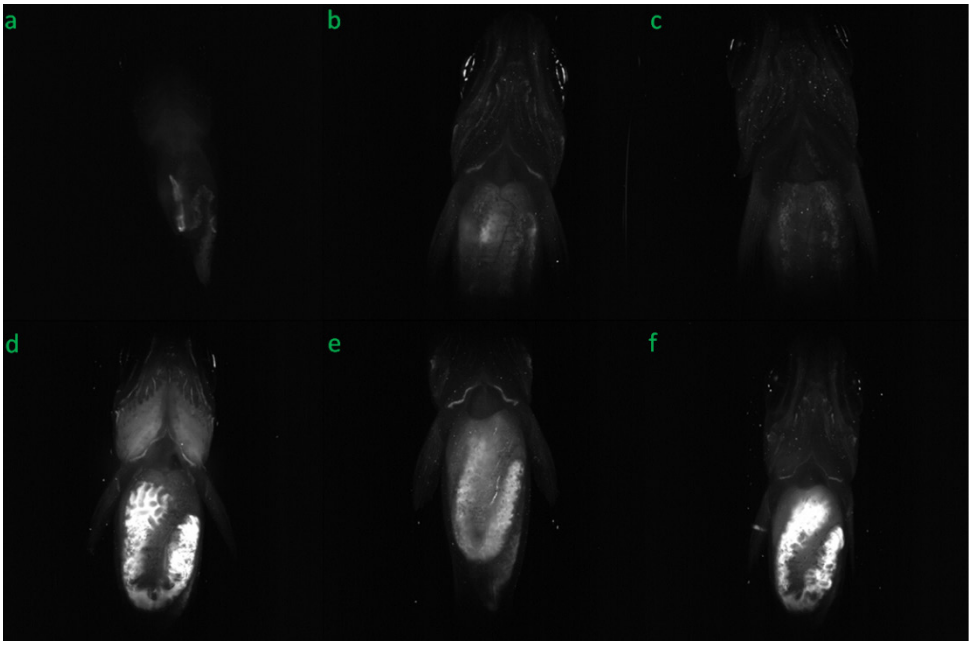


**Figure 14:** Max intensity light sheet microscopy images of zebrafish exposed to 1mg Au/L fluorescent Au ENP functionalized with a FITC probe at 0.5% for 1, 3 and 7 days through aqueous phase (a, b and c) and through diet (d, e and f) with *A. salina* pre-exposed to 1 mg Au/L Au ENP functionalized with a FITC probe at 0.5%. Bright white signal corresponds to fluorescent polystyrene beads, grey signal corresponds with autofluorescence of the zebrafish (Skjolding et al., 2015 – Paper IV).

# 5 Discussion

The body of literature regarding effects and behavior of ENPs using standardized and more exploratory test methods is expanding. However, differences are reported even for similar ENPs in terms of e.g. uptake or body burden, as presented for fish in section 4.5/4.6 making it challenging to carry out chemical safety assessment of ENPs.

One of the main obstacles is the general lack of specific TGs for ENPs. Currently there is no minimum requirement for characterization for example reporting state of aggregation (both initially and during testing) thus probably masking parameters, which could explain the discrepancy in results reported. With the dynamic behavior of ENPs as described in section 3, characterization becomes pivotal for the basic understanding of controlling parameters. Characterization of physical (e.g. aggregation kinetics) and chemical (e.g. dissolution kinetics) behavior of ENPs is to some extent predicted under different conditions. However, in ecotoxicological studies the characterization reported is rarely comprehensive e.g. only reporting primary particle size and not behavior in exposure media. Consequently, interpretation of results is often speculative, and comparison with other studies becomes difficult.

With the huge diversity (both physical and chemical) in ENPs used in consumer products as outlined in section 2, it is simply not practically possible to test all ENPs and combinations of those on a case-by-case basis (Handy et al., 2012b). Consequently, a basic understanding of how intrinsic properties of the ENPs (e.g. size, shape, coating and functionalization), test conditions (e.g. pH, hardness, salinity and dosing), test organism (e.g. feeding traits, habitat and physiology), impact the end points assessed in a given test setup is necessary.

In the following sections a discussion of the intrinsic properties of ENPs, the test conditions, the choice of test organism and the influence on outcome of the end point assessed, and how it differs from conventional chemicals in relation to current OECD TGs.

Fate and transport models for organic and inorganic chemicals are well established tools based on chemical partition coefficients (Mackay, 2001; Scheringer, 2002). However, for ENPs such well-defined systemic description for behavior is still not established. This is due to the double nature of the ENPs, which consist both of a chemical identity (e.g. the inorganic core) and a phys-

ical identity (e.g. nano-size). The chemical identity is the basis for OECD TGs. However, influence of the physical identity have previously not been necessary to the extend present for ENPs thus generally TGs are lacking a description of how to carry out this type of characterization. This was also recognized during an OECD expert meeting on ecotoxicology and environmental effects in 2013 where the summary report included a set of specific recommendations regarding characterization of the physical identity of ENPs and what key parameters to consider (OECD, 2014). However, as these physical effects with regards to specific behavior as a function of the decreasing particle size are not included in the OECD TGs it was concluded that the TGs were applicable to ENPs (OECD, 2014).

## 5.1 Influence of instability of engineered nanoparticles in test systems

Paper I, II, III and VI of this thesis highlighted the impact of instability of ENPs on the resulting concentration in the test setup (Skjolding et al., 2014a – Paper I; Skjolding et al., 2014b – Paper II; Mackevica et al., 2015 – Paper III; Thit et al., 2015 – Paper VI). Different processes cause this decrease in concentration during the test. In the case of soluble ENPs the dissolution would change the configuration of the ENP. The OECD TGs deals well with soluble metal salt thus the dissolved fraction of ENPs is accounted for. However, dissolution kinetics and modelling approaches have yet to be established for ENPs. Additionally, partly soluble ENPs, as presented in paper II, III and VI, where uptake of ENPs and subsequent dissolution in the organism or cell could exert a "Trojan horse" effect that could cause concern (Luoma, 2008; Limbach et al., 2007; Lubick, 2008). Even without internalization, adsorption of ENPs to internal structures and subsequent release of ions resulting in high localized concentrations have raised concern (Luoma et al., 2014). Consequently, the assumption of toxicity only related to the release of ionic species is not always valid, and a the "nano-specific" effect have been observed in different organisms and ENPs for example studies blocking ionic metal uptake, but uptake continues (Zhao and Wang, 2010), greater uptake of ENPs then predicted for dissolved metals (Croteau et al., 2014; Skjolding et al. 2015 – Paper II), higher toxicity from ENPs than can be explained by dissolved metal concentration (Buffet et al., 2011; Cong et al., 2011; Meyer et al., 2010), different fate of ENPs compared to dissolved metal after internalization (García-Alonso et al., 2011). These examples are just some of the "nano-specific" endpoints where the chemical identity would not suffice in

describing the endpoint assessed. Another effect directly related to the particle nature of ENPs is the high interaction with surfaces. A high adsorption to the test vessels were observed resulting in a decrease in concentration (Skjolding et al., 2014a – Paper I). This highlights the need to carefully monitor the concentration throughout the test period especially for long term testing. Modification of the test medium has been proposed to maintain a stable suspension thus keeping the ENPs in suspension and avoid aggregation resulting in sedimentation (main focus for FP7 Project MARINA, Grant no. 263215). However, in the case of filter feeders the uptake from the aqueous phase could be substantial thus effectively lowering the surrounding concentration in static test setups, such effects would not be averted through stable test conditions (Skjolding et al., 2014a - Paper I). Another approach to maintain constant aqueous phase concentration could be a semi static test system. Such a testing system was successfully employed resulting in a fulfillment of the ±20% of nominal concentration criteria stated in e.g. OECD TG 305 (Jang et al., 2014; Mackevica et al., 2015 – Paper III). Various other systems including for example stirring during the test have also been proposed (Boyle et al., 2015), however the successful application of such novel test systems to multiple ENPs have yet to be reported.

## 5.2 Influence of intrinsic properties of engineered nanoparticles on bioaccumulation

Paper I identified some of the challenges in regards to size and uptake of ENPs. Differences in uptake rate and residual body burden as a function of size and coating was reported for ENPs of similar chemical identity (Au ENPs) (Skjolding et al., 2014a – Paper I). It was found that smaller ENPs were taken up faster than larger ones for one coating (MUDA). Consequently, different sizes of ENPs could cause different potential for bioaccumulation. In OECD TGs such an influence of different sizes is not included, due to the lack of inclusion of the physical identity for dissolved chemicals it was established for. Considering the size threshold for cellular internalization presented in section 4.1 it is clear that size inherently could influence internalization.

Results presented in paper V did however not indicate such internalization of Au ENPs. The lack of uptake could be due to short exposure time since seemingly more Au ENPs were found associated with the microvilli (Jensen et al., 2015 – Paper V) compared to other studies using a shorter time of exposure (Lovern et al., 2008; Khan et al., 2014). While internalization of ENPs was not found in Paper V, other studies have identified internalization of ENPs *in*

*vivo* (García-Alonso et al., 2011; Heinlaan et al., 2011; Feswick et al., 2013). The discrepancy could be due to the use of inert model ENPs. Additionally, the coating applied to the ENPs was not expected to promote cellular internalization as for example ENPs used in cancer treatment (Jolck et al., 2015). While Paper V did not present evidence of internalization it should be noted that only one coating was used. The studies that observed internalization used ENPs varying in terms of either size, chemical composition, coating or functionalization from the studies with Au ENPs (Lovern et al., 2008; Khan et al., 2014; Jensen et al., 2015 – Paper V). Consequently, it is still not clear how size, coatings or functionalizations influence internalization *in vivo*. However, Paper II indicated that different functionalizations could indeed influence the uptake, but it was unclear due to the method applied whether internalization occurred.

Additionally, it should be mentioned that while most studies conducted have used pristine, or rather simple configurations of ENPs the constant tailoring with more advanced and sophisticated roles of ENPs, e.g. for drug delivery should raise concern for end points such as bioaccumulation. While tests carried out for pristine ENPs are useful for the basic understanding of mechanisms they will probably not qualify as a predictor for risk of emerging ENPs. Furthermore, it should be noted that the coating or functionalization of ENPs could be dynamic thus coating with proteins or NOM would also influence the fate and effects of ENPs. While those coatings could be characterized as a chemical identity they are not considered in the OECD TGs in relation to behavior or effects of ENPs.

In Paper II it was clearly illustrated that the functionalization of ZnO ENPs affected their uptake, with one functionalization (ZnO-OH) decreasing uptake compared to pristine ZnO ENPs and one functionalization (ZnO-octyl) increasing uptake compared to pristine ZnO ENPs (Skjolding et al. 2014b – Paper II). Different coatings have also been shown to cause different uptake and internalization for QDs (Feswick et al., 2013). It has been proposed that negatively charged ENPs are taken up more than positively charged ENPs (Feswick et al., 2013). However, this is contrary to conventional knowledge that e.g. clathrin-mediated endocytosis favors positively charged particles (Harush-Frenkel et al., 2007; Nam et al., 2009). A reduction in negative charge of Au ENPs by addition of amine groups to the surface resulted in higher ingestion efficiency in daphnia (Wray and Klaine, 2015). Naturally occurring particles generally have negative surface charges. For filter feeders that have a high ingestion of particles it could be beneficial to have mecha-

nisms to exclude unwanted particles. For *D. magna* the mesh size of the filtering apparatus is one mechanism for size exclusion. Additionally, the filtering apparatus have a negative surface charge thus promoting repulsion of smaller particles. This could propose a higher adsorption of positively charged ENPs to the filtering apparatus. Thus, the influence of surface charge could both promote and decrease the potential for uptake *in vivo*.

Consequently, when determining the influence of surface charge one must consider interactions in relation to both the intrinsic properties of the ENPs and the test organism used.

Another issue that could influence bioaccumulation is that of aging. However, OECD TGs is only for testing of the original material hence this matter is considered beyond the scope of this thesis.

For organic chemicals the internalization is governed by diffusive transport across biological membranes. From section 4 it is clear that this type of distribution to organisms will generally not hold for exposure to ENPs. However, toxic effects of ENPs following uptake could possibly be exerted without internalization. Indeed, protrusion of gut epithelia in fish was observed after exposure to $TiO_2$ ENPs without evidence of internalization (Kwon et al., 2015). Furthermore, physical effects of ENPs situated in the gut could be associated with for example reduction of food intake and energy inputs, changes in gut mobility, or effects on nerves or smooth muscle fibers (OECD, 2014). However, such end points have not been carefully studied. Also, the effect of physical interactions with exterior of test organism and its possible interactions with for example behavior is not well understood. While Paper IV did not show significant effects compared to that of control, one study found reduced number of lateral line neuromasts in zebrafish after exposure to Cu ENPs (McNeil et al., 2014). In the same study effects on the rheotaxis behavior was also observed for exposure to both Cu ENPs and Ag ENPs (McNeil et al., 2014). Current OECD TGs do not consider this type of physical or behavioral endpoints and could possibly overlook toxic effects that could cause indirect effects in the environment.

## 5.3 Influence of exposing ENPs through different pathways

In the OECD TG 305 it is stated that "*... revision of Test Guideline 305 is two-fold. Firstly, it is intended to incorporate a dietary bioaccumulation test suitable for determining the bioaccumulation potential of substances with*

*very low solubility*" (OECD, 2012). Most ENPs inherently is of very low solubility thus testing exposure through the dietary phase could be an obvious choice. However, for stable suspensions exposure through aqueous phase could be mimicked and could possibly be an equally important exposure regime (OECD, 2014).

A basic understanding of how the different exposure routes could influence different test organisms is necessary to determine whether one exposure route would constitute a larger risk. In this regard it is important to understand the biology of the different test organisms used. Such understanding is well developed for standardized test organisms e.g. *D. magna* and *D. rerio*. However, the interaction between the biological traits of test organisms and bioaccumulation has not played a major role in relation to conventional chemicals due to diffusive driven bioaccumulation correlated with for example fat content of the test organism for organic chemicals (OECD, 2012).

For ENPs it was highlighted that e.g. the mesh size of the filtering apparatus would create a threshold value where larger particles would be taken up faster than small particles (Kwon et al., 2014). However, for stable ENPs in the size range of 1-100 nm higher uptake of smaller ENPs would be expected as discussed in previous section (Skjolding et al., 2014a – Paper I; Wray and Klaine, 2015). Furthermore, evolutionary traits based on feeding behavior for daphnia promoting repulsion of negatively charged ENPs is another example of biological traits which could result in contradictive results compared to the expected behavior of ENPs based on assumptions for test systems without test organisms. Furthermore, through the feeding behavior of daphnia contact with large amounts of suspended solids already occur thus daphnia could be better adapted to exposure of e.g. aggregated ENPs. While fish would have some mechanisms (e.g. excretion of mucus) to coop with particle exposure the system could be less efficient. These types of differences for each trophic level of test organisms is to some extend implemented in OECD TGs by testing multiple trophic levels. However, the influence of differences in ENPs exposure at different trophic levels is not yet comprehensively understood.

After exposure of *D. rerio* to FPB through aqueous phase and diet differences in localization was observed (Skjolding et al., 2015 – Paper IV). Fluorescent signal was observed in gills, head region, and gut after aqueous exposure while the dietary exposure was mainly associated with the gut (Figure 13 and 14). Consequently, endpoints like behavior (McNeil et al., 2014; Skjolding et al., 2015 – Paper IV) and excessive mucus excretion in the gill region could

be associated with aqueous exposure whereas results of dietary exposure could be effects in the gut region (Skjolding et al., 2015 – Paper IV).

This type of discrepancies shows that one route of exposure would possibly not cover the potential effects of ENPs and that the endpoints to be associated with each exposure pathway would most likely be different too.

In summary, it should be stated that for any successful comparison of studies using ENPs and determining parameters influencing endpoints a comprehensive characterization of the physical identity (e.g. size, state of aggregation, surface charge) have to be carried out initially, and throughout the testing period while also considering the influence on bioaccumulation as presented in this thesis. Effects related to the chemical identity (e.g. ionic exposure due to dissolution) is well covered by OECD TGs and fate modelling while the "nano-specific" effect as discussed herein is not assessed. Furthermore, it has been shown in this thesis that coating and functionalization influence bioaccumulation thus a characterization of this identity would be necessary throughout the test period. When introducing the biological identity (test organisms) the influence on the other parameters is largely unknown. However, using standardized and well characterized test organisms as presented in TGs would enhance our general understanding of mechanisms that could influence endpoints such as bioaccumulation. While this would inherently minimize the value of the results obtained in relation to environment scenarios it would help minimizing the amount of variables in a given test setup thus isolating and identifying novel effects related to the physical identity of ENPs resulting in enhanced interpretation of endpoints assessed.

# 6 Conclusion and recommendations

In order to improve the understanding of intrinsic properties of ENPs and how they influence bioaccumulation in aquatic organisms this thesis have used different particle sizes, coatings and functionalization on two trophic levels (primary consumers (*D. magna*) and secondary consumers (*D. rerio*)). Additionally, different microscopy techniques were used to assess internalization of ENPs in *D. magna* and *D. rerio*. Furthermore, different exposure pathways (aqueous phase and dietary) were assessed for *D. rerio* to investigate possible differences in localization.

This thesis highlighted the need for characterization of both the physical and chemical identity of ENPs opposed to conventional chemicals where the behavior can be largely predicted from partition coefficients based on the chemical identity. It was found that size could affect uptake and depuration in aquatic organisms with smaller ENPs being taken up faster than larger ENPs. However, this behavior was not found for all coatings tested. Furthermore, functionalizations were found to either increase or decrease the uptake potential. Consequently, size, coating and functionalization were shown to affect uptake and depuration.

No internalization in *D.* magna was indicated for the ENPs using multiple microscopy techniques (LSM, FIB-SEM and TEM). It was highlighted that elemental analysis is necessary for correct interpretation of results.

Localization of ENPs in *D. rerio* was investigated using LSM. It was found that aqueous exposure resulted in uptake in gills, head region and gut while dietary exposure resulted in uptake in the gut. Consequently, ecotoxicological tests should be carried out for different exposure routes so possible effects are not overlooked due to the exposure route employed.

As pointed out throughout this thesis a need for comprehensive characterization of the ENPs behavior both initially and while testing is necessary for comparison without misinterpreting results. Consequently, a list describing “minimum characterization” should be implemented for OECD TGs thus also promoting this list of “minimum characterization” in the scientific community. A list of “minimum characterization” could consist of:

- Concentration of ENPs at beginning and end of test.
- A mass balance of ENPs in the test system to account for sorption processes.

- Changes in size of ENPs for the duration of test period in appropriate test medium.
- Dissolution kinetics of ENPs in appropriate test medium.
- Characterization and description of coating or functionalization in terms of potential for detachment, toxic effects, surface charge etc.

While such a list of "minimum characterization" would not account for biological interactions it would create a basis for comparison for example in terms of size which in this thesis and in the literature have been shown to influence bioaccumulation. Furthermore, comprehensively describing the physical identity of ENPs would enable the vast biological information on standardized test species to elucidate biological traits that could explain discrepancies in results.

So far discrepancies in reported results hamper determination of what parameters have the most influence in relation to different ecotoxicological endpoints e.g. toxicity and bioaccumulation. However, it seems most likely that understanding of chemical, physical and biological interactions would be more feasible to achieve in standardized ecotoxicological test systems where a certain level of control is established before expanding to more complex test systems.

Finally, it should be stressed that successful interpretation of all ecotoxicological tests with ENPs will ultimately rely on comprehensive characterization of the ENPs used, especially in relevant test media. This also underlines the immediate need for implementation of some level of physical characterization in OECD TGs.

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# 8 Appendix

**Table 1:** Overview of literature from Web of Knowledge using the search phrase: (topic) bioaccumulation AND (topic) nano* AND (year published) 2013-2015, manually refined to contain papers with aquatic invertebrate or protozoa and aquatic vertebrates. Papers are presented with ENPs used and relevant characterization properties (e.g. dissolution, aggregation, sedimentation), exposure condition, and major findings with regards to bioaccumulation. Abbreviations: d.w. (dry weight), w.w. (wet weight), PVP (polyvinylpyrrolidone), PEG (polytheylene glycol), MH (medium hard), PAAH (poly(allylamine hydrochloride)), PAA (poly(acrylic acid sodium salt) and DLS (Dynamic Light Scattering).

| Category | Organism | ENPs & Properties | Exposure condition | Major findings | Ref. |
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| Aquatic invertebrate or protozoa | *Dreissena polymorpha* | $TiO_2$ ENPs, primary particle size 10.4±3.3 nm, immediate aggregated size after addition to test media ~2500 nm. Sedimentation decreased the concentration in the aqueous phase to ~40% of the nominal concentration. | Exposure for 1h through aqueous phase (concentration 7-120 µg/L), through dietary phase (concentration 4-830 µg/L) or through dietary phase (concentration 100 µg/L) followed by 72h depuration with food source. | Increasing Ti tissue concentration with increasing exposure concentration. Low concentration 1h aqueous exposure 1.2 µg Ti/g mussel, exposure with food present yielded 0.2 µg Ti/g mussel. $TiO_2$ ENPs were easily depurated during 72h when food was administered. | Bourgeault et al. (2015) |
| | *Tetrahymena thermophila* | QDs, polymer coated CdSe/ZnS core/shell structure with terminal carboxyl groups (Qdot® 655 ITK™ Carboxyl Quantum Dots, Life Technologies Europe B. V.), 12 nm. | Exposure for 1h at a concentration of 10 nM QDs. | Time and concentration dependent uptake was observed. The QDs was internalized in food vacuoles. QDs were still present even after 20h of depuration in clean media. | Mortimer et al. (2014) |
| | *Scrobicularia plana* | Cadmium sulfide QDs, 3 to 10 nm. Limited aggregation of larger particles (100-300 nm). 10% Cd ions released after 7d in $NaNO_3$ solution. | Exposure for 14d at 10 µg Cd/L. | No difference in bioaccumulation potential between ionic form (290±70 µg Cd/kg) or particulate form (311±52 µg Cd/kg). | Buffet et al. (2015) |
| | *Macoma balthica* | Ag ENPs, 20 and 80 nm with a PVP coating (Nanostructured & Amorphous Materials, Inc. U.S.A) and a micron sized Ag particle 2-3.5 µm (Sigma Aldrich). | Exposure for 35d at 200 µg Ag/g d.w. sediment. | Body burden increased with time up till day 35 and trended with size: ionic > 20 nm = 80 nm > micron sized particles. | Dai et al. (2013) |

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| *Macoma balthica* | CuO ENPs <100 nm (National Histroy Museum, U.K.), and a micron sized CuO particle <5 µm (Sigma-Aldrich). | Exposure for 35d at 200 µg Cu/g d.w. sediment followed by 15d of depuration in clean media. | Body burden increased with time up till day 35 and trended with size: ionic > 20 nm = 80 nm > micron sized particles. Depuration in soft tissue was only significant for ionic Cu. | Dai et al. (2013) |
| *Drissena polymorpha* | $CeO_2$ ENPs, 3±1 nm, in stock suspension hydrodynamic diameter 8 nm. | Exposure for 94h at concentrations 10 and 100 µg/L/d. | A time dependent increase in body burden was observed peaking after 4 days of exposure (low conc. 8 µg/g d.w., high conc. 15 µg/g d.w.). No significant difference in body burden was observed between the two exposure concentrations. | Garaud et al. (2015) |
| *Mytilus galloprovincialis* | Au ENPs, coated with citrate or PEG in distilled water mean size of 15 and 50 nm respectively. Aggregation in seawater to ~1200 nm (citrate coated) and 850 nm (PEG coated). | Exposure for 24h at concentrations of 23.6 mg Au/L through aqueous or dietary exposure. | Uptake was significantly increased for aqueous exposure compared to dietary exposure. Independent of exposure pathway PEG coated Au ENPs (aqueous and dietary 0.9 and 0.04 and Au/g tissue w.w. respectively) showed higher uptake compared to citrate coated Au ENPs (aqueous and dietary 0.4 and 0.015 mg Au/g tissue w.w. respectively). | Larguinho et al. (2014) |
| *Mytilus galloprovincialis* | QDs with a CdTe core coated with carboxyl groups (-COOH) (PlasmaChem GmbH, Berlin), 2-7 nm. Aggregated in natural seawater (Salinity 36.3±0.07 ‰) to 563±435 nm at pH 8. After 12h of exposure the concentration in the water phase was ~30% of the initial concentration. | Exposure for 21d at a concentration of 10 µg Cd/L followed by a 50d depuration period in the presence of natural seawater. | Cd content in soft tissue was increased for both ionic (~40 µg Cd/g d.w.) and QDs (~18 µg Cd/g d.w.) exposure after 21d compared to control. No significant depuration of QDs was observed over the 50d of depuration. | Rocha et al. (2015) |

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| *Leptocheirus plumulosus* | ZnO ENPs (Meliorum Technologies, Rochester, NY, U.S.A), 20-30 nm. >65% dissolved in seawater after 3d. | Exposure for 10d at a concentration of 500, 1000, 1500 and 2000 µg/g sediment d.w. | A concentration dependent increase in body burden was observed peaking at 1000 µg/g sediment d.w. with a body burden of ~500 µg Zn/g d.w. (no data for >1000 µg/g sediment d.w. due to low survival). | Hanna et al. (2013) |
| *Leptocheirus plumulosus* | CuO ENPs (Sigma-Aldrich), 200-1000 nm. 1% dissolved in seawater after 90d | Exposure for 10d at a concentration of 500, 1000, 1500 and 2000 µg/g sediment d.w. | A concentration dependent increase in body burden was observed peaking at 2000 µg/g sediment d.w. with a body burden of 585±9 µg Cu/g d.w.. | Hanna et al. (2013) |
| *Leptocheirus plumulosus* | NiO ENPs (Sigma-Aldrich) 13.1±5.9 nm. 21% dissolved in seawater after 28d. | Exposure for 10d at a concentration of 500, 1000, 1500 and 2000 µg/g sediment d.w. | A concentration dependent increase in body burden was observed peaking at 1000 µg/g sediment d.w. with a body burden of 1028±44 µg Ni/g d.w.. | Hanna et al. (2013) |
| *Daphnia magna* | Fullerenes ($C_{60}$, 99.9% pure; Yongxin Sci & Tech), 139±10 nm in MilliQ. Changed less than 20% from nominal concentration after 24h pre-equilibration. | Exposure for 48h at a concentration of 0.2 mg/L and depurated for 48h in clean media. | A plateau in body burden was reached within 8 hours. After 48h the body burden was (2268±158 mg/kg). Rapid depuration was observed within the first 6h reaching a plateau with less than 20% of $C_{60}$ taken up. | Chen et al. (2014) |
| *Daphnia magna* | Single-wall carbon nanotubes (cheaptubes.com, Brattleboro, VT), length 750-2000 nm and diameter ~2 nm. Functionalized with either silicon dioxide, poly aminobenzenesulfonic acid or polyethylene glycol. | Exposure for 96h at a concentration of 1 mg/L with daily renewal and feeding. | All tested SWCNTs were taken up in the gut tract. No internalization was observed for any of the tested SWCNTs. | Edgington et al. (2014) |

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| *Daphnia magna* | Graphene layers, sandwich-like $FePO_4$/dodecylamine, Fe concentration <0.005 mg/L. The concenrration decreased 20-25% of the initial after 24h due to sedimentation. | Exposure for 48h at concentrations 50, 100, 250 µg graphene/L followed by a depuration period of 24h. | Concentration and time dependent increase in body burden peaking at 24h (~7400 mg grapheren/kg daphnia d.w. at 250 µg grapehene/L). 90% of the graphene taken up was depurated after 24h in the presence of algae. Significant transfer to offspring was observed. | Guo et al. (2013) |
| *Daphnia magna* | Au ENPs, 20 nm citrate stabilized, after 5h in media aggregated to 291±18nm. | Exposure for 5h at a concentration of 0.6 mg Au/L followed by a depuration period of 24h with food present. | The body burden after 5h reached 400±129 mg Au/kg d.w.. No transepithelial alimentary uptake was observed using TEM. | Khan et al. (2014) |
| *Daphnia magna* | Ag ENPs, coated with citrate, polyvinylpyrrolidone or polytheylene glycol. The order of solubility was PEG Ag ENPs (28.7±0.1%) > citrate Ag ENPs (20.7±1.0%) = PVP Ag ENPs (19.7±0.5%). PEG Ag ENPs released significantly more ions than the other ENPs tested. | Exposure for 24h at concentrations 0-5.5 ug/L as PVP Ag ENPs, 0-18 ug/L as PEG Ag ENPs and 0-7.5 ug/L as citrate Ag ENPs followed by 5d of depuration with food present. | The order of uptake rates was dissolved Ag (6.2±0.07 L/g/d) > PVP Ag ENPs (1.65±0.56 L/g/d) > citrate Ag ENPs (0.87±0.31 L/g/d) > PEG Ag ENPs (0.26±0.09 L/g/d). Fast initial depuration (<1d) was observed followed by a slow depuration (>2d) resulting in ~10% residual body burden after 4d of depuration for all coatings. | Khan et al. (2015) |
| *Daphnia magna* | $TiO_2$ ENPs (P25, Evonik GmbH, Germany). Increase to micron size ~1000 nm within 10h in MH water. | Exposure for 24h at a concentration of 45 mg $TiO_2$/L | Highest uptake shown for aggregated ENPs compared to stable ENPs. A cut-off size for uptake of 280 nm was proposed for *Daphnia magna*. | Kwon et al. (2014) |

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| *Daphnia magna* | $TiO_2$ ENPs (commercially available P25, Evonik GmbH, Germany and laboratory synthesized), 23±7 nm and 5±2 nm respectively. P25 aggregated to several microns in MH water while citrate treated laboratory synthesized $TiO_2$ ENPs had a hydrodynamic diameter of 12±3 nm after 48h. | Exposure for 48h at a concentration of 25 mg $TiO_2$/L | Uptake was correlated with hydrodynamic size, larger $TiO_2$ ENPs were taken up faster than well dispersed $TiO_2$ ENPs. No uptake past the gut epithelia was observed with TEM. | Kwon et al. (2015) |
| *Daphnia magna* | Au ENPs, 10 nm citrate coated. Aggregated to 129±8 nm in tris-acetate phosphate medium. | Exposure for 24h through dietary pathway (algae body burden 71.55±25.33 µg Au/g d.w.). | Au ENPs were taken up through the food source and resulted in a body burden of 76.23±17.63 µg Au/g d.w.. | Lee et al. (2015) |
| *Daphnia magna* | Ag ENPs, polymer coated (90% polymer) (SkySpring Nanomaterials, Houston, U.S.A), 15 nm. In high phosphate (1 mM $PO_4^{-3}$) TAP media 7.1% of the initial concentration dissolved within 1h. Limited aggregation (26.79±1.81 nm) was observed after 5d in high phospoate TAP media. | Exposure for 5d through dietary pathway (algae pre-exposed to 100 µg Ag/L at two different phosphate concentrations high and low, 1.0 and 0.1mM $PO_4^{-3}$ respectively) followed by depuration for 24h in the presence of clean algae. | No difference in total Ag body burden (~0.15 ng/daphnia) was observed in Daphnia fed algae grown at the two phosphate concentrations. Decreased feeding rate was observed for both treatments. | McTeer et al. (2014) |
| *Daphnia magna* | Au ENPs, 10 nm and 30 nm, with two different coatings citrate and MUDA. Increase in size to 229±60, 279±53, 188±48 and 328±61 nm for MUDA 10nm, MUDA 30nm, citrate 10nm and citrate 30nm respectively after 24 incubation in Elendt M7 media. | Exposure for 24h at a concentration of 0.5 mg Au/L followed by a 24h depuration phase. | Significantly different Au content was found after 24h for all exposures. Residual body burdens of 16.1±10.3, 1.2±0.76, 11.2±3.2 and 1.7±1.0 for MUDA 10nm, MUDA 30nm, citrate 10nm and citrate 30nm respectively. Smaller primary particle sizes generally lead to higher residual body burdens. | Skjolding et al. (2014a – Paper I) |

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| *Daphnia magna* | Au ENPs, 6, 20, 30 nm with a citrate coating, and two rods (17.82±2.03 x 58. 08±5.31 nm) with PAAH and PAA coating respectively. Slight aggregation of 6 nm Au ENPs was observed after 24h in MH water (increase from 6 to 13 nm). | Exposure for 48h at concentrations 149 nM to 2522 nM (6 nm Au ENPs), 32 nM to 2399 nM (20 nm Au ENPs), 100nM to 2429 nM (30 nm Au ENPs). Au rods 2.47 nM to 576 nM (PAA) and 33 nM to 733 nM (PAAH) followed by depuration for 48h in presence of food. | Highest uptake rate was observed for 6 nm Au ENPs (5.14±0.39 L/g organism/d) followed by PAAH coated rods (4.63±0.83 L/g organism/d), 20 and 30 nm spheres (2.77±0.25 and 2.68±0.12 L/g organism/d), and PAA coated rods (1.55±0.04 L/g organism/d). Similar pattern was observed for the depuration rates. Generally, surface charge and core diameter were more influential than shape. | Wray and Klaine (2015) |
| *Daphnia magna* | Cu ENPs (purity 99.8%, Io-LiTecGmbh Heibronn, Germany), 50 nm, aggregated to 879±228 after incubation for 24h in ISO standard test medium. After 1h incubation at 0.1 mg/L in ISO standard test medium ~19% of the Cu ENPs were dissolved. | Exposure for 48h at concentrations 0.1 and 0.05 mg/L. | Concentration dependent uptake was observed resulting in a Cu body burden of 264±60 µg Cu/g d.w. at 0.1 mg/L. At 0.1 mg Cu/L exposure 72±12 % of the accumulated Cu was by particle uptake. | Xiao et al. (2015) |
| *Daphnia magna* | ZnO ENPs (purity 99.5% Io-LiTecGmbh Heibronn, Germany), 43 nm, aggregated to 1647±129 after incubation for 24h in ISO standard test medium. After 1h incubation at 1 mg/L in ISO standard test medium 59% of the ZnO ENPs were dissolved. | Exposure for 48h at concentrations 1 and 0.5 mg/L. | Concentration dependent uptake was observed resulting in a Zn body burden of 264±60 µg Zn/g d.w. at 1 mg/L. At 1 mg/L exposure 64±3 % of the accumulated Zn was by particle uptake. | Xiao et al. (2015) |
| *Daphnia magna* | ZnO ENPs, 30 nm, functionalized with OH groups, and octyl groups. After 24h incubation in Elendt M7 the aggregated size was 2020±120, 6460±1660 and 5520±1220 nm for ZnO ENPs, ZnO-OH ENPs and ZnO-octyl ENPs respectively. | Exposure for 24h at a concentration of 1 mg Zn/L. | Higher body burden of all ZnO ENPs was observed compared to ionic exposure (1660±1020 mg Zn/kg d.w.) except for ZnO-OH ENPs where uptake was not significantly different from control. Zn content after 24h uptake was 7690±3580 and 37230±2460 mg Zn/kg d.w. for ZnO ENPs and ZnO-octyl ENPs. | Skjolding et al. (2014) – Paper |

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| *Moina macrocopa* | QDs, CdSe core, ZnS shell, and polymer layer ($COO^-$), 4.6 nm. Aggregated in media to 14±3 nm. No release of Cd was measured (detection limit 0.1 µg/L). | Exposure for 5h through diet to pre-exposed algae (8.79±1.65 ug Cd/g d.w.) | Body burden after 5h was 18.34±23.4 µg/g d.w.. | Lee and An (2015) |
| *Moina macrocopa* | Ag ENPs (Dongyang (HK) International Group Limited, Hong Kong, China), 84.60±14.38 nm. | Exposure for 48h at concentrations 0-3.88 mg Ag/L. | Body burden after 48h was higher for ionic exposure (170.99±22.36 µg Ag/g) than Ag ENPs (61.86±82.75 µg Ag/g) exposure. | Yoo-iam et al. (2014) |
| *Ceriodaphnia dubia* | $TiO_2$ ENPs (99.7% anatase, SD Fine Chemicals Ltd., India), <25 nm. | Exposure for 48h in the presence of algae at a concentration of 1-64 mg/L. | A concentration dependent uptake was observed peaking at 8 mg/L (20±0.003 g/kg) and decreasing in a concentration dependent manner hereafter. The majority (nearly 70%) of the uptake was through diet. | Dalai et al. (2014) |
| *Ceriodaphnia dubia* | $Al_2O_3$ ENPs (Sigma-Aldrich), <50 nm, Aggregates of 871 to 1420 were observed after 48h incubation with test media. Remaining concentration after 24h in test media was in the range of 65.2 to 88.6% of nominal concentration. | Exposure for 72h through dietary exposure with pre-exposed algae (concentration range 20-120 mg/L resulting body burden 0.32±0.09 to 17.28±0.16 µg/g). | A dose and time dependent increase in $Al_2O_3$ ENPs content in daphnia was observed after dietary exposure with body burdens ranging from 0.08±0.01 to 1.94±0.04 mg/kg w.w.. Feeding was skewed towards algae not pre-exposed to ENPs. Reproduction was not impaired after exposure. | Pakrashi et al. (2014) |
| *Gammarus roeseli* | $CeO_2$ ENPs, 3±1 nm, in stock suspension hydrodynamic diameter 8 nm. | Exposure for 94h at concentrations 10 and 100 µg/L/d. | A fast uptake was observed for both concentrations. The high concentration peaked after 1d (59 ug/g d.w.) while the low concentration peaked after 4d (~5 ug/g d.w.). The body burden from the high concentration was found to be statistically different from the body burden found with the low concentration. | Garaud et al. (2015) |

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| *Chironomus* (larvae) | Ag ENPs, <100 nm (Sigma-Aldrich). No dissolution was observed | Exposure for 6, 24, 30 and 48h at concentrations of 0.1, 1 and 10 mg Ag/L | Increasing body burden with increasing concentration (0.1, 1, 10 mg Ag/L) after 48h (7.44±0.18, 152.5±5.2, 1249±33 mg Ag/kg d.w., respectively). | Asztemborska et al. (2014) |
| *Chironomus spp.* | Ag ENPs (Dongyang (HK) International Group Limited, Hong Kong, China), 84.60±14.38 nm. | Exposure for 48h at concentrations 0-3.88 mg Ag/L spiked into sediment. | Body burden after 48h was similar for ionic exposure (38.75±2.05 µg Ag/g) and Ag ENPs (44.94±7.76 µg Ag/g) exposure. | Yoo-iam et al. (2014) |
| *Lymnaea stagnalis* | CuO ENPs, 7 nm, in deionized water a hydrodynamic diameter of 77±5 nm. | Exposure for 24h at concentrations from 4 nM to 31 µM for aqueous exposure and contaminated diet with concentrations 50 nmol/g to 50 µmol/g. | The bioavailability of CuO ENPs was lower than ionic Cu when exposed through water. When exposed through diet, bioavailability of CuO ENPs exceeded that of ionic Cu. | Croteau et al. (2014) |
| *Lymnaea stagnalis* | Ag ENPs, PVP coated, aggregated to a hydrodynamic diameter of 36±1 nm in MH water after 24h of incubation. | Exposure for 2-4h through dietary exposure to pre-exposed algae (412-586 nmol/g d.w.) followed by 48h depuration with food present and three levels of water hardness (10-800 mg $CaCO_3$/L. | Concentration dependent uptake of Ag. There was not found any difference in uptake rates of Ag by change in water hardness. | Oliver et al. (2014) |
| *Peringia ulvae* | ZnO ENPs, 7.8±1.2 nm and ZnO bulk (<2 µm). Aggregates of ~100 nm (~10% by itensity) and 1200 nm (~90% by intensity) was observed after 3h in 17‰ artificial estuarine water. After 7h ZnO ENPs could not be detected in the solution by DLS. 60% of the ZnO ENPs dissolved within 48h. | Exposure for 7d at a concentration of 20 µg Zn/L followed by depuration for 28d with food present. | Higher uptake of ionic Zn and bulk ZnO compared to ZnO NP. Slow release of all forms of Zn presented to the snail (<10% depurated after 28d). | Khan et al. (2013) |

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| *Peringia ulvae* | QDs of CdS and CdSe, 3.1±0.4nm and 4.2±0.8nm, respectively. | Exposure for 4d with 3h feeding per day, with food spiked at different concentrations 50, 100, 200 and 400 µg Cd/g d.w. followed by 96h depuration with food present. | A concentration dependent increase in uptake was observed. Body burden after ionic Cd exposure were higher than both types of QDs. However, the depuration of ionic Cd was likewise faster than both QDs. The uptake for both types of QDs was similar. | Khan et al. (2013) |
| *Potamopyrgus antipodarum* | CuO ENPs, three different sizes 6 nm, 100 nm (Natural History Museum, London, UK) and bulk (<5 µm) (Sigma-Aldrich). Hydrodynamic diameter measured to by 19 nm, 204±1 nm and 813±141 respectively. | Exposure for 14d at a concentration of 240 ug Cu/g d.w. sediment followed by a gut purging step for 24h. | Size significantly affected the Cu body burden. Bulk CuO particles did not differ from control group while 6 and 100 nm CuO ENPs differed from control. Similar body burden for 6 and 100 nm CuO ENPs was found (~320 µg Cu/g d.w.). | Pang et al. (2013) |
| *Potamopyrgus antipodarum* | CuO ENPs, three different shapes; spherical (7±1 nm), rods (8±1 nm x 40±10 nm), platelets (1140±240 nm x 270±50 nm x 30±10 nm). The concentration in the overlying water after 24h of sedimentation in MH water was only significantly different from control after addition of spherical CuO ENPs (42.1±2.2 µg Cu/L). | Exposure for 14d at a mean concentration of 207 µg Cu/g d.w. sediment followed by a gut purging step for 24h and a depuration phase for 14d. | Increased Cu content in tissue (approximate average 210 µg Cu/g d.w. tissue) and shell (approximate average 90 µg Cu/g d.w. shell) was observed for all shapes tested compared to control. However, no difference between shapes or ionic exposure was observed during the 14d of uptake. No significant depuration of Cu occurred over the course of the depuration period. Growth was decreased for platelets and spheres compared to control. | Ramskov et al. (2015) |
| *Potamopyrgus antipodarum* | CuO ENPs, three different shapes; spherical (7±1 nm), rods (8±1 nm x 40±10 nm), platelets (1140±240 nm x 270±50 nm x 30±10 nm). | Exposure for 14d at a concentration of 240 µg Cu/g d.w. sediment followed by gut purging step for 24h. | Increased Cu content in tissue (approximate average 230 µg Cu/g d.w. tissue) were observed for sphere and platelets whereas rods did not show a significant increase in Cu content compared to control. | Ramskov et al. (2014) |

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| Aquatic vertebrate | *Danio rerio* | Ag ENPs, <100 nm (Sigma-Aldrich). No dissolution was observed | Exposure for 8d, and given 2d to evacuate the gut content at concentration of 0.05 mg/L through aqueous or dietary exposure. | Majority of the Ag fraction remains in the gut. After aqueous exposure 57.7±3.6 mg Ag/kg d.w. with the gut content, 0.43±0.06 mg Ag/kg d.w. without gut. Dietary exposure 6477±149 mg Ag/kg d.w with gut content, 0.36±0.09 mg Ag/kg d.w without gut content. | Asztemborska et al. (2014) |
| | *Danio rerio* | Fullerenes ($C_{60}$, 99.9% pure; Yongxin Sci & Tech), 139±10 nm in MilliQ. Changed less than 20% from nominal concentration after 24h pre-equilibration. | Exposure for 7d at concentrations 0.2 mg/L and 2.0, followed by depuration for 48h in clean media. | Concentration dependent increase in $C_{60}$ uptake. Body burden reached 222±30 mg/kg after 3 days of exposure but decreased from day 3 to 7. The residual body burden after depuration was ~30% of the $C_{60}$ taken up. | Chen et al. (2014) |
| | *Danio rerio* | Au ENPs with citrate coating, 14 nm. | Exposure for 20d, at sediment concentrations 16 mg/kg and 55 mg/kg resulting in a water phase concentration of 0.25µg/L and 0.8 µg/L. | Concentration dependent uptake was observed. Highest concentration of Au was observed in the digestive tract (low conc. 0.22±0.03 mg/kg, high conc. 1.4±0.3 mg/kg) followed by gills (low conc. 0.014±0.0008 mg/kg, high conc. 0.029±0.001 mg/kg). No detectable concentration was observed in brain or muscle. | Dedeh et al. (2015) |
| | *Danio rerio* | QDs, CdSe core, ZnS shell, and polymer layer ($COO^-$), 4.6 nm. Aggregated in media to 14±3 nm. No release of Cd was measured (detection limit 0.1 µg/L). | Exposure for 48h with contaminated food (18.34±23.4 µg Cd/g d.w.). | Body burden of Cd was measured to 0.85±1.32 µg Cd/g d.w.). QDs was found in the intestinal bulb and in feces using intravital multiphoton laser scanning microscopy. | Lee and An (2015) |

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| *Danio rerio* | MWCNTs, 4 nm inner and 5 to 20 nm outer diameter, and a length og several µm. The concentration decreased to ~20% of the nominal concentration after 48h | Exposure for 14d at a concentration of 1 mg MWCNTs/L followed by 7d of depuration both in the presence and absence of food. | Highest MWCNTs content was observed after 14d (25 µg). 99.4% was found in the gut, 0.4% in the gills and skin+filet and only 0.01% in blood. After 7d of depuration only 0.4% remained in the fish. Generally, a higher body burden was found in the unfed fish, proposedly due to limited depuration of the gut content. | Maes et al. (2014) |
| *Danio rerio* | $Fe_2O_3$ and $Fe_3O_4$ ENPs, purity >99% with an average size of 80-90 nm and 140-160 nm respectively. After dispersion in test system the Fe content decreased by ~30% for $Fe_2O_3$ at 4.0 mg/L and 10 mg/L. While Fe content in $Fe_3O_4$ exposures decreased by 61% and 79% at 4.0 mg/L and 10 mg/L respectively. | Exposure for 28d at concentrations 4.0 and 10.0 mg/L followed by 24d depuration period with food present. | Maximum Fe content in fish was 1.32±0.37, 1.15±0.26, 1.25±0.27 and 0.90±0.29 for 4.0 mg/L $Fe_2O_3$ ENPs, 10 mg/L $Fe_2O_3$ ENPs, 4.0 mg/L $Fe_3O_4$ ENPs and 10 mg/L $Fe_3O_4$ ENPs respectively. The elimination after 24d was 96.4%, 100%, 100% and 86.0% for 4.0 mg/L $Fe_2O_3$ ENPs, 10 mg/L $Fe_2O_3$ ENPs, 4.0 mg/L $Fe_3O_4$ ENPs and 10 mg/L $Fe_3O_4$ ENPs respectively. | Zhang et al. (2015) |
| *Danio rerio* | ZnO ENPs, 30 nm, functionalized with OH groups, and octyl groups. After 24h incubation in Elendt M7 the aggregated size was 2020±120 and 5520±1220 nm for ZnO ENPs and ZnO-octyl ENPs respectively. | Exposure for 14d through dietary exposure with pre-exposed daphnia (exposed to 1 mg Zn/L for 24h before feeding) followed by 7d depuration period with clean food present. | Body burden at 14d of exposure reached 890±180 mg Zn/kg d.w. and 2170±410 mg Zn/kg d.w. for ZnO ENPs and ZnO octyl ENPs. | Skjolding et al (2014) – Paper II |
| *Danio rerio (embryo)* | Ag ENPs, coated with citrate and starch (0.1% and 0.1% respectively), 50 to 100 nm. Negligible dissolution. | Exposure for 48h at concentrations 100 and 1000 µg Ag/L followed by 24h depuration period in clean media. | A concentration and time dependent uptake was observed peaking at 48h of exposure (body burden low conc. 59.9 µg Ag/kg w.w. and high conc. 1162.0 µg Ag/kg). No depuration was observed during the 24h period. | López-Serrano et al. (2014) |

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| *Barbonymus gonionutus* | Ag ENPs (Dongyang (HK) International Group Limited, Hong Kong, China), 84.60±14.38 nm. | Exposure for 48h at concentrations 0-3.88 mg Ag/L. | Body burden after 48h was higher for ionic exposure (6.89±0.45 µg Ag/g) compared to Ag ENPs (2.79±0.05 µg Ag/g) exposure. | Yoo-iam et al. (2014) |
| *Carassius auratus* | CuO ENPs (Skyspring Nanomaterials, Houston, TX), 20-75 nm, in test media 280-1172 nm (mean 490 nm). 98% dissolution at 1.0 mg/L and 17.5% dissolution at 10 mg/L. | Exposure for 21d, through aqueous (1.0 and 10 mg/L or dietary exposure (1.1-1.3 and 9.3-10.1 mg/L). | Difference in dissolution percentage dependent on exposure route. Accumulation in intestine (261-3183 µg/kg), gills (91-337 µg/kg) and liver (53-153 µg/kg) for dietary exposure and for aqueous exposure intestine (3400-32583 µg/kg) gills (170-1274 µg/kg) and liver (123-350 µg/kg). | Ates et al. (2014) |
| *Carassius auratus* | ZnO ENPs (Skyspring Nanomaterials, Houston, TX)<br>16-50 nm, in test media 423-1275 nm (mean 658 nm). 55% dissolution at 1.0 mg/L and 10% dissolution at 10 mg/L. | Exposure for 21 days, through aqueous (1.0 and 10 mg/L or dietary exposure (1.0-1.3 and 9.7-10.6 mg/L). | Difference in dissolution percentage dependent on exposure route. Accumulation in intestine (295-3710 µg/kg), gills (99-269 µg/kg) and liver (63-199 µg/kg) for dietary exposure and for aqueous exposure intestine (3594-30591 µg/kg) gills (153-1174 µg/kg) and liver (113-350 µg/kg). | Ates et al. (2014) |
| *Cyprinus Carpio* | ZnO ENPs (Beijing DK nano technology Co. Ltd, China), 30 nm, hydrodynamic diameter 850±40. 1% of the initial concentration was found as dissolved after 30 min in 50 mg/L suspension. | Exposure for 30d at a concentration of 50 mg/L. | Zn contents were found to significantly increase in gill (~3950 mg Zn/kg tissue d.w.), liver (~6200 mg Zn/kg tissue d.w.), intestine (~950 mg Zn/kg tissue d.w.) and brain tissue (62 mg Zn/kg tissue d.w.), but not in muscle tissue after 30 days of exposure. ZnO ENPs caused higher level of Zn in all tissue except intestine compared to ZnO bulk. | Hao et al. (2013) |

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| *Oreochromis mossambicus* | Ni ENPs, in MilliQ hydrodynamic diameter 50.69 nm. 84% of the initial concentration remained in the aqueous phase after 24h at 10 mg/L. | Exposure for 14d at concentrations 0.1, 1.0, 10 mg Ni/L. | A time and concentration dependent uptake was observed with highest concentration found in liver (170 mg Ni/kg w.w.), followed by gill (95 mg Ni/kg w.w.) and skin 75 mg Ni/kg w.w.). Alterations in histopathology and morphology were observe din liver, gill and skin tissue. | Jayaseelan et al. (2014) |
| *Oncorhynchus mykiss* | Ag ENPs (Nanocid, Nano Nasb Pars Co., Tehran, Iran), 16.6 nm, a hydrodynamic diameter ranging from 3.9 to 163.5 nm was observed in distilled water. | Exposure for 14d at concentrations 3.2, 10 and 32 mg Ag/L and different salinities (6±0.3 ppt and 12±0.2 ppt) | Ag content in liver, kidneys and gills were significantly different at high salinity compared to moderate salinity at all concentrations tested. In muscle the Ag content was only significantly different in concentrations >3.2 mg Ag/L. | Salari Joo et al. (2013) |
| *Oryzias melastigma* | Ag ENPs (Ted Pella), 80nm with a citrate coating. Average aggregation rates of 46-54.2 nm/h was observed for salinities of 1-30 psu) | Exposure for 6h at a concentration of 87 µg Ag/L at four different salinities (1, 5, 15 and 30 psu) | A reserve correlation was observed between uptake and salinity, with lowest uptake at highest salinity. Highest Ag content (95 ng Ag/g w.w.) was observed after 5h at 5 psu. | Wang and Wang (2014) |
| *Fundulus heteroclutus* *(embryo)* | Citrate coated Ag ENPs, 3 nm. In 10‰ Artificial sea water aggregates between 300 and 20.000 nm after 48h. Dissolution <4 wt% in 10‰ Artificial sea water after 48h. | Exposure for 48h at a concentration of 5.0 mg Ag/L | Ag ENPs accumulated mainly on the surface of the embryo. Accumulation on the surface accounted for 76±2% and 58±4% of the total silver associated with the embryo. | Auffan et al. (2014) |
| *Xenopus laevis* | Ag ENPS, acid coated with proprietary polyacrylic and a carboxylic acid surface functionalization, 2-6 nm. Aggregation in media to ~40 nm. The exposure concentration after 28d in two types of media was 20.0±5.0% and 51.7±11.7% of nominal concentration. | Exposure for 28d at concentrations of 0.018, 0.18 and 1.8 µg Ag/L. | Ag tissue concentration increased in a dose-dependent manner. Tissue content was ~600-1000 and 500 times the measured aqueous Ag concentration for premet-stage and promet-stage respectively. | Carew et al. (2014) |

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# 9 Papers

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**VI** Thit, A., **Skjolding, L. M.**, Selck, H., Sturve, J., 2015, *In vitro* and *in vivo* effects of copper oxide nanoparticles and copper ions to Zebrafish (*Danio rerio*): Effects on Cells, Embryos and Fry. (*Manuscript*)

In this online version of the thesis, the papers **I-VI** are not included but can be obtained from electronic article databases e.g. via www.orbit.dtu.dk or on request from.

DTU Environment
Technical University of Denmark
Miljøvej, Building 113
2800 Kgs. Lyngby
Denmark
info@env.dtu.dk.

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**DTU Environment**
**Department of Environmental Engineering**
Technical University of Denmark

Miljoevej, building 113
2800 Kgs. Lyngby
Denmark

Phone: +45 4525 1600
Fax: +45 4593 2850
e-mail: info@env.dtu.dk
www.env.dtu.dk