Trophic transfer of metal nanoparticles in freshwater ecosystems

should we be concerned?

Tangaa, Stine Rosendal

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Trophic transfer of metal nanoparticles in freshwater ecosystems – should we be concerned?

PhD Thesis

Stine Rosendal Tangaa

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Supervisors

Henriette Selck

Margrethe Winther-Nielsen

Department of Science and Environment

Roskilde University, Denmark

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Preface

The work described in this thesis was mainly conducted at Roskilde University (RUC), Department of Science and Environment (INM), DK and DHI, Department of Environment and Toxicology (EAT), DK, from November 2014 to October 2017. The project was supervised by Professor Henriette Selck (RUC) and PhD Margrethe Winther-Nielsen (DHI). In addition, a 7-month research stay at U.S. Geological Survey (USGS), Menlo Park, California, USA was part of the project. Here, collaboration with PhD Marie-Noële Croteau resulted in the work presented in Paper III.

The thesis includes 4 research papers (two published, one submitted and one draft). In addition, a popular science paper (Danish, published) and a workshop paper has been conducted during the PhD.

Acknowledgements

This PhD project was jointly funded by Roskilde University and DHI through the performance contract with the Danish Ministry of Higher Education and Science. In addition, travel funding was provided by Knud Højgaards Fond and Oticon Fonden, as part of the 7-month research stay in California.

Above all I would like to thank my two supervisors, Professor Henriette Selck and PhD Margrethe Winther-Nielsen. You have pushed me along and made me realize my own potential as a researcher. Our meetings and discussions have been essential for the work carried out during the past three years, and I am forever grateful for the opportunity you gave me by hiring me for this PhD project. And Henriette, thank you so much for standing by me and guiding me through tough times, bot scientifically and personally.

My colleagues at RUC, including our research group Environmental Dynamics, the Ecotox-group, the PhD network and our fantastic laboratory staff, have all contributed to making this PhD an unforgettable journey. A special thanks goes to my office mate and partner in crime, Ronja Windfeld – without you, I would never have survived all the conferences, lab-work or long days at the office. Amaile Thit Jensen, a huge thank you for our scientific discussions and amazing times at conferences and over a cup of coffee in the late afternoons. You two have made work so much fun!

During my research stay at USGS, I met some wonderful people, who all contributed to making the 7-month stay both scientifically fascinating and personally developing. Thank you to Marie-Nöele Croteau for hosting me and helping me with everything from lab-work to interpreting data. I highly appreciate our collaboration and hope to do more work together with you in the future. A special thanks to Samuel Luoma for scientific inputs and great times at conferences. Also, a huge thanks to David Barasch, Matthew Turner and Dominic Ponton – you guys made my stay unforgettable and extremely fun. I will always appreciate our time together!

I would also like to thank Mette Flodgaard and Torben Knudsen for help in the laboratory; Mette Albrektsen and the lab-team at DHI for help with fish cultures; Bjarne Christensen for developing lab-devices for my experiments; Lars Skjolding for scientific discussions over a beer at conferences; Farhan Khan for scientific collaborations; Tim van Driel for endless moral support and technical assistance, and Per Meyer Jepsen for good vibes at the office.

Last, but definitely not least, a huge thanks and appreciation to my friends and family for their understanding and patience, support and comfort during the past three years.

Stine Rosendal Tangaa, 2017

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Abbreviations

ENP	Engineered nanoparticle
Me-ENP	Metal-based ENP
BDM	Biodynamic model
FP	Food packages
dw	dry weight
ww	wet weight
DL	Detection limit
OC	Organic content
NOM	Natural organic matter
IS	Ionic strength
AVS	Acid volatile sulfides
POC	Particulate organic carbon
BCF	Bioconcentration factor
BAF	Bioaccumulation factor
BSAF	Biota-sediment accumulation factor
BMF	Biomagnification factor
TAM	Trophically available metal
ERA	Environmental risk assessment
CSA	Chemical safety assessment
OECD	Organization for Economic Cooperation and Development
ECHA	European Chemicals Agency
REACH	Registration, Evaluation and Authorization of Chemicals
NANoREG	EU-funded project; www.nanoreg.eu
ProSafe	EU-funded project; www.h2020-prosafe.eu

Abstract

Metal-containing engineered nanoparticles (Me-ENPs) are used in a wide range of products, such as inks, plastics, consumer products, lubricants, electronics and bioactive coatings. Silver (Ag) ENPs are one of the most used Me-ENPs to date, primarily due to its antibacterial effects. When entering the aquatic ecosystems, Ag ENPs will undergo several transformation processes, ultimately leading to particles settling out of the water column. This will likely result in an increased concentration of ENPs in the sediment. In fact, predicted environmental concentrations of Ag ENPs in Danish and European freshwater ecosystems range from a few ng/L in surface waters and up to mg/kg in sediments. Several studies have shown Ag ENPs to be toxic, bioaccumulative and harmful to aquatic biota within these concentration ranges. However, research on potential trophic transfer of Ag ENPs is limited.

To investigate the effects and trophic transfer capability of Ag ENPs, a range of experiments was conducted. This includes sediment exposure of worms, biodynamic modelling and trophic transfer of Ag ENPs from worms to fish. In addition, effect assessments via investigation of burrowing, accumulation and mortality of Gold (Au) and graphene oxide (GO) ENPs were carried out. Results were used to interpret how Me-ENPs affects biota in freshwater environments, and if we should be concerned about their transport up the food chain.

Firstly, behavioral patterns of the sediment-dwelling oligochaete *Tubifex tubifex* during sediment exposures to Me-ENPs was investigated. This model species was chosen based on its life-history traits and presence in freshwater environments around the world. *Secondly*, uptake and elimination processes of Ag added as Ag ENPs and $AgNO_3$ after waterborne and sediment exposures in *T. tubifex* was examined. The biodynamic modelling approach was used to characterize Ag uptake from the two different uptake routes as well as to describe the elimination of Ag after waterborne exposures to the two Ag-forms. *Thirdly*, trophic transfer of silver Ag ENPs in a simple freshwater food web, including sediment, sediment dwelling worms (*T. tubifex*) and pelagic fish (*Danio rerio*) was investigated. *T. tubifex* was exposed to sediment amended with Ag ENPs, converted into food packages, and fed to *D. rerio*. In addition, food packages were created from uncontaminated worm-homogenate spiked with Ag ENPs, to test if this standard method gave similar results compared to the sediment exposed worms.

The main results showed that 1) uptake route and Ag-form are important when assessing the bioavailability of Ag to *T. tubifex*; 2) the dietary uptake of Ag is slow, mainly because Ag is not very bioavailable from sediment; 3) using the biodynamic model reveals that diet is more important for Ag ENP uptake at environmentally relevant conditions, and that Ag from $AgNO_3$ is more bioavailable regardless of uptake route; 4) exposure time impacts Ag accumulation following exposure to Ag ENPs, such that *T. tubifex* accumulates higher degrees of Ag added as Ag ENPs from sediment,

when exposure time is increased (i.e. from 8 hours to 15-21 days); 5) Ag was detected in fish after exposure to worm food packages, indicating that trophic transfer of Ag ENPs from sediment to biota is possible; 6) Ag ENPs embedded in sediment and accumulated in worms showed the highest biomagnification factor (BMF = 0.32) in fish; 7) behavioral end-points such as burrowing are highly useful for detecting stress in sediment-dwelling worms.

The main recommendations based on the experimental data produced during this thesis, is that sediment should be incorporated as the main exposure route for assessing bioaccumulation and trophic transfer of Me-ENPs. Natural fluctuating parameters will result in Me-ENPs accumulating in the sediment, causing uptake in benthic organisms, likely leading to re-introduction of Me-ENPs from the abiotic sediment to the biotic freshwater food web. Future studies should concentrate on the internal distribution of Me-ENPs after uptake in both prey and predator, as this will increase the understanding of fate and effects of Me-ENPs on aquatic biota. Trophic transfer studies including more trophic levels, and higher pelagic organisms, are needed to elucidate if and to what degree Me-ENPs will biomagnify.

Keywords: Nanoparticles, Silver, Sediment, Bioavailability, Bioaccumulation, Trophic Transfer, *Tubifex tubifex*, *Danio rerio*

Sammenfatning (Danish abstract)

Menneskeskabte, metal baserede nanopartikler (Me-ENP'er) bliver anvendt i mange forskellige produkter, f.eks. maling, plastik, cremer, elektronik og overfladebehandlings- og forbruger produkter. Sølv (Ag) ENP'er er en af de mest anvendte typer af Me-ENP'er, primært på baggrund af deres antibakterielle egenskaber. Når Ag ENP'er udledes til det akvatiske miljø, sker der en række transformeringsprocesser, som resulterer i at partiklerne fælder ud af vandsøjlen. Denne udfældning vil højst sandsynligt lede til en stigning i ENP-koncentrationen i sedimentet. De miljømæssige koncentrationer af Ag ENP'er i danske og europæiske ferskvandssystemer er modelleret til at ligge mellem få ng/L i overfladevand og op til mg/kg i sedimentet. Mange studier har vist at Ag ENP'er er giftige, bioakkumulative og farlige for akvatiske organismer indenfor disse koncentrationer. Men studier der undersøger trofisk transport af Ag ENP'er er imidlertid meget få.

For at undersøge effekterne og muligheden for trofisk transport af Ag ENP'er, blev der udført en række eksperimenter. Disse inkluderede sediment eksponering af orme, biodynamisk modellering og trofisk transport af Ag ENP'er fra orm til fisk. Derudover blev effekten af Guld (Au) og graphene oxid (GO) ENP'er på ormenes nedgravningsevner, bioakkumulering og dødelighed undersøgt. Resultaterne blev brugt til at forstå hvordan Me-ENP'er påvirker organismer i ferksvands økosystemer, samt om vi bør være bekymrede for deres videre transport op igennem fødekæden.

Først, blev adfærdsmønstrene hos den sediment-levende oligochæt *Tubifex tubifex* undersøgt som funktion af eksponering til sediment tilsat Me-ENP'er. Denne model organisme blev valgt på baggrund af dens biologiske karaktertræk og tilstedeværelse i ferskvandsmiljøer over hele verden. *Dernæst*, blev optag og udskillelse af Ag tilsat som Ag ENP'er eller $AgNO_3$ undersøgt fra både vand og sediment ved brug af *T. tubifex*. Den biodynamiske model blev anvendt til at karakterisere Ag optag fra de to forskellige optagelsesveje (vand og sediment), samt til at beskrive udskillelse af Ag efter vandeksponering til de to Ag-former. *Til sidst*, blev trofisk transport af Ag ENP'er undersøgt i en simpel, ferskvands fødekæde bestående af sediment-levende orme (*T. tubifex*) og pelagiske fisk (*Danio rerio*). *T. tubifex* blev eksponeret til sediment tilsat Ag ENP'er, hvorefter ormene blev omdannet til fødepakker og fodret til fiskene. Derudover blev der fremstillet fødepakker af ukontaminerede orme-homogenat spiket med Ag ENP'er, for at teste om denne standard metode gav forskellige resultater ifht. de sediment eksponerede orme.

Resulaterne viste at, 1) optagelsesvej og Ag-form er vigtige for biotilgængeligheden af Ag for *T. tubifex*; 2) optag fra føde (sediment) er langsom, især fordi Ag ikke er særlig biotilgængeligt når det først er tilsat sedimentet; 3) den biodynamiske model viste at føde bliver mere vigtigt for optag af Ag ENP'er under miljømæsigt relevante forhold, og at Ag fra $AgNO_3$ er mere biotilgængeligt uanset optagelsesvej; 4) eksponeringstid har en

effekt på bioakkumuleringen af Ag, således at *T. tubifex* optager mere Ag fra Ag ENP'er tilsat sedimentet når eksponeringstiden forlænges (i.e., fra 8 timer til 15-21 dage); 5) Ag kunne detekteres i fisk efter eksponering til fødepakker, hvilket indikerer at trofisk transport af Ag ENP'er fra sediment til akvatiske organismer er muligt; 6) Ag ENP'er tilsat sediment og akkumuleret i orme resulterede i den højeste biomagnificerings-faktor (BMF = 0,32) i fisk; 7) end-points relateret til adfærd (f.eks. nedgravningsevne) er brugbare til at bestemme om *T. tubifex* er påvirkede af eksponering til sediment-bundne metaller og Me-ENP'er.

Baseret på de eksperimentielle resultater i denne afhandling, er de vigtigste anbefalinger at sediment skal medtages som optagelsesvej når bioaakumulering og trofisk transport af Me-ENP'er skal bestemmes. Naturlige fluktuerende parametre vil resultere i at Me-ENP'er akkumulerer i sedimentet, hvilket forårsager optag i bentiske organismer. Dette kan meget vel føre til re-introduktion af Me-ENP'er fra det abiotiske sediment til den biotiske fødekæde. Fremtidige studier skal fokusere på intern distribution af Me-ENP'er efter optag i både bytte og rovdyr, da en sådan viden vil udvide forståelsen af hvordan Me-ENP'er påvirker akvatiske økosystemer. Trofisk transport med flere trofiske niveauer og organismer længere oppe i den akvatiske fødekæde er nødvendige for at belyse hvis og i hvilken grad Me-ENP'er biomagnificerer.

Nøgleord: Nanopartikler, Sølv, Sediment, Biotilgængelighed, Bioakkumulering, Trofisk Transport, *Tubifex tubifex*, *Danio rerio*

List of Papers

Papers

The work carried out during and presented in this thesis is based on the four manuscripts listed below:

Paper I: Trophic transfer of metal-based nanoparticles in aquatic environments: a review and recommendations for future research focus; **Stine Rosendal Tangaa**, Henriette Selck, Margrethe Winther-Nielsen, Farhan R. Khan; *Environmental Science:Nano, 2016, 3:966-981 (DOI: 10.1039/c5en00280j)*

Paper II: Bioaccumulation and effects of sediment-associated gold- and graphene oxide nanoparticles on *Tubifex tubifex*; Panhong Zhang; Henriette Selck, **Stine Rosendal Tangaa**, Chengfang Pang, Bin Zhao; *Journal of Environmental Sciences (China), 2017, 51:138-145 (https://doi.org/10.1016/j.jes.2016.08.015)*

Paper III: A biodynamic understanding of dietborne and waterborne Ag uptake from Ag NPs in the sediment-dwelling oligochaete, *Tubifex tubifex*; **Stine Rosendal Tangaa**, Marie-Nöele Croteau, Margrethe Winther-Nielsen, Henriette Selck; *Submitted to NanoImpact (September 2017)*

Paper IV: Trophic transfer of Ag ENPs from sediment to fish in a simple freshwater food web; **Stine Rosendal Tangaa**, Margrethe Winther-Nielsen, Henriette Selck; *Manuscript draft*

Additional studies

In addition, the following publications were concluded during the thesis:

Study I: (Popular science piece; Danish): Ophobes nanopartikler i fødekæden? **Stine Rosendal Tangaa**, Henriette Selck, Margrethe Winther-Nielsen, Farhan R. Khan; *Vand & Jord, 2017, 1:16-19*

Study II: Assessing and managing multiple risks in a changing world - The Roskilde recommendations; Henriette Selck, Peter B. Adamsen, Thomas Backhaus, Gary T. Banta, Peter K. H. Bruce, Allan G. Burton, Michael B. Butts, Eva Boegh, John J. Clague, Khuong V. Dinh, Neelke Doorn, Jonas S. Gunnarsson, Henrik Hauggaard-Nielsen, Charles Hazlerigg, Agnieszka D. Hunka, John Jensen, Yan Lin, Susan Loureiro, Simona Miraglia, Wayne R. Munns, Farrokh Nadim, Annemette Palmqvist, Robert A. Rämö, Lauren P. Seaby, Kristian Syberg, **Stine R. Tangaa**, Amalie Thit, Ronja Windfeld, Maciej Zalewski, Peter M. Chapman; *Environmental Toxicology and Chemistry, 2017, 36:7-16*

Chapter 1: Introduction

Nanoparticles and nanomaterials are substances in the size range of 1-100 nm. Their small size and large surface area-to-volume ratio, are likely making them highly reactive compared to their bulk counterparts (Holsapple et al., 2005). Nanoparticles are found in various shapes and sizes, both naturally occurring and as engineered materials (Klaine et al., 2008; Luoma, 2008). From an ecotoxicological point of view, the engineered nanoparticles (ENPs) are of most interest, as they might affect the natural environments in an unprecedented manner. Metal-based ENPs (Me-ENPs) have attained a high degree of attention during the past decades, as they possess many different application characteristics. They are used in a wide range of products such as inks, plastics, consumer products, lubricants, electronics and bioactive coatings (Vance et al., 2015). These particles enter the aquatic environment (Gottschalk and Nowack, 2011) e.g. via use, waste and effluents, posing unknown threats to biota. Studies have shown that Me-ENPs cause organelle damage, DNA-damage, oxidative stress, apoptosis and alter protein regulation after cellular uptake (Limbach et al., 2007; Midander et al., 2009; Cronholm et al., 2013). Thus, a way to detect and determine the effects of these particles to the environment is highly necessary.

How Me-ENPs interact and affect pelagic organisms in water-only exposure studies are relatively well-known (e.g. (Griffitt et al., 2008; Fabrega et al., 2011)) however, information on the risks and impact on the sediment community is scarce. When ENPs enter the aquatic environment, they will likely interact with components in the water (i.e. natural organic matter (NOM) and different ions) causing them to agglomerate or aggregate. This will result in particles settling out of the water column and ending up on the sediment surface, increasing ENP concentration in the sediment compartment. Thus, the benthic organisms living in the sediment will be exposed, likely affecting their natural behavior. Sediment-dwelling organisms serve many purposes in the benthic environment, such as bioturbation (i.e., particle mixing and irrigation). Deposit-feeding oligochaetes feed head down and excrete fecal pellets on the sediment surface (Rhoads, 1974), contributing to mixing of the sediment, including exchanging interstitial water, dissolved gasses and particles (natural and engineered). In addition, the feeding behavior of worms create burrows, that stabilizes the sediment compartment (Rhoads, 1974). Thus, benthic organisms affect factors such as sediment compaction, porosity and water content, as well as oxidize the top centimeters of the otherwise anaerobic sediment (i.e., the oxygenated zone becomes deeper). This increases microbial degradation, impacting nutrient cycles and biodegradation of contaminants (i.e., surface layer/coatings on ENPs) (Batley et al., 2013). As sediment is an important sink (and accumulation site) for Me-ENPs, the sediment compartment was a main focus in this thesis. Besides using sediment as the main exposure matrix, the importance of uptake route (water vs sediment (diet)) for bioaccumulation and bioavailability of Me-ENPs was tested. By getting a mechanistic understanding of uptake and elimination from different exposure

routes, a better understanding of the mechanisms controlling ENP bioaccumulation and toxicity in benthic biota can be achieved. This will help support risk assessment regarding environmental effects of these relatively new contaminants.

Some studies have investigated trophic transfer of ENPs in the environment, however information is scarce and many knowledge gaps exist (as reviewed in Paper I). In addition, most of the studies examining trophic transfer of ENPs have concentrated on the pelagic food web (i.e. algae, daphnids and fish, e.g. (Bouldin et al., 2008; Skjolding et al., 2014a)), but the benthic community have been largely left out. The overall aim for this thesis was therefore to test whether Me-ENPs can be transported from the abiotic sediment into the aquatic food chain using a simple benthic food web system: sediment to oligochaetes to fish. The experimental part was setup according to OECD guidelines regarding ENPs (e.g. (OECD, 2008, 2014, 2017a)), to ensure results comparable to other studies within the field. By exploring the transfer of Me-ENPs from the abiotic sediment compartment, through the benthic organisms and up to the next food chain level, a broader understanding of the impact of Me-ENPs on the environment was achieved.

Silver (Ag) ENPs are one of the most used ENPs, primarily due to its antibacterial affects (Vance et al., 2015). Several studies have shown Ag ENPs to be toxic (Cong et al., 2014), bioaccumulative (Croteau et al., 2011a) and harmful (Mackevica et al., 2015) to aquatic biota. However, knowledge on the underlying mechanisms controlling uptake of Ag ENPs, as well as their trophic transfer potential in the aquatic food chain is scarce. Ag ENPs was therefore chosen as the Me-ENP in focus for this thesis. Besides detecting the bioaccumulation and trophic transfer potential of Ag ENPs, two other ENP types was chosen as test materials: Gold (Au) and graphene oxide (GO) ENPs. These particles represent different groups of ENPs compared to Ag. Au ENPs are considered an inert tracer, i.e., useful as a particle specific reference (e.g., as described in (Unrine et al., 2010)). Graphene oxide ENPs represents a non-metal group of nanoparticles, employed to get a broader perspective on how ENPs affect the benthic community.

Several factors including environmental (i.e. temperature, pH) and particle (i.e. size, coating) specific characteristics will have an impact on the bioaccumulation potential and bioavailability of Me-ENPs, which will in turn determine if and to what degree Me-ENPs affect biota. Thus, many processes must be accounted for when assessing the risk and exposure of these materials. Previously, Me-ENPs have been treated as their bulk counterparts (i.e. metal ions) in a regulatory manner, even though studies have shown that ENPs can react differently (e.g. (Cong et al., 2014; Thit et al., 2015)). However, projects such as NANoREG and the Horizon 2020 ProSafe have recently resulted in a report with a range of recommendations for reducing uncertainties in regulatory assessment of nanomaterials (ProSafe, 2017), improving the application of established safety measures for ENPs (see section 2.2). The challenges with including these recommendations in environmental risk assessment (ERA), is the lack of consensus in

the scientific community with regards to appropriate test and characterization methods, including a lack of readily available analytical equipment (i.e. as discussed in (Hansen et al., 2016)).

1.1 Project aim

The main goal of this thesis was to examine the availability of sediment-associated Me-ENPs to the deposit feeding oligochaete *Tubifex tubifex*, as well as the trophic transfer of Me-ENPs from worm (*T. tubifex)* to zebrafish (*Danio rerio*). The biodynamic modelling approach was used to assess uptake and depuration kinetics in *T. tubifex* following waterborne and dietborne (i.e., sediment) exposures to Ag ENPs and $AgNO_3$. In addition, bioavailability and bioaccumulation potential of Ag ENPs compared to $AgNO_3$ after addition to sediment was assessed for worms (Paper III). The effect of ENPs on burrowing, mortality and avoidance was assessed, to get a broader perspective on how ENPs affect the benthic community (Paper II). Standard test guidelines by OECD combined with in-house designed experimental setups, was used to investigate trophic transfer of Ag ENPs from sediment – to worms – to fish (Paper IV). In addition, a general look into knowledge, and especially knowledge gaps, regarding trophic transfer of Me-ENPs in aquatic ecosystems created the baseline for the experimental work carried out during this thesis (Paper I). A schematic overview of the framework is presented in Figure 1.

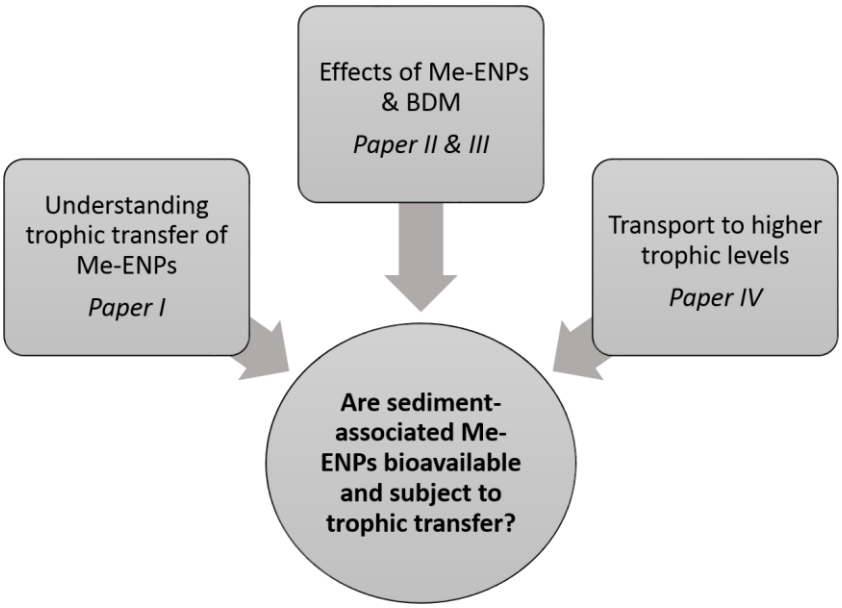


Figure 1: Schematic representation of the framework used in this thesis, displaying the 3 subtopics that support the main question of this thesis, in increasing complexity (from left to right), as well as the papers associated with each. BDM: Biodynamic modelling; Me-ENPs: Metal-based engineered nanoparticles

Chapter 2: Background

2.1 Nanoparticles: interactions and environmental factors

As mentioned, Ag ENPs were used as the main model Me-ENP in this thesis. Ag ENPs are incorporated into products such as textiles, food storage containers and disinfectants, primarily due to its antibacterial effects (Luoma, 2008). Monitoring programs for Ag ENPs in the environment are currently lacking, but modelled predicted environmental concentrations of Ag ENPs in the aquatic environment are in the range of 0.09-320 ng/L for water and 0.15 μg/kg to 14 mg/kg for the sediment compartment (Blaser et al., 2008; Mueller and Nowack, 2008; Gottschalk et al., 2009). Studies have shown that Ag ENPs within these concentration ranges can be accumulated in aquatic biota (e.g. (Cong et al., 2011; Croteau et al., 2011a)), possibly leading to detrimental effects for the ecosystem. In addition to Ag ENPs, Au and GO ENPs were used to test the effects of a broader range of ENPs. Au and GO ENPs are widely used in industry, as both are unique materials for nano-medicine applications such as drug delivery (Zhang et al., 2010; Dykman and Khlebtsov, 2016). Studies have shown that Au and GO ENPs are bioaccumulated and affecting aquatic biota (e.g. (Skjolding et al., 2014b; Cano et al., 2017)), however studies including the benthic community is lacking. To the best of our knowledge, there is no published information on environmental (sediment) concentrations of either Au or GO ENPs. Throughout the thesis, focus will be on Ag ENPs, as Au and GO ENPs were included primarily as reference materials.

When ENPs enter the aquatic environment, they undergo several transformation processes altering their form, including speciation, coating and size (as reviewed in Paper I). Metal-based ENPs are prone to transformations such as dissolution, agglomeration/aggregation and sedimentation. Dissolution of Me-ENPs is affected by particle properties, such as size, surface chemistry and constituent metal. In addition, the composition of the environmental media plays an important role. The interaction of the two will affect the degree and characteristics of dissolution, with values reported in the range of 1-90% for Ag ENPs in various environmentally relevant media (Misra et al., 2012b). Factors such as pH, particle coating and size all affects the degree of dissolution, complicating the understanding of the dissolution potential of Ag ENPs (Misra et al., 2012b). Arguably, dissolution is the most important transformation that Me-ENPs can undergo, changing the metal from a nano-scale structure into its ionic form.

Besides dissolution, factors such as natural and dissolved organic matter (NOM/DOM) and their interactions with Me-ENPs can create new particulate bilayers, affecting the behavior of the particles (i.e. stability in the environment) and the interactions with biota (Philippe and Schaumann, 2014). Thus, when an organism comes into contact with Me-ENPs in the environment, it is not the inert particle but the altered surface that is

"seen" by this organism (Lynch et al., 2007; Lundqvist et al., 2008). Proteins, and especially apolipoproteins, have been shown to adsorb to ENP-surfaces creating coatings known as a "protein corona"(Cedervall et al., 2007). The presence of a surface layer or corona changes the properties and "biological identity" of the ENP, likely promoting particle uptake (i.e. as described in Paper I and (Kim et al., 2007)).

Agglomeration and aggregation is the interaction between particles and refers to the structure of the particle clusters, i.e., loosely or strongly bound, respectively. Aggregation and agglomeration processes affect the size and shape of the particles, and will often result in settling, thereby moving the particles from the water column and into the sediment (Handy et al., 2008b). Aggregation and agglomeration is affected by particle specific factors as well as environmental conditions such as water composition, pH and the presence of NOM (Handy et al., 2008b). Especially agglomeration is affected by the presence of NOM, which can interact with ENPs and increase agglomeration by flocculation or decrease it by electrostatically stabilizing the particles (as reviewed by (Philippe and Schaumann, 2014)). Homo-aggregation occurs when particles of the same material combine and form new and larger particles that have different properties than the single ENP. This process is especially affected by pH as well as the presence (or lack) of coating on the specific particle. Hetero-aggregation is when particles of different composition (e.g. an ENP and colloids (clay and NOM)) interact and create new particular structures (Praetorius et al., 2014). This interaction can also lead to surface alterations, affecting the particles to different degrees. In general, these processes change the bioavailability of the particles, complicating the risk assessment of such materials.

Due to agglomeration/aggregation and the following sedimentation processes ENPs undergo when entering the aquatic environment, the concentration of these ENPs will likely be increased in the sediment compartment. As Ag ENPs enter the sediment, factors such as oxygenation level, concentration of acid volatile sulfides (AVS), and particulate organic carbon (POC), affects their form and bioavailability. Sulfidation is believed to be the most important transformation process for Ag ENPs in sediments (Levard et al., 2011) creating particulate Ag_2S structures (Dale et al., 2013). Depending on the sediment characteristics, and the amount of AVS, POC and oxygen, Ag ENP/Ag_2S particulates may persist in the sediment for several decades (Dale et al., 2013). Due to a lack of readily available analytical methods, the identification and characterization of Ag ENPs after introduction to the sediment is complicated. Thus, we can only assume that the added Ag ENPs remain as a mixture of mainly ionic silver, ENPs and Ag_2S structures when mixed into the sediment matrix. Either way, the addition of Ag ENPs to sediment can result in bioaccumulation of Ag in benthic invertebrates through ingestion or via porewater following dermal uptake (Cong et al., 2014; Ramskov et al., 2015a), increasing the likelihood of trophic transport of these particles through the aquatic food chain.

2.2 Environmental risk assessment

Briefly, environmental risk assessment (ERA) is the frame-work used to predict and prevent risks of anthropogenic contaminants. Risk is based on hazard and exposure, taking both the chemical characteristics of the contaminant and the actual exposure scenario into account (NRC, 1983). When conducting an ERA, three steps are normally incorporated: hazard assessment, exposure assessment and risk characterization (Chapman, 2002). ERA is highly important, as it protects the ecosystems from adverse effects, by evaluating anthropogenic chemicals before they are released into the environment. In Europe, several organizations are in involved in the regulatory process of chemicals, including OECD and ECHA. OECD are responsible for creating test guidelines on measurements and analysis of how hazardous a chemical is (i.e. persistence, toxicity and bioaccumulation potential (PBT)). ECHA is the driving force behind implementing the chemical legislation in the EU, thereby helping producers live up to the regulations set by REACH (van Leeuwen and Vermeire, 2007). REACH is the European chemical legislation, used to regulate chemicals produced within EU. These organizations work closely together to improve the process of risk assessing chemicals. Historically, risk assessment has been based on aquatic scenarios, using pelagic organisms and water-only exposures. However, in the 1980's and 1990's sediment was added to the framework, including the first test protocols for sediment and benthic organisms (as described in (Long and MacDonald, 1998)). Today, OECD have test guidelines (TGs) for sediment-associated contaminants, recommending benthic organisms such as *T. tubifex* as test species (OECD, 2008). However, when considering nanomaterials, specific TGs or ERA approaches have been largely lacking. In 2010, OECD published their first, updated guidance manual with recommendations for testing nanomaterials (OECD, 2010), which was followed up in 2017 with the first actual TG on Ag ENPs (OECD, 2017b). Some of the recommendations for testing ENPs includes characterization data (i.e. composition, morphology and surface chemistry), physical-chemical properties (i.e. aggregation/agglomeration, solubility/dissolution and particle size) and environmental fate (i.e. degradability, adsorption to sediment and bioaccumulation potential) (OECD, 2010). In addition to TG's being updated, large projects on nanomaterials have been conducted over the past years. For example, the EU funded projects NANoREG (www.nanoreg.eu) and Horizon 2020 ProSafe (www.h2020-prosafe.eu) have put great efforts into creating recommendations for new TGs, regulation aspects regarding environmental health and safety of nanomaterials, as described in the newly published report "*Towards a more effective and efficient governance and regulation of nanomaterials"* (ProSafe, 2017). This will improve future ERA on nanomaterials, and create more consensus in the scientific field, by offering more standardized approaches to testing ENPs, both for human health and the environment.

2.3 Bioaccumulation

Bioaccumulation is defined as the net sum of all processes related to contaminant uptake, internal distribution, metabolism (organic contaminants) and elimination by an organism (Ratte, 1999). In simple terms, bioaccumulation equals how much material is taken up minus how much is eliminated. Generally, bioaccumulation is described by a ratio between the concentration of contaminant in the organism ($[M]_{org}$) and the surrounding media ($[M]_{water/sediment}$). Depending on the scenario in question (e.g. water or food/sediment exposure), bioaccumulation is described via a Bioconcentration Factor (BCF), Bioaccumulation Factor (BAF), Biota-Sediment Accumulation Factor (BSAF) or Biomagnification Factor (BMF). All factors are calculated based on an assumption that steady-state is obtained between the organisms and the surrounding environment (i.e. when the concentration inside organism tissue is no longer changing with time) (Spacie and Hamelink, 1995). As presented in Table 1, BCF describes uptake from the water-phase only, including dermal absorption and respiratory intake. This factor is normally used for contaminants dissolved in water. BAF is considering absorption of contaminants from all uptake routes (i.e. water and diet), and is primarily used for monitoring, taking a measurable water-concentration as a proxy for how much an organism will bioaccumulate trough both food and water (Arnot and Gobas, 2006). BSAF describes sediment as a route of uptake, and has been introduced to account for the hydrophobic contaminants and metals often found in the sediment compartment. This value is highly useful for benthic organisms, and can be calculated based on organism lipid content, organic carbon in sediment or simply as the ratio between contaminant concentration in organism vs that found in sediment (OECD, 2008). As sediment is rarely included in risk assessment, no clear thresholds are given for BSAF-values with regards to risk assessment. BMF is describing if a contaminant is increasing in concentration when going from food item to organism, i.e. biomagnification, taking the trophic level into account (Arnot and Gobas, 2006; Hou et al., 2013). BMF can also be calculated based on assimilation efficiency (AE), ingestion rate (IR) and elimination rate constant (k_e) (OECD, 2012), (see section 2.4 and Paper IV for detail)s. These bioaccumulation factors are often used in ecotoxicological studies, as they can give an idea of how concerned we should be if a given contaminant is released to the environment. That is, if the given threshold is exceeded, contaminants are considered bioaccumulative, posing a risk towards biota.

Table 1: Overview and description of factors regarding bioaccumulation of contaminants in accordance with REACH. $[M]_{org}$: metal concentration in organism (µg/g); $[M]_{water}$: metal concentration in water (µg/L); $[M]_{diet}$: metal concentration in food (i.e., sediment) (µg/g); $[M]_{sediment}$: metal concentration in sediment (µg/g); $[M]_{prey}$ and $[M]_{predator}$: metal concentration in organisms (µg/g); f_{lipid}: lipid content of organism (g); f_{TOC}: total organic carbon content of sediment (g); AE: assimilation efficiency (%); IR: ingestion rate (g/g/d); k_e: elimination rate constant (d^{-1}). --- = no clear threshold (Luoma and Rainbow, 2008)

Factor	Description	Calculation	Threshold
BCF	Bioconcentration Factor	$\mathrm{BCF} = \frac{[\mathrm{M}]_{\mathrm{org}}}{[\mathrm{M}]_{\mathrm{water}}}$	BCF>500-5000
BAF	Bioaccumulation Factor	$\mathrm{BAF} = \frac{[\mathrm{M}]_{\mathrm{org}}}{[\mathrm{M}]_{\mathrm{diet}}}$	BAF>500-5000
BSAF	Biota-Sediment Accumulation Factor	$\mathrm{BSAF} = \frac{[\mathrm{M}]_{\mathrm{org}}/\mathrm{f}_{\mathrm{lipid}}}{[\mathrm{M}]_{\mathrm{sediment}}/\mathrm{f}_{\mathrm{TOC}}}$	---
BMF	Biomagnification Factor	$\mathrm{BMF} = \frac{[\mathrm{M}]_{\mathrm{prey}}}{[\mathrm{M}]_{\mathrm{predator}}}$ $\mathrm{BMF} = \frac{\mathrm{AE} \cdot \mathrm{IR}}{\mathrm{k}_{\mathrm{e}}}$	BMF>1 (OECD, 2012)

2.4 Bioavailability and the Biodynamic Model

Bioavailability can be described as "*how much of a compound that is available for uptake/accumulation by an organism summed across all possible uptake routes*"(Luoma et al., 2014). For example, the bioavailability of a metal can be described by how much (i.e. in percent) of the total metal concentration in sediment that is taken up and accumulated in a benthic organism. Factors affecting bioavailability and bioaccumulation includes ingestion rate (IR; g sed/g org/d), gut passage time and assimilation efficiency (AE; % metal assimilated in org). AE represents the proportion of metal that is assimilated after ingestion, and can be used as a proxy to infer metal bioavailability (Wang and Fisher, 1999). These factors are species specific and highly dependent on the organism. *T. tubifex* have been reported to have an IR of 0.43 g dw sed/g org/d (Cammen, 1980), and a recommended gut purging time of 6 h (OECD, 2008) when assessing bioavailability of contaminants. Gut purging time refers to the time organisms need to process one gut-full of sediment (i.e., time from ingestion to egestion). The recommended gut clearance time for *T. tubifex* in uncontaminated media (water or sediment) is 24 h (Gillis et al., 2004; OECD, 2008), thus worms need 24h to empty their gut completely of ingested contaminant after transfer to uncontaminated conditions. *T. tubifex* have been reported to have AE-values of up to 70%, when exposed to sediment amended with Selenium (Se) for 28 days (Dubois and Hare, 2009). In addition, AE-values of 0.1% for Cd and 26% for Zn have been reported after 7-10 days of exposure (Redeker et al., 2004). In

general, the mean AE-value for metals are reported as 4.1% for these worms (Brinkhurst and Austin (1979), cited in (Méndez-Fernández et al., 2014)).

Section 4.1 provides an overview of how natural fluctuating parameters influence the bioavailability of nanomaterials after release to the aquatic environment. Briefly, key environmental factors affecting bioavailability of nanoparticles include pH, ionic strength, NOM and UV-radiation. Natural fluctuating parameters as well as particle specific characteristics all play a role in how bioavailable Me-ENPs are to biota (see Chapter 4 for further discussion).

Bioavailability and bioaccumulation (i.e. body burden) is believed to be the predictors of negative effects of metals and Me-ENPs, such as toxicity responses (i.e. mortality) (Peijnenburg and Jager, 2003). Also, bioavailability is used to describe how likely it is, that a metal or Me-ENP are available for trophic transfer, e.g. Trophically Available Metal (TAM) (Luoma and Rainbow, 2008). In this thesis, bioavailability of Ag ENPs was investigated via use of the biodynamic model (BDM). The model was used to define unidirectional uptake and elimination rates of Ag in two forms (Ag ENPs and $AgNO_3$), giving insights into how Ag are being handled by benthic organisms. The difference between water and diet (sediment) as primary uptake route was tested, using *T. tubifex* as model organism (Paper III). In addition, BDM-parameters determined in Paper III, was used to estimate accumulation potential of Ag in worms (prey) in Paper IV.

The Biodynamic Model

The biodynamic model and associated formulas can be used as an overall description of the flow of a contaminant, by separating the observed concentration in the organism into individual components. Following this formulation, experiments can be carefully designed to target and measure the concentrations and rates described by the formulas independently, thereby accounting for the full flow of a given contaminant as a sum of its contributors. In general terms, the biodynamic model is a tool to separate and investigate the mechanistic processes controlling contaminant bioaccumulation (Luoma and Rainbow, 2005). As presented in Paper III, the model can be used to determine unidirectional metal uptake and elimination by organisms. The uptake and elimination rate constants are determined experimentally, and further used to estimate the overall metal influx to the organism during exposure. Generally, the model can be expressed as:

$$(1)\quad [\mathrm{M}]_{\mathrm{org}} = \underbrace{k_{uw} \cdot [\mathrm{M}]_{w}}_{waterborne\ uptake} + \underbrace{k_{uf} \cdot [\mathrm{M}]_{f}}_{dietborne\ uptake} - \underbrace{k_{e} \cdot [\mathrm{M}]_{org}}_{elimination} - \underbrace{k_{g} \cdot [\mathrm{M}]_{org}}_{growth\ dilution}$$

where $[M]_{org}$ is metal concentration in the organism (nmol/g), $[M]_w$ is metal concentration in water or exposure media (nmol/L) and $[M]_f$ is metal concentration in food or sediment (nmol/g); k_{uw} and k_{uf} are the unidirectional metal uptake rate constants from solution (L/g/d) and food (g/g/d), respectively; k_e is the rate constant for physiological loss (d^{-1}) and k_g the rate constant for growth dilution (d^{-1}) (Croteau et al., 2014b). Most biodynamic experiments are conducted over short time periods, with

no substantial growth of the organisms, making k_g insignificant (Luoma and Rainbow, 2005). By using the determined uptake and elimination rate constants, the metal concentration in exposed organisms at steady-state ($[M]_{ss}$ in nmol/g) can be determined as:

$$(2) \quad [M]_{ss} = \frac{k_{uf} \cdot [M]_f}{k_{ef} + k_g} + \frac{k_{uw} \cdot [M]_w}{k_{ew} + k_g}$$

where k_e is differentiated between the two uptake routes as k_{ef} (from food) and k_{ew} (from water) (Croteau et al., 2014b). The $[M]_{ss}$ expression is highly useful, as it can predict if and when an organism will experience a detrimental internal metal concentration. This is highly relevant for environmental risk assessment, as well as for predictions of possible bioaccumulation scenarios in the field. This way, a relatively short-term exposure experiment can be used to predict the effects of metal or Me-ENP contamination in an area, increasing the protection of the aquatic ecosystems.

Uptake: The uptake rate constant from food (k_{uf}) can be described via the AE and IR of the metal:

$$(3) \quad k_{uf} = AE \cdot IR$$

As mentioned, IR is a measure of the amount of food-associated metal ingested by the organism (g/g/d) and AE represents the proportion of the metal that is assimilated after ingestion (%). IR and AE can be estimated as:

$$(4) \quad IR = \frac{(M_{org} + M_{feces})}{[M]_f \cdot wt_{org} \cdot t}$$

$$(5) \quad AE = \frac{M_{org}}{M_{org} + M_{feces}} \cdot 100\%$$

where M_{org} is the amount of metal within the organism after depuration (ng); M_{feces} the amount of metal in feces after depuration (ng); $[M]_f$ is metal concentration in food or sediment (nmol/g); wt_{org} the dry weight of the organism after depuration (ng) and t is exposure time (d) (Luoma and Rainbow, 2008).

Elimination: The elimination rate constants (k_{ef} and k_{ew}) are determined in experiments where organisms are exposed to a metal, and then allowed to depurate the accumulated metal in clean media. The physiological loss of metal accumulated in tissues can be described as:

$$(6) \quad [M]_{org}(t) = [M]^f_{org} \cdot e^{-kf \cdot t} + [M]^s_{org} \cdot e^{-ks \cdot t}$$

where $[M]_{org}$ is the metal concentration in the organism at a given time, t, during the elimination (nmol/g); $[M]^f_{org}$ and $[M]^s_{org}$ are the metal concentrations in the fast and slow exchanging compartments, respectively (nmol/g); t is depuration time (d); k_f and k_s represents the fast and slow rate constants of loss (d^{-1}) (Khan et al., 2012).

2.5 Trophic transfer

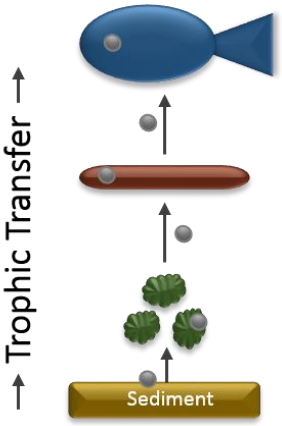


Figure 2: Schematic depiction of trophic transfer of Me-ENPs (grey dots) from sediment to fish via primary producers (i.e. algae, green dots) and benthic organisms, such

In very simple terms, trophic transfer is the transport of contaminants from one level in the food chain to the next (see Figure 2). During trophic transfer, through digestion and respiration, it is estimated that about 10% energy is transferred from prey to predator (Spacie and Hamelink, 1995). Studies have shown that conventional metals biomagnify along the food chain (e.g. (Croteau et al., 2005; Zhao et al., 2013; Cardoso et al., 2014)), and based on such research, the passage of metals through aquatic food webs can be described by two main processes; (1) the accumulation of metal from the surrounding environment by prey organisms followed by (2) assimilation of metal in predators (Rainbow et al., 2006a). When investigating trophic transfer of metals, the concept of TAM is often used. TAM describes the amount of metal that is available for trophic transfer, based on the assumption that the physiochemical form of accumulated metal in prey affects the assimilation of metals by the predator (Rainbow et al., 2011). TAM is highly dependent on the prey-predator relations, as well as the type, form and location of the metal. As the movement of Me-ENPs in the food chain is relatively poorly understood, the factors affecting trophic transfer of conventional metals can be useful in pointing towards the most important processes to study for Me-ENPs (as described in Paper I).

Determining the movement of intact particles in aquatic food webs is difficult due to the transformations occurring after particles enter the aquatic environment, as described in Section 2.1 and Paper I. However, studies have shown that Me-ENP trophic transfer can occur in aquatic food webs (e.g. (Bouldin et al., 2008; Holbrook et al., 2008)). Yet, the number of studies remain low and most have been conducted with simple, two-step food chains including only pelagic organisms (see Table 1 in Paper I). In addition, biomagnification factors, when reported, are variable. When biomagnification is not detected (i.e. BMF<1), that indicates that transfer of Me-ENPs to higher-level organisms are not likely. However, many factors remain unknown, including the mechanistic processes that control trophic transfer and biomagnification of Me-ENPs.

When addressing trophic transfer of Me-ENPs, it is important to clarify when a Me-ENP is considered trophically transferred. In this thesis (e.g. Paper I & IV), all Me-ENPs associated with prey, was considered as available for trophic transfer. This included Me-ENPs adsorbed to the outer surface of prey, found in prey gut lumen, absorbed into prey and taken up into prey cells. Thereby, despite how the particles were associated with prey, if the prey was consumed by a predator, the Me-ENPs was considered to be trophically transferred

Chapter 3: Materials and Methods

3.1 Experimental overview

The experimental work carried out in this thesis, was based on recommendations and TGs provided by OECD. As no actual TGs were available for ENPs at the beginning of this project, we made our best efforts to adapt present TGs. The updates to existent TGs regarding nanomaterials was also considered. To get an overview of the knowledge (and knowledge gaps) within ERA of nanomaterials, an E-learning course regarding these issues was developed in 2015. Together with the review on trophic transfer of Me-ENPs (Paper I), the course was used as baseline for the experimental designs. Environmental realism (i.e. using low exposure concentrations) was implemented, and the question of how natural fluctuating parameters affect the bioavailability of Me-ENPs in the environment considered. The experimental part of the thesis was divided into two main sections:

I) Assessing the uptake and accumulation of Me-ENPs in a model benthic organism (*T. tubifex*)
   a. Effect assessment via investigation of burrowing, accumulation and mortality of ENPs (Paper II)
   b. Mechanistic understanding of Ag ENP accumulation via use of the biodynamic model (Paper III)
   c. Assessment of long-term effects, via 21 days sediment exposure to Ag ENPs

II) Investigating trophic transfer of Me-ENPs
   a. In-depth literature study on trophic transfer of Me-ENPs in aquatic ecosystems (Paper I)
   b. Food package preparation of pre-exposed *T. tubifex*
   c. Dietborne exposure of zebrafish (*D. rerio*) as a proxy for trophic transfer (Paper IV)

In addition, the particles (Ag ENPs) were characterized in artificial freshwater (FW) within the experimental time-frame in Paper III. Au and Go ENPs were characterized as part of Paper II.

Exposure scenarios

To address part I, sediment and water-only exposures of *T. tubifex* were carried out. In Paper II, the main objective was to study general behavior and bioaccumulation in the experimental organism, *T. tubifex*, during sediment exposure to Au and GO ENPs. This was carried out to get a better understanding of the organism, so deviations from its normal behavioral patterns would be easier to detect in the key experiments. In addition, the bioaccumulation study with Au ENPs was setup to assess how worms coped with

and accumulated Me-ENPs from sediment. To test the experimental design, as well as the bioaccumulation pattern of Ag ENPs in worms, a small study was carried out as a collaboration with a bachelor student from Copenhagen University (Ditte Paludan Secher, see (Paludan, 2015)). In Paper III, a mechanistic investigation of uptake and elimination of Ag was carried out. Worms were exposed via water-only or sediment setups, and $AgNO_3$ used as reference treatment as suggested in e.g. (Selck et al., 2016). All exposures were acute (i.e. short time frame). Following uptake from water, worms were allowed to depurate Ag under uncontaminated conditions, to follow their elimination pattern. Paper III is a main part of the thesis, as the understanding of how *T. tubifex* accumulates Ag ENPs is highly important for the understanding of the effects such particles have in freshwater ecosystems. As results from Paper III showed that worms did not accumulate Ag to a high degree under the experimental conditions, long-term exposures (i.e. 21 days at 15°C in complete darkness) of worms were carried out. This data was used to elucidate long-term effects of Ag ENP exposure to *T. tubifex*. In addition, the long-term exposures served as basis for Paper IV, where *T. tubifex* were used as feed for *D. rerio* to detect the degree of trophic transfer of Ag ENPs.

To address part II, a review on trophic transfer of Me-ENPs in aquatic ecosystems was created (Paper I). By taking point-of-departure in the large knowledge base on trophic transfer of conventional metals, this review highlighted the most important factors to address for future studies regarding trophic transfer of Me-ENPs. In addition, the review, and knowledge gained from writing it, was used as baseline for the experimental preparation for the final trophic transfer experiment (Paper IV). To investigate trophic transfer, exposed worms were turned into food packages, by adjusting the method described by (Palmqvist et al., 2006). In addition, food packages were created from spiked worm-homogenate using un-contaminated worms (see section 2.4 and Paper IV for details) and the two methods compared based on the bioaccumulation and biomagnification data from uptake in fish.

An overview of the different exposure scenarios used during the experimental work (i.e. Paper II-IV) is provided in Figure 3.

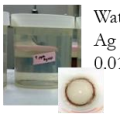
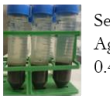
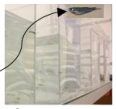
Organism(s)	Paper	Compound	Setup	Exposure time
 *T. tubifex*	II	Au ENPs GO ENPs	 Sediment exposure Au: 10 & 60µg/g GO: 20 & 180µg/g	5 days
 *T. tubifex*	III	Ag ENPs $AgNO_3$	 Water exposure Ag ENPs & $AgNO_3$: 0.01-47nmol/L  Sediment exposure Ag ENPs & $AgNO_3$: 0.4-480nmol/g	4 hours 5-8 hours
 *T. tubifex*  *D. rerio*	IV	Ag ENPs	 Sediment exposure Ag ENPs: 20µg/g  Food packages  Dietborne exposure $2µg/g_{(sedi)}$ $10µg/g_{(C1)}$ $500µg/g_{(C2)}$  +  Spiking w/ Ag ENPs [Ag]: 10µg/g & 500µg/g	Worms in sediment: 21 days Fish: 14 days U & 14 days D

Figure 3: Overview of the different exposure scenarios carried out during the thesis and used for Paper II-IV. ENPs: engineered nanoparticles; Au: gold; GO: graphene oxide; Ag: silver; U: uptake, D: depuration

3.2 Test organisms

Uptake and accumulation patterns of Ag ENPs in *T. tubifex* created the first steps towards understanding if and how these particles affect the aquatic ecosystems. In addition to this sediment-dwelling worm, the freshwater fish *D. rerio* (zebrafish) was used as model predator organism in the trophic transfer study (Paper IV). The origin and culturing methods of both organisms are described below.

Tubifex tubifex

Figure 4: Private photo of T. tubifex

T. tubifex is an omnipresent, sediment-dwelling oligochaete found in freshwater environments worldwide (Lazim and Learner, 1986). It lives in the sediment-water interface, burrowing its head in the sediment and keeping the tail in the overlaying water (Brinkhurst and Jamieson, 1971). Worms create burrows in the sediment, feeding with their head down, and keeping oxygenated via the upright tail (Guérin and Giani, 1996). The main nutrient intake in these worms is via ingesting sediment and extracting any accessible organic material (Cammen, 1980). As sediment consists largely of

inorganic sand and clay grains, the worms must ingest large amounts of sediment to meet their nutritional needs (Lopez and Levinton, 1987). *T. tubifex* have been shown to selectively feed on the smaller sediment particles (<63µm), first noted by Wagner (Wagner, 1968). This is interesting from a bioavailability point of view, as most metals will be associated with the smaller (silt and clay) fractions of the sediment (Rodriguez et al., 2001). The feeding behavior of worms, defecating on the sediment surface, result in a fecal layer on the sediment surface consisting of particles with higher organic content (Rodriguez et al., 2001). This can also result in metals being re-introduced to the sediment-water interface, as they are depurated on the sediment surface (Guérin and Giani, 1996). OECD recommends *T. tubifex* as test organism when assessing bioaccumulation from sediment (OECD, 2008). In addition, these worms serve as prey for higher organisms such as demersal fish (Chapman, 2001), making them highly relevant for the studies carried out during this thesis.

Culturing

T. tubifex were purchased from a local pet shop (Bonnies Dyrecenter, Rødovre, DK) (Paper II & IV) or from Niles Biological Inc. (Sacramento, CA, USA) (Paper III). They were reared in two culture setups, depending on the experimental approach: The aqueous culture consisted of artificial freshwater (FW), prepared according to OECD guideline 203 as recommended when using *T. tubifex* (OECD, 2008), see section 3.3 for details; the sediment culture consisted of natural sieved (<250µm or <125µm) sediment and FW (see section 3.3 for details). Worms in the aqueous culture were fed twice a week with finely ground Tetramin®, just after two thirds of the FW had been renewed with freshly made oxygenated FW. Two thirds of the overlaying water in the sediment culture was renewed once a month. In theory, worms should be able to live of the sediment, however to ensure an appropriate nutrient level, finely ground Tetramin® was added to the sediment culture once a month. For Paper II & IV, worms were kept at 19±2°C, with a light:dark cycle of 16:8h; for Paper III, worms were kept at 15±2°C, in complete darkness. The difference in culturing methods are due to the experiments being carried out at different locations (Paper II & IV at RUC; Paper III at USGS). The literature shows that *T. tubifex* can be cultured successfully in both settings (Redeker et al., 2004).

Danio rerio

Figure 5: Private photo of D. rerio

D. rerio (zebrafish), is an omnivorous freshwater fish, primarily found in tropical regions. Its diet is highly diverse and consists of aquatic and larval form of insects, phytoplankton and zooplankton etc. (Spence et al., 2008). In captivity, it grows up to a length of 5 cm and has a life-span of two to three years (Reed and Jennings, 2011). *D. rerio* is a well-known and highly used model organism in (eco)toxicology, and have been used for toxicity studies over the past several decades. It has been widely studied in several fields, including nanomaterial bioaccumulation studies (Maurer-Jones et al., 2013), and a large database on its physiology, toxicity response and bioaccumulation pattern are accessible

from the scientific literature. Routes of uptake for ENPs (and other contaminants) includes the gut (drinking of water, ingestion of food) and the gills (Handy et al., 2008a). *D. rerio* is recommended as a test organism by OECD with regards to bioaccumulation of different contaminants from food or diet (OECD, 2012). In addition, *D. rerio* is natural prey to higher, predatory fish such as snakeheads (*Channa* spp.) and garfish (*Xenentodon cancila*) (Spence et al., 2008). Together, these biological traits made zebrafish the obvious choice for the trophic transfer setup.

Culturing

Adult zebrafish were purchased from Credo Fish Aps (Nørresundby, DK) and cultivated at DHI (Hørsholm, DK). One to two weeks prior to experimental start, fish were acclimated to the experimental conditions. Fish were kept in aquaria containing FW, prepared in the same manner as for *T. tubifex* and according to official guidelines (OECD, 2012). Organisms was kept in a controlled climate room with a light:dark cycle of 14:10h. Temperature, pH and O_2 was measured regularly, and kept within the limits of 23±2°C, 7.8±0.2, min. 90%, respectively (ISO/TC-147, 1996). The fish loading was kept within the recommended range of 0.1-1.0 g wet weight (ww) fish per liter of water (OECD, 2012). Fish were fed Tetramin® or food packages (FPs) created from *T. tubifex* (see section 3.5 for details). The same conditions were used for fish during acclimation and experimental setups.

3.3 Exposure media

The benthic community was central for this thesis, making sediment a great part of the experiments carried out. In the OECD guidelines, it is recommended to use artificial sediment, to keep the setup as standardized as possible. However, in order to increase the environmental realism and keep the exposure environment as natural as possible, sediment collected in the field was used. Treatment of sediment included sieving, rinsing and determination of organic carbon content (OC). The dry weight (dw)/ww ratio was determined for spiking reasons, as concentrations are normally provided based on dry weight. By providing these parameters (i.e. grain size, OC and dw/ww), it is possible to compare the results presented in this thesis (i.e. Paper II-IV), with other sediment-based exposure studies. The origin, collection and treatment methods for the sediment used during the experiments are provided below.

Sediment

For Paper II & IV, sediment was collected at Isefjorden (Munkholm, Denmark; 55°40'27"N, 11°48'53"E), located away from any potential point sources of contamination. Sediment from this location have been used as control sediment (i.e. uncontaminated) in several studies (i.e. (Dai et al., 2013; Ramskov et al., 2015a)).

For Paper III, sediment was collected from Searsville Lake, a freshwater reservoir located in San Mateo County, CA, USA (37°24'N, 122°14'16"W), which is a part of the

Jasper Ridge Biological Preserve. This location is a known scientific study site, without high levels of metal contamination (Coleman, 2004).

Isefjorden: Surface sediment was scraped off and transported to Roskilde University. Sediment was rinsed and sieved (<125 μm) with deionized (DI) water, and left to settle. After 3-5 days, overlaying water was removed and sediment frozen (-20°C) for min. 24h. Sediment was thawed, rinsed twice in FW, homogenized and left to settle (3 days, room temperature). Overlaying water was removed and the sediment stored at room temperature until use (i.e., within a day). Percentage dry weight and organic carbon content (OC) of sediment were 39±1% and 6±0.6%, respectively. Background silver concentration in sediment was 0.07 μg/g dw.

Searsville Lake: Surface sediment was sampled with an Ekman grab (6x6x6"), and transported to the USGS laboratories. Sediment was rinsed and sieved (<250 μm) in DI-water, homogenized and frozen (-20°C). After 5 days, sediment was thawed, rinsed twice with FW, homogenized and left to settle (3 days, 15°C). Sediment was stored at 15°C in darkness until use. Percentage dry weight and OC of sediment were 40±0.2% and 6±0.4%, respectively. Background silver concentration in sediment was 0.02 μg/g dw.

Artificial freshwater

Artificial freshwater (FW) was prepared according to OECD guideline 203 (ISO 6341-1892), as recommended for *T. tubifex* (OECD, 2008). The same media was used for *D. rerio*, as recommended by the International Organization for Standardization (ISO) (ISO/TC-147, 1996). Briefly, DI-water was mixed with four types of salts (294 mg/L $CaCl_2 \cdot 2H_2O$, 123.3 mg/L $MgSO_4 \cdot 7H_2O$, 63 mg/L $NaHCO_3$, 5.5 mg/L KCl), to obtain a water hardness of 250 mg/L ($CaCO_3$) and a pH between 6.0 and 8.5 (preferably within 7.8±0.2). The FW was aerated for 24-48 h before use, and kept at the temperature employed for the experimental setup (see Paper II-IV for details).

3.4 Test chemicals

Besides the Ag ENPs used as the main model Me-ENP, Au and GO ENPs were used in Paper II. Detailed characterization information on all three ENP-types can be found in Paper III (*Supplementary Information*; Ag) and Paper II (Au, GO), respectively. To be able to determine whether the effects of Ag ENPs was more or less bioavailable or toxic than the conventional metal form, a reference treatment was included in the form of an ionic silver salt, $AgNO_3$. In addition, the use of isotopically labeled Ag was incorporated in Paper III, to increase the detection limit and differentiate newly accumulated Ag from the background concentration in sediment. A brief overview of the test chemicals used throughout the thesis is provided in the following.

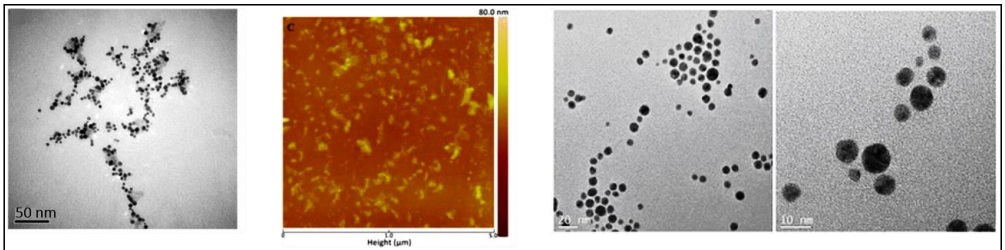
Silver nanoparticles

Paraffin coated Ag ENPs were purchased from Amepox Microelectronics (Lodz, Poland). Particles were provided in a water-solution, with a silver concentration of 1500 ppm (provided by the supplier). Information regarding stability, coating etc. was provided by the supplier, and hydrodynamic diameter, size distribution and aggregation monitored over time periods representative of the exposure setups used in Paper III. Analytical tools included Dynamic Light Scattering (DLS) (Zetasizer Nano, ZS90, Malvern) and UV-vis (UV-1800 Shimadzu). The dissolution of Ag NPs was assessed in FW by centrifugal ultrafiltration (Millipore, Amicon, 3KDa membrane filters).

Gold and Graphene Oxide nanoparticles

Au ENPs were synthesized as part of Paper II, using a method described by (Brust et al., 1994, 1995). Au ENPs were stored in solution creating a stock with a theoretical concentration of 55 µg Au/mL. Characterization of Au ENPs included primary particle size (TEM; JEM-1011, Japan), hydrodynamic diameter and zeta potential in MilliQ-water (Zetasizer Nano, ZS90, Malvern).
GO ENPs were synthesized as part of Paper II, according to a modified method developed by (Hummers and Offeman, 1958) creating plate-formed, multi-layered ENPs. GO ENPs were stored in solution creating a stock with a concentration of 2.09 mg GO/mL. Characterization of GO ENPs included particle size (AFM; NanoScope IIIA Veeco, USA), hydrodynamic diameter and zeta potential in MilliQ-water (Zetasizer Nano, ZS90, Malvern).

An overview of the three used Me-ENPs is provided in Figure 6.



Au ENPs	GO ENPs	Ag ENPs
Spherical	Plates	Spherical
4.9±0.14 nm (TEM)	150 nm (AFM)	10 nm (TEM)
63±0.34 (DLS, MQ)	0.8 nm thick	2-8 nm (size distribution)
-34.4±1.2 (ζ, MQ)	121±3 nm (DLS, MQ)	15-30 nm (UV-vis; FW)
	-60.2±1.2 (ζ, MQ)	148-174 nm (DLS; FW)

Figure 6: Overview of the Me-ENPs used in the thesis (i.e., Paper II-IV). TEM: Transmission Electron Microscopy; DLS: Dynamic Light Scattering; ζ: Zeta-potential; MQ: MilliQ-water; AFM: Atomic Force Microscopy; FW: artificial freshwater

Silver nitrate (reference treatment)

An ionic counterpart to Me-ENPs are often used as reference material when conducting ecotoxicological experiments, to evaluate if the effects of the particles can be related to the released ions. In addition, many studies have been conducted with trace and dissolved metals, creating a large database to compare results with. In this thesis, $AgNO_3$ was chosen as the Ag^+ counterpart to Ag ENPs (Paper III). By comparing the results obtained from the Ag ENPs exposures with that gained from exposures with $AgNO_3$, a relative conclusion can be made regarding toxicity and bioaccumulation. That is, if the effects of Ag ENPs are more or less hazardous to the environment than conventional ionic Ag, and if the effects seen are particle specific. By comparing the two Ag-forms, it can be determined if Ag in particulate form is more or less bioavailable than $AgNO_3$.

For Paper III, the $AgNO_3$ standard (10 ppm in 2% HNO_3) was purchased from High-Purity Standards (Charleston, SC, USA) and used as reference treatment in waterborne exposures. The Ag speciation in FW was estimated at two different Ag-concentrations (1 and 25 nM) using PHREEQC (pH-redox-equilibrium concept) software, developed to model metal speciation in aquatic systems (Parkhurst and Appelo, 2013). Results showed that only 10% of the added $AgNO_3$ was present as freely dissolved Ag (i.e. Ag^+). The main silver complex in FW was estimated to be $AgCl_x$ complexes. No precipitation was observed in the concentrations used, indicating that these complexes were all dissolved in the FW.

In addition to using $AgNO_3$ as reference treatment, the use of isotopically labelled compounds can lower detection limits, and enhance the differentiation between newly accumulated metal from Me-ENPs and background metal (Misra et al., 2012a; Croteau et al., 2014a). In Paper III, isotopically labelled ^{109}Ag was added to sediment as reference treatment in the dietborne exposures, to enhance detection and to distinguish it from background concentrations, as described in (Croteau et al., 2014a). $^{109}AgNO_3$ was purchased from Trace Sciences International (Wilmington, DE, USA) and dissolved in HNO_3 to a final concentration of 171 ppm.

3.5 Experimental procedures

In general, all relevant experimental work carried out during this thesis is described in Paper II-IV, however a brief overview of the procedures is provided here. A schematic overview of the experimental setups is provided in Figure 3.

Sediment spiking

In paper II, sediment from Isefjorden (<125 μm) was spiked with Au or GO ENPs, by adding known amounts of the stock solutions to separate beakers containing wet sediment. Spiked sediment was mixed by hand, covered with parafilm and left on a shaking table for 24h, to obtain homogenous suspensions of sediment and Au or GO

ENPs, respectively. Nominal concentrations of 10 and 60 μg Au/g dw sediment, and 20 and 180 μg GO/g dw sediment were chosen based on data from Au ENPs in soil and GO ENPs in sediment (see Paper II for details).

In paper III, sediment from Searsville Lake (<250 μm) was spiked with Ag ENPs or $^{109}AgNO_3$ stock solutions, by addition to Falcon tubes containing wet sediment. Spiked sediment was mixed on a tube rotator (12rpm) for 24h in complete darkness. Concentrations of 0.4-480 nmol Ag/g dw sediment was obtained for both Ag-forms (see Paper III for details).

In Paper IV, sediment from Isefjorden (<125 μm) was spiked with Ag ENPs, by adding stock solution directly to wet sediment, in order to obtain a nominal concentration of 20 μg Ag/g dw sediment. Beakers were covered with foil and left on the shaking table for 24h. More details can be found in Paper IV.

For all sediment exposure setups, a control with uncontaminated sediment and FW were prepared in the same manner, by adding MilliQ-water instead of Me-ENP stock solutions to wet sediment.

Exposure setups with T. tubifex

Prior to exposure, spiked sediments were divided into smaller exposure beakers and FW added. After ~2h of settling, overlaying water was gently renewed with freshly prepared and aerated FW and worms added. Number of worms depended on the specific setup.

In Paper II, bioaccumulation, mortality, avoidance and burrowing behavior was assessed. Worms were exposed to sediment spiked with Au or GO ENPs for 5 days at 19±2 °C in a controlled climate room (light:dark cycle of 16:8h). All exposure beakers were gently aerated. After 5 days of exposure to GO ENP spiked sediment, worms were rinsed in FW and transferred to uncontaminated sediment. Burrowing behavior was recorded after 3min, 1, 2, 12 and 24h. All exposed worms were rinsed and transferred to uncontaminated FW to empty their guts (6h). Hereafter worms were terminated by freezing (-20°C, 24h). Bioaccumulation was determined by AAS analysis of worm tissue after Au ENP exposure (see section 3.6). Avoidance was assessed by counting the number of worms on the sediment surface at different time slots (1, 12, 24, 48, 72, 96 and 120h) during 5 days exposure to Au or GO ENP spiked sediment. Mortality was determined as number of dead worms at the end of exposure.

In (Paludan, 2015), *T. tubifex* were exposed to Ag ENP spiked sediment in an environmentally relevant concentration (7.1±0.6 μg Ag/g dw sediment). Worms were exposed for 15 days followed by 7 days of depuration in uncontaminated sediment, inspired by recommendations by OECD (OECD, 2008).

In Paper III, *T. tubifex* were exposed to Ag ENPs or $AgNO_3$ added to water (FW) or sediment, to elucidate the influence of uptake route (water vs sediment) and Ag-form

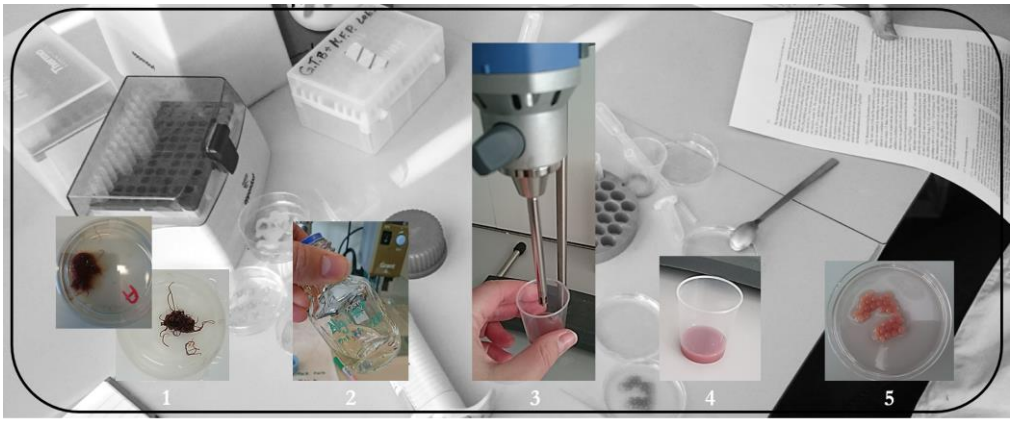
(ENPs vs ions) on bioaccumulation and bioavailability. Worms were exposed to contaminated sediment for 5h ($^{109}AgNO_3$) or 8h (Ag ENPs) at 15°C in a controlled cold room (complete darkness). After exposure, a subsample of worms was rinsed in FW and individually transferred into 1.5 mL centrifuge tubes, in which ~1 g ww of clean sediment had been added along with ~1 mL of FW. Worms were allowed to depurate any unassimilated Ag during a 24h period. Hereafter worms were terminated by freezing (-20°C, 24h). Remaining worms were terminated directly, for determination of bioaccumulation over the different exposure concentrations. Bioaccumulation was determined by ICP-MS analysis of worm tissue (see section 3.6). For waterborne exposure, worms were exposed for 4h to each Ag-form separately, at a concentration range of 0.01-47 nmol Ag/L FW. In addition, elimination after waterborne exposure was assessed, by letting worms exposed to Ag ENPs (58±1 nmol Ag/L) or $AgNO_3$ (3±1 nmol Ag/L) for 48h, depurate any accumulated Ag in uncontaminated FW for up to 20 days.

In Paper IV, long-term bioaccumulation of Ag ENPs was determined, prior to food package (FP) creation (see below for details). Worms were exposed for 21 days at 15±2°C in a controlled climate cabinet (complete darkness). All exposure beakers were gently aerated. After exposure, worms were rinsed and transferred to uncontaminated FW to empty their guts (6h). Hereafter worms were terminated by freezing (-20°C, 24h). Bioaccumulation was determined in a sub-sample of worms by ICP-MS analysis of worm tissue after Ag ENP exposure (see section 3.6). Remaining worms were kept frozen until FP production.

Food packages for trophic transfer study

Food packages were prepared after a method described in Palmqvist *et al.* (Palmqvist et al., 2006), adjusted to fit the used experimental conditions. Two approaches were used to turn *T. tubifex* into food packages for the zebrafish. The reason for using food packages and not live worms for the trophic transfer experiment, was to ensure uniformity in the exposure concentration for the fish. This was obtained by homogenizing exposed worms and using equal amounts of worm homogenate for each food package. In the second approach (i.e. spiking of food packages), uncontaminated worm homogenate was spiked to obtain two different Ag-concentrations in the final food packages. The method is briefly described below.

Figure 7: Overview of the method used to create food packages of T. tubifex *(inspired by* (Palmqvist et al., 2006)). *Numbers 1-5 refer to the order of the steps carried out and described below.*



Frozen worms were thawed and mixed with FW (2 mL per 1.2g worms). Sodium alginate was prepared by mixing 159 mg alginic acid sodium salt (Sigma-Aldrich, CAS: 9005-38-3) with 10 mL FW, heating until dissolution and cooled down (<35°C). Worms were homogenized using a micro homogenizer (VWR, VDI 12). The alginate-solution was added to the worm homogenate in the ratio of 1:1 and mixed well. A 2% $CaCl_2$-solution was prepared in DI-water and added to a petri dish (enough to cover the bottom). Small droplets of the worm:alginate mixture was added to the $CaCl_2$-solution, creating gelated, spherical food packages. Food packages were stored cold and dark in FW to keep their form and consistency intact. FPs had a mass of 2.0±0.8 mg ww and a diameter of 1.1±02 mm (n=60) (see Figure 7).

Exposure setup with D. rerio

Before conducting the final trophic transfer experiment, a pilot study was carried out to test whether the experimental conditions were optimal (i.e., exposure time and feeding process). *T. tubifex* was exposed to natural sediment (<125µm) spiked with Ag ENPs for 21 days, at a concentration of 77.9±1.4 µg Ag/g dw sediment. Food packages (FPs) were created as described above. The main aim was to test whether fish would eat the FPs and if it was possible to detect any Ag in fish after exposure. Fish were exposed together in 20L tanks and fed FPs created from uncontaminated or sediment exposed worms. Results showed that Ag was detectable in fish gut/intestinal tissue after 10 days of exposure (i.e., $[Ag]_{gut}$: 3.47±0.38 µg Ag/g dw). Fish did eat the FPs provided, however, the setup prevented a controlled feeding, and hence it was not known if all fish were fed with the same amount of FPs. Therefore, the final experimental setup was altered so fish were exposed individually.

For the final trophic transfer experiment (Paper IV), fish were exposed for 14 days to four different treatments including control (AgS: FPs from sediment exposed worms (1.8µg/g ww); AgC1: FPs from spiked worm homogenate (10µg/g ww); AgC2: FPs

from spiked worm homogenate (500µg/g ww)), followed by 14 days of depuration where all fish were fed uncontaminated FPs. The exposure was carried out in a flow-through system, with water being fully renewed in all aquaria once per day. Fish were kept in 20L glass aquaria, equipped with a splitting device, creating 6 compartments for fish in each aquarium (one fish in each compartment). This was done to ensure individual exposure, and thereby decrease the degree of uncertainty when analyzing the results. The splitting devices were created at RUC by Bjarne Christensen, and added to the aquaria (i.e. attached with aquarium sealant and fixed with aquarium vat). Each device contained holes covered with net, to ensure exchanges of water and O_2, as well as keeping fish inside their own compartment. Before introducing fish to the system, all aquaria were rinsed twice in FW (i.e., filled and emptied) and fresh FW added. All aquaria were aerated, and temperature, pH and O_2 measured regularly, and kept within the limits of 23±2°C, 7.8±0.2, min. 90%, respectively (ISO/TC-147, 1996). Pictures of the device are presented in Figure 8.

Figure 8: Splitting device added to aquaria to keep fish separated during the experiment, thereby ensuring a more controlled feeding process.

3.6 Chemical analysis

As bioaccumulation and trophic transfer of Ag ENPs was a focus of this thesis, techniques suitable for detecting metal concentrations in biological samples were used throughout the experimental phase. For Paper III, analyses were carried out at the equipment available at USGS, whereas apparatus at RUC were used for Paper II and IV.

ICP-MS

Inductively Coupled Plasma-Mass Spectrometry (ICP-MS) was used to identify the level of Ag bioaccumulation in all samples (Paper III and IV). This technique is highly useful for characterizing low levels of metals in biological samples, and can detect several different elements and isotopes in one sample. This technique is crucial when working with low, environmentally relevant concentrations. For the biodynamic modelling study (Paper III), ICP-MS (NexION 300Q, Perkin-Elmer; DL: 10 ng Ag/L) was used to both

identify total Ag concentration in sediment, water and worms, as well as distinguish any newly accumulated Ag via use of isotopically enriched ^{109}Ag. In the trophic transfer study (Paper IV), ICP-MS (Agilent 7900; DL: 0.8 μg Ag/L) was used to identify Ag bioaccumulation in sediment, fish, food packages and water samples. Digestion procedures are described in Paper III and IV.

AAS

Graphite Furnace Atomic Absorption Spectroscopy (GF-AAS; GTA 120, Varian) was used to identify Au level in sediment and worm samples in Paper II. Digestion procedures are described in Paper II. AAS is similar to ICP-MS, however only one element can be detected at a time, and isotopes are not distinguishable. In general, this is a good technique for samples which are believed to contain high amounts of metal, as the detection limit is generally higher for AAS compared to ICP-MS.

3.7 Statistical analysis

All statistical analyses were performed using SYSTAT (version 13.1). Datasets were tested for normal distribution and variance equality using Kolmorogov Smirnoff and Levenes Test, respectively. One-way analysis of variance (ANOVA) was used when conditions were met. Otherwise, the non-parametric Kruskal-Wallis test was used. Tukeys test or Conover-Inman was used for comparison among treatments, if ANOVA or Kruskal-Wallis showed a significant effect, respectively (Paper II-IV). For pairwise comparisons, a two-sample t-test was performed. Regression analyses were used to determine if rate constants differed significantly from zero (Paper III). Statistical significance was obtained if $p \leq 0.05$.

Chapter 4: Results and Discussion

To sum up the key findings, main results obtained in Paper I-IV of this thesis are presented and discussed here. In addition, the interactions between Me-ENPs and naturally fluctuating parameters with regards to bioavailability is highlighted. To sum up the entire thesis, a section on ERA and how the results obtained herein can be used to optimize the procedures for testing Me-ENPs is presented.

4.1 Natural fluctuating parameters and bioavailability of Me-ENPs

The effects of natural fluctuating parameters on bioavailability of Me-ENPs once released to the aquatic environment were investigated and presented in a method document for internal use at DHI. Four key factors (i.e. NOM, pH, ionic strength (IS) and UV-radiation) were chosen based on a literature review. The main effects are presented in Figure 9.

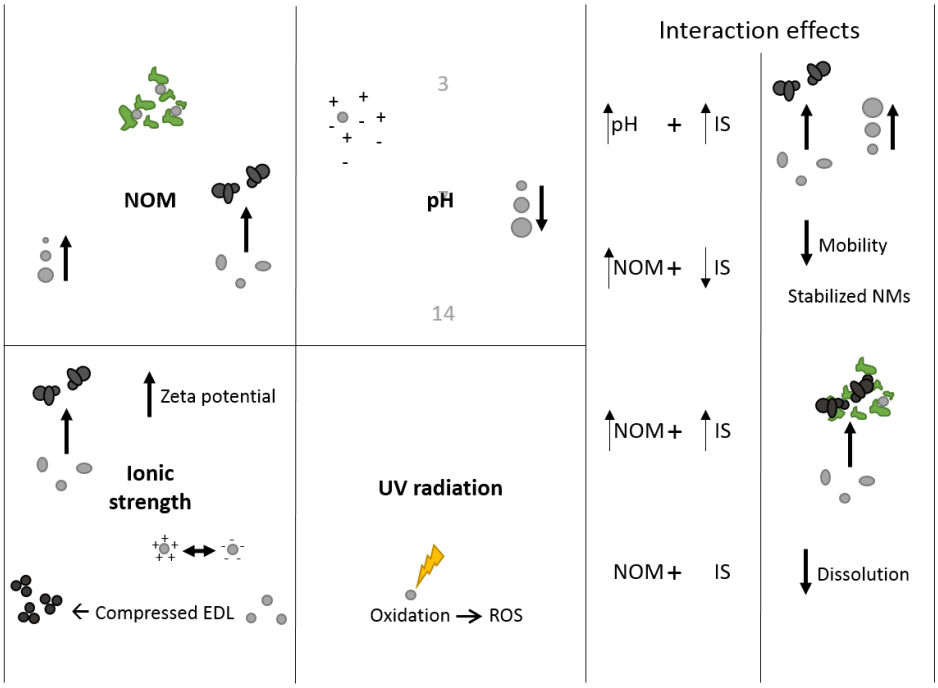


Figure 9: Schematic representation of how the four key natural parameters influencing bioavailability (NOM, pH, Ionic Strength (IS) and UV-radiation) interacts and affects Me-ENPs in the aquatic environment. Light grey represents single particles; dark grey indicates aggregated/agglomerated particles; green represents NOM; arrows indicates direction of processes (i.e. increased or decreased; smaller or larger particle sizes; leads to). EDL = electric double layer; ROS = reactive oxygen species; NMs = nanomaterials.

Depending on the Me-ENP type, different factors will affect the transformation processes (e.g. aggregation, dissolution), however some general traits exist.

NOM: For Ag ENPs increased amounts of NOM in the water will likely result in increased stabilization of particles, due to production of new coatings. This could keep ENPs in suspension longer, resulting in higher bioavailability for pelagic organisms (Cumberland and Lead, 2009; Wagner et al., 2014). In addition, the new surface layer could result in less dissolution thereby decreasing toxicity (i.e. less free Ag-ions) (Cross et al., 2015). The addition of NOM (i.e. oxalic and adipic acids) to Me-ENP surfaces, might lead to less mobility in the sediment, increasing the risk of "hot spots" for Ag ENP contamination (Cross et al., 2015).
Ionic strength (IS): Increased ionic strength (IS) can lead to compression of the electric double layer (EDL), thereby reducing electrostatic repulsion and increasing aggregation of the particles. This can lead to both increased hetero- and homoaggregation (Cross et al., 2015). Furthermore, increased IS can lead to increased instability and aggregation of Ag ENPs, which could result in increased sedimentation and thus increased availability for benthic organisms (Cumberland and Lead, 2009).
pH: Changes in pH are known to affect Me-ENPs. For Ag ENPs an increase in pH (i.e. from 5 to 8) in aquatic solution results in agglomeration/aggregation and thereby decreased bioavailability for pelagic organisms (Cumberland and Lead, 2009). On the contrary, a decreased pH (i.e. <7) results in increased dissolution, likely causing higher toxicity to pelagic organisms (e.g. (Liu and Hurt, 2010)).
UV-radiation: UV-radiation is known to degrade Ag, and it has been shown that Ag ENPs exposed to UV-light reduces toxicity causing altered bioavailability of the particles. Furthermore, Cheng *et al.* showed that Ag ENPs aggregated irreversibly when exposed to sunlight, with the UV part of the light being the driver of this aggregation (Cheng et al., 2011).
Besides each parameter affecting bioavailability, some interaction effects between IS and NOM have been detected, increasing the complexity of understanding how environmental factors affect Me-ENPs (see Figure 9). For example, increased NOM and decreased IS can result in less mobility of ENPs, as well as a higher stability of particles in solution (Cumberland and Lead, 2009). This might lead to higher bioavailability for pelagic organisms.

Besides natural fluctuating parameters affecting bioavailability of Me-ENPs, organism characteristics will have an impact on how much metal that is accumulated within their tissue. That includes feeding strategy (e.g. filter feeder or sediment dweller), primary habitat (e.g. water, sediment or both) and uptake mechanisms (e.g. endocytosis, subcellular distribution). In Paper III, we observed AE-values of <1 % for *T. tubifex* exposed to sediment amended with Ag ENPs (0.1-0.8%). This low bioavailability might be due to the exposure setup or the organisms. Other studies have shown higher AE for Me-ENPs, for example for the benthic snail *Lymnea stagnalis* (AE(Ag): 49-58 %, (Croteau et al., 2011a)) or the sediment-dwelling oligochaete *Lumbriculus variegatus* (AE(CuO): 24-41 %, (Ramskov et al., 2015b)). This highlights that organism type does have an influence on the bioavailability of Me-ENPs, however other factors such as metal-type and exposure scenario will also affect these differences. The low AE-value observed for

T. tubifex in Paper III indicates that these worms are not good bioaccumulators of Me-ENPs from sediment under the used experimental conditions.

Generally, bioavailability is an important factor to asses when dealing with anthropogenic contaminants. For example, if Me-ENPs are released to the environment, but not accumulated or taken up by biota, the risk of their use might be non-existing. Therefore, the many factors influencing bioavailability of Me-ENPs, or at least the most important ones when working with the aquatic ecosystems (as presented in Figure 9) should be accounted for in experimental setups. This is especially important when trying to compare data between studies, as different exposure scenarios will have a big influence on the bioavailability and bioaccumulation potential of ENPs. Therefore, dissolution and particle size in the experimental media (i.e. artificial FW) were determined for the used Ag ENPs prior to exposure (Paper III). In addition, speciation was estimated in FW using specialized computer software (as described in Chapter 3 and Paper III).

4.2 Assessing the uptake and accumulation of Me-ENPs in *T. tubifex*

To gain knowledge on how the chosen model organism, *T. tubifex*, accumulated and handled Me-ENPs, a few studies was carried out (i.e. Paper II and (Paludan, 2015)). This way, an understanding of how benthic worms cope with Me-ENP contamination was achieved, giving insights into valuable end-points when dealing with Me-ENPs found in sediment. Furthermore, a range of experiments designed to assess the unidirectional uptake and elimination rate constants of Ag in *T. tubifex* were setup (Paper III), to get a mechanistic understanding of how such worms handle exposure to toxic metals and metal nanoparticles.

4.2.1 Observations on behavior & Experimental design

Results from Paper II showed that worms were capable of bioaccumulating Au ENPs from sediment after 5 days of exposure, with no sign of stress or mortality observed. In addition, worms were negatively affected by the presence of GO ENPs in sediment, as they showed decreased burrowing behavior after exposure. To our knowledge this study was the first to show that these worms are bioaccumulating metal originating from sediment spiked with Me-ENPs, with high body burdens (12.5 and 65.8 μg Au/g dw tissue at exposure concentrations of 8.5±2 and 70±7 μg Au/g dw sed., respectively) detected after only 5 days of exposure. These results highlight that an inert metal like Au is available for uptake in *T. tubifex* and that burrowing is a good, non-lethal endpoint highly useful of detecting if worms are stressed.

In addition to Paper II, results from (Paludan, 2015) showed that worms did in fact accumulate Ag from sediment, and this uptake was conducted in a two-phase manner. Worms were accumulating Ag fast within the first part of the exposure, followed by a

leveling off resulting in a slower accumulation profile. Maximum body burden in worms were detected after 15 days of exposure (5.6 μg Ag/g dw tissue). In addition to accumulating Ag, worms were able to eliminate at least part of the metal again when transferred to uncontaminated sediment. As with uptake, depuration was fast in the beginning, but leveled off after 3 days, with body burdens being somewhat stable at around 40% of the Ag concentration in tissue after 15 days of exposure (i.e. end body burdens of 2.5 μg Ag/g dw tissue).

A two-compartment elimination pattern was also observed for *T. tubifex* exposed to sediment spiked with Ni, Cd and Pb (Gillis et al., 2004), indicating that these worms store metal and Me-ENPs in both a loose and more tightly bound pool. This could have consequences for predators feeding on *T. tubifex*, as metal tightly bound will remain in organisms, increasing the likelihood of trophic transfer. Other studies have shown *T. tubifex* to be an efficient bioaccumulator of metals such as Ni, Cd and Pb during both short (96h) (Bouché et al., 2000) and long term (6 weeks) sediment exposures (Gillis et al., 2004). In addition, impacted burrowing activity has been observed for *T. tubifex* exposed to metal-contaminated field sediment (Méndez-Fernández et al., 2014).

4.2.2 Importance of exposure route for Ag uptake in T. tubifex

After determining that *T. tubifex* accumulates Ag ENPs from sediment, the underlying mechanisms responsible for this accumulation were investigated in Paper III. Results showed that worms accumulated Ag from $AgNO_3$ more efficiently than from Ag ENPs during waterborne exposure, i.e., the Ag uptake rate constants from water was 8.2 L/g/d for $AgNO_3$ and 0.34 L/g/d for Ag ENPs. Silver accumulated from either form was efficiently retained in tissues as no significant loss of Ag was detected after up to 20 days of depuration in uncontaminated media. High mortality (~50%) during depuration (i.e. after 17 days) was only observed for worms exposed to waterborne $AgNO_3$ (3 nmol/L). Sediment exposures to both Ag forms resulted in low accumulation, i.e., the uptake rate constants for $AgNO_3$ was 0.002 g/g/d and that for Ag ENPs was 0.005 g/g/d. Inference of bioavailability from estimations of Ag assimilation efficiencies suggest that Ag from both forms (AE: 3-12% for $AgNO_3$ and 0.1-0.8% for Ag NPs) is poorly bioavailable from sediment in these worms. Body burdens (in nmol Ag/g dw tissue) of 0.7±0.2 to 168±15 for $AgNO_3$, and from 0.6±0.2 to 16±2.4 for Ag ENPs were observed after exposure to waterborne Ag at a concentration range of 0.01 to 47 nmol Ag/L. For sediment, body burdens of 0.1±0.03 to 0.2±0.2 for $^{109}AgNO_3$, and 0.5±0.1 to 1.2±0.5 for Ag ENPs were detected, after exposure to contaminated sediment (0.4 to 480 nmol Ag/g dw sediment). Overall, results from Paper III indicates that uptake route and Ag form are important for the bioaccumulation and bioavailability of Ag in *T. tubifex*. $AgNO_3$ was more bioavailable regardless of uptake route compared to ENPs, under the used experimental conditions.

Studies on metal accumulation from sediment in *T. tubifex* have shown that these worms are not the best bioaccumulators of metals such as Zn, Mg, Cu and Pb, which the

authors attributed to the high defecation and metabolic rates of worms (Kaonga et al., 2010). This is consistent with the low accumulation of Ag from sediment observed in Paper III. In contrast, Kaonga *et al.* observed that *T. tubifex* accumulated Cd in significant amounts from contaminated field sediment (Kaonga et al., 2010). Likewise, we observed a significant accumulation of Au ENPs and Ag ENPs from sediment in Paper II and (Paludan, 2015), respectively. These results indicate that both the metal type and exposure time has an influence on the metal bioaccumulation pattern in worms.

As mentioned, worms exposed to $AgNO_3$ showed deterioration and mortality after 17 days of elimination in uncontaminated FW. The degeneration might be a way for the worms to protect themselves from increased internal metal concentrations (Lucan-Bouché et al., 1999). Degeneration was not observed for worms pre-exposed to waterborne Ag ENPs, suggesting a different internal fate for Ag from Ag ENPs compared to $AgNO_3$. DeJonge *et al.* showed that *T. tubifex* stores metals in different subcellular compartments, depending on the metal type. Cd, Pb, Ni and Cr were mainly stored as biological detoxified metal (i.e., in heat stable proteins and metal rich granules), whereas Cu, Zn, As and Ag were generally found in the metal sensitive fraction (i.e., organelles and heat denatured proteins) (De Jonge et al., 2011). This could explain the high mortality observed for worms exposed to $AgNO_3$, i.e., if worms were simply not able to eliminate or detoxify the accumulated Ag, when present as ionic Ag. However, subcellular fractionation was not part of this thesis, so further studies are needed to elucidate if this is the case. In (Paludan, 2015), worms showed a slow elimination pattern during a depuration period of 7 days in uncontaminated sediment. Together with the slow elimination pattern for Ag ENPs after waterborne exposure in Paper III, these results indicate that if worms accumulate Ag ENPs (from water or sediment), they will retain at least part of the Ag (i.e., ~40% in (Paludan, 2015) and ~70% in Paper III). Thus, trophically transporting Ag ENPs (or intracellularly dissolved Ag ENPs) from worms to fish is possible.

Behavioral differences were observed for worms exposed to the two Ag-forms, with $AgNO_3$ resulting in avoidance (i.e. worms stayed on the sediment surface during exposure), followed by decreased burrowing behavior when transferred to uncontaminated sediment. This pattern was not detected for worms exposed to Ag ENPs (or in control). A similar behavior was observed for the sediment-dwelling worm *Neries diversicolor* after exposure to Cu-spiked media. Here ionic Cu had a negative effect on burrowing time, which was not observed for CuO ENPs (Buffet et al., 2011). However, the opposite trend was seen when *N. diversicolor* was exposed to sediment spiked with Ag, where only the nanoparticulate form affected burrowing (Cong et al., 2014). This might be due to the difference in exposure scenario, where Cong *et al.* used sediment exposure and Buffet *et al.* used water-only exposure prior to adding worms to uncontaminated sediment and monitoring burrowing. Méndez-Fernández *et al.* exposed *T. tubifex* to field sediments contaminated with different metals (Cu, Co, Ni, Zn and Pb),

and observed a weak burrowing activity for all worms (i.e., high avoidance). Visual observations revealed that worms were primarily on the sediment surface (i.e. avoidance) during the exposure (Méndez-Fernández et al., 2014). This is consistent with observations in Paper II and III, indicating that *T. tubifex* are affected by both metal-ions and Me-ENPs mixed into sediment. In addition, Méndez-Fernández *et al.* observed no fecal pellets on the sediment surface, indicating that worms were not feeding during exposure, likely a result of the high avoidance and low burrowing activity observed. This is similar to what we observed in Paper III, where it was not possible to detect or retrieve fecal pellets for worms exposed to either Ag-form, after transfer to uncontaminated sediment. This is likely the reason for the low feeding rate and uptake rate constants from food detected in this study. Overall, these results indicate that worms are able to detect metals and Me-ENPs in sediment, and by avoiding the contaminated sites, decrease their sediment intake and thereby their metal accumulation.

The biodynamic model proved useful in predicting steady state concentrations in worms. Incorporation of the uptake rate constants from water and food (k_{uw} and k_{uf}), and the elimination rate constant from water (k_{ew}) into the biodynamic model, along with environmentally realistic Ag-concentrations in water (0.1 μg/L) and sediment (10 μg/g), yielded steady-state Ag body burdens of 840 μg/g and 2.8 μg/g for $AgNO_3$ and Ag ENPs, respectively. Biodynamic modelling suggested that 1) water is the primary route of uptake for $AgNO_3$ in *T. tubifex* regardless of distribution coefficient (k_d); 2) uptake of Ag from sediment becomes more important at higher k_d; 3) regardless of uptake route, $AgNO_3$ exposure results in higher body burdens than similar exposure to Ag ENPs. Uptake of Ag from sediment becomes equally important for both Ag forms at a k_d-value of 10^7. This would correspond to a sediment concentration of 1000 μg/g when $[Ag]_{water}$ is 0.1 μg/L, which is a factor of 10 to 100 higher than what has been reported for Ag in natural sediments (i.e. (Luoma et al., 1995; Luoma, 2008). This was also observed for *L. variegatus*, where sediment became the dominant exposure route as Cu partitioning from water to sediment increased (Ramskov et al., 2015b). In a study incorporating the biodynamic modelling approach, *T. tubifex* was exposed to water or sediment spiked with Cd or Zn. Results showed that both compartments were important for the overall metal accumulation in worms, however when the gut of worms was taken into account, sediment became the main exposure route for both metals (i.e. >50% metal in worms originated from the sediment) (Redeker et al., 2004). In addition, Méndez-Fernández *et al.* determined that influx rate from food (i.e. sediment) could predict metal accumulation in *T. tubifex* (Méndez-Fernández et al., 2014). Overall, use of the biodynamic model highlighted that sediment is an important route of uptake under environmentally relevant conditions. This highlights the importance of incorporating sediment as exposure route in ecotoxicological studies.

When using the biodynamic parameters to predict steady state concentrations in worms, it highly underestimates the uptake when compared to what was actually measured (27.4±11.3 μg Ag/g dw) after long-term exposure (21 days; Paper IV), see Table 2).

$[Ag]_{worm}$ was calculated based on AE & IR or k_{uf} resulting in body burdens of 6.9 and 8.2 µg Ag/g dw tissue, respectively. The lower body burden predicted for worms is likely due to the difference in experimental conditions. In Paper III, worms were exposed for a short period of time, transferred to uncontaminated sediment to eliminate for 24h and then kept in FW overnight to empty their guts of sediment. In Paper IV, worms were exposed for 21 days and allowed to empty their guts of sediment in FW overnight. Thus, the elimination in sediment was not included in the long-term exposure, likely resulting in the higher body burden. The longer exposure time, as well as the lack of elimination, was done to ensure a detectable Ag-concentration in worms that was high enough to be used as a fish food (i.e., palatable FPs). Using the measured Ag-concentration found in worms after 21 days of exposure to estimate an uptake rate constant (k_{uf}) results in a k_{uf}-value an order of magnitude higher than what was found in Paper III (see Table 2).

Table 2: BDM-parameters determined for Ag ENP exposed worms in Paper III (left column) and used for the predicted $[Ag]_{worm}$; Long-term exposure data from Paper IV (right column); Predicted Ag-concentrations in worms using BDM-parameters (bottom). IR: Ingestion rate; AE: Assimilation efficiency; k_{uf}: uptake rate constant from food; k_e: elimination rate constant

Data from BDM-study (Paper III)			**Data from long-term exposure** (Pilot study, Paper IV)		
Parameter		*unit*	Compartment	[Ag]	*unit*
IR	0.6	g/g/d	Sediment	77.9±1.4	µg/g dw
AE	0.7	%	Worms	27.4±11.3	µg/g dw
k_{uf}	0.005	g/g/d			
k_e	<0.001	d^{-1}			

Predicted $[Ag]_{worm}$ (21d) using BDM-parameters compared with measured $[Ag]_{worm}$

Calculations based on	$[Ag]_{worm}$	*unit*
AE & IR	6.9	µg/g dw
k_{uf}	8.2	µg/g dw
Measured Ag	27.4	µg/g dw
k_{uf} (predicted)	0.02	g/g/d

The underestimation of $[Ag]_{worm}$ highlights the need for more studies incorporating the biodynamic model when working with Me-ENPs. Especially the parameters IR and AE showed very low values for *T. tubifex*, likely due to the short exposure times in sediment (Paper III). Khan *et al.* designed a setup to increase the dietborne exposure time when assessing biodynamic parameters, by exposing the organisms (*Perengia ulva*) to several, short (3h) contamination series (Khan et al., 2013). This approach may well be useful for future studies, however, the extra handling of worms (i.e. removing and re-introducing

them to sediment several times) may introduce other stress factors which could impact uptake. As a model is only as valid as the input data, the data presented in Table 2 highlights that the parameters from the short-term study are too uncertain to predict long-term results. That is, they are only based upon one study, thereby not representative for all the factors influencing bioaccumulation in *T. tubifex* (e.g., exposure conditions, time and media). The overall aim with Paper III was to determine unidirectional uptake constants, i.e. it was necessary to estimate uptake without interference of elimination. More studies are needed to create model parameters describing the broad range of effects and factors impacting uptake and elimination of Ag ENPs in benthic organisms such as *T. tubifex*.

4.2.3 Bioaccumulation factors

BAF and BCF-values were estimated by making the assumption that worms had reached steady-state during exposure. While this is most likely untrue, it makes it possible to do a more general comparison between the studies. As lipid determination were not determined for worms, BSAF-values could not be estimated. Bioaccumulation factors were estimated as described in Chapter 2, Table 1. In Paper II and (Paludan, 2015), worms were only exposed via the sediment route, so BAF-values was estimated. In Paper III, worms were exposed to either water- or sediment-associated Ag in two forms ($AgNO_3$, Ag ENPs), making it possible to estimate a BCF and BAF-value for worms. As the exposure times were not equal between studies, body burdens were normalized to an exposure time of 24h. The estimated bioaccumulation factors are presented in Table 3.

Table 3: Estimated bioaccumulation factors for worms exposed in the three studies (Paper II and III, and (Paludan, 2015)*). Exposure concentrations are normalized to 24h in all three studies. BCF and BAF values for worms exposed in Paper III are estimated as an average over the exposure concentration range.*

Study	Metal	Exposure conc.	Factor	
Paper II	Au ENPs	8.5 μg Au/g dw	BAF	0.3
	Au ENPs	70 μg Au/g dw	BAF	0.2
(Paludan, 2015)	Ag ENPs	7.1 μg Ag/g dw	BAF	0.4
Paper III	$AgNO_3$	0.01-3.5 μg Ag/L	BCF	**6.7±1**
		9-50 μg Ag/g dw	BAF	~0.002
	Ag ENPs	0.1-5 μg Ag/L	BCF	0.4±0.1
		11-52 μg Ag/g dw	BAF	~0.01

As seen from the estimated factors, worms are generally not good bioaccumulators of Me-ENPs (all values below 1), when sediment is the main exposure route. In fact, the only exposure scenario resulting in a bioaccumulation factor above 1, is when worms were exposed to waterborne $AgNO_3$. This is in accordance with the uptake rate constants determined in Paper III, where worms accumulated Ag from $AgNO_3$ from water much faster than from Ag ENPs. Likewise, the very low accumulation observed for worms exposed to sediment amended with either Ag-form in Paper III, is reflected in the low factors estimated here. The low values observed during sediment exposure, are similar to BAF-values observed for *N. diversicolor* (BAF<0.2, (Cong et al., 2011) and *P. antipodarum* (BAF<0.4, (Ramskov et al., 2015a)) exposed to sediment amended with Ag ENPs. However, when exposing the sediment-dwelling polychaete *Capitella teleta* to sediment amended with Ag ENPs, BAF-values of up to 4 was estimated (Ramskov et al., 2015a). These differences between species might be due to differences between studies (i.e. in exposure time, concentration and conditions) or organism characteristics, as noted by (Ramskov et al., 2015a). However, when comparing among *T. tubifex* only, the main differences are in exposure time and conditions, including sediment characteristics. BAF-values are likely affected by sediment characteristics such as organic content (OC) and grain size. In Paper II, worms were exposed to natural sediment collected in Denmark, with a size fraction <125µm. In Paper III, sediment was collected in CA, USA, an sieved to a size fraction <250µm, and in (Paludan, 2015) artificial sediment was prepared according to OECD guidelines. As the OC in sediment differed between studies (i.e., ~6% in natural sediments and ~2% in artificial sediment), this might explain the difference observed. As *T. tubifex* lives off the organic content within the sediment (Lopez and Levinton, 1987), a low OC will result in organisms ingesting more sediment to meet their nutritional needs. This leads to more sediment going through their system, increasing the likelihood of accumulating sediment-bound contaminants. Thus, the lower OC found in artificial sediments, could explain the higher BAF-value observed for these worms. If following the general guidance on bioaccumulative contaminants (i.e., as presented in Chapter 2, Table 1), none of these metal-forms (i.e. Au and Ag ENPs, $AgNO_3$) would be considered highly bioaccumulative from the sediment compartment. However, metal body burdens show that worms do accumulate some metal from sediment and obtain higher body burdens compared to control organisms. Thus, Me-ENPs are to some degree bioavailable to worms when added to sediment, even though their bioaccumulation potential might not be high.

In summary

The main message from these studies is that uptake route (and metal form) are important when assessing bioaccumulation in benthic organisms, such as *T. tubifex*. The biodynamic model showed that Ag is most bioavailable when added to water, and in the form of $AgNO_3$. Also, Ag was accumulated to a low degree from sediment, regardless of form. However, using the model parameters to predict steady state Ag-

concentrations in worms, showed that sediment becomes more important when the partitioning from water to sediment increases and resemblances what is found in nature (k_d: 10^5 (Luoma and Rainbow, 2008)). In addition, it was shown that burrowing is a good sub-lethal end-point to use, in order to detect if worms are stressed during exposure. *T. tubifex* are able to accumulate Me-ENPs (Au and Ag) from sediment, during exposure times of 5 and 15 days, respectively. Thus, Ag ENPs are bioavailable when added to sediment, even at low, environmentally realistic concentrations. Furthermore, after accumulation of Ag ENPs from water (Paper III) or sediment ((Paludan, 2015)) *T. tubifex* do not eliminate all Ag when transferred to uncontaminated conditions. Estimating BAF-values to compare results among studies, highlighted that *T. tubifex* is not a good bioaccumulator of metal and Me-ENPs from sediment. However, uptake of Me-ENPs was detected in worms, despite the low bioavailability. Together, these findings indicate that Ag ENPs added to sediment will accumulate in benthic worms, suggesting that worms may act as a mediator of metals from the sediment to higher organisms. Thus, trophic transfer from these sediment-dwelling organisms are worth investigating further.

4.3 Investigating trophic transfer of Ag ENPs

Trophic transfer of Me-ENPs has been scarcely studied, and especially studies including the benthic community are lacking (as described in Paper I). Therefore, the transfer of Ag ENPs from sediment, to benthic worms and further up the food chain to fish, was investigated as the second part of this thesis. In addition, the review on parameters and processes important for trophic transfer of Me-ENPs (Paper I) was used as a predecessor for the experimental work in Paper IV. A general overview and summation of the results obtained in Paper I and IV are presented and discussed below.

4.3.1 Background knowledge on trophic transfer

In Paper I, knowledge regarding trophic transfer of Me-ENPs in aquatic ecosystems was scrutinized and the most important factors influencing this transfer highlighted. In general, not much is known on the underlying mechanisms and processes responsible for trophic transfer. Studies involving the lower food chain levels (i.e. algae and zooplankton) are numerous (e.g. (Pakrashi et al., 2014; Chen et al., 2015)) but not many have included higher, predatory organisms such as fish. By considering the literature on trophic transfer of conventional metals, it was seen that trophic transfer and even biomagnification is occurring to higher food chain levels (e.g. (Woodward et al., 1994; Harada, 1995)). This could also be possible for Me-ENPs. Based on the large knowledge on trophic transfer of conventional metals (i.e. in ionic form) and the particle-specific effects found, 4 key factors for understanding and investigating trophic transfer of Me-ENPs was determined. Briefly, they involved (1) environmental transformations of Me-ENPs, (2) uptake and accumulation in prey, (3) internal fate and localization in prey and (4) digestive physiology of the predator. Using the available information, a schematic

overview of the different processes affecting trophic transfer of Me-ENPs once they enter the aquatic environment was created (see Paper I, Figure 1). In addition, the review highlighted the knowledge gaps that exist regarding trophic transfer of Me-ENPs. It was pointed out that future studies should focus on higher organisms and more complex food chains, as well as the internal localization of Me-ENPs in both prey and predator. A few studies have addressed this (e.g. (García-Alonso et al., 2011; Thit et al., 2015)), however much information is still needed. Likewise, the digestive physiology of the predator could be highly valuable in understanding the fate of Me-ENPs inside organisms, providing insights on bioaccumulation patterns and bioavailability. Last, but not least, it was pointed out how important the sediment compartment is, not just as a sink, but as an accumulation site for Me-ENPs and as such the most likely route of entry for trophic transfer of intact particles.

As previously mentioned and discussed in Paper I, the likely fate for Me-ENPs released to the aquatic environment is association with sediments, as a result of agglomeration/aggregation and following sedimentation out of the water column. Sediment-dwelling organisms such as *T. tubifex* and Nereid polychaetes ingest sediments to consume nutritious organic matter, incidentally ingesting sediment-associated contaminants at the same time. Studies have shown that *N. diversicolor* accumulated up to 50% Ag from ingestion of sediments spiked with Ag ENPs (García-Alonso et al., 2011), whereas *Nereis succinea* bioaccumulated 95% Ag from $AgNO_3$ spiked sediment (Cozzari et al., 2015). These organisms are important prey items for many different invertebrates, fish and bird species, thereby providing a pathway for Me-ENPs to move from the abiotic sediment to the aquatic food web. However, whether the particles remain intact or are prone to dissolution within worm gut cavity or tissue is unknown. García-Alonso *et al.* visualized Ag ENPs in endosomes and small vesicles in gut epithelial cells of *N. diversicolor* after exposure to Ag ENP spiked sediments. The particles appeared to have been endocytosed as intact particles (García-Alonso et al., 2011). But, as described in Paper I, different endocytotic mechanisms can lead to lysosomal degradation leading to particle dissolution. Either way, the presence of intact ENPs in the tissue of this common prey item, increases the probability of Me-ENP trophic transfer.

Assuming that some Me-ENPs persist in particulate form in prey tissue, achieving trophic transfer will depend on how those ENPs are handled when ingested by a predator. Dietary exposure of predatory organisms such as fish, have shown that Me-ENPs are accumulated within organism tissue (e.g. (Ramsden et al., 2009; Skjolding et al., 2014a)), however the mechanisms responsible for the further allocation and internal distribution are relatively unknown. Gaiser *et al.* showed that fish (*Cyprinus carpio*) exposed to Ag ENPs via water-only ingested the particles, resulting in accumulation in the gastrointestinal tract. Furthermore, a translocation of Ag ENPs was observed from the gut to other organs (liver, intestine and gall bladder), indicating that such particles can internally distribute in organism tissue after ingestion (Gaiser et al., 2012). Generally, the largest knowledge gaps regarding Me-ENPs bioaccumulation and bioavailability

relates to higher, predatory organisms such as fish. Therefore, more emphasis should be put on this part of the food web in the future.

4.3.2 Trophic transfer of Ag ENPs

Results from Paper IV showed that Ag ENPs were accumulated in fish during the 14 days of dietary exposure. Ag was primarily detected in gut/intestinal tissue of fish, with no significant increase in Ag-concentration in remaining fish tissue for AgS and AgC1 exposed fish (0.01±0.00 and 0.01±0.01 μg Ag/g dw, respectively). For fish exposed to AgC2 FPs, some Ag was measurable in the remaining fish tissue, however no clear trend was observed with regards to exposure and depuration time (i.e., $[Ag]_{fish}$ was in the range of 0.21±0.08 to 0.97±0.67 μg Ag/g dw). Fish exposed to FPs spiked with the highest Ag-concentration (i.e. AgC2; 500 μg Ag/g ww), experienced the highest uptake, with body burdens of 127±74 μg Ag/g dw tissue after 14 days of exposure. The AgS and AgC1 FP treatments resulted in lower and similar body burdens, with values of 2.5±1 and 2.9±0.5 μg Ag/g dw tissue, respectively. Besides detecting trophic transfer of Ag, it was shown that fish were able to eliminate the accumulated Ag when transferred to uncontaminated conditions. Here, fish exposed to AgS FPs showed the fastest elimination, with Ag concentrations in gut tissue returning to that of control worms after just 1 day of depuration. For the AgC1 and AgC2 FP treatments, it took 2 and 14 days, respectively. All data is presented in Paper IV.

Zebrafish are known to accumulate metal from Me-ENPs during dietary exposure (Geffroy et al., 2012; Ladhar et al., 2014). When exposed to Ag ENPs embedded in *Chironomid* larvae, zebrafish showed a higher uptake compared to waterborne exposure (Asztemborska et al., 2014). Authors highlighted that trophic transfer of Ag ENPs from larvae to fish was observed, when larvae was pre-exposed to Ag ENPs in water. To our best knowledge, Paper IV is the first study to show that Ag amended as Ag ENPs to sediment, can be transferred to higher pelagic organisms. Results also indicate that Ag concentration in food or prey item does influence the actual transfer to predators, as well as the elimination pattern post exposure. Zebrafish have been shown to eliminate accumulated Ag during 2 days in uncontaminated conditions. The authors concluded that Ag was not accumulated into tissue, but only present in the gut regions of fish (Asztemborska et al., 2014), similar to what was observed in Paper IV. Fish are known to create mucus layers within their gut/intestinal systems, used to eliminate contaminants by excreting the mucus-cells (Handy et al., 2000; Khan and McGeer, 2013). In addition, zebrafish have been shown to depurate TiO_2 ENPs (Zhu et al., 2010), Cd QDs (Lewinski et al., 2011) and ZnO ENPs (Skjolding et al., 2014a) post dietborne exposure (i.e. trophic transfer from prey to fish). So, in a scenario where fish are able to move away from the contamination source, transfer of ENPs further up the food chain is unlikely.

By assuming that fish had reached steady state during the 14 days of exposure, biomagnification factors were calculated for the exposed fish. As no fecal matter was

collected from fish during the experiment, BMF was estimated according to OECD TG 305 (OECD, 2012). In addition, steady-state body burdens ($[Ag]_{ss}$) in fish were estimated using the BDM approach (Croteau et al., 2014b). AE and IR was estimated based on feeding rate (i.e., 1.5% of fish ww per day) and Ag-concentrations detected in fish tissue during depuration (see Chapter 2, Table 1 and Paper IV for details). Results suggest that none of the treatments resulted in biomagnification (BMF<1), however FPs created from pre-exposed worms (AgS) resulted in a BMF an order of magnitude higher than the spiked treatments (see Table 4). This could be due to the concentration difference in exposure concentrations (i.e. AgS being a factor of ten lower than AgC1), however this trend is not consistent when taking AgC2 into account (a factor of 100 higher than AgS). We therefore speculate, that Ag ENPs embedded into sediment and turned into palatable FPs are more bioavailable than Ag ENPs spiked directly into worm homogenate. Previous studies with zebrafish and Me-ENPs showed no sign of biomagnification, with BMF-values of 0.04 and 0.1 for Cd QDs (Lewinski et al., 2011; Lee and An, 2014). The compartmentalization of metal in *T. tubifex* can affect how much of a metal that is assimilated by a predator (common carp, *C. carpio*) (Redeker et al., 2007). Thus, the degree of biomagnification could be both particle, metal and prey-type dependent. Fathead minnows (*Pimephales promelas*) fed metal-contaminated *T. tubifex* and *Daphnia magna* assimilated Tl and Ni from both prey-types. The authors suggested that the metal concentration in prey had an influence on the overall accumulation in predator, possibly due to saturation of metal-transport sites with increasing concentration in food (Lapointe et al., 2009). This could also be the case in Paper IV, with lower BMF-values in fish when prey items contained more Ag.

Table 4: Biomagnification factors (BMF) calculated as described in OECD TG 305; Steady-state Ag-concentration in fish gut/intestinal tissue ($[Ag]_{ss}$) calculated based on the biodynamic model (BDM) and measured Ag-concentration in fish tissue after 14 days of depuration ($[Ag]_{fish}$). AgS: FPs created from sediment-exposed worms; AgC1 and AgC2: FPs created from spiked worms at concentration 1(10µg/g) and 2(500µg/g); AE: assimilation efficiency; I: ingestion rate constant; k_2: depuration rate constant.

Estimated parameters according to OECD				**Calculated**		**Measured**
Treatment	AE (%)	I (g/g/d)	k_2 (d^{-1})	BMF	$[Ag]_{ss}$ (µg/g)	$[Ag]_{fish}$ (µg/g)
AgS	-0.09	0.015	-0.004	0.32	0.57	0.71
AgC1	-0.24	0.015	-0.124	0.03	0.30	0.39
AgC2	-0.12	0.015	-0.162	0.01	5.63	6.58

Use of the BDM approach indicated that fish had not reached their predicted steady-state body burdens after 14 days in uncontaminated conditions (i.e., $[Ag]_{ss} < [Ag]_{fish}$). The estimated AE-values predict that Ag from AgS FPs are most bioavailable, followed by AgC2 and AgC1. The depuration rate constants (k_2) indicate that Ag are eliminated fastest from fish exposed to AgC2 FPs, followed by AgC1 and AgS FPs (see Table 4).

These results suggest, that Ag from AgS FPs will be most harmful to fish, i.e. higher accumulation due to larger bioavailability and slower elimination. However, the estimated steady-state BB's indicates that fish will experience the greatest accumulation when exposed to AgC2 FPs (i.e., the highest exposure concentration), consistent with measured data. This highlights that no model parameter can stand alone, and must be considered in a broader perspective, taking both the remaining parameters and actual data into account.

We expected the spiked treatments to result in higher uptake in fish, both due to the higher Ag concentration and the assumption that Ag ENPs spiked into worm-homogenate would be bound "less" than Ag ENPs taken up into worm tissue (Khan et al., 2010). As mentioned, higher BB's was seen for fish exposed to FPs with the highest Ag-concentration (AgC2), as well as when applying BDM. However, the BMF-values indicate that exposure conditions for prey should be considered when assessing trophic transfer, as fish exposed to AgS FPs showed the highest BMF. This is highly important, as dietborne exposure studies today are conducted using the spiking method we applied for the treatments AgC1 and AgC2 (OECD, 2012), which resulted in the lowest BMF-factors. The standard method could therefore, in worst-case, underestimate the bioaccumulation potential of dietary Me-ENPs, similar to what was shown for Cd embedded into natural (*Lumbriculus variegatus*) and commercial feed and fed to rainbow trout (*Oncorhynchus mykiss*) (Ng and Wood, 2008). However, more studies are needed before such conclusions can be made.

The results highlight the need for more studies taking the sediment compartment into consideration, when assessing the risks of Me-ENP exposure and trophic transfer. As other studies have shown that the internal metal compartmentalization affects the bioavailability of metal in prey, more focus should be put on investigating this for Me-ENPs. Both in prey and predator, such information would increase the understanding of how hazardous Me-ENPs are when released to the aquatic environment and accumulated in biota. Environmentally relevant exposure concentrations should be employed, as studies have shown that the internal metal concentration in prey affects the uptake in predators (i.e. higher $[M]_{prey}$ results in lower $[M]_{predator}$) (Dumas and Hare, 2008; Lapointe and Couture, 2009). Likewise, studies including intracellular location of ENPs (i.e., subcellular fractionation and several microscopy techniques, as described in (Jensen et al., 2017)) are needed in order to elucidate if Me-ENPs are crossing the cell-barriers in the gut, or just adsorbed to the intestinal surfaces. It is important to highlight, that we do not know if the Ag ENPs was actually found as ENPs after addition to sediment or worms in Paper IV, and especially not when transferred into fish. All these factors must be addressed in future studies, if we want to get a step closer to fully understanding the risk of use and release of Me-ENPs to the environment.

In summary

Many factors affect if and to what degree trophic transfer of Me-ENPs occur. However, mechanisms known from trophic transfer studies with conventional metals will be helpful as starting point for future Me-ENPs studies. Trophic transfer of Ag ENPs from sediment – to worm – to fish is occurring, and to our best knowledge, our study is the first to prove this point. The highest BMF-factor is seen when fish are exposed to FPs created from pre-exposed worms, highlighting that the contamination process of prey or food item is important when assessing trophic transfer of Me-ENPs. Fish were able to depurate any accumulated Ag again when fed uncontaminated FPs, regardless of the body burdens obtained during exposure. This indicates that further transport up the food chain is unlikely, if fish can move away from the contamination source. As fish did not show any distress during feeding on contaminated prey in Paper IV, it is likely that they would continue to eat, which may result in even higher body burdens than we observed. This could lead to transfer further up the food chain and have consequences for higher organisms predating on fish, such as humans. However, many factors remain unknown, and more studies are needed to make definitive conclusions regarding these issues.

4.4 ERA and appropriate test conditions for ENPs

When considering the results presented in this thesis, some interesting points regarding regulatory chemical safety assessment (CSA) of ENPs comes to mind: 1) should sediment (or diet) be used as the main exposure route, when working with Me-ENPs?; 2) should benthic organisms be included in CSA when assessing the effects of Me-ENPs?; 3) would the incorporation of more food web levels increase the usefulness of CSA for Me-ENPs?

As shown in Paper II and III, Me-ENPs added to sediment are bioaccumulated in benthic organisms, however to different degrees, highly dependent on exposure time. As sediment is believed to be the main sink for Me-ENPs after release to the aquatic environment, these result underlines and supports that sediment should be incorporated in standard TGs and used as exposure site when assessing the risks of Me-ENPs to aquatic biota. In addition, benthic organisms are highly useful in this assessment, as they are the primary accumulation "site" due to their feeding behavior. By incorporating these organisms into ERA of nanomaterials (i.e. as they already are in OECD TG 315), the quality of the assessment would be increased (i.e. as described in (Chapman, 2001) and (ProSafe, 2017)). Benthic worms are prey to many higher organisms such as demersal fish, making them extremely valuable in assessing both the effects of ENPs on the benthic community, but also on the trophic transfer capability of these materials. As shown in Paper IV, Ag ENPs associated with sediment are trophically transported to fish, when benthic worms are the biotic link. In addition, Ferry *et al.* showed that Au ENPs would distribute between several food chain steps, when amended to an estuarine mesocosm (Ferry et al., 2009). Thus, incorporation of more complex food webs and the

sediment compartment when conducting ERA of nanomaterials is highly recommended. And even though the biodynamic modelling study (Paper III) showed that *T. tubifex* was not a great bioaccumulator of Ag ENPs from sediment, it was food packages created from sediment-exposed worms that resulted in the highest BMF-value (Paper IV). Therefore, the exposure conditions for prey should be considered, when assessing trophic transfer, in order to mimic environmentally relevant situations. Future studies should use sediment exposure of benthic organisms, and use them as feed directly after exposure (i.e. without a depuration or elimination period). This way, contaminated sediment still present in prey guts and intestinal regions would be considered, and not only ENPs accumulated into prey tissue. As predators will eat a prey when needed (i.e. not wait for them to empty their guts) this is likely a more realistic approach towards assessing trophic transfer in aquatic environments.

Generally, the results presented herein are valuable to risk assessors, as they provide new evidence for ENP-related end-points and effects. For example, the non-lethal end-point of burrowing proved useful in detecting levels of stress in exposed worms. This is not new, however results from Paper II and III underlines that Me-ENPs will cause a change in behavior, making it valuable in determining how Me-ENPs are affecting the benthic community. Results in this thesis showed that Me-ENPs amended to sediment was accumulated to larger degrees over long exposure times (i.e. 5, 15 and 21 days) compared to short (acute) exposure durations (5-8h). Thus, short exposure times might not be appropriate for bioaccumulation tests with ENPs. This is in accordance with OECD TG 315, where 28 days is recommended as exposure time, with the notion that experimental work has shown 12-14 days of uptake to be sufficient for several contaminants (OECD, 2008). The overall aim with Paper III was to determine unidirectional uptake constants, i.e. it was necessary to estimate uptake without interference of elimination. This entails short exposure times, as *T. tubifex* are known to ingest and excrete sediment within 5-8h (Redeker et al., 2004). Thus, short exposure times was crucial, but might have resulted in underestimation of the actual uptake potential in worms. In relation to exposure route, aquatic organisms and setups are often used, however results herein underlines that Me-ENPs will not be found in the water column for long, decreasing the risk for pelagic organisms (i.e. Paper I). Several studies have shown that sediment or diet is a more appropriate route of exposure when assessing the risks of Me-ENPs (i.e. (Croteau et al., 2011a; Skjolding et al., 2014a; Ramskov et al., 2015b), and even though results from Paper III showed that Ag was taken up faster from water, sediment was the main exposure route for Ag ENPs under environmentally realistic conditions. Thus, we recommend that future ERA focus on diet or sediment, including benthic organisms, in accordance with (ProSafe, 2017).

Chapter 5: Conclusions and Outlook

When searching for papers on trophic transfer of Me-ENPs in freshwater ecosystems in 2014/15, less than 20 studies came up (see Table 1, Paper I). The knowledge base was scarce, and no studies including sediment as exposure source were available. Most studies had not detected biomagnification of Me-ENPs when working with higher organisms such as fish. In addition, only a few benthic organisms had been included (i.e. mussels). Thus, the overall aim of this thesis was to investigate the possibility of transporting Me-ENPs from the abiotic sediment to pelagic organisms in a freshwater food chain. By including how natural fluctuating parameters affects the bioavailability of Me-ENPs in aquatic ecosystems, a broad understanding of the most important factors to consider in an experimental setup was achieved. The studies on behavior of the model benthic organism *T. tubifex* and general accumulation of Me-ENPs from sediment in these worms, gave insight into how the benthic community are responding to ENP contamination. Use of the biodynamic model resulted in unidirectional uptake and elimination rate constants, providing information on how *T. tubifex* bioaccumulates Ag from different environmental compartments (i.e. water and sediment) in a more mechanistic manner. Lastly, the experimental work on trophic transfer from sediment – to worms – to fish, proved as one of the first studies that Ag from Ag ENPs can in fact be transferred from the abiotic to the biotic parts of the freshwater ecosystem. Even though biomagnification was not detected, this opens for the possibility of transporting Me-ENPs further up the food chain, e.g. to predatory fish and humans.

To answer the overall aim of the thesis, the results presented herein clearly shows that yes, trophic transfer of Me-ENPs is occurring, even after the particles are mixed into the sediment compartment. It is therefore recommended to add the sediment as an experimental unit in future studies, including the organisms living herein. In addition, more studies should be investigating the biomagnification potential of Me-ENPs, when sediment is the primary exposure source. However, the results presented in this thesis also questions if trophic transfer of Me-ENPs should be a point of concern, as fish were able to eliminate accumulated Ag relatively fast, when fed uncontaminated food. In addition, results did not show any transport of Ag ENPs from gut/intestinal area to remaining fish tissue, indicating that these particles might just be ingested and egested, without further incorporation into tissue. Therefore, it is recommended that future studies investigate how higher organisms such as fish handle the accumulated Me-ENPs (e.g. are they stored in the gut or taken up into cells). Also, continued exposure might lead to higher body burdens in fish than observed in Paper IV (i.e., steady-state not reached). Hence, long-term exposure studies with predatory organisms should be prioritized in the future.

The main recommendations for future studies are primarily to include sediment as exposure source, and not only focus on the water phase. Also, using sediment-dwelling organisms as prey items provide an environmentally realistic scenario for trophic

transfer of intact Me-ENPs. Easily accessible methods and analysis tools for detecting and characterizing Me-ENPs (and ENPs in general) after they enter the sediment are highly needed. Also, more information on how Me-ENPs distribute and speciate internally in organisms will give insights into how these contaminants affects organisms at a more detailed level. Overall, the newest addition to ERA of nanomaterials (i.e., (ProSafe, 2017)) is pulling the regulators and risk assessors in the right direction. Thus, we are on the right track with regards to protecting our environment from unnecessary harm, and will hopefully only benefit from the exciting possibilities within the nanotechnology industry in the future.

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Papers

Paper I

Trophic transfer of metal-based nanoparticles in aquatic environments: a review and recommendations for future research focus

Stine Rosendal Tangaa, Henriette Selck, Margrethe Winther-Nielsen, Farhan R. Khan

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- Conducting the literature analysis
- Writing the manuscript

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Trophic transfer of metal-based nanoparticles in aquatic environments: a review and recommendations for future research focus

Stine Rosendal Tangaa,*[ab] Henriette Selck,[a] Margrethe Winther-Nielsen[b] and Farhan R. Khan*[a]

Metal-containing engineered nanoparticles (Me-ENPs) are used in a wide range of products including inks, plastics, personal care products, clothing and electronic devices. The release of Me-ENPs has been demonstrated from some products, and thus, particles are likely to enter the aquatic environment where they have been shown to be taken up by a variety of species. Therefore, there is a possibility that Me-ENPs will enter and pass through aquatic food webs, but research on this topic is limited. In this tutorial review, we discuss the factors contributing to trophic transfer of Me-ENPs, and where this information is scarce, we utilize the existing literature on aqueous metal trophic transfer as a potential starting point for greater mechanistic insight and for setting directions for future studies. We identify four key factors affecting trophic transfer of Me-ENPs: (1) environmental transformations of Me-ENPs, (2) uptake and accumulation in prey organisms, (3) internal fate and localization in the prey, and (4) digestive physiology of the predator. Whilst much research has been conducted on the first two of these factors, key knowledge gaps exist in our understanding of how Me-ENP trophic transfer is affected by the internal distribution in prey organisms and the digestive physiology of the predator. Additionally, we suggest that the ENP association with sediments may be a key process that results in the transfer of intact particles within aquatic food webs.

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Nano impact

Studies on the trophic transfer of Me-ENPs remain scarce, and the factors leading to this transfer are poorly understood. Here, we describe four processes that influence trophic transfer and suggest that the trophic transfer of aqueous metals is a logical starting point for future research involving Me-ENPs. We suggest that the initial uptake of Me-ENPs through the sediments is a likely source for intact Me-ENPs to enter the aquatic food web, and more focus should be directed here. To fully understand the potential for Me-ENP trophic transfer, future research needs to address the internal fate and localization of Me-ENPs in the prey organisms and the impact of the predator's digestive physiology.

1. Introduction: trophic transfer, trace metals and metal-containing nanoparticles

Trophic transfer, described as the movement of toxicants up through the food web *via* ingestion of prey organisms by predators, has been widely recognized and remains a much studied eco-toxicological issue. In the case of trace metals in aqueous form (a term used here to include all metal species (ionic, dissolved, complexed) that exist in the water after addition of the metal salt), high profile incidences affecting both human health (*e.g.* methylmercury poisoning in the city of Minamata, Japan[1]) and piscine health have increased public and regulatory awareness. An example of the latter is provided by studies at the Clark Fork River in Montana, USA, which has received inflows of metal-rich mine effluents since the late 19th century. Young rainbow and brown trout readily accumulated Cd, Cu, Pb and As from diets of benthic invertebrates leading to reduced survival, growth and feeding activity.[2,3] Moreover, studies show that trace metals biomagnify along the food chain,[4–6] where biomagnification is a measure of contaminant transfer from lower to higher trophic levels and a biomagnification factor (BMF) of >1 indicates an increasing concentration up the food chain. Based on such research, the passage of trace metals through aquatic food webs is broken down into two main processes: (1) the accumulation of metals from the surrounding environment by prey organisms (*i.e.* net accumulation of metal into tissues

[a] Department of Science and Environment, Roskilde University, DK-4000 Roskilde, Denmark. E-mail: stangaa@ruc.dk, frkhan@ruc.dk; Tel: +45 4674 2899, +45 4674 3735
[b] Department of Environment and Toxicology, DHI, Agern Allé 5, DK-2970 Hørsholm, Denmark

via all available uptake routes), followed by (2) assimilation of metals in predators (*i.e.* the efficiency with which the metal is extracted from ingested food and absorbed into the body).[7] Whilst the movement of aqueous metals in the aquatic food chain is well known and relatively well understood,[2–4,8,9] studies on the potential trophic transfer of particulate metals, metal oxides and metal mixtures in the nano-size range, formulated as engineered nanoparticles (Me-ENPs), are scarce but are increasingly subject to similar concerns.

The unique properties of Me-ENPs result from the combination of the inherent properties of the metal and the novel properties related to the nanoscale morphology such as size, shape, high surface to volume ratio, surface functionalization and surface charge.[10,11] As such, Me-ENPs have found use in a wide range of products including cosmetics (Ag, TiO_2, ZnO), medicine (Ag, CeO_2), electronics (Cu, Au, Cd (as quantum dots)), bioactive coatings (Ag, CuO) and inks (Au, Ag, TiO_2). Due to the increasing production and use of Me-ENPs, their release into the aquatic environment is inevitable and has already been demonstrated.[12,13] Several studies show that metals introduced to organisms as ENPs are taken up from the abiotic compartments from both water and diet,[14–17] commonly with ENPs mixed into sediments[18–22] or added to

Stine Rosendal Tangaa

Stine Rosendal Tangaa is a Ph.D. student at Roskilde University and DHI, Denmark. She obtained her Bachelor's and Master's degrees in Ecotoxicology, with emphasis on bioaccumulation of metal-based engineered nanoparticles (Me-ENP) from sediment systems, at Roskilde University. She began her Ph.D. within the subject of trophic transfer of Me-ENPs in 2014, under the supervision of Dr. Henriette Selck and Dr. Margrethe Winther-Nielsen. Her research work includes experimental and theoretical understanding of the fate and behavior of Me-ENPs in freshwater ecosystems, including biodynamic modelling, bioaccumulation patterns and uptake from sediment in benthic and aquatic organisms.

Henriette Selck

Henriette Selck is an Associate Professor and head of the Graduate School of Environmental Stress Studies, Roskilde University, Denmark. She obtained her Ph.D. degree in Ecotoxicology from Roskilde University in 2003. She was a Fulbright guest scientist at the US Geological Survey, CA, USA, in 2012, where she worked on the impact of metal nanoparticles in sediment systems. Her research is focused on environmental risk assessment of chemicals (e.g. metal nanoparticles), including the effects of benthic infauna on the fate of contaminants and the effects of uptake route (water, diet) on chemical uptake, internal fate and elimination in the aquatic environment.

Margrethe Winther-Nielsen

Margrethe Winther-Nielsen is an R&D Program Manager at DHI, Denmark. She obtained her Ph.D. degree in Environmental Engineering from the Technical University of Denmark (DTU) in 1992. She has held Research & Development Engineer positions at the Risoe National Laboratory, Denmark, at Superfos Biosector A/S, Denmark and at the Institute of Biotechnology, DTU. Since 1991, she has been employed at DHI as a consultant and a researcher within the field of environmental fate, especially transformation as well as the hazardous effect of chemical substances, which also includes particulate pollutants such as nanoparticles and microplastics.

Farhan R. Khan

Farhan R. Khan is an Assistant Professor at Roskilde University, Denmark. He obtained his Ph.D. degree in Environmental Science from King's College London in 2008, where he studied trace metal detoxification strategies in aquatic organisms and their effects on trophic transfer. He held Post-Doctoral Research positions at Wilfrid Laurier University (Ontario, Canada), Natural History Museum (London, UK) and Heriot Watt University (Edinburgh, UK). Since 2010, his research has focused on utilizing biodynamic/bioaccumulation models to understand how metal-containing nanoparticles interact with organisms in the aquatic environment, as well as looking at extending the biodynamic approach to novel pollutant scenarios such as microplastic pollution.

Table 1 List of published studies (to date) conducted on trophic transfer of Me-ENPs in aquatic media. Columns 1 and 2 show the Me-ENP type and size as presented by the authors, column 3 describes the used test organisms with arrows indicating the trophic transfer pathway, column 4 shows the exposure time for the used trophic links, column 5 summarizes the main study findings, and all references are listed in column 6. Abbreviations: h = hours; d = days; U = uptake; D = depuration; QDs = quantum dots

ENP type	Primary particle size (nm)	Organism(s)	Exposure time	Main findings	Ref.
CdSe/ZnS QDs	10–25	Algae → daphnia	96 h & 48 h	The coating provides protection against toxicity, leading to increased trophic transfer potential	Bouldin, 2008 (ref. 28)
CdSe/ZnS QDs (different surface groups)	6–12	Ciliates → rotifers	Up to 7 d	Dietary transfer of QDs important for higher trophic organisms	Holbrook, 2008 (ref. 27)
Au (amine coated)	10 ± 0.5	Algae → bivalve	24 h & 7 d	Bioaccumulation & uptake in cells *via* gill penetration and the intestinal epithelia in bivalve. Biological removal of coating caused oxidative stress	Renault, 2008 (ref. 148)
Au (rods)	65 × 15	Marine mesocosm Entire food web	12 d	Transfer from water & sediment to organisms. Highest bioaccumulation in clams & biofilms	Ferry, 2009 (ref. 37)
TiO_2	21	Daphnia → zebrafish	24 h & 14 dU + 7 dD	Dietary transfer of TiO_2 ENPs from daphnids to zebrafish. No biomagnification	Zhu, 2010 (ref. 145)
CdSe/ZnS QDs	—	Daphnia (artemia) → zebrafish	24 h & 14 dU + 7 dD	Dietary transfer of QDs from daphnids to zebrafish. No biomagnification	Lewinski, 2011 (ref. 146)
CdSe QDs (bare)	5	Bacteria → protozoa	Up to 16 h	Trophic transfer of QDs led to biomagnification of Cd in protozoans. Non-degraded QDs in protozoans might increase risk of Me-ENP contamination in higher organisms	Werlin, 2011 (ref. 34)
ZnO	—	Algae → copepods	7 d & 7 d	Decreased copepod survival due to trophically transferred ZnO. Impaired fecundity in the highest dietary ZnO concentration	Jarvis, 2013 (ref. 31)
TiO_2 (heterogeneous)	6.4–73.8	Bacteria → ciliates	24 h	Dietary transfer of TiO_2 ENPs led to reduced growth rate and population yield in ciliates. TiO_2 NP detected in food vacuoles. No biomagnification	Mielke, 2013 (ref. 30)
CuO, ZnO	40, 10–30	Brine shrimp → goldfish	24 h & 21 d	Accumulation of CuO and ZnO in intestine, liver and gills, however no significant increase in concentrations in muscle, heart or brain after dietary (or waterborne) exposure	Ates, 2014 (ref. 147)
CeO_2 (rods)	67 ± 8 × 8 ± 31	Phytoplankton → blue mussel	5 weeks (37 d)	Trophic transfer of CeO_2 ENPs from phytoplankton to mussel. No difference in bioaccumulation in regard to the exposure method (water *vs.* diet)	Conway, 2014 (ref. 149)
SnO_2, CeO_2, Fe_3O_4, SiO_2	61, 50–105, 20–30, 4–40	Algae → sea urchin larvae	48 h & 15 d	Decreased larval survival after dietary exposure to SnO_2 & CeO_2 ENPs. Developmental effects due to trophic transfer of all NPs from algae to larvae	Gambardella, 2014 (ref. 32)
Au (citrate or PEG coating)	Differs between media	Algae → blue mussel	24 h & 24 h	Au only detected in digestive gland after dietary exposure. Coating affected bioaccumulation	Larguinho, 2014 (ref. 102)
CdSe/ZnS QDs (polymer coating)	4.6	Protozoa → zooplankton → zebrafish	48 h & 48 h & 48 h	QDs observed in all 3 organisms by IMP-SLM, thus trophic transfer of QDs between the 3 tested levels occurred. No biomagnification in fish	Lee, 2014 (ref. 35)
Al_2O_3	40–100	Algae → daphnia	48 h & up to 72 h (OECD 202)	Dietary exposure caused alterations in daphnid feeding behavior, which could lead to a disrupted energy flow in the ecosystem	Pakrashi, 2014 (ref. 168)
ZnO (bare or octyl-coated)	30 ± 17	Daphnia → zebrafish	24 h & up to 14 dU + 7 dD	Trophic transfer of ZnO from daphnids to zebrafish. Tenfold higher bioaccumulation compared to water exposure	Skjolding, 2014 (ref. 33)

Table 1 *(continued)*

ENP type	Primary particle size (nm)	Organism(s)	Exposure time	Main findings	Ref.
TiO_2	21 (250.5)	Algae → daphnia	72 h & 35 d	Trophic transfer of TiO_2 ENPs from algae to daphnids, with apparent biomagnification (BMF > 1). Addition of SDBS (anionic surfactant) increased ENP dispersion and enhanced accumulation in both species	Chen, 2015 (ref. 29)
Ag (PVP, PEG or citrate coating)	~11 nm (core size)	Algae → daphnia	4 h & 40 min to 24 h	Diet is the primary route of uptake for Ag ENPs. Complete depuration of Ag ENPs from daphnids was not obtained, thus trophic transfer to higher levels is possible. Starch granules act as storage sites for ENPs in algae (*C. vulgaris*)	Kalman, 2015 (ref. 169)
Au	10 ± 1	2 algae types → daphnia	48 h & 24 h	Trophic transfer of Au from both algae types to daphnids. Highest accumulation of Au in *E. gracilis* probably due to lack of cell wall. Different accumulation patterns in the prey leads to a difference in the amount of Au transferred to the predator	Lee, 2015 (ref. 36)

food sources such as algae.[23,24] This uptake of Me-ENPs creates a link between the abiotic environmental compartments and organisms in the aquatic food chain. Once taken up in the biota, as either an aqueous metal or Me-ENPs retained in the gut or absorbed over the epithelia, predation of these organisms potentially leads to ingestion and retention of Me-ENPs or at least the constituent metal ions in cases of particle dissolution. Describing the movement of intact particles in aquatic food webs is difficult due to the transformations that can occur after particles enter the environment, especially the aquatic environment. There is evidence to indicate that Me-ENP trophic transfer occurs in aquatic food webs,[19,22] and although currently there are only a handful of studies on this topic, it warrants further investigation. Furthermore, a few studies have examined trophic transfer of Me-ENPs in terrestrial environments, with movement of intact Au ENPs from tomato and tobacco plants to the tobacco hornworm (*Manduca sexta*). Au ENPs were significantly accumulated in hornworms when passed on from the lower trophic level (*i.e.* accumulated in leaves) but not when particles were only sorbed (*i.e.* added to leaf surfaces) to the leaves.[25,26] These studies demonstrate the possible movement of intact Me-ENPs up the food chain, as well as how accumulation in the predator differs based on how Me-ENPs are taken up by the prey.

Based on the current literature on trophic transfer of Me-ENPs in the aquatic environment, we here assess the existing knowledge with the aim of highlighting knowledge gaps and suggesting directions for key future research areas. The trophic transfer of Me-ENPs in aquatic ecosystems is still a topic in its infancy, with <20 studies published to date (summarized in Table 1). Thus, we provide relevant analogies to the wealth of research that already exists regarding trophic transfer of aqueous metals. We recommend areas of research that require greater investigation to better understand how Me-ENPs that enter the aquatic environment may firstly move from the abiotic to biotic compartments and then be subject to food web transfer.

2. Current investigations of trophic transfer of Me-ENPs in the aquatic food web

The first reported studies on trophic transfer of Me-ENPs utilized the fluorescence properties of Cd-containing quantum dots (QDs) to visualize transfer in aquatic food webs.[27,28] Cd QDs were shown to pass between the ciliate *Tetrahymena pyriformis*, used as the prey item, and the rotifer *Brachionus calyciflorus* in a simple two-level invertebrate food chain. Ciliates exposed to a suspension of Cd QDs for up to seven days were offered as a contaminated food source to the rotifers leading to intracellular detection of Cd QDs in ciliates, as well as in the gut and body cavity of the rotifers.[27] Similarly, Bouldin *et al.* (2008)[28] exposed a green algae (*Pseudokirchneriella subcapitata*) to Cd QDs for 96 h and then offered them as feed to a crustacean (*Ceriodaphnia dubia*). Cd QDs were detected within the algae cells, followed by morphological changes in *P. subcapitata*, such as altered cell integrity, structure and shape. The dietary transfer of Cd QDs from algae to daphnia was detected within the experimental time frame, with Cd QDs primarily found in the daphnids' digestive tract.[28] Both studies revealed transfer of Cd QDs from the lower food chain level (bacteria, algae) to higher organisms (rotifers, daphnia); however, there was no evidence of biomagnification within the experimental time frames used, suggesting that although the QDs did pass to the predating organisms, there was no up-concentration of ENPs in the tissue.

Following these initial studies, most research into this topic has been conducted with relatively simple, mainly

pelagic food webs consisting of the minimum number of two trophic levels. These studies utilize relatively short exposure durations, typically 24–96 hours for prey and up to 14 days for predators. As shown in Table 1, the most frequently used organisms are algae and daphnids, and the ENPs tested are primarily metal oxides, Cd QDs or Au ENPs. As a general trend, it is reported that transfer of Me-ENPs does occur between the investigated trophic levels; however, the extent of trophic transfer is dependent on various factors including the predator and prey species, the exposure route of the prey, as well as the ENP characteristics, including the constituent metal and the presence of functionalization on the surface (as summarized in Table 1).

Chen *et al.* (2015)[29] observed BMFs of almost 8 for daphnids (*Daphnia magna*), after dietary exposure to TiO_2 ENP contaminated algae (*Scenedesmus obliquus*). Different sub-lethal effects, such as reduced growth rate,[30] impaired fecundity[31] and developmental changes,[32] also resulted from the transfer of metal-oxide ENPs. Additionally, a 10-times higher body burden was detected in zebrafish (*Danio rerio*) after dietary transfer of ZnO ENP from daphnids (*D. magna*) compared to waterborne exposure.[33] For Cd QDs, Werlin *et al.* (2011)[34] detected biomagnification from bacteria (*Pseudomonas aeruginosa*) to protozoans (*Tetrahymena thermophila*), and since protozoans stayed physically intact after Cd QD accumulation, the authors suggested that non-degraded Cd QDs in protozoans could be transported to higher organisms. However, Lee and An (2014)[35] did not detect biomagnification of Cd QDs in fish (*D. rerio*), after transport of these particles from protozoans (*Astasia longa*) to zebrafish, highlighting the difficulties in making general conclusions based on single studies. Lee *et al.* (2015)[36] examined whether food type (different algae species) influenced the degree of Au ENP trophic transfer, resulting in the highest accumulation in daphnids (*D. magna*) when Au ENPs were associated with the algae *Euglena gracilis*. As the main difference between the food types tested was physiological, the authors suggested that the observed bioaccumulation patterns were likely due to *E. gracilis*' lack of a cell wall.[36] Only a few examples exist in the literature including more trophic levels and complex systems. For instance, Ferry *et al.* (2009)[37] added Au ENPs to a marine mesocosm that included both sediment and water and several trophic levels. Au ENPs accumulated in the food web, with the highest bioaccumulation observed in clams (*Mercenaria mercenaria*) and biofilms.[37] In addition, as organisms such as clams and biofilms constitute a great part of the food for predatory invertebrates and demersal fish, the bioaccumulation of Au ENPs in these organisms could potentially be transferred further up the food web. A comparison of Me-ENP BMFs to that of the corresponding aqueous metal form would indicate whether the particulate metal is more or less biomagnified. Unfortunately, the literature cited does not include a metal reference (*e.g.* the salt form of the metal), and therefore, direct comparisons are not possible. Biomagnification is considered specific to both abiotic (*e.g.* environmental parameters) and biotic (*e.g.* organism physiology, food web structure, feeding relationships, analyses of whole body *vs.* single organ concentrations) factors, which makes comparison to the published literature on aqueous metals difficult, if not impossible. In fact, metal BMFs vary considerably and depend strongly on these factors, and we encourage readers to visit chapter 7.4.1. in Luoma and Rainbow (2008)[38] for a more elaborate discussion. This clearly illustrates the need and importance of including reference treatments in any study of metal ENP uptake kinetics and effects.

The current state of the literature would indicate that the trophic transfer of Me-ENPs appears to occur, but biomagnification factors, when reported, are variable. Where biomagnification does not occur (*i.e.* BMF < 1), this would suggest that there may be no transfer of ENPs to higher-level organisms. Yet, caution must be taken, as most studies have included relatively short exposure durations and few trophic levels. These studies do highlight the importance of the dietary exposure route, which results in a higher body burden[39–42] and differential levels of toxicity[40–42] when compared to water-only exposure. Despite the evidence for trophic transfer, the main factors and mechanistic processes that control this, in the case of Me-ENPs, remain largely unknown. It is our contention in this tutorial review that the processes known to be involved in trophic transfer of trace metals in aqueous form may provide insights into the movement of Me-ENPs. Hence, the literature pertaining to the former is considered alongside our review of the Me-ENP trophic transfer literature.

3. Factors affecting trophic transfer of Me-ENPs

A multitude of factors may affect whether, and in what form, Me-ENPs are transferred between trophic levels. Based on the existing literature that has investigated this directly (described in section 2 and Table 1) as well as the wealth of literature on the trophic transfer of metals, we identify four broad key factors affecting trophic transfer of Me-ENPs. These factors, depicted in Fig. 1, are (1) the environmental transformations of Me-ENPs, (2) the uptake and accumulation in prey organisms, (3) the internal fate and localization in the prey, and (4) the digestive physiology of the predator. The relevance of each of the four factors and their relationship to the trophic transfer of Me-ENPs are detailed in the following sections.

3.1. Environmental transformations of Me-ENPs

Me-ENPs enter the environment *via* several routes including untreated wastewater, accidental spills and intentional usage such as environmental remediation.[43,44] Once in the environment, Me-ENPs will undergo a variety of transformation processes that influence their biotic interactions:[45–47] (a) dissolution resulting in the release of metal ions,[48–50] (b) alterations of the ENPs through association with environmental ligands and/or the formation of possible coatings/bilayers,[51–53] and (c) aggregation/agglomeration leading to precipitation and

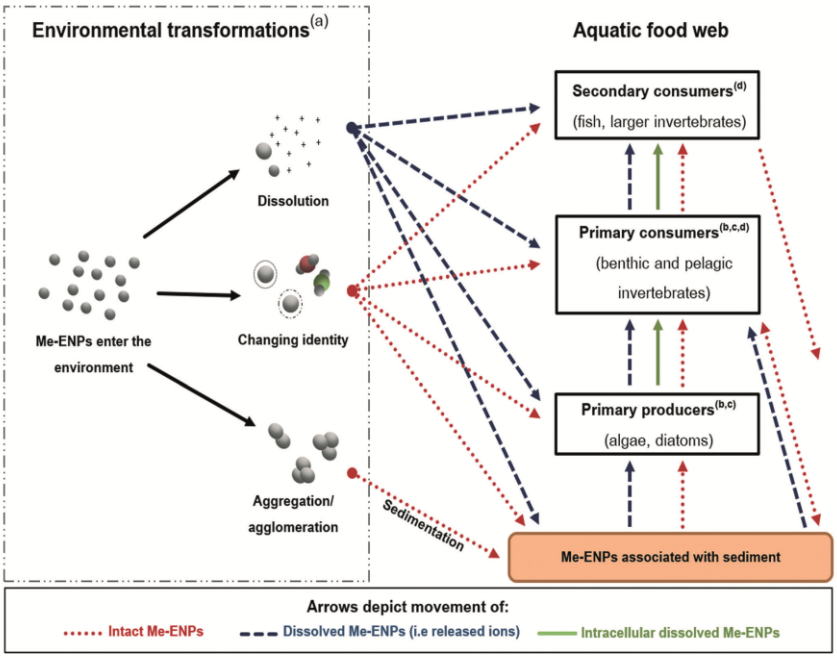

Fig. 1 Schematic presentation of exposure pathways and environmental transformation processes relevant to aquatic ecosystems in regard to trophic transfer of Me-ENPs. After introduction of Me-ENPs to the environment, particles undergo different environmental transformation processes: dissolution leads to release of aqueous metal ions, changing identity refers to the association with ligands and formation of oppositely charged bilayers/coatings, and aggregation/agglomeration results in sedimentation of particles. All transformations lead to different mechanisms for trophic transfer, depicted in the right side of the figure. After sedimentation, Me-ENPs can react with different constituents or undergo transformation processes within the sediment, *e.g.* sulfidation, leading to changed bioavailability of the particles. Furthermore, Me-ENPs can be excreted by organisms after intake, creating an indirect release to the environment. (a)–(d) Refers to section 3: (a) environmental transformations, (b) uptake by the prey, (c) internal fate and subcellular localization in the prey, and (d) digestive physiology of the predator. The red arrows indicate movement of intact particles (direct and indirect release), the blue arrows indicate movement of dissolved Me-ENPs (as metal ions), and the green arrows indicate movement of intracellular dissolved particles (metal ions) in the prey to the predator.

likely sedimentation.[54–56] These transformations are likely to occur simultaneously,[45] and the combination of these transformative processes will profoundly affect any subsequent trophic transfer.

Several studies show that dissolution is of central importance to the accumulation and toxic potential of Me-ENPs. This is particularly the case for ZnO,[16,57–59] CuO[60–62] and Ag[50,63–66] ENPs, although for the latter there is also a weight of evidence to suggest nano-specific uptake mechanisms (*i.e.* endocytosis).[19,23,67] Dissolution of Me-ENPs is affected by the inherent properties of the particle, such as size, constituent metal and surface chemistry as well as the composition of the environmental media. The interaction of these two factors will add to the variety of scenarios under which dissolution occurs, as described by Misra *et al.* (2012).[68] Dissolution in various environmentally relevant media is reported in the range of 1–80% for CuO, Ag and ZnO ENPs, and this wide range again highlights the importance of thorough and exposure-specific characterization during experiments,[68] although it is recognized that characterization is not available for all environmental matrices (*e.g.* sediments) owing to the lack of analytical methodologies. Arguably, however, dissolution is the most important transformation that a Me-ENP can undergo, changing the metal from a nano-scale structure into its ionic form. In terms of environmental safety and risk assessment, it has been suggested that the dissolution of the particle may represent the best case scenario as the ecotoxicological consequences would be likely no different from those of the constituent ion.[69] This would be equally true for potential trophic transfer of Me-ENPs as the uptake and accumulation of trace metals by various trophic levels has been widely investigated.[70–78]

Alterations to the surface of Me-ENPs will take place at different degrees and will depend on factors such as particle coating and the presence of binding or complexing agents in the environment in which particles are released. Surface alterations, as have been shown, affects the behavior of the Me-ENPs in the environment and, subsequently, how they interact with organisms. In terms of changing the ENP surface, sulfidation has been reported to be an important process, particularly in the case of Ag ENPs. It likely causes a decreased dissolution rate and mobility, which would have an impact on the toxicity of the particles and their interactions with organisms.[79] The sulfidation of Ag ENPs results in the formation of Ag_2S adsorbed to the particles as coating[55] or in the formation of new silver-sulfide nanostructures[80] similar to those observed in field samples.[81] Similarly, salinity, natural organic matter (NOM) and dissolved organic matter (DOM) all affect Me-ENP surfaces. Interactions between particles and NOM or DOM can create new particulate bilayers, which, like sulfidation, would affect the behavior of the particle (*i.e.* stability in the environment) and its interactions with biota. In their review of how Me-ENPs (termed as inorganic colloids) interact with DOM, Philippe & Schaumann (2014)[82] outlined several adsorption mechanisms that control the degree of sorption, stability and aging. Their main findings were that DOM dynamics, bridging, and aggregation–disaggregation mechanisms are all influenced by the presence of humic substances, polysaccharides, and proteins found in natural waters and must be understood to describe colloid stability.[82] Studies on ENPs and protein interactions have highlighted that it is not the inert particle that is in contact with biological systems (*i.e.* epithelial cells of the gill or intestine) during uptake, but in fact the altered particle surface.[51,52,83,84] Proteins, and especially apolipoproteins, have shown to adsorb to ENP surfaces creating coatings known as a "protein corona".[85] It is this corona that the epithelial cells "see" and interact with when ENPs are taken up. The presence of a surface layer or corona changes the properties and 'biological identity' of the ENP, and in the case of the protein, the corona is likely to promote particle uptake.[86] These findings illustrate the importance of characterizing particles in the environment or test media, in particular, as the composition of the media is highly influential in determining the fate of the particle. The chance of finding pristine particles in the aquatic environment is highly unlikely.

Combinations of environmental processes and particle characteristics will cause aggregation and/or agglomeration of Me-ENPs, which results in the likely sedimentation of the particles. For instance, pH, ionic strength, surface coating and surface charge will all influence the degree of aggregation. Furthermore, salinity changes the time taken for sedimentation and aggregation from days in seawater to months in freshwater.[87–89] The relatively slow sedimentation in freshwater can result in a greater dispersion time in the water column with possible uptake and effects to pelagic species. Conversely, the faster sedimentation in more saline waters will lead to higher concentrations of ENPs in the sediment, resulting in increased risk for benthic and sediment-dwelling organisms. When natural disturbance is taken into consideration, using setups mimicking turbulent systems, sedimentation rates in the same order of magnitude for different ENPs under different salinity and aging time is seen.[90] This is in contrast with the reported data from Garner and Keller (2014)[91] highlighting the complexity of determining these factors for Me-ENPs. Furthermore, it illustrates that a greater number of potentially competing processes must be considered when the environmental fate and behavior of Me-ENPs are studied.

Praetorius *et al.* (2014)[92] reported that hetero-aggregation (the interaction between different particle types, both organic and inorganic) is more important than homo-aggregation (interaction of the same particle type) for TiO_2 ENPs in natural environments. Furthermore, attachment efficiency, reflecting the likelihood of particles "sticking" together, is highly important and should be implemented in future environmental fate models.[92] Attachment efficiency may describe the creation of primary or secondary aggregates, that is aggregates created between primary particles (*i.e.* TiO_2 ENPs) or aggregation of already aggregated particles and other particulates (*i.e.* organic matter), thereby producing secondary (larger) clusters.[92] Primary and secondary aggregates are likely to be found in natural environments, making this an important observation for future studies. Me-ENPs such as TiO_2 ENPs will likely exist as aggregates in diverse forms, increasing their sedimentation rates and thereby the likelihood of finding them within the sediment compartment. Again, media composition and turbulence will also play an important role. Dale *et al.* (2015)[93] modelled the environmental fate of ZnO and Ag ENPs in the James River Basin (Virginia) and found that due to high mobility, sediment transport and streamflow, ENPs would be removed downstream from the River Basin. However, estimations also suggest that ENPs would eventually accumulate and persist in sediments. In extreme cases, particles may persist for over a century.[93] Hence, depending on the system (static *vs.* turbulent), media (fresh *vs.* seawater) and particle type (coated *vs.* un-coated), sedimentation rates can be highly variable, but sedimentation appears to be a transformation of importance, thus highlighting the sediment compartment as a realistic environmental exposure route to ENPs.

With the differing sedimentation rates in mind, it is important to acknowledge that Me-ENPs will firstly be available for organisms in the water phase, after which particles settle out of the water column. Most studies agree that the final destination for Me-ENPs released into the aquatic environment is the sediment, making this an important sink (and source) for these contaminants.[53,90,94] Interactions with sediment-dwelling organisms are therefore important to characterize. The most obvious being the incidental ingestion of particles by these organisms[19] and whether (and how) they are subsequently internalized into the tissue. However, sediment dwellers are also likely to influence the distribution of the ENPs through the excretion of unassimilated particles re-

entering the environment and *via* bioturbation, where through the movement through sediment grains and irrigation of burrows these organisms may recycle Me-ENPs back into the water column.[95]

Whilst a great deal of research has been conducted into the environmental transformations Me-ENPs may undergo in the environment, there are still many knowledge gaps that constitute future research needs including reverse reactions, transformation rates and the implications of aged or altered particles. However, we do know that numerous environmental transformations will occur simultaneously, leading to Me-ENPs in the aquatic environment existing as a mixture of released ions, particles with altered surfaces (potentially altered biological identities), agglomerates and aggregates. Thus, it is of the utmost importance to differentiate between the uptake and trophic transfer of intact Me-ENPs, which, although may have been modified through aggregation/agglomeration, sulfidization or surface alteration, are still nano-scale structures, and the metal which, although may have entered the environment in nano-form, is present in aqueous form. As a significant sink for settled intact Me-ENPs, sediments may be the most important entry point for intact Me-ENPs entering the aquatic food webs.

3.2. Uptake and accumulation by the prey organism

After environmental release, the Me-ENPs in the aforementioned forms (released ions, ENPs with altered surfaces, agglomerates and aggregates, associated with sediments) will be available for uptake by organisms at the base of various food webs. The mechanisms of uptake will highly influence the likelihood of the particles being passed onwards to prey organisms.

As mentioned, ENP dissolution has been described as the best case scenario as the risk of aqueous metals are largely established.[69] If ions are released, then uptake will be achieved in the same way as ions originating from aqueous metals. Ion uptake is achieved by membrane transporters that can transport metal ions directly into the cell.[71] Essential metals, such as Cu, Zn and Fe, use established pathways, whereas non-essential metals often employ ionic mimicry using transporters intended for similarly sized and charged ions. For instance, Ag is taken up by Cu transporters in mammalian cells[96] and *via* Na channels in freshwater fish.[97] As Me-ENPs release ions, such mechanisms may also facilitate metal uptake from Me-ENPs. Any subsequent trophic transfer of this metal to the next trophic level would occur in the same ways that have been already studied.[2–4,8,9]

However, it is internalization of intact ENPs into lower trophic level organism tissues, or adsorption to body surfaces (*e.g.* on snail shells[18,21]), accumulation in gut lumen (either adsorbed to gut epithelia or as aggregates/agglomerates), and their subsequent transfer to their predators that presents a novel scenario. Regardless of how ENPs are associated with the prey (sorbed to shell or epithelia layers or internalized), they will be transferred to the predator; however, the availability for assimilation will depend on the ENP association with the prey. One hypothesis is that sorbed ENPs will be more readily available for assimilation than internalized ENPs. Me-ENP uptake into epithelial cells that face the external environment (*i.e.* those of the gill or gut) can be facilitated *via* different pathways, yet consensus amongst studies shows that primary uptake mechanisms for intact Me-ENPs is *via* endocytotic processes.[19,65,67,98] Nanomedicine has shown that the different endocytotic processes (*i.e.* clathrin- and caveolae-mediated endocytosis or pinocytosis) can lead to the internalization of single particles and aggregates in the size range of 10 nm to 5 μm.[99] Khan *et al.* (2014)[100] showed that the endocytotic uptake of Ag ENPs presented to a mud snail (*Peringia ulvae*) through waterborne exposure occurred *via* multiple routes that included both clathrin- and caveolae-mediated endocytosis, as well as ion channels and/or transporter proteins for the dissolved Ag fraction. Endocytotic processes would lead to uptake of intact particles, which would be followed by different intracellular outcomes such as intracellular ion release, creation of ENP-containing vesicles or disruption of normal cell function.[99,100] In primary producers such as unicellular algae, uptake mechanisms depend on both cell wall characteristics and particle size. Plant cell walls are semipermeable, including pores with diameters between 5 and 20 nm, meaning that Me-ENPs within this size range might be allowed to pass the barrier and move into the plasma membrane.[101] Again, cellular uptake is predominantly *via* endocytotic processes;[98] however, Me-ENPs could also employ ion channels or protein carriers to cross the membrane.[101]

As discussed, ENPs have been shown to interact with proteins and ligands within biological fluids, creating a biological surface coating on the particle, possibly enhancing cellular uptake.[52] Other particle characteristics can also affect how Me-ENPs are taken up and accumulated. In the study by Bouldin *et al.* (2008),[28] the organic polymer coating on Cd QDs protected the algae against direct toxic effects, leading to an increased transfer of Cd QDs from algae to primary consumers such as daphnids, as the algae continued to be an attractive food source.[28] Likewise, coating affected bioaccumulation in a study by Larguinho *et al.* (2014)[102] where a bivalve (*Mytilus galloprovincialis*) fed algae (*Dunaliella salina*) pre-exposed to PEGylated Au ENPs showed a higher Au content, compared to algae exposed to citrate-capped particles.[102] Cellular uptake mechanisms are also affected by particle characteristics such as size and coating. Smaller particles (5 nm) and organic coated particles (tannic acid and citrate) are taken up in cells to a higher degree than larger (50–100 nm) and PVP-coated Me-ENPs.[13,103] Thus, both the physiochemical properties of the ENP and the physiological characteristics of the species will affect the pattern of accumulation and must be considered when investigating the internalization of particles.

Several factors influence the particle fate after organism uptake, when the route of uptake is dietary. Feeding rate affects the time a metal or Me-ENP is retained in the gut (the

gut passage time (GPT)) of the prey, and therefore, the time allowed to, for example, disaggregation/dissolution and absorption over the gut epithelia. GPT is inversely related to feeding rate, and the assimilation efficiency (AE) increases with increasing GPT until a 'steady-state' level is reached.[104–107] Metal assimilation efficiencies (AE) from diets are generally high (ranging from 65 to 97%) and depend on both the test organism and the selected metal.[75,108,109] Examples of AE values for Me-ENPs are reported in the same range, from 41 to 93%.[23,110,111] If metal or Me-ENP assimilation is high in the prey, the possible transfer of the accumulated and assimilated metal to the predator is increased.

Daphnia magna is a well-established laboratory species and natural prey to many aquatic organisms. Thus, a number of studies have investigated how daphnids accumulate metals and Me-ENPs from the surrounding media and food.[112–115] Daphnids filter particles between 0.1–5 μm,[116] making aggregated ENPs available for ingestion in these organisms. Recent studies on uptake and depuration mechanisms in *D. magna* after short-term exposure to Au ENPs demonstrated that the elimination rate is increased when daphnids have access to food[117] and a bi-phasic elimination pattern during the depuration phase with food present results in more than two thirds of the ingested Au being excreted within the first hour of depuration.[115] Thus, the presence of food enhances the elimination of Me-ENPs from daphnids, whereas limited or no access to food decreases particle excretion.[118] The retention of intact Me-ENPs in the gut of daphnids may not constitute uptake in the sense of being incorporated into the tissue (and nor would ENPs adsorbed to the external carapace[118]), but if daphnids containing ENPs are predated upon, then those intact particles are subject to transfer to the predator as discussed above.

Pelagic zooplankton, however, whilst well studied are perhaps not where investigations of trophic transfer should focus. As previously discussed, the likely eventual fate for Me-ENPs released into the environment is to associate with sediments, although the time to sedimentation may vary with environmental conditions. *Nereid polychaetes* provide an example of deposit-feeding animals that ingest sediments to consume nutritious organic matter but will also incidentally ingest sediment-associated contaminants. Up to half the silver uptake in *Nereis diversicolor* resulted from the ingestion of sediments,[119] whereas for *Nereis succinea* the figure was 95%.[120] Such organisms are an important prey item for a variety of large invertebrates, fish and bird species and thus provide a pathway for ENPs to move from the abiotic compartment into the aquatic food web. The caveat with this is of course whether the ENPs remain intact or whether they are prone to dissolution within the worm tissue or gut cavity. García-Alonso *et al.* (2011)[19] visualized the ENPs in endosomes and small vesicles in gut epithelial cells at the base of the microvilli upon exposure to citrate-coated Ag NPs mixed in estuarine sediments. These ENPs appeared to have been endocytosed as intact particles, but as described in the following section different endocytotic mechanisms can lead to different intracellular fates, including lysosomal degradation leading to particle dissolution. Although the exact nature of endocytosis was not investigated by those authors, the presence of intact ENPs in the tissue of common prey items does dramatically increase the probability of Me-ENP trophic transfer.

The biological processes utilized in the uptake of intact Me-ENPs can influence the internal fate of the particles. Intracellular dissolution in prey organisms will negate the transfer of intact Me-ENPs to predators, whereas slower dissolution could result in a relatively higher transfer of ENPs through the food chain. Thus, it is important to distinguish the trophic transfer of ions released by Me-ENPs internally and the movement of the ENPs themselves up the food chain. In order for actual trophic transfer of Me-ENPs to occur, the uptake by or adsorption to the prey followed by further transfer to predators should be of the intact Me-ENPs. In this regard, the uptake route that starts with the incidental ingestion of ENPs from food sources, such as algae or from sediment, may present the greatest likelihood of intact ENPs passing up the food chain.

3.3. Internal fate and subcellular localization in the prey

When Me-ENPs are taken up by prey organisms, different processes will occur depending on species- and tissue-specific physiology and the mechanism of Me-ENP uptake. For example, the interactions between gastric acid and Ag ENPs show accelerated dissolution,[64] whereas the release of silver ions from Ag ENPs in simulated lung media is negligible after 96 h, and aggregation of particles increases with ionic strength.[121] Thus, whilst the former may limit the potential for trophic transfer, the latter in which Ag NPs remain intact may offer greater potential for food web passage. Me-ENPs might degrade or form complexes with substances present within biological media, such as gut or cellular fluids, altering their toxicity and bioavailability[63,122] to both the organism in question and those that predate it.

As mentioned, the primary mechanism for intact particles to enter tissues appears to be endocytotic in nature and could potentially take place at the epithelia of the digestive or respiratory systems. The exact mechanism may be an important determinant of the Me-ENP's intracellular fate: NPs endocytosed through the clathrin-mediated pathway are likely destined for lysosomal degradation.[123] This pathway may be responsible for the much described nanoparticle "Trojan horse" effect in which intracellular toxicity results in the presence of high concentrations of labile metal ions.[124] Conversely, if uptake is achieved *via* caveolae-mediated endocytosis or macropinocytosis, the ENP is not directed to the lysosome. Instead, intracellular vesicles (known as caveosomes in the case of the caveolae pathway) may fuse with the cell membrane and deliver the NPs out of the cell (exocytosis), so that NPs pass through the cell (transcytosis[98]). ENPs within macropinosome vesicles are not directed to the lysosome either but may remain in the cell in

particulate form. Whilst internalization *via* these mechanisms may lead ENPs to novel intracellular locations where they might induce toxic responses,[98] this does suggest that intact ENPs do remain in the tissue and potentially may be bioavailable to the predatory organisms.

Whilst different uptake mechanisms potentially lead to different internal fates, the key aspect of this topic is to address whether intact Me-ENPs in the tissue are trophically available. Within this review, we have proposed that previous research into the trophic availability of aqueous metals provides a guide to understand whether and how Me-ENPs move in aquatic food chains. It is perhaps in this topic area that studies with aqueous metals are most useful. Subcellular fractionation (differential centrifugation) protocols have been used to examine the internal distribution of metals and in a few studies with Me-ENPs. Commonly, the following subcellular fractions are collected from tissue homogenates: metal-rich granules (MRG), cellular debris, organelles (*i.e.* lysosomes and mitochondria), cytosolic heat sensitive proteins ('enzymes') and cytosolic heat stable proteins ('metallothionein-like proteins' (MTLP)).[125–127] These fractions can be grouped according to solubility (insoluble MRG, debris and organelles *vs.* soluble enzymes and MTLP), toxic potential (detoxified metal within the MRG and MTLP fractions and other fractions considered as metal sensitive), and on the basis of trophic bioavailability of metals ('trophically available metal' (TAM), considered to include MTLP, enzymes and organelles).[128] This latter division has been shown to be largely consistent amongst a variety of prey organisms and predators when exposing the prey to aqueous metals,[129–136] but TAM is not a universally defined fraction and differences occur based on the metal in question, the physiology and internal metal handling of the food item and the feeding animal.[128,137]

The described fractionation method was employed on the endo-benthic ragworm, *N. diversicolor*, following exposure to citrate-coated Ag ENP spiked sediments.[19] Ag ions were used as reference, and tissue homogenates from different exposure scenarios were examined and showed differences between Ag forms. Ag ions were detected in the MTLP fraction, whereas Ag ENPs were found in MRG, organelles and enzyme fractions. The difference in the distribution of Ag administered as particulate and aqueous forms was demonstrated and indicated that Ag ENPs did not follow the same subcellular distribution as Ag^{+}, suggesting that the Ag ENPs did not dissolve internally.[19] Similarly, sediment exposure of *N. diversicolor* to different forms of Cu (Cu ions, CuO micro- and nanoparticles) resulted in differential distribution of Cu between the subcellular fractions. Following exposure to sediment spiked with Cu ions, Cu was primarily found in MRG, to sediment spiked with CuO-micro, Cu was distributed equally among all five fractions and to sediment spiked with CuO-nano, Cu was primarily present in cellular debris.[22] Thus, the subcellular fractionation protocol established for aqueous metals may also work for Me-ENPs, but nano-specific considerations need to be taken into account. The drawback is that such operational processes might introduce artefacts, as shown for trace metals.[138] For instance, ENPs might combine with fractions based on mass during centrifugation rather than biological association, giving a false impression of the actual subcellular distribution of the Me-ENPs. Yet, given the importance of metal localization in prey organisms in regard to bioavailability to the next trophic level, this method, with appropriate nano-specific considerations, may provide initial guidelines into determining internal fate and trophic availability.

Additional tools for characterizing Me-ENPs in different sample types offer approaches for determining internal fate. Qualitative analysis *via* transmission electron microscopy (TEM) or scanning electron microscopy (SEM) has been used to visualise the location of ENPs in tissue,[19,139] and even light microscopy and TEM have been used to detect Me-ENPs in *D. magna*. Au ENPs were observed in the midgut of organisms with no cellular uptake detected, indicating that particles were not moving past the intestinal barrier.[115] Synchrotron X-ray radiation tools have been applied to nanomaterials science to measure ENP size, agglomeration state and surface structure *in situ*.[140] This technique appears very promising for investigating the internal fate of Me-ENPs in tissue samples, as well as ENP behaviour in different media such as water or sediment. Other visualization techniques include the use of fluorescent particles (*e.g.* QDs) together with flow cytometry[35] and confocal laser scanning microscopy (CLSM).[141] The advantage of these newer techniques compared to TEM and SEM is that particles can be tracked inside whole organisms, diminishing the artefacts related to sample preparation. Furthermore, imaging particles *in vivo* will increase our qualitative understanding of how Me-ENPs are accumulated and handled within tissues.

Determining the internal fate of Me-ENPs is still a relatively novel research area. Protocols known to work for aqueous metals, such as differential centrifugation, could also be applicable for Me-ENPs, whilst nano-specific methods will undoubtedly build on initial data. In combination, these tools should be employed to understand the mechanisms controlling internal localization of Me-ENPs. Within prey organisms, this is likely key to determining whether and how Me-ENPs move through aquatic food chains.

3.4. Digestive physiology and accumulation mechanisms of the predator

The preceding sections have described how Me-ENPs may be subject to transformations both following environmental release and within prey organisms. With the assumption that some ENPs persist in particulate form, the remaining barrier to achieving trophic transfer will be how those ENPs within the tissue of the prey are handled once ingested by the predator. At this point, it is important to consider what constitutes dietary uptake and/or assimilation efficiency when dealing with particulate contaminants. For non-particulate contaminants, including trace metals, the common understanding is

that the term includes the proportion of the ingested contaminant that crosses the gut lumen and is present in the tissue.[7,8] This is predominantly determined by measuring tissue burdens following a suitable depuration period. However, it is not clear whether this requirement also applies to Me-ENPs. Many studies with Me-ENPs determine the presence of metal ions in tissue digests, where the metal has been introduced as an Me-ENP,[14–24] but this is not the same as determining the presence of the nanoparticle itself. Thus, in many cases where ENPs are introduced *via* food, it is not possible to determine whether i) the ENP has crossed the intestinal epithelium, ii) the intact ENP remains in the lumen or iii) the ENP undergoes complete or partial dissolution in the lumen and the ions are translocated into the tissue. Included within this is the possibility that particulate forms may associate with luminal material and persist beyond the depuration period as seen in some invertebrate models.[115,118] Thus, for the purposes of our discussion on trophic transfer, we suggest the widest definition of uptake and assimilation, which also encompasses the retention without assimilation of ENPs in the gut lumen of the higher trophic level organisms.

Like prey, predatory organisms differ in their feeding mode, gut residence time and digestive physiology, all affecting how metals are taken up and assimilated within the organism.[142] Gut pH varies among different organisms, with invertebrates having a somewhat neutral pH, most fish having acidic gut conditions (pH<2) and some polychaetes experiencing higher gut pH (>8).[38] This leads to an enhanced or decreased metal uptake, as pH is believed to influence ion release.[68] Whilst the trophic transfer of metals has been shown to be affected by factors such as assimilation, internal localization, gut physiology and concentration of metals within both prey and predator,[7,143,144] much less is known for Me-ENPs. Some studies with Me-ENPs have included secondary consumers, such as zebrafish[33,35,145–147] or bivalves,[102,148,149] when investigating trophic transfer (Table 1). Based on the published results to date, evidence suggests that biomagnification is not of concern at this level, thereby decreasing the contamination risk for higher, predatory organisms such as carnivorous fish or humans. However, knowledge at these trophic levels is limited, and studies describing factors and processes responsible for trophic transfer of Me-ENP to higher organisms are scarce.

The major predator in the pelagic food web is fish, and studies have looked into how metals and Me-ENPs are being taken up and accumulated in these organisms.[17,150,151] Fish can, like daphnids, accumulate metals and Me-ENPs in their gut from the surrounding media, as they drink metal-contaminated water.[152,153] Dietary uptake of trace metal ions may result in physiological alterations of the gut,[154,155] affect reproductive output[156] and possibly cause cell damage.[135,136] The mechanism(s) responsible for metal transport in predatory fish have been shown *via in vitro* and *in vivo* exposures of the African catfish (*Clarias gariepinus*), revealing that mucosal cells within the intestinal regions were responsible for the highest Cu accumulation.[157,158] Fish were able to eliminate metals by increasing their intestinal mucus production and excrete mucosal cells. Metals can also be translocated from gut cells to organs such as intestine, brain and gills,[74,159] thereby increasing the metal concentration within internal organs. With regard to Me-ENPs, the same kind of translocation was observed in a freshwater fish (*Cyprinus carpio*) exposed to waterborne Ag ENPs. A significant Ag uptake in liver, intestine and gallbladder was due to translocation of Ag ENPs from the gastrointestinal tract.[112] Hence, the mechanisms responsible for trace metal accumulation in predatory fish could be applicable for Me-ENPs, but many factors remain unclear.

Besides fish, bivalve mollusks are considered a top predator, primarily in the benthic food web. They are often used as bio-indicators in aquatic ecosystems, and several studies have examined metal accumulation and effects on these organisms,[160–162] including subcellular distribution.[125–127,163] As suspension feeders, bivalves are at high risk of Me-ENP exposure. Due to their enhanced processes of cellular internalization of natural particles in the micro- and nano-size ranges, their physiological system is susceptible to ENP uptake.[164] For example, the bivalves *Mytilus edulis* and *Crassostrea virginica* capture and retain natural particles <100 μm in size during certain times of the year, making aggregated ENPs highly available for uptake.[165] As reviewed by Canesi *et al.* (2012),[164] bivalve mollusks are valuable model organisms for understanding the risks and effects of ENPs on aquatic invertebrates. *In vivo* and *in vitro* studies show that ENPs may target the immune system, and agglomerates and aggregates translocated from gill to the digestive gland lead to intracellular uptake and oxidative stress.[166,167] This makes these organisms sensitive to the increasing ENP contamination and, due to their placement in the food web, also an important predatory organism to encounter in trophic transfer studies.

Amongst the four key processes we outline as factors that may affect the potential for Me-ENP trophic transfer, the role played by the digestive physiology of the predator is the least studied. The likelihood of intact ENPs moving to this level of the food chain decreases at each step, due to environmental and *in vivo* transformations that take place before and after uptake by primary consumers. However, given the effects caused by dietary trace metals, more research needs to be conducted on potential outcomes following Me-ENP passage up the food chain. Future studies should aim at describing the fate of Me-ENPs at this food chain level in more detail, in order to increase the understanding of mechanisms responsible for transport to higher trophic levels.

4. Recommendations for future research & conclusions

Trophic transfer of Me-ENPs has become an increasingly researched area, yet many factors remain unknown. As shown in Fig. 1, numerous processes and mechanisms are likely to influence Me-ENP transfer, and these can be grouped into the four broad categories that we propose, (1) environmental

transformations of Me-ENPs, (2) uptake and accumulation in prey organisms, (3) internal fate and localization in the prey, and (4) digestive physiology of the predator. Most research has been conducted within the first step(s) of the food web. Primary producers and consumers have been thoroughly investigated with regard to uptake, bioaccumulation and nano-specific effects. These organisms create the largest pool of knowledge for further ENP studies, but as we continue up the food web, less information is available and we rely more and more on indications and qualified guesses.

Currently, little is known about the trophic transfer of ENPs; therefore, we propose that mechanisms, processes and factors controlling trophic transfer of trace metals may provide a good starting point for increasing our understanding with the acknowledgement that nano-scale specificities must also be considered. Examining the species-specific characteristic of lower and higher trophic-level organisms, including uptake routes, accumulation characteristics and subcellular distribution could provide the first steps towards a better description of trophic transfer of Me-ENPs in aquatic food webs. The internal fate and behavior of Me-ENPs, particularly in those organisms that constitute food items, are understudied, yet highly important. Subcellular fractionation can give an indication of where particles reside within organisms following uptake and bioaccumulation; however, the link between subcellular distribution and trophic availability requires verification for Me-ENPs. Moreover, very little research has focused on how the digestive physiology of the predator influences the uptake of Me-ENPs at the higher trophic levels, and related to this, it may become necessary to revise our understanding of what constitutes uptake for particulate contaminants if they remain within the digestive system without necessarily achieving trans-epithelial uptake.

Amongst the relevant accumulation routes, sediment exposures arguably provide the greatest likelihood of intact ENPs being subject to trophic transfer. Although pelagic zooplankton has been shown to take up particles from the water column *via* filter feeding, the contact with water-borne particles is time-limited since particles are generally assumed to sediment. Thus, both through sediments being an eventual sink for ENPs and the potential persistence of the particle, sediment dwelling-organisms have the greatest exposure duration. Furthermore, it has been demonstrated that benthic organisms may incidentally ingest ENPs during their consumption of sediment, and that, at least in some cases, these particles can be endocytosed within the gut and remain relatively untransformed for a period of time. From this scenario, the potential for Me-ENP food web transfer is maximal but will ultimately depend on the fate of the particle in the prey and the digestive physiology of the predator. These two factors constitute areas where more research focus is required, but sediment exposures could be regularly employed as the most likely exposure route to consistently load prey food items with Me-ENPs.

In this tutorial review, we highlight four broad key factors in describing trophic transfer of Me-ENPs, which all should be studied further to give a better understanding of this phenomenon. Trophic transfer of Me-ENPs occurs under some circumstances, but the underlying processes responsible are poorly understood. Emphasis on digestive physiology of predators is needed, as well as studies including several trophic levels and more complex systems. For both greater scientific understanding and risk assessment needs, the present research into the trophic availability of trace metals is likely to be an important guide. However, nano-specific deviations from this must be recognized and understood.

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Paper II

Bioaccumulation and effects of sediment-associated gold- and graphene oxide nanoparticles on *Tubifex tubifex*

Panhong Zhang, Henriette Selck, Stine Rosendal Tangaa, Chengfang Pang, Bin Zhao

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- Supervising the experimental work
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Bioaccumulation and effects of sediment-associated gold- and graphene oxide nanoparticles on *Tubifex tubifex*

Panhong Zhang[1,2], *Henriette Selck*[3,*], *Stine Rosendal Tangaa*[3], *Chengfang Pang*[1], *Bin Zhao*[1,*]

1. *State Key Laboratory of Environmental Chemistry and Ecotoxicology, Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences, University of Chinese Academy of Sciences, Beijing 100085, China Email: panhongzhang2015@yeah.net*
2. *Sino-Danish Center for Education and Research, Beijing 100190, China*
3. *Department of Science and Environment, Roskilde University, P.O. Box 260, 4000 Roskilde, Denmark*

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ABSTRACT

With the development of nanotechnology, gold (Au) and graphene oxide (GO) nanoparticles have been widely used in various fields, resulting in an increased release of these particles into the environment. The released nanoparticles may eventually accumulate in sediment, causing possible ecotoxicological effects to benthic invertebrates. However, the impact of Au-NPs and GO-NPs on the cosmopolitan oligochaete, *Tubifex tubifex*, in sediment exposure is not known. Mortality, behavioral impact (GO-NP and Au-NP) and uptake (only Au-NP) of sediment-associated Au-NPs (4.9 ± 0.14 nm) and GO-NPs (116 ± 0.05 nm) to *T. tubifex* were assessed in a number of 5-day exposure experiments. The results showed that the applied Au-NP concentrations (10 and 60 μg Au/g dry weight sediment) had no adverse effect on *T. tubifex* survival, while Au bioaccumulation increased with exposure concentration. In the case of GO-NPs, no mortality of *T. tubifex* was observed at a concentration range of 20 and 180 μg GO/g dry weight sediment, whereas burrowing activity was significantly reduced at 20 and 180 μg GO/g dry weight sediment. Our results suggest that Au-NPs at 60 μg Au/g or GO-NPs at 20 and 180 μg GO/g were detected by *T. tubifex* as toxicants during short-term exposures.

Introduction

Engineered nanoparticles (ENPs) are widely applied in diverse fields, such as medicine, cosmetics, renewable energy, food industry, electronic devices and environmental remediation (Dong and Feng, 2007; Fabrega et al., 2011; Kachynski et al., 2008; Lens, 2009; Pavasupree et al., 2006; Tungittiplakorn et al., 2004; Wei et al., 2008). Among various engineered nanomaterials, gold (Au) and graphene oxide (GO) nanoparticles are widely used. Both Au-NPs and GO-NPs are unique materials for nano-medicine applications such as drug delivery (Dykman and Khlebtsov, 2012; Zhang et al., 2010) and thermodynamic therapy (Lytton-Jean and Mirkin, 2005; Wang et al., 2011). Au-NPs have been used in materials science, electron microscopes and biological sensors (Dreaden et al., 2012; Lim et al., 2011; Panyala et al., 2009; Zeng et al., 2011), and GO-NPs have been applied in energy storage, electronics and bioenvironmental materials (Park and Ruoff, 2009; Wang et al., 2011; Zhao et al., 2012). The widespread use of Au-NPs and GO-NPs is likely to increase their release into the aquatic environment via wastewater discharges. Once these nanoparticles are released into the aquatic environment, they will likely undergo transformation processes including dissolution, aggregation, agglomeration, and eventually settle into the sediment (Thit et al., 2015; Zhao et al., 2014). Therefore, sediment may become an ultimate

* *Corresponding authors.* E-mail: selck@ruc.dk (Henriette Selck), binzhao@rcees.ac.cn (Bin Zhao).

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reservoir for ENPs. As a result, nanoparticles may be ingested by deposit-feeding benthic invertebrates and potentially be bio-magnified within the food chain (Ferry et al., 2009; Judy et al., 2011), which may pose a high risk to invertebrates and higher trophic level organisms.

Toxicity and bioaccumulation of sediment-associated Ag-NPs and CuO-NPs to sediment-dwelling invertebrates have been investigated (Cong et al., 2011, 2014; Dai et al., 2013; Pang et al., 2012, 2013; Ramskov et al., 2014; Thit et al., 2015). CuO-NPs with concentrations ranging from 30 to 240 μg CuO/g dry weight sediment negatively affected the specific growth rate, feeding rate, bioaccumulation and reproduction of the freshwater snail *Potamopyrgus antipodarum*, whereas the survival of *P. antipodarum* was not affected (Pang et al., 2012, 2013; Ramskov et al., 2014). It was reported that Ag-NPs of 1 to 50 μg Ag/g dw sediment caused DNA damage and genotoxicity in the marine polychaete *Nereis diversicolor* (Cong et al., 2014). Furthermore, the burrowing behavior of *N. diversicolor* was impaired by Ag-NPs at a concentration of 150 μg Ag/g dw sediment without affecting survival (Thit et al., 2015). Dai et al. (2013) investigated the toxic effects of sediment-associated Ag-NPs and CuO-NPs on the mussel *Macoma balthica* and found no negative effects on genotoxicity, mortality, condition index, or burrowing behavior at concentrations from 150 to 200 μg Ag or CuO/g dw sediment (Dai et al., 2013).

Studies investigating the toxicity of Au-NPs to organisms have mainly focused on water and soil exposure. Soil exposure showed that Au-NP concentrations up to 37.5 μg Au/g dw did not impact the survival and reproduction of the grindal worm *Enchytraeus bucchholzi* (Voua Otomo et al., 2014). In contrast, Unrine et al. (2010) showed that Au-NPs were accumulated in earthworms (*Eisenia fetida*) exposed to 5–50 μg Au/g dw soil and caused adverse effects on reproduction (Unrine et al., 2010). In water exposures, 100 μg Au-NP/L induced metallothionein production as a response to metal contamination, and increased activities of catalase, superoxide dismutase and glutathione S-transferase for the bivalve *Scrobicularia plana*. In addition, the burrowing behavior of *S. plana* was impaired when transferred from Au contaminated seawater to clean sediment (Pan et al., 2012). GO-NPs have been found to cause negative effects in aquatic invertebrates during development (Mesarič et al., 2013), and induce significant adverse effects on vertebrates, protozoa and microbial communities (Ahmed and Rodrigues, 2013; Chen et al., 2012; Hu et al., 2015). However, few studies have been conducted with sediment.

The sediment-dwelling Oligochaete, *Tubifex tubifex* was selected as model organism, because they are widely distributed in the freshwater eco-system, and have a feeding behavior that includes ingesting large amounts of fine particles (<60 μm) and extract organic matter associated with ingested sediment. Due to their bioturbation activities (i.e., irrigation and particle mixing), high tolerance for polluted ecosystems, intermediate position in the trophic network and the ease of breeding in the laboratory, they are widely used as a standard model organism in ecotoxicological studies (Bouche et al., 2000; Lagauzere et al., 2009; Mosleh et al., 2007). However, due to difficulties in tracing NPs in the sediment compartment, the ecotoxicity of NPs on benthic invertebrates in sediment exposure media is scarce and studies with Au-NPs and GO-NPs have not been reported to our knowledge. In the present study, Au-NPs and GO-NPs-induced mortality, avoidance, burrowing behavior and bioaccumulation in *T. tubifex* are investigated via sediment exposure.

1. Materials and methods

1.1. Animal collection and culturing

T. tubifex were reared in aquaria added sediment and freshwater with frequent additions of extra food (mortared Tetramin® Tetra, Germany) in the laboratory at Roskilde University. The body length of *T. tubifex* ranged from 4 to 5 cm. One day before experimental setup, all worms were carefully picked out of the culture and placed in artificial *T. tubifex* media (see below) to empty their guts overnight. During exposure periods, worms were kept in natural sieved sediment without additional food supply.

1.2. Synthesis and characterization of nanoparticles

1.2.1. Synthesis of graphene oxide nanoparticles

Graphene oxide nanoparticles (GO-NPs) were synthesized according to the modified Hummers method (Hummers and Offeman, 1958). Graphite flakes (1 g, 99.8%, Alfa Aesar, China) and NaCl (35 g, Sinopharm Chemical Reagent Ltd., China) were ground into powder with a mortar and pestle. The powder was dissolved in deionized water (18 MPa, Milli-Q water), the solution filtered with filter paper (50 μm) and dried in an oven at 60°C for 24 hr. Subsequently, the dry graphite was dissolved in H_2SO_4 (23 mL, Sinopharm Chemical Reagent Ltd., China), and $KMnO_4$ (3 g, Sinopharm Chemical Reagent Ltd., China) was slowly added. The mixture was stirred for 30 min at 37°C, followed by 45 min stirring at 70°C. Afterwards, 5 mL deionized water was added, and the solution was heated and stirred for 10 min at 70°C followed by an addition of 40 mL water and heating for 15 min at 100°C. Finally, deionized water (140 mL) was added, followed by H_2O_2 (10 mL, 30%, Sinopharm Chemical Reagent Ltd., China) in order to obtain the brownish graphite oxide. The graphite oxide was purified by centrifugation at 8000 r/min for 5 min, followed by washing with 5% HCl and deionized water 6 times, successively. In order to enhance electrostatic repulsion, NaOH (1.8 g) and deionized water (10 mL) were added to the above solution, then left in an oil bath and stirred for 4 hr. Afterwards, pH was adjusted to <1 by addition of HCl (5 mL, 36%, Sinopharm Chemical Reagent Ltd., China). The solution was centrifuged at 8000 r/min for 5 min five times with DI water. Afterwards, the solution was sonicated for 45 min on ice to obtain GO. Finally, the GO solution was concentrated by centrifugation at 13,000 r/min for 5 min to give a final concentration of 2.09 mg/mL.

1.2.2. Synthesis of gold nanoparticles

Gold nanoparticles (Au-NPs) were synthesized using citrate reduction of $HAuCl_4$ as described by (Brust et al., 1994, 1995). Briefly, 3 mL of 10 mmol/L $HAuCl_4$ (AR, Sinopharm Chemical Reagent Ltd., China), 2 mL of 38.8 mmol/L citrate (AR, Sinopharm Chemical Reagent Ltd., China) and 1 mL of

0.075 wt.% $NaBH_4$ (AR, Sinopharm Chemical Reagent Ltd., China) were successively added to 80 mL deionized water at 1 min intervals with constant stirring. The mixture was stirred for 15 min at room temperature to obtain 5 nm Au-NPs. The theoretical Au-NP concentration was 55 μg/mL.

1.2.3. *Characterization of Au-NPs and GO-NPs*

The primary particle size of Au-NPs in MilliQ water was assessed by Transmission Electron Microscopy (TEM) (JEM-1011, Japan) operating at 80 kV. The particle size of GO-NPs was examined using Atomic Force Microscopy (AFM) (NanoScope IIIA Veeco, USA). Hydrodynamic diameters (in suspension) and Zeta potential of Au-NP and GO-NP suspensions prepared in deionized water were measured by Zetasizer Nano (ZS90Malven, UK).

1.3. Sediment and *T. tubifex* media preparation

Sediment was collected at Munkholmbroen in Holbæk, Denmark, sieved to <125 μm using deionized water and left to settle for two days. The overlying water was carefully removed through a plastic tube and the sediment was frozen at −20°C in order to kill micro- and macro-organisms. Afterwards, the sediment was thawed, washed with *T. tubifex* media once, left to settle and then overlaying water was removed. The ratio of dry weight to wet weight and organic carbon content in the sediment were measured by first placing the wet sediment in the oven for 24 hr at 105°C, and then heating the dry sediment for 4 hr at 550°C. *T. tubifex* media was prepared with 80 mmol/L $CaCl_2 \cdot 2H_2O$, 20 mmol/L $MgSO_4 \cdot 7H_2O$, 31 mmol/L $NaHCO_3$ and 3 mmol/L KCl according to OECD 203, ISO 6341-1982, and then aerated for 48 hr prior to use.

1.4. Sediment spiking and experimental setup

Sediment was spiked by adding a known amount of Au-NP stock suspension, GO-NP stock suspension or MilliQ water (controls) to 6 separate glass beakers containing wet sediment, to final nominal concentrations of 10 or 60 μg Au/g dw for Au-NPs, and 20 or 180 μg GO/g dw for GO-NPs, respectively. There is to the best of our knowledge no published information on environmental sediment concentrations of either Au-NP or GO-NP. The concentrations selected is based on published studies on Au-NP (soil: (Unrine et al., 2010; Voua otomo et al., 2014)) and C_{60}-NP (sediment: (Van der ploeg et al., 2011)) exposures. Sediments were mixed and covered with Parafilm, then left on a shaking table for 24 hr in order to obtain a homogeneous Au-NP and GO-NP distribution, respectively. Hereafter, spiked sediment was transferred to experimental glass beakers (3 replicates for each treatment) and *T. tubifex* media were gently added. The system was left to settle overnight. Hereafter, *T. tubifex* media were removed and 40 mL of fresh *T. tubifex* media were added before introducing *T. tubifex* to the beakers. Worms (20 or 5) were carefully transferred to each beaker to study the toxicities of sediment-associated Au-NPs and GO-NPs on *T. tubifex*, respectively. After 5 days sediment exposure, the worms were transferred to clean *T. tubifex* media and left for 6 hr to empty their gut. The experiment was carried out at (17 ± 2)°C in a controlled climate room for 5 days. Air was supplied to overlying water from pumps through plastic tubes and pipette tips.

1.5. Sample analysis

Au-NP concentrations in the start-sediment were measured by flame atomic absorption spectrometry (FAAS, SpectrAA-220 VARIAN Mulgrave, Australia). Au-NP concentrations in worm tissue were determined by graphite AAS (GTA 120 VARIAN Mulgrave, Australia). Samples were lyophilized at −50°C overnight, weighted and digested in a microwave oven. Samples were heated in a mixture of HNO_3 (2.25 mL, 35%) and HCl (0.75 mL, 35%) in the microwave oven at 250, 400, 650 and 250 W for 6 min at each step. Afterwards, samples were transferred into a water bath at room temperature and cooled for 30 min. Finally, samples were passed through pre-washed filters (Volume: 35%, HNO_3:MilliQ water =1:1 (V:V)) into 25 mL volumetric flasks. A series of standard Au solution (0, 5, 10, 20, 40, 60, 80 and 100 μg/L) were used for calibration of Au concentrations.

1.6. Mortality and avoidance response

The ability of *T. tubifex* to avoid sediment spiked with either Au-NPs or GO-NPs was tested by recording the number of worms on the surface of the sediment at different time slots (1 hr, 12 hr, 24 hr, 48 hr, 72 hr, 96 hr and 120 hr) during 5-day exposure. At the end of exposure (day 5), the dead worms were counted. Mortality was calculated using the ratio as the number of dead worms on day 5 divided by the number of worms initially added.

1.7. Burrowing behavior

After 5 days of exposure to GO-NP spiked sediment, worms were transferred to beakers containing 2 cm uncontaminated natural sediment and 40 mL *T. tubifex* media. Burrowing behavior was recorded at 30 min, 1 hr, 2 hr, 12 hr and 24 hr.

1.8. Statistical analysis

Data is presented as mean ± standard deviation (SD) of three replicates except for the data of Au bioaccumulation due to the insufficient biomass of worms. One- and two-way analysis of variance (ANOVA) was employed to detect significant differences among samples. Prior to ANOVA, Levene's Test was used to check homogeneity of variances, and normality of distributions was tested with Kolmogorov–Smirnov. Data were analyzed using SPSS version 19. Significant difference was accepted at a p value <0.05. Mortality data were arcsin transformed prior to statistical analyses.

2. Results

2.1. Characterization of Au-NPs and GO-NPs

TEM images of Au-NPs showed an average primary particle size of (4.9 ± 0.14) nm (86% were between 4 and 6 nm) using Nano measure 1.2 software. The particles were spherical and

relatively monodisperse and particle size was normally distributed (Fig. 1a, b). AFM images revealed that the particle size of GO-NPs fragments ranged from 1to 350 nm with the majority being around 150 nm (Fig. 1c, d). Thus, the thickness of GO-NPs were more than the 0.8 nm which is the typical thickness of single-layer GO sheets (Schniepp et al., 2006), indicating that the synthesized GO-NPs were multi-layered. The hydrodynamic diameter of 50 μg/mL Au-NPs and 200 μg/mL GO-NPs suspensions was (63 ± 0.34) and (121 ± 3) nm, respectively. The zeta potentials for Au-NPs and GO-NPs in MilliQ water were (−34.4 ± 1.2) and (−60.2 ± 1.2) mV, respectively, indicating that both suspensions were stable.

2.2. Sediment properties

The ratio of dry weight to wet weight of sediment was 0.38, the organic matter content was 0.9% and the background Au concentration was lower than the detection limit (<5 μg Au/g dw sediment). The concentration of Au in Au-NPs spiked sediment (n = 4) was (8.58 ± 1.55) μg Au/g and (70.27 ± 6.70) μg Au/g dw, respectively, which was close to the nominal concentrations (i.e., 10 and 60 μg Au/g dw). For GO-NPs, nominal concentrations of 20 μg/g dw or 180 μg/g dw were chosen, and 0.47 or 4.26 mL of the concentration GO-solution was added to the sediment, respectively. The final concentration of GO-NPs in sediment was not determined due to insufficient analysis methods.

2.3. Effects of Au-NPs to T. tubifex

2.3.1. Mortality and avoidance behavior

The mortality of *T. tubifex* was 5% in the control, 3.33% in 10 μg/g and 11.67% in 60 μg/g dw. No significant difference in mortality between treatments was observed (p = 0.124). During 5 day exposure, *T. tubifex* exhibited avoidance behavior to sediment treated with the three concentrations of Au-NPs (i.e., 0, 10 or 60 μg/g dw) (Fig. 2). Au concentration and exposure duration did not interact to affect the avoiding behavior (p > 0.05). However, there was a tendency that high exposure concentration resulted in a stronger avoiding behavior, especially in the beginning of the exposure period (Fig. 2).

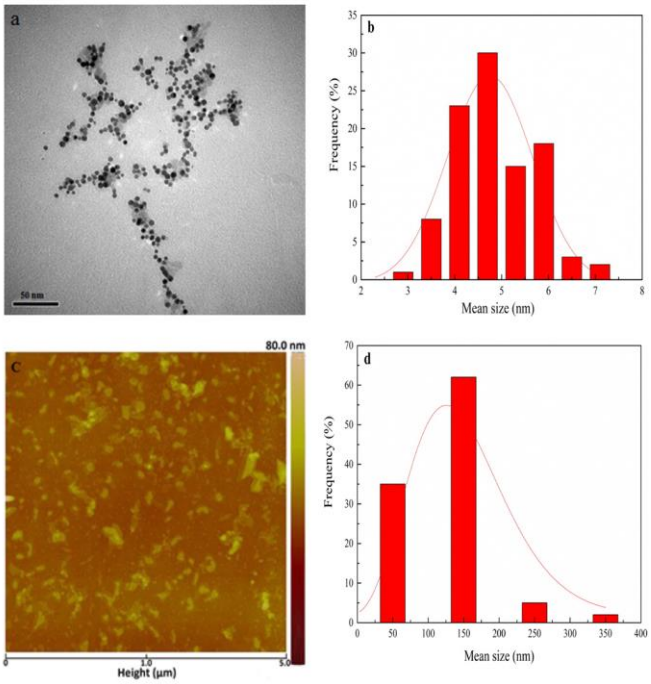

Fig. 1 – Transmission electron microscopy (TEM), atomic force microscopy (AFM) and size distribution of Au-NPs and GO-NPs in MilliQ water: (a) TEM images of 5 nm Au-NPs, (b) size distribution of Au-NPs, (c) AFM images of GO-NPs, (d) size distribution of GO-NPs. Au: gold; NPs: nanoparticles; GO: graphene oxide.

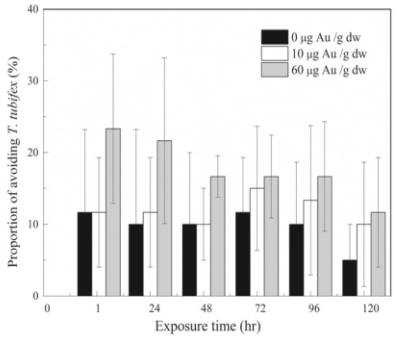

Fig. 2 – Avoidance response of *T. tubifex* during 5-day sediment exposure with Au-NPs (*n* = 3). The bars represent standard deviation (SD).

2.3.2. *Bioaccumulation of Au-NPs in T. tubifex*

Au accumulated in *T. tubifex* tissue during the 5-day sediment exposure. In the control group, the Au body burden was lower than the detection limit. The body burdens of Au in *T. tubifex* after 5 days was 12.49 and 65.84 μg/g dw when exposed to 10 and 60 μg/g dw sediment, respectively. However, to reach above the detection limit in worm tissue, worm biomass was pooled among three replicates leaving one data point per exposure concentration (0, 10, 60 μg AuNP/g dw sediment, *n* = 1 containing up to 20 samples). Thus, no statistical test could be performed, however, the data does show a clear tendency of a concentration-dependent accumulation of Au in *T. tubifex* worms.

2.4. Effect of GO-NPs on *T. tubifex*

2.4.1. *Mortality*

No worm mortality was observed after 5-day sediment exposure to 20 and 180 μg GO/g dw sediment.

2.4.2. *Avoidance and burrowing behavior*

Avoidance. There was no interaction between Au concentration and exposure duration on avoidance behavior of *T. tubifex* during 5 days of exposure ($p > 0.05$). During the first hour of exposure to GO-NP spiked sediment, only a few *T. tubifex* were visible at the sediment surface in the control treatment, while 20% and 24% of *T. tubifex* were observed on the surface of the 20 μg GO/g dw sediment and 180 μg GO/g dw sediment, respectively (Fig. 3). Yet, no significant avoidance was detected among treatments ($p > 0.05$).

Burrowing behavior. After *T. tubifex* were transferred into clean sediment, time for all organisms to fully burry into the clean sediment was significantly dependent on the pre-exposure concentration, such that time increased with increasing sediment concentration of GO-NPs ($p = 0.005$). *T. tubifex* took 1 hr to completely burrow into the sediment in the control treatment, while 8 and 24 hr was needed to completely burrow into the sediment for worms pre-exposed to 20 and 180 μg GO/g dw, respectively (Fig. 4).

3. Discussion

3.1. *Au-NP effects and Au bioaccumulation in T. tubifex*

Generally, sediment-associated Au-NP showed low mortality to *T. tubifex*. Au-NPs with a concentration of 10 and 60 μg Au/g dw had no significant effects on the avoidance behavior of *T. tubifex* during the short-term exposure. However, there was a tendency for higher avoidance for worms exposed to higher concentration of Au-NP and that the lack of significance may be related to a high variation in data. Avoidance responses induced by metal nanoparticles have not been examined greatly in the aquatic environment. Ramskov et al. (2014) reported an avoidance response of the freshwater snail, *P. antipodarum*, exposed to 100 μg Ag-NPs/g dw sediment for 14 days. An avoidance behavior of the polychaete *N. diversicolor* was also observed during exposure to 100 μg Ag-NP/g dw and 150 μg CuO-NP/g dw for 10 days, respectively (Cong et al., 2014; Thit et al., 2015), indicating that benthic invertebrates are able to detect and avoid nanoparticles in sediment exposure, this is in accordance with the present studies on the avoidance behavior of *T. tubifex* to nanoparticles. Furthermore, studies exist examining soil exposures of Oligochates, such as the earthworms *Eisenia fetida* and *Enchytraeus albidus*. *E. fetida* consistently avoided soil spiked with Ag-NPs in concentrations of 6.97–54 μg Ag/g dw, Al_2O_3--NPs with concentrations of 5000–10,000 μg Al_2O_3/g dw and TiO_2-NPs with concentrations of 1000–5000 μg TiO_2/g dw over 48 hr, respectively (Coleman et al., 2010; McShane et al., 2012; Shoults-Wilson et al., 2011). Likewise, *E. albidus* significantly avoided Cu-NPs with a concentration of 43–241 μg Cu/g dw during 48 hr of soil exposure, and the $EC_{50\text{-avoidance}}$ was 241 μg Cu/g dw (Amorim and Scott-Fordsmand, 2012).

Metal nanoparticle bioaccumulation in benthic invertebrates in sediment exposure has been studied in a number of benthic invertebrates including the clam *M. balthica* (Dai et al., 2013), the freshwater snail *P. antipodarum* (Pang et al., 2012, 2013; Ramskov et al., 2014), the estuarine worm *H. diversicolor*/*N. diversicolor* (Buffet et al., 2011, 2014; Cong et al., 2014). In sediment exposure, *P. antipodarum* accumulated 40–155 μg Cu/g dw after a long term exposure (8 weeks) to 30–240 μg CuO-NPs/g dry weight sediment, and the Cu body burden increased with increasing exposure concentration (Pang et al., 2012). For Ag-NPs, the clam *M. balthica* were able to accumulate 200–250 μg Ag/g dw after 35 days of exposure to 200 μg/g dw sediment spiked with Ag-NPs (20–80 nm) (Dai et al., 2013). Likewise, in a short-term sediment exposure, *N. diversicolor* accumulated approximately 2–9 μg Ag/g dw tissue after exposure to Ag-NPs (5–100 μg Ag-NPs/g dw sediment), and accumulated Ag increased with exposure concentrations (Cong et al., 2014). These results suggested that benthic invertebrates could accumulate nanoparticles like CuO-NP and Ag-NP. There exist to our knowledge no reported Au bioaccumulation data following sediment-exposure to Au

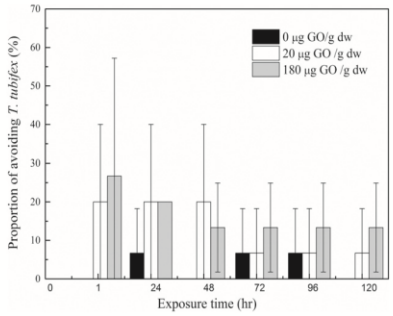

Fig. 3 – Avoidance response of *T. tubifex* during 5-day sediment exposure with GO (*n* = 3). The bars represent standard deviation (SD).

NPs, but results are available for Tellinid clams and earthworms following water and soil exposures, respectively. The clam *S. plana* accumulated Au in their soft tissues and the mean concentrations reaching 10.5, 12.0 and 17.7 μg Au/g, respectively for *S. plana* exposed to 100 μg/L of Au-NPs with the size of 5, 15 and 40 nm for 16 days (Pan et al., 2012). The earthworm *E. fetida*, accumulated 0.3–1.5 μg Au/g fresh tissue after 28 days of exposure to 10 μg Au/g dw of Au-NPs, and Au bioaccumulation followed in a dose-dependent manner (Unrine et al., 2010). In the present study, there was a tendency that bioaccumulation of Au-NPs in *T. tubifex* increased with increasing exposure concentration, and bioaccumulated Au in *T. tubifex* was up to 65.84 μg Au/g following exposure to 60 μg Au/g dw sediment of Au-NPs in

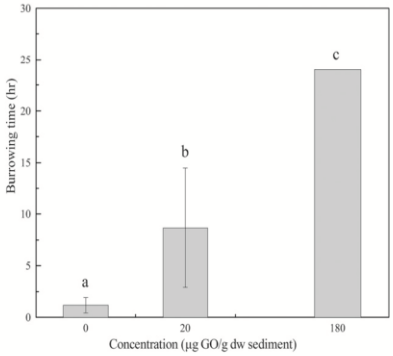

Fig. 4 – Time for all *T. tubifex* in each beaker to completely burrow in clean natural sediment after 5-day exposure to GO-NPs. The bars represent standard deviation (SD) (n = 3). Different letters indicate statistical differences at $p < 0.05$ level.

a short term period (5 days). Thus, there exist data indicating that benthic invertebrates are able to accumulate CuO-NP, Ag-NP as well as Au-NP on sediment exposure.

3.2. Impaired burrowing behavior of *T. tubifex* following exposure to sediment-associated GO-NPs

The GO-NPs with a concentration of 20 and 180 μg GO/g dw in the sediment did not affect the survival of *T. tubifex* following 5-day exposure in this study. This is in accordance with earlier published results showing low or no mortality in organisms exposed to other carbon nanomaterials (Liu et al., 2014; Pakarinen et al., 2011; Petersen et al., 2008). For example, the mortality of the Oligochate *Lumbriculus variegatus* was not affected by exposure (28 days) to 50 μg fullerenes/g dw sediment, 0.03 μg SWCNT (single-wall carbon nanotubes)/g dw sediment (SWCNT) or 0.03 μg MWCNT (multi-wall carbon nanotubes)/g dw sediment (Pakarinen et al., 2011; Petersen et al., 2008). GO-NPs has also showed low mortality in other test systems like water exposure, where Liu et al. (2014) reported that 1–100 mg GO/L had no adverse effects on the survival of zebrafish embryos after 96 hr exposure (Liu et al., 2014).

The burrowing behavior of *T. tubifex* in clean sediment was significantly affected following exposure to 20 and 180 μg GO/g dw in the present study. Similar observations were reported for *N. diversicolor*, and *S. plana* exposed to different nanoparticles such as CuO-NP, Ag-NP, CdS-NP and ZnO-NP (Cong et al., 2014; Boldina-Cosqueric et al., 2010; Buffet et al., 2012, 2013a, 2013b, 2014; Thit et al., 2015). Boldina-Cosqueric et al. (2010) discussed the origins of impairments of burrowing behavior in *S. plana*. They found that the reduced burrowing speed of clams from a clean site exposed to contaminated sediment may be interpreted as an avoidance response (Boldina-Cosqueric et al., 2010), In addition, the burrowing behavior was significantly decreased for worms (*H. diversicolor*) exposed to 10 μg Cu/L of CuO NP for 14 days (Buffet et al., 2013a), suggesting that burrowing behavior of benthic invertebrates may be a more sensitive endpoint in behavior tests, and likely of particularly importance when considering NP effects. Since *T. tubifex* plays an important role in biogeochemical processes through its burrowing and irrigation activity, the impairment of burrowing behavior may lead to ecologically detrimental effects, such as an increase in the susceptibility of sediment-dwelling species to predation. This could lead to an increased predation of contaminated worms by fish, possibly biomagnifying NPs up the food chain, thereby affecting the entire ecosystem. However, the concentration range from 20 to 180 μg GO-NPs/g dw used in this study is not expected in the environment, making considerations like this predominantly theoretical.

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Paper III

A biodynamic understanding of dietborne and waterborne Ag uptake from Ag NPs in the sediment-dwelling oligochaete, *Tubifex tubifex*

Stine Rosendal Tangaa, Marie-Nöele Croteau, Margrethe Winther-Nielsen, Henriette Selck

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- Developing the experimental idea and plan together with Marie-Nöele Croteau
- Conducting the experimental work and data-analysis
- Writing the manuscript

A biodynamic understanding of dietborne and waterborne Ag uptake from Ag NPs in the sediment-dwelling oligochaete, *Tubifex tubifex*

Stine Rosendal Tangaa[abc], Marie-Noële Croteau[c], Margrethe Winther-Nielsen[b] & Henriette Selck[a]

[a]Roskilde University, Dept. of Science and Environment, Roskilde, Denmark

[b]DHI, Dept. of Environment and Toxicology, Hørsholm, Denmark

[c]US Geological Survey, Menlo Park, CA, USA

Abstract

Metal nanoparticles (Me-NPs) are increasingly used in various products, such as inks and cosmetics, enhancing the likelihood of their release into aquatic environments. An understanding of the mechanisms controlling their bioaccumulation and toxicity in aquatic biota will support environmental risk assessment. We characterized unidirectional parameters for uptake and elimination of silver (Ag) in the sediment-dwelling oligochaete *Tubifex tubifex* after waterborne (0.01-47 nmol Ag/L) and dietborne (0.4-480 nmol Ag/g dw sed.) (i.e., sediment) exposures to Ag NPs and $AgNO_3$, respectively. Worms accumulated Ag from $AgNO_3$ more efficiently than from Ag NPs during waterborne exposure, i.e., the Ag uptake rate constants from water were 8.2 L/g/d for $AgNO_3$ and 0.34 L/g/d for Ag NPs. Silver accumulated from either form was efficiently retained in tissues as no significant loss of Ag was detected after up to 20 days of depuration in clean media. High mortality (~50%) during depuration (i.e. after 17 days) was only observed in worms exposed to waterborne $AgNO_3$ (3 nmol/L). Sediment exposures to both Ag forms resulted in low accumulation, i.e., the uptake rate constants were 0.002 and 0.005 g/g/d for $AgNO_3$ and Ag NPs, respectively. The lack of avoidance behavior during exposure to sediment amended with Ag NPs, and the biodynamic model predictions of sediment being the most important route of uptake for Ag NPs at environmentally relevant k_d, could lead to increased accumulation of Ag NPs. However, inference of bioavailability from our estimations of Ag assimilation efficiencies (AE) suggests that Ag from both forms (AE: 3-12% for $AgNO_3$ and 0.1-0.8% for Ag NPs) is weakly bioavailable from sediment for this species. Thus, Ag amended to sediment as NPs might not pose greater problems than "conventional" Ag for benthic organisms such as *T. tubifex*.

Keywords: *nanoparticles, metals, silver, bioavailability, sediment*

1. Introduction

Environmental exposures to metal-based engineered nanoparticles (Me-NPs) pose unknown threats to aquatic ecosystems. Due to their many application possibilities, Me-NPs are increasingly used in consumer products such as inks, textiles and cosmetics (Vance et al., 2015). As a result, Me-NPs are likely released into aquatic environments (Gottschalk and Nowack, 2011) posing risks to biota. Several abiotic transformation processes may lead to their removal from the water, including agglomeration and aggregation. These processes will increase the sedimentation rates of Me-NPs (Velzeboer et al., 2014), thereby increasing their concentration in the sediment. Silver nanoparticles (Ag NPs) are one of the most frequently used Me-NPs, primarily due to their antibacterial effects (Vance et al., 2015). Although monitoring programs for Ag NPs in the environment are currently lacking, their predicted environmental concentrations in surface water and sediment range from 0.09 to 320 ng/L and 0.15 µg/kg to 14 mg/kg, respectively (Mueller and Nowack, 2008; Gottschalk et al., 2009). Most of the published literature on Ag NP bioavailability relates to water-only exposure studies involving pelagic species, such as *Daphnia magna* (e.g.(Mackevica et al., 2015)). Studies have shown that Ag NPs can be both toxic and bioaccumulative (e.g.(Gaiser et al., 2011; Kalman et al., 2015)). However, the bioavailability and fate of Ag NPs in the sediment remain largely unknown (e.g. as reviewed in (Tangaa et al., 2016)). This is partly due to the difficulties with handling and characterizing Me-NPs in the complex sediment matrix (Rajala et al., 2016). Yet, Me-NPs will accumulate in this environmental compartment (Lowry et al., 2012). Thus, understanding how these particles interact, interfere and affect benthic organisms is crucial. Studies published so far with benthic organisms such as gastropods and clams highlight some of the Me-NP effects on the benthic community (Croteau et al., 2011a; Dai et al., 2013). Based on the few sediment exposure studies available, Ag associated with sediment is bioavailable to benthic organisms including the sediment dwelling ragworm, *Nereis diversicolor* (Cong et al., 2014) and the polychaete, *Capitalla teleta* (Ramskov et al., 2015a). These worms accumulated Ag after exposure to sediment amended with both Ag NPs and $AgNO_3$. However, knowledge of the underlying mechanisms controlling Ag uptake by these benthic organisms is lacking (Rajala et al., 2016).

We ask if uptake route (water vs sediment) and Ag form ($AgNO_3$ vs Ag NPs) influence bioaccumulation in the freshwater sediment-dwelling worm, *Tubifex tubifex*. Worms were exposed in controlled laboratory experiments to both Ag forms separately at exposure concentrations chosen to reflect the predicted environmental concentrations of Ag (Mueller and Nowack, 2008; Gottschalk et al., 2009). Key physiological processes controlling the uptake and elimination of Ag were parameterized using the precepts of a bioaccumulation model (Luoma and Rainbow, 2005). Given the lack of adequate and

standardized analytical methods for detecting NPs in sediment (Gottschalk et al., 2013), Ag NPs were characterized only in the aqueous media prior to addition to sediment.

2. Material and methods

2.1 Biodynamic modeling approach

The biodynamic model considers metal uptake from both food (including sediment) and water, and accounts for elimination and organismal body growth dilution when predicting bioaccumulation (Luoma and Rainbow, 2005). The model is expressed as:

$$[M]_{org} = k_{uw} \cdot [M]_w + k_{uf} \cdot [M]_f - k_e \cdot [M]_{org} - k_g \cdot [M]_{org} \qquad \text{(eq. 1)}$$

where $[M]_{org}$ is the metal concentration in the organism (nmol/g), $[M]_w$ is the aqueous metal concentration (nmol/L) and $[M]_f$ is the metal concentration in food or sediment (nmol/g); k_{uw} and k_{uf} are the unidirectional metal uptake rate constants from water (L/g/d) and food (g/g/d), respectively; k_e is the rate constant for physiological loss (d^{-1}) and k_g the rate constant for growth (d^{-1}). k_e can be determined after waterborne or dietborne exposures, allowing parameterization of elimination rate constants specific to each uptake route (k_{ew} and k_{ef}, respectively). When experiments are conducted over time periods short relative to the life span of the studied species, k_g is considered negligible (Luoma and Rainbow, 2005).

The elimination rate constants (k_{ef} and k_{ew}) are determined in experiments where organisms are exposed to a metal, and then allowed to depurate the accumulated metal in clean media. The physiological loss of metal accumulated in tissues can be described as:

$$[M]^t_{org} = [M]^f_{org} \cdot \exp(-kft) + [M]^s_{org} \cdot \exp(-kst) \qquad \text{(eq. 2)}$$

where $[M]^t_{org}$ is the metal concentration in the organism at a given time (nmol/g) and $[M]^f_{org}$ and $[M]^s_{org}$ the metal concentrations in the fast and slow exchanging compartments, respectively (nmol/g); *t* is depuration time (d); k_f and k_s represents the fast and slow rate constants of loss (d^{-1}) (Khan et al., 2012).

The uptake rate constants (k_{uw} and k_{uf}) are determined from the slope of the linear relationship between metal influx and metal exposure concentration (i.e. in either water or sediment) (Croteau et al., 2011a). The uptake rate constant from food (k_{uf}), represents the combined influence of food ingestion rate (IR in g/g/d) and metal assimilation efficiency (AE in %) (Croteau et al., 2011b). AE represents the proportion of metal that is assimilated after ingestion, and can be used as a proxy to infer metal bioavailability from food (Wang and Fisher, 1999). Both IR and AE are determined by mass balance calculations (see the Supplementary Information (SI) for more information).

Steady state body burdens can be predicted using eq. 1: (Croteau et al., 2014b)

$$[M]_{SS} = (k_{uf} \cdot [M]_f)/(k_{ef} + k_g) + (k_{uw} \cdot [M]_w)/(k_{ew} + k_g) \qquad \text{(eq. 3)}$$

where $[M]_{ss}$ is the metal concentration in the organism at steady-state (nmol/g).

2.2 Chemicals and analyses

Paraffin coated Ag NPs were purchased from Amepox Microelectronics (Lodz, Poland). Information provided by the supplier indicated particle sizes ranging from 3 to 8 nm (TEM, Laser Diffraction, Figure S1 and Table S1). The stock solution had a nominal silver concentration of 1500 ppm. The hydrodynamic size, size distribution and aggregation were monitored over time periods representative of the experimental exposures (0-240 min) using Dynamic Light Scattering (DLS) (Zetasizer Nano ZS90, Malvern) and UV-vis (UV-1800 Shimadzu) (Figure S2 and Table S2). Measurements were carried out with Ag NPs dispersed in artificial freshwater (FW), see section 2.3 for details. Dissolution of the Ag NPs was assessed in FW by centrifugal ultrafiltration (Millipore, Amicon, 3KDa membrane filters). Silver concentrations in the filtrate and in the original solution were determined by Inductively Coupled Plasma-Mass Spectrometry (ICP-MS; see section 2.4.4 for details). Ag speciation in FW was estimated at two different Ag-concentrations (1 and 25 nM) using PHREEQC (pH-redox-equilibrium concept) software, developed to model metal speciation in aquatic systems (Parkhurst and Appelo, 2013). The $AgNO_3$ standard (10 ppm in 2% HNO_3) was purchased from High-Purity Standards (Charleston, SC, USA) and used as the aqueous counterpart in waterborne exposures. Isotopically enriched Ag (99% ^{109}Ag) was purchased from Trace Sciences International (Wilmington, DE, USA) and dissolved in HNO_3 to reach a final concentration of 171 ppm. Isotopically enriched ^{109}Ag was added to sediment as the ionic counterpart in the dietborne exposures, to enhance detection and circumvent the confounding influence of background concentrations, as described in (Croteau et al., 2014a).

2.3 Test organism and experimental media

The model species *T. tubifex* is an oligochaete omnipresent in freshwater environments worldwide (Brinkhurst and Jamieson, 1971). Worms live within the sediment-water interface, interacting with the sediment as well as both the overlying- and pore water. The main nutrient intake in these worms is via ingesting sediment and extracting any accessible organic material (Cammen, 1980). This species play an important role in the food chain, serving as prey for higher trophic organisms such as demersal fish (Redeker et al., 2004).

Artificial freshwater was prepared according to OECD guideline 203 (ISO 6341-1892 (294 mg/L $CaCl_2 \cdot 2H_2O$, 123.3 mg/L $MgSO_4 \cdot 7H_2O$, 63 mg/L $NaHCO_3$, 5.5 mg/L KCl;

pH 7.8±0.2; hardness 250 mg/L)), as recommended when using *T. tubifex* (OECD, 2008). All constituents were weighed and mixed with deionized (DI) water, and aerated 24 to 48h before use. Worms were purchased from a commercial supplier of biological organisms, Niles Biological Inc. (Sacramento, CA, USA), and reared in two culture setups. The "aqueous culture" consisted of worms reared in tanks filled with FW. These worms were used for the waterborne exposure experiments. The "sediment culture" consisted of worms reared in tanks containing natural sieved (<250 μm) sediment and FW. These worms were used for the sediment (i.e. dietborne) exposures. All cultures were kept at 15°C in complete darkness with constant aeration. Worms in both cultures were fed once a week with finely grounded fish feed (Tetramin®). Two third of the FW was renewed twice a week in the aqueous culture and once a month in the sediment culture. Literature shows that *T. tubifex* can be cultured successfully in both settings (Redeker and Blust, 2004).

Sediment was collected from Searsville Lake, a freshwater reservoir located in San Mateo County, CA, USA (37°24'N, 122°14'16"W), which is part of the Jasper Ridge Biological Preserve. Surface sediment was sampled with an Ekman grab (6x6x6"), and transported to the laboratory. Sediment was sieved through a 250 μm sieve with use of DI water, homogenized and frozen (-20°C). After 5 days sediment was thawed, rinsed twice with FW, homogenized and left to settle (3 days, 15°C). Sediment was stored at 15°C in darkness until use. Percentage dry weight (dw) and organic carbon content (OC) of sediment were 40±0.2% and 6±0.4%, respectively. Background silver concentration in sediment was 0.02 μg/g dw.

2.4 Experimental approach

The overall aim with this study, was to determine unidirectional uptake and elimination rate constants for Ag ($AgNO_3$ and Ag NPs) from water and diet in *T. tubifex*. Thus, the experimental setup was divided into three main parts: a waterborne (aqueous exposure), a dietborne (sediment exposure) and an elimination experiment.

To minimize metal contamination, laboratory ware was soaked in acid (10% HNO_3, 5% HCl) for a minimum of 3 days, rinsed in MilliQ-water (18.2 MΩ·cm) and dried under a laminar-flow hood prior to use.

2.4.1 Waterborne exposure

Prior to exposure, worms were removed from the aqueous culture and rinsed in FW. Ten worms were exposed in 1 L acid-washed Nalgene™ jars to Ag concentrations ranging from 0.01 to 47 nmol Ag/L. A control was also included (FW only). Silver was added as either Ag NPs or $AgNO_3$, and stock solutions of each Ag form were prepared with FW prior to exposure. Worms were exposed in the dark at 15°C for 4h (Ag NPs) or at ambient light at room temperature for 4h ($AgNO_3$), without addition of food. The

light and temperature conditions were tested in a parallel study in which worms were exposed to 5 nmol/L $AgNO_3$ for 1h (i.e., at 15°C and darkness or room temperature and ambient light). After exposure, all organisms were removed, rinsed with DI water and transferred individually to acid washed centrifuge tubes, and frozen (-20°C). Water samples (2 mL, unfiltered) were taken before and after exposure and acidified with double distilled 16N HNO_3 (1%).

2.4.2 Dietborne exposure

The dietborne experiment had two phases, i.e., an exposure phase and a depuration phase. In the exposure phase, worms were exposed to sediment spiked with Ag NPs or $^{109}AgNO_3$ for 5-8 hours. One group of worms were kept for bioaccumulation assessment (i.e. metal-analyses of worm tissue) and another group of worms were transferred to uncontaminated sediment to assess depuration (24 hours).

Exposure to spiked sediment: One day prior to exposure, worms were removed from the sediment culture and placed in sterile polystyrene multi-well plates presoaked in tap water (Nunclon, Becton Dickinson Labware, North Carolina, USA) to empty their guts overnight (Gillis et al., 2004). Each plate consisted of 12 circular 6 mL wells (d: 2.2 cm) with 5 mL FW per well. Approximately 20 g wet sediment was transferred to sterile 50 mL Falcon tubes (n=3). Silver was added as either Ag NPs or $^{109}AgNO_3$ in FW to obtain Ag concentrations ranging from 0.4 to 480 nmol Ag/g dw sed. A control was also included (sediment without Ag addition). Spiked sediments were mixed in the dark at 15°C for 24h on a tube rotator (12 rpm) (Figure S3). The overlying water was renewed with clean FW prior to the onset of the experiment to ensure a saturated oxygen level and to minimize metal concentration in the overlying water. Worms (n=10-15) were transferred to the sediment containing tubes and exposed in the dark at 15°C to either $^{109}AgNO_3$ for 5h or Ag NPs for 8h (see Figure S4). The number of worms added per tube was determined based on recommendations from OECD 315 (OECD, 2008). Exposure time was kept short to avoid or minimize depuration of Ag during exposure, as *T. tubifex* is known to ingest and egest sediment within 5-8h (Redeker et al., 2004). Sediment sub-samples were taken from each test tube before adding the worms. After exposure, overlying water was discarded and the sediment was sieved to facilitate the sampling of worms. The sieved sediment was kept for analysis (see 2.4.4).

Depuration in clean sediment: After exposure, worms were rinsed in FW and individually transferred into 1.5 mL centrifuge tubes, in which ~1 g ww of clean sediment had been added along with ~1 mL of FW. Worms were allowed to depurate any unassimilated Ag during a 24h period, which is the recommended gut purging time for *T. tubifex* (OECD, 2008). After depuration, worms were transferred to multi-well plates (1 worm per well) containing 5 mL FW. Worms were allowed to empty their guts

overnight. Worms were rinsed in DI water, placed individually in acid washed centrifuge-tubes and frozen (-20°C) until analyses.

2.4.3 Elimination

To determine unidirectional elimination rates, worms were first exposed to waterborne Ag NPs or $AgNO_3$ for 48h. Hereafter, worms were transferred to clean conditions and allowed to depurate any accumulated Ag. Worms were fed finely ground, uncontaminated Tetramin® regularly over the entire elimination period of up to 20 days.

Prior to exposure, worms were removed from the aqueous culture and rinsed in FW. Worms (n=100) were then exposed in 2 L Nalgene™ jars to either 3 nmol/L $AgNO_3$ or 58 nmol/L Ag NPs in FW. The difference in exposure concentration was chosen based on results from the waterborne exposure experiment. Worms were exposed in the dark at 15°C for 48h, without addition of food. The test solution was renewed after 24h to ensure a constant exposure concentration during the exposure. Water samples (2 mL, unfiltered) were taken from each exposure jar prior to adding worms and after exposure. After 48h, water was removed and worms collected and rinsed in FW.

The setup for the elimination experiment followed that described by (Croteau et al., 2011c). Briefly, exposed worms were divided into groups of 10, and each group was transferred into a 150 mL acid-washed polypropylene vial. A total of 9 vials were used per Ag form. Each vial had two 4 cm diameter holes opposite of each other, covered with 63 μm acid-washed mesh. The mesh allowed for exchanges of water and oxygen with the surrounding media, and prevented worms from escaping (see Figure S5). All vials were closed with a lid and submerged in a 40 L glass tank filled with 30 L FW, creating an enclosed, recirculated aerated freshwater system. A coal filter and pump were used in the depuration tank to keep the excreted metal concentration below detectable background levels (i.e., 10 ng Ag/L) (Wang et al., 1996). The experiment was carried out as a static test, i.e. no renewal of water in the depuration tank during the 20 days of elimination. One group of worms (i.e. 1 vial with 10 worms) was sampled on day 0, 1, 2, 3, 5, 7, 10, 14 and 20, respectively. Sampled worms were rinsed in DI water, placed individually in acid washed centrifuge tubes and frozen (-20°C). Water samples (2 mL, unfiltered) were taken from the depuration tank on each sampling day. All water samples (from exposure and elimination) were acidified with double distilled 16N HNO_3 (1%).

2.4.4 Sample preparation and metal analysis

Sediment samples were placed in 20 mL scintillation vials, and dried in the oven at 80°C for 5 days. The dried sediment samples were then digested at room temperature in HCl using an extraction method derived from (Sutherland, 2002). Briefly, 12 mL of 0.6 N

HCl (Omnitrace) were added to each sample. Samples were then shaken by hand every 20min. for 2h, and filtered through 0.45 µm PVDF filters. This weak digestion procedure was chosen to extract the bioavailable fraction of Ag added to sediment (Luoma, 2008).

Frozen worms were transferred to acid-washed Teflon-sheets and dried in the oven at 40°C for 3 days. Dried worms were weighed on a microbalance (Sartorius, Model M20) and inserted into a 6 mL Teflon vial. Worms were digested with double distilled 16N HNO_3 (200 µL per sample) in a pressure cooker for 3h (125°C, 20 psi). All digested samples were diluted with MilliQ-water to obtain a 5% HNO_3 solution. Samples were filtered through 0.45 µm PVDF filters. Similar weight samples of the certified reference material DOLT-3 (Dogfish liver tissue from the National Research Council Canada) were processed similarly, in addition to procedural blanks. Germanium (Ge) was added as an internal standard (3 ppm Ge, 8 µL per mL sample) to all samples (water, tissue and sediment) to account for change in sensitivity and instrumental drift. Samples were analyzed for Ag (^{107}Ag and ^{109}Ag) by ICP-MS (NexION 300Q, Perkin-Elmer), as described in (Croteau et al., 2011b). Calibration curves (^{107}Ag and ^{109}Ag) were created from external standards serially diluted (0.01-40 µg/L). Additional quality controls were done by running independent standards (0.1 or 1 µg/L) every 10 samples. The limit of detection of the ICP-MS for Ag was 10 ng/L. All data is shown as actual, measured Ag concentrations unless stated otherwise.

2.4.5 Calculation of Ag concentrations

Newly accumulated ^{109}Ag was calculated as described in (Croteau et al., 2014a). The equations for calculating $\Delta[^{109}Ag]_{org}$ are provided in the SI. Because the newly accumulated Ag ($\Delta[^{109}Ag]_{org}$) for the control worms included negative values, a value of 0 was ascribed to these samples. Since feces could not be distinguished from the sediment, the amount of Ag in the worm feces ($[Ag]_{feces}$) was indirectly determined using the total amount of Ag detected in sediment from each depuration chamber ($[Ag]_{dep.sed}$), minus the background Ag found in uncontaminated sediment samples ($[Ag]_{BG.sed}$), see SI eq. S6.

2.5 Statistical analyses

All statistical analyses were performed using SYSTAT (version 13.1). Datasets were tested for normal distribution and variance equality using Kolmorogov Smirnoff and Levenes Test, respectively. One-way analysis of variance (ANOVA) was used when conditions were met. Otherwise, the non-parametric Kruskal-Wallis test was used. Tukeys test or Conover-Inman was used for comparison among treatments, if ANOVA or Kruskal-Wallis showed a significant effect, respectively. For pairwise comparisons, a two-sample t-test was performed. Regression analyses were used to determine if rate

constants differed significantly from zero. Statistical significance was obtained if $p \leq 0.05$. Data is presented as mean ± standard deviation (SD) unless stated otherwise.

3. Results

3.1 Characterization of nanoparticles in exposure media

TEM images showed spherical particles with sizes below 10 nm. UV-vis analysis of Ag NPs dispersed in FW and MilliQ-water showed peaks corresponding to Ag NPs (Figure S1 & S2) (Bhui et al., 2009). DLS measurements indicated that the particles had an initial (after 10min) average hydrodynamic diameter of 148 nm in FW, with a Polydispersity Index (PdI) of 0.2 (Table S2). Agglomeration/aggregation was observed over the time course of the exposure (up to 240min), with an average particle diameter of 174 nm (PdI = 0.2) after 240min in FW. Dissolution was less than 2%, regardless of the Ag concentration. PHREEQC modeling predicted that 90% of the Ag added as $AgNO_3$ in FW occurred as AgCl-complexes; the remaining 10% was Ag^+. Based on these data, we speculate that ionic Ag was the least dominant form in both treatments (i.e. for both Ag-forms). $AgNO_3$ formed complexes with Cl-species in the waterborne experiments, but no precipitates were observed in the exposure beakers.

3.2 Silver concentrations in water and sediment

Table 1: Measured Ag concentrations in the waterborne (nmol/L, n=3, mean±SD) and dietborne (nmol/g dw, n=5, mean±SD) experiments, as well as in the elimination experiment (nmol/L, n=3, mean±SD).

Waterborne Exposure			Dietborne Exposure		Elimination	
Ag-form (nmol/L)			Ag-form (nmol/g dw)		Ag-form (nmol/L)	
$AgNO_3$	Ag NP	*Treatment*	$^{109}AgNO_3$[a]	Ag NP	$AgNO_3$	Ag NP
0.01±0.005	0.02±0.005		0	0.4±0.07	3±1	58±1
0.1±0.004	1.0±0.01	A	64±32	101±3		
0.6±0.01	5.2±0.03	B	261±6	244±12		
2.4±0.2	9.9±0.3	C	454±29	293±16		
9.5±0.7	24±0.7	D	463±34	482±17		
32±1.7	47±0.3					

[a]*Newly added ^{109}Ag (Ag background subtracted)*

Silver concentrations (in nmol Ag/L) in the waterborne exposures ranged from 0.01 to 32 and from 0.02 to 47 for $AgNO_3$ and Ag NPs, respectively. In the dietborne exposures, Ag concentrations (in nmol Ag/g dw sed) ranged from 0 to 463 for $^{109}AgNO_3$, and from 0.4 to 482 for Ag NPs. The Ag concentrations were significantly different ($p<0.05$, Table 1) among treatments for both Ag-forms, except for the two highest treatments for $AgNO_3$ in the dietborne exposure. Thus, a concentration gradient was obtained for both Ag forms for both exposure routes. Because the Ag concentrations for the two highest $AgNO_3$ sediment treatments (i.e. C & D in Table 1) were similar, bioaccumulation data for these two treatments were pooled. Due to the difference in bioaccumulation observed for worms in Ag NP and $AgNO_3$ experiments (see 3.3.1), worms in the Ag NP elimination experiment were exposed to a higher silver concentration compared to the $AgNO_3$ elimination experiment to ensure that the tissue concentrations were above detection limit.

3.3 Accumulation of silver

3.3.1 Uptake from water

Worms accumulated Ag from the aqueous phase, regardless of the Ag-form added, with Ag from $AgNO_3$ being accumulated to the largest extent. Body burdens (in nmol Ag/g dw worm) varied from 0.1±0.03 to 30±3 for $AgNO_3$, and from 0.6±0.3 to 16±2 for Ag NPs dispersed in FW. Worms exposed for 4h to both Ag forms accumulated significant amounts of Ag compared to control worms ($p<0.05$). Ag uptake rates into worm tissues increased with exposure concentrations regardless of Ag form ($p<0.05$, Figure 1). Ag uptake rates (in nmol/g dw/d) increased from 0.7±0.2 to 168±15 for $AgNO_3$, and from 0.6±0.2 to 16±2.4 for Ag NPs. The uptake rate constant from water (k_{uw}) was nearly 25-times greater for $AgNO_3$ (k_{uw}= 8.2±1.1 L/g dw/d) compared to Ag NPs (k_{uw}= 0.34±0.6 L/g dw/d). k_{uw} for Ag NPs was not statistically significantly different from zero ($p>0.10$). The averaged dry weight of worms (after exposure) used in both treatments was 2.4±0.5 mg dw. Silver from $AgNO_3$ was taken up faster and to a higher degree when the exposure was conducted at 15°C in darkness than when conducted in ambient light and at room temperature, see SI for details.

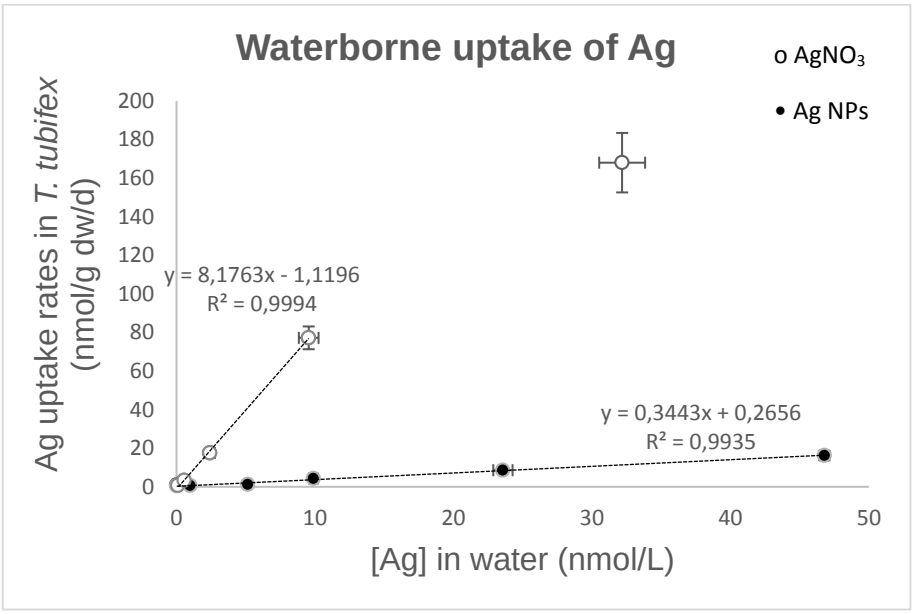

Figure 1: Silver uptake rates in T. tubifex *exposure to aqueous Ag added as $AgNO_3$ (open circles, ±SD) or Ag NPs (closed circles, ±SD). The SDs are generally very low and, therefore, not all are visible.*

3.3.2 Uptake from sediment

Worms exposed to sediment spiked with either $^{109}AgNO_3$ or Ag NPs weakly accumulated Ag. Body burdens (BB; in nmol Ag/g dw worm) ranged from 0.1±0.03 to 0.2±0.2 for $^{109}AgNO_3$, and from 0.5±0.1 to 1.2±0.5 for Ag NPs. Similarly, Ag uptake rates (in nmol Ag/g dw worm/d) ranged from 0.2±0.1 to 0.7±1.0 for $AgNO_3$, and from 1.6±0.4 to 3.7±1.4 for Ag NPs. Although worms exposed to Ag NP spiked sediment appeared to accumulate Ag at a faster rate than worms exposed to sediments amended with $^{109}AgNO_3$ (Figure 2, Table 2), this difference was not significant. The uptake rate constants from food (k_{uf}; g/g dw/d) were not statistically different from zero for either Ag form ($p>0.10$; $^{109}AgNO_3$ k_{uf}: 0.002±0.7; Ag NPs: 0.005±0.6) (Figure 2, Table 2). For worms exposed to sediment spiked with Ag NPs, Ag uptake was higher at the two highest exposure concentrations compared to the lowest treatment and the control ($p<0.05$). Worms used in both experiments were of similar sizes (1±0.2 mg dw) after exposure.

Silver AE and IR were estimated for each Ag-form at each exposure concentration, based on the Ag content in sediment and fecal matter from the depuration chambers (Table 2). AE ranged from 3 to 12% for worms exposed to $^{109}AgNO_3$, and did not exceed 1% for worms exposed to Ag NP. Worm IR was, in general, lowest for worms in the $^{109}AgNO_3$ treatments (0.4-0.5 g/g dw/d) and highest for worms in the Ag NPs treatments (0.6-1.6 g/g dw/d). The ingestion rates were not significantly affected by the

Ag concentration for $^{109}AgNO_3$ ($p>0.10$), whereas worms in Ag NP treatments had significantly lowered their IR at the three highest concentrations ($p=0.01$, Table 2).

Worms exposed to sediment spiked with $^{109}AgNO_3$ appeared to avoid the sediment, staying on the surface instead of burrowing into the sediment. This behavior was especially prevalent at the highest Ag exposure concentration in the sediment. This avoidance behavior was not observed for worms exposed to Ag NPs. Also, worms exposed to the highest $^{109}AgNO_3$ concentration remained at the surface of the sediment once transferred to clean sediment, suggesting impaired burrowing capability. The lack of burrowing activity was not observed for worms pre-exposed to Ag NPs. Likewise, no indicators of stress (i.e. avoidance or lack of burrowing) was observed for control worms.

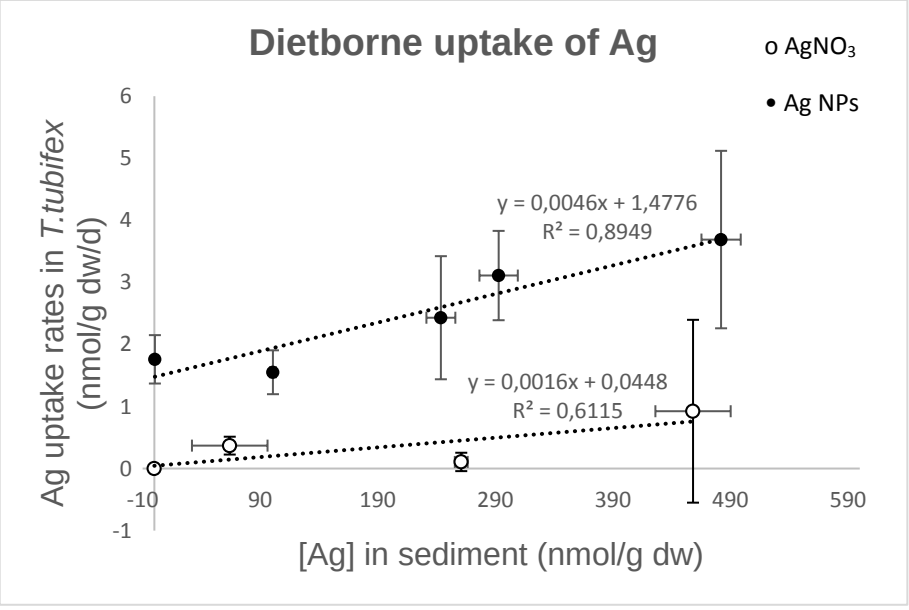

Figure 2: Dietborne uptake rates for ^{109}Ag (open circles, ±SD) and Ag NPs (closed circles, ±SD) in T. tubifex.

3.4 Elimination after waterborne exposure

Once accumulated into tissues Ag was eliminated very slowly regardless of the Ag form. Worms exposed to $AgNO_3$ did not lose a significant amount of Ag after up to 17 days of depuration ($p>0.10$), impeding estimation of the rate constant of loss. The value of 0.001 d^{-1} was thus ascribed when modeling Ag bioaccumulation (see 2.5). In contrast, loss of Ag accumulated after Ag NP exposure was detectable after 7 days of depuration. The rate constant of loss was 0.03±0.01 d^{-1}.

After 48h of exposure to waterborne Ag, worms had achieved mean body burdens (BB; in nmol Ag/g dw worm) of 12.6±2.5 for $AgNO_3$ and 8.4±1.1 for Ag NPs, respectively

(see Figure S8). The minor increase in Ag body burden in worms exposed to Ag NPs after 1 day of depuration (BB = 12±4 nmol Ag/g dw worm) was not significantly different than day 0 of the depuration, and probably reflected the large individual variation in Ag uptake by worms (see Figure S8). After 17 days of depuration, only 5 % of the accumulated Ag from $AgNO_3$ exposure had been eliminated. The elimination experiment was ended for the $AgNO_3$ exposed worms at day 17 because worms were impaired, showing 50 % mortality. For Ag NPs, worms slowly lost Ag from day 0 to 7, but no detectable loss was detected thereafter (Figure 3). No mortality was observed during 20 days of elimination for worms pre-exposed to Ag NPs. For worms pre-exposed to $AgNO_3$, there was no change in biomass from day 0 to 14. Likewise, no change in biomass was observed throughout the depuration for worms pre-exposed to Ag NPs (Figure S9). This indicates that feeding with Tetramin® was sufficient to keep the worms alive during the elimination period. Worms had similar sizes (1.6±0.3 mg dw) after elimination between experiments. No Ag was detected in the water samples from the elimination tank (i.e. all water samples were below DL of the ICP-MS), confirming that worms were not exposed during elimination, and that the Ag detected in worm tissue was from the 48h exposure period only.

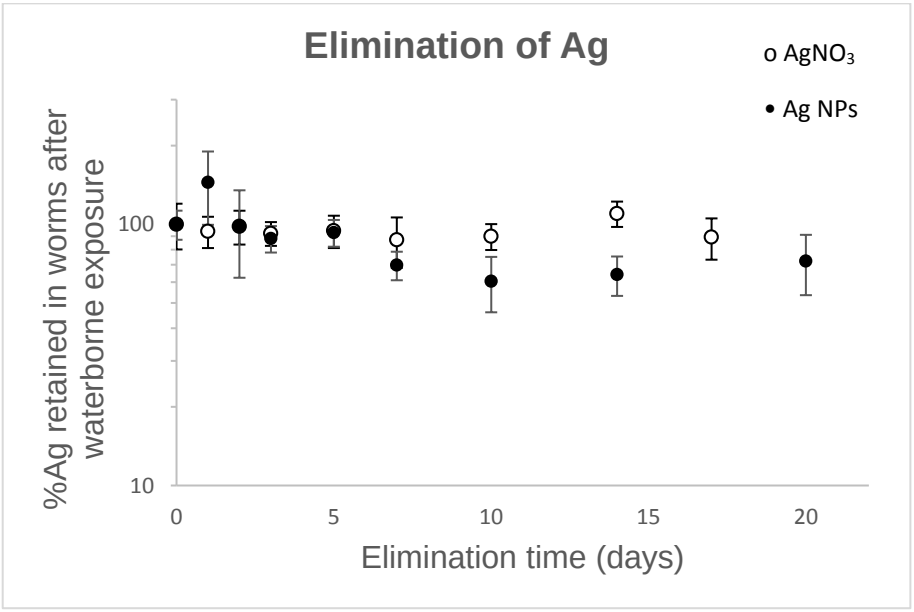

Figure 3: Ag elimination following 48h waterborne exposure to 3±1nmol/L $AgNO_3$ (open circles, ±SD) or 58±1nmol/L Ag NPs (closed circles, ±SD) expressed as % retained Ag in worm tissue over time. % Ag retained in worms (y-axis data) is shown on logarithmic scale.

3.5 *Biodynamic parameters*

The parameters required and estimated for the biodynamic model are presented in Table 2.

Table 2: Biodynamic parameters for both Ag forms. k_{uw}, k_{ew}, k_{uf}, k_{ef} (mean±SE); AE, IR (mean±SD). Numbers in parentheses indicates number of individual worms used for the estimation. A-D refers to each exposure treatment, as given in Table 1. (*) indicates that numbers are not significantly different from zero.

Biodynamic parameters			Ag form	
			$AgNO_3$	Ag NPs
k_{uw} (L/g/d)	*Uptake rate constant from water*		8.2±1.1	0.3±0.6*
k_{ew} (d^{-1})	*Elimination rate constant from water*		<0.001*	0.03±0.01
k_{uf} (g/g/d)	*Uptake rate constant from food (i.e. sediment)*		0.002±0.7*	0.005±0.6*
k_{ef} (d^{-1})	*Elimination rate constant from food (i.e. sediment)*		---	---
AE (%)	*Assimilation efficiency*	*A*	12±9 (3)	0.1±0.02 (3)
		B	---	0.8±0.4 (13)
		C	2.9±1.9 (5)	0.7±0.6 (10)
		D	---	0.8±0.5 (13)
IR (g/g/d)	*Ingestion rate*	*A*	0.5±0.5 (3)	1.6±0.5 (3)
		B	---	0.6±0.2 (13)
		C	0.4±0.7 (5)	0.6±0.2 (10)
		D	---	0.6±0.1 (13)

--- Not estimated due to low accumulation from sediment

The weak Ag accumulation from sediment along with the limited number of data points (i.e. BB below DL) impeded estimating AE and IR for the $AgNO_3$ at all exposure concentrations. Due to the low accumulation of Ag from sediment for both Ag forms, it was not possible to perform an elimination experiment in sediment.

4. Discussion

4.1 Particle characterization

UV-vis analysis of Ag NPs dispersed in both MilliQ-water and FW showed peaks corresponding to Ag, supporting the presence of Ag NPs in the exposure medium. In FW, particles showed hydrodynamic sizes varying from 148 to 174 nm (mean size 164 nm) over a time course equivalent to that used in the experiments (up to 4h). Agglomeration and/or aggregation occurred when particles were added to the exposure medium, which is often reported in nano-ecotoxicity studies (e.g. (Tejamaya et al., 2012)). The actual form of Ag NPs after addition to sediment is unknown. However, some studies have highlighted the possibility that NPs remained in some (nano)particulate form after entering the sediment (e.g. (Dale et al., 2013)). Our data emphasizes the need of using more than one characterization method, as the information on pristine NPs provided by the supplier are not accurately describing the size of the NPs dispersed in our experimental media (i.e., FW; see SI). The importance of proper particle characterization by several techniques has been described elsewhere (e.g. (Petersen et al., 2014)).

4.2 Uptake from water

The greater Ag uptake in worms following waterborne exposure to $AgNO_3$ compared to Ag NPs suggests that particulate Ag is less bioavailable to *T. tubifex* than Ag from $AgNO_3$, under the used experimental conditions. The difference in exposure conditions might explain in part this difference as Ag uptake from $AgNO_3$ is faster at lower temperature and darkness than at ambient light and room temperature (see SI for details). *T. tubifex* is known to thrive in a wide range of temperatures, although Rathore *et al.* showed that these worms are more sensitive to heavy metals at higher temperatures (>15°C) (Rathore and Khangarot, 2002). Nonetheless, the results obtained for $AgNO_3$ at both temperatures show that Ag from $AgNO_3$ is taken up more efficiently than Ag from Ag NPs, when water is the main exposure route. This difference in bioaccumulation potential between Ag forms is consistent with other findings. For example, Croteau *et al.* found that silver added as $AgNO_3$ to water was taken up faster by the freshwater snail *Lymnaea stagnalis* than silver added as citrate-coated or humic acid-coated Ag NPs (17±5 and 13±3 nm (TEM)) (Croteau et al., 2011a). Similar findings were observed for the estuarine snail *Perengia ulva* when exposed to dissolved and particulate Ag (16.5±4.5 nm (TEM)), where uptake of Ag from $AgNO_3$ was 10-

times faster than Ag uptake from NPs (Khan et al., 2012). To our knowledge, our study is the first documenting waterborne uptake of Ag from Ag NPs in *T. tubifex*.

4.3 Uptake from sediment

Generally, worms accumulated low amounts of Ag from sediment, regardless of the Ag form added. Uptake rates for Ag NPs was generally higher than for $AgNO_3$ (Figure 2), but detection of a significant difference in Ag uptake rate constants between Ag forms was hampered by the limited amount of data. Body burdens in worms exposed to the two highest concentrations of Ag NPs added to sediment were significantly higher than control worms, which was not seen for $AgNO_3$ exposed organisms. This might indicate that nanoparticulate Ag is taken up to a higher degree than Ag from $^{109}AgNO_3$, when sediment is the main exposure route. The higher uptake of Ag from Ag NPs compared to that from $AgNO_3$ has also been observed for *Potamopyrgus antipodarum* when exposed to sediment amended with either $AgNO_3$ or Ag NPs (13.9±3.2 nm (DLS)) (Ramskov et al., 2015a). This suggests that exposure route (water or sediment) affects the uptake of these two forms of Ag in benthic species. However, the very low uptake rate constants observed for both Ag forms indicate that Ag is either not bioavailable or have very low bioavailability to *T. tubifex* when added to sediment, under the used experimental conditions. *T. tubifex* have been shown to accumulate significant amounts of metals (Cd, Pb; (Gillis et al., 2004)) and Me-NPs (Au; (Zhang et al., 2017)) from sediment, when the exposure duration was longer (4 weeks and 5 days, respectively). But, since the main aim of this study was to determine unidirectional uptake rates of Ag from water and sediment, the use of short exposure durations to avoid loss was necessary. Khan *et al.* designed a setup to increase the dietborne exposure time when assessing biodynamic parameters, by exposing the organisms (*P. ulva*) to several, short (3h) contamination series (Khan et al., 2013). This approach may be useful for future studies, however, the extra handling of worms (i.e. removing and re-introducing them to sediment several times) may introduce other stress factors which could impact uptake.

Difference in Ag AEs between the two forms of Ag suggests that Ag bioavailability from the sediment was greater for $AgNO_3$ than for Ag NPs. However, IR-values showed the opposite pattern, with worms exposed to sediment amended with Ag NPs ingesting greater amounts of sediment compared to worms exposed to sediment amended with $AgNO_3$. Enhanced ingestion of sediment with Ag of a lower bioavailability yielded higher Ag BB. This indicates that AE alone is not a good predictor of Ag bioaccumulation from sediment as food IR is inversely related to AE.

In general, the Ag assimilation efficiency observed in this study was lower (for both Ag forms) in *T. tubifex* (3-12 % for $^{109}AgNO_3$; 0.1-0.8 % for Ag NP) compared to other benthic organisms such as *L. stagnalis* (~70 % for $AgNO_3$; 40-60 % for Ag NPs <30 nm (TEM)) (Croteau et al., 2011a, 2014a). This indicates that *T. tubifex* is not an efficient

bioaccumulator of Ag from sediment, under the experimental conditions of this study. The slow feeding rates along with the avoidance behavior observed in worms exposed to sediment spiked with $^{109}AgNO_3$ suggest that worms detected Ag when presented as $^{109}AgNO_3$. This effect seems perpetuated after transferring worms to clean sediment. These findings are in accordance with other studies (e.g. (Buffet et al., 2014; Ramskov et al., 2015b)), supporting the hypothesis that benthic organisms are able to detect Ag from $AgNO_3$ but not from nanoparticles. This could result in higher accumulation of Ag NPs (BB is greater for Ag NPs than $AgNO_3$; Figure 2), leading to unknown consequences for the benthic community.

4.4 Elimination after waterborne exposure

Worms exposed to either form of Ag in water barely lost Ag after up to 20 days of depuration. Similarly, low loss of accumulated metal after waterborne exposure to Cu in two forms (^{65}Cu-aq & ^{65}CuO NPs) in the sediment-dwelling oligochaete *Lumbriculus variegatus* have been observed (Thit et al., 2016). Loss of Ag by the snail *L. stagnalis* was also negligible after waterborne exposure to $AgNO_3$, and that of Ag from Ag NPs was minimal (~5 % per day) (Croteau et al., 2011a). Slow elimination of Ag could have adverse consequences for higher trophic levels, as *T. tubifex* is prey for demersal fish (Redeker et al., 2004). Further, the high mortality and deterioration of worms observed after 17 days in uncontaminated FW during depuration suggest a delayed toxic effect of $AgNO_3$. The degeneration might be a way for the worms to protect themselves from increased internal metal concentrations (Lucan-Bouché et al., 1999). Degeneration was not observed for worms pre-exposed to waterborne Ag NPs, suggesting a different internal fate for Ag from Ag NPs compared to $AgNO_3$.

4.5 Predictions of bioavailability

Incorporation of the uptake rate constants from water and food (k_{uw} and k_{uf}), and the elimination rate constant from water (k_{ew}) into the biodynamic model (eq. 3), along with environmentally realistic Ag-concentrations in water (0.1 µg/L) and sediment (10 µg/g), yielded steady-state Ag body burdens of 840 µg/g and 2.8 µg/g for $AgNO_3$ and Ag NPs, respectively. The relative importance of each uptake route was evaluated across a range of sediment:water distribution coefficients (k_d) for Ag, including the reported value in freshwater systems ($k_d = 10^5$) (Luoma and Rainbow, 2008), as shown in Figure 4.

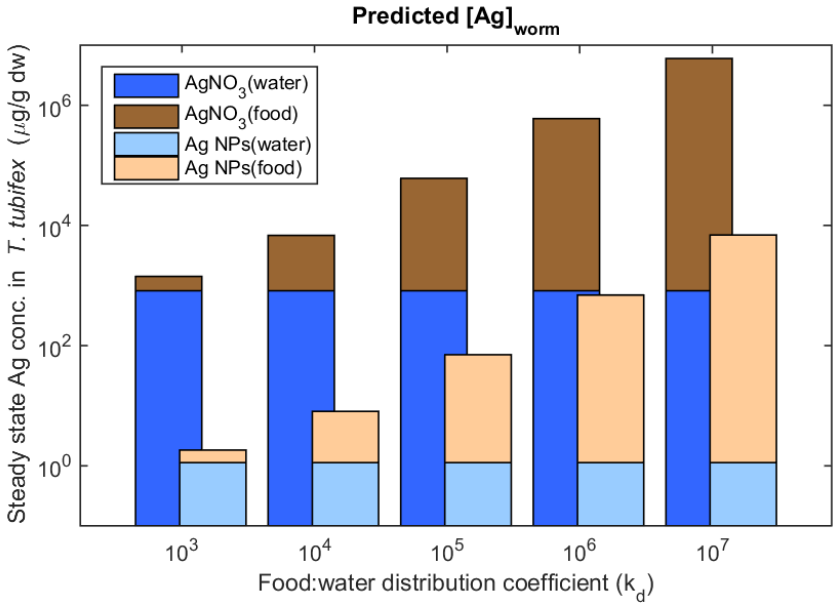

Figure 4: Model predictions using the biodynamic parameters presented in Table 2, eq.2, and k_d-values (10^3 to 10^7). $[Ag]_{water}$ = 0.1 µg/L, $[Ag]_{diet}$ was derived via use of the environmentally relevant k_d-values (i.e. around the natural k_d for Ag, 10^5(Luoma and Rainbow, 2008)*). $k_e(AgNO_3)$ was set at 0.001 d^{-1}. Blue is for waterborne uptake; brown is for dietborne uptake; dark colors represent the contribution of each uptake route for $AgNO_3$, while the lighter colors represent the contribution of each uptake route for Ag NPs.*

Biodynamic modelling suggests that 1) water is the primary route of uptake for $AgNO_3$ in *T. tubifex* regardless of k_d; 2) uptake of Ag from sediment becomes more important at higher k_d; 3) regardless of uptake route, $AgNO_3$ exposure results in higher body burdens than similar exposure to Ag NPs. Based on the reported distribution coefficients for Ag in natural aquatic ecosystems (10^4 to 10^5; (Smith and Flegal, 1993; Wang et al., 1999)), sediment appear as the most important source of Ag for the Ag NPs for *T. tubifex*. Uptake of Ag from sediment becomes equally important for both Ag forms at a k_d-value of 10^7. This would correspond to a sediment concentration of 1000 µg/g when $[Ag]_{water}$ is 0.1 µg/L, which is a factor of 10 to 100 higher than what has been reported for Ag in natural sediments (i.e. (Luoma et al., 1995; Luoma, 2008). If Ag NPs are released to the environment and accumulate in sediments, as have been predicted in other studies (e.g. in (Dale et al., 2013)), they could be accumulated in benthic organisms, such as *T. tubifex*. However, our results show that bioavailability of Ag from the sediment is low for worms under the used experimental conditions. This indicates that this species does not accumulate Ag from Ag NPs to a great extent, especially when the particles are mixed into the sediment.

5. Conclusion

Uptake route and Ag form are both important for the overall bioaccumulation of Ag in *T. tubifex*. Specifically, Ag uptake rates from water are faster for $AgNO_3$ than for Ag NPs. The dietary uptake of Ag is slow for both Ag forms, mainly because Ag is not highly bioavailable from the sediment under the experimental conditions. The overall aim was to determine unidirectional uptake constants, i.e. it was necessary to estimate uptake without interference of elimination. Thus, short exposure times was crucial, but might have resulted in underestimation of the actual uptake potential in worms. The avoidance behavior and decreased burrowing activity observed for worms exposed to sediment spiked with $AgNO_3$, in combination with the high mortality and caudal deterioration observed after waterborne exposure to $AgNO_3$, indicate that Ag from $AgNO_3$ is impairing the worms. The low Ag AE values and slow uptake of Ag NPs from either exposure route, indicate that the lower bioavailability of Ag NPs mitigates the adverse effects of Ag. The slow loss of Ag after waterborne exposure to either form highlights the bioaccumulation capability for these worms, which might have consequences for organisms higher up the food chain. Biodynamic modelling reveals that water is most important for $AgNO_3$ uptake, but that sediment contributes significantly to the overall bioaccumulation when k_d increases. For Ag NPs, sediment uptake is more important at environmentally realistic k_d-values. Overall, $AgNO_3$ is more bioavailable regardless of the uptake route compared to the particulate form, under the used experimental conditions. Thus, Ag from Ag NPs might not pose a greater problem than "conventional" Ag forms (e.g., like $AgNO_3$) for benthic organisms such as *T. tubifex*. However, issues such as bioaccumulation over longer exposure times, trophic transfer capability and fate of Ag NPs once they enter the sediment needs to be elucidated, and should be prioritized in future studies.

Conflict of interest

The authors do not report any conflict of interest.

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names is for descriptive purposes only and does not imply endorsement by the U.S. Government.

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Supplementary information to Tangaa et al.

A biodynamic understanding of dietborne and waterborne Ag uptake from Ag NPs in the sediment-dwelling oligochaete, *Tubifex tubifex*

Stine Rosendal Tangaa[*abc], Marie-Noële Croteau[c], Margrethe Winther-Nielsen[b] & Henriette Selck[a]
[a]Roskilde University, Roskilde, Denmark, Dept. of Science and Environment
[b]DHI, Hørsholm, Denmark, Dept. of Environment and Toxicology
[c]US Geological Survey, Menlo Park, CA, USA

*Corresponding author e-mail: stangaa@ruc.dk

9 pages (9 figures, 3 tables, 6 equations, 4 references)

1. Biodynamic Model equations

Ingestion rate (IR) and assimilation efficiency (AE) can be estimated as:

$$IR = \frac{(Ag_{org.} + Ag_{feces})}{[Ag]_f \cdot wt_{org.} \cdot t} \quad \text{(eq. S1)}$$

$$AE = \frac{Ag_{org.}}{Ag_{org.} + Ag_{feces}} \cdot 100\% \quad \text{(eq. S2)}$$

where $Ag_{org.}$ is the amount of silver within the organism after depuration (ng); Ag_{feces} the amount of Ag in feces after depuration (ng); $[Ag]_f$ the silver concentration in food (i.e. sediment) (ng g dw^{-1}); $wt_{org.}$ is dry weight of the organism after depuration (ng) and *t* is exposure time (d).

2. Characterization of the Ag NPs

TEM-images provided by the supplier showing particles less than 10nm in size. The size distribution analysis indicates a size distribution from 2-8nm, with an average size of 7nm.

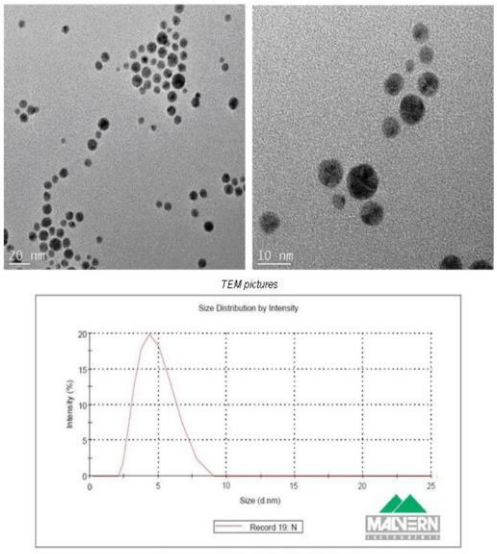

Figure S1: TEM images and size distribution from supplier (Amepox)

Table S1 provides information from the supplier such as Ag concentration and coating.

Table S1: Ag NP specifications given by the supplier (Amepox)

Solvent	Silver nanoparticles in water medium
Consistency	Fluid with low viscosity
Color	Dark brown
Concentration	1500 ppm (0,1%)
Average particle size	3 – 8 nm
Protective shell	Paraffin
Viscosity *(Brookfield LVDVII+CP;100rpm; 25°C.)*	1.0 ± 0.3 mPas
Specific gravity	1.0 ± 0.2 g/cm³
Storage	Without limitation with closed container.

UV-vis

Figure S2 shows UV-vis spectrum of 100ppb dilutions of Ag NP stock in FW (left) and MilliQ-water (MQ) (right), measured over time. Samples were run every 40min (T1-T6) over a period of 240min. Peaks around 410nm correspond to Ag NPs, and wavelengths from 410-450 indicate a size range of 15-30nm [1], as seen for the particles in both MQ-water and FW.

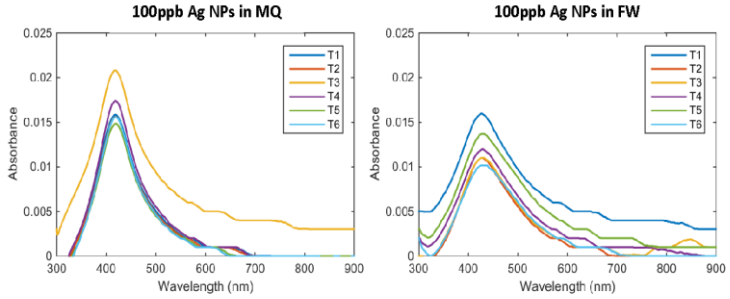

Figure S2: UV-vis spectrum of a 100ppb dilution of Ag NP stock in FW (left) or MilliQ-water (right). Absorbance is measured at different time-intervals (T1-T6) over a period of 240min.

In MQ-water, the main peak is around 413-423nm. In FW, the main peak appears around 431-448nm, corresponding to a right shift of the peak compared to particles suspended in MQ-water. This could be due to a higher degree of particle aggregation in FW compared to MQ-water. The peak intensity decreases over time for both suspensions, but the curves follow the same pattern. There is a slight broadening of the peak over time, indicating agglomeration/aggregation of NPs. The stability over time suggests that the particle suspension is quite stable, regardless of dissolution media.

Hydrodynamic size

The Ag NP hydrodynamic size is quite different than what was detected by UV-vis (Table S2). The size distribution is broader, and the averages varied from 148 nm to 174 nm in FW, indicating aggregation over time.

Table S2: Hydrodynamic size measurements (DLS, Zetasizer) of Ag NPs dispersed in OECD-media and MilliQ-water over time. PdI = Polydiversity Index

Freshwater (pH≈7.8)			MQ-water (pH≈5.5)		
Time (min)	Z-average (d.nm)	PdI	Time	Z-average (d.nm)	PdI
10	147,8	0,179	10	214,2	0,361
60	158,9	0,205	20	218,2	0,342
145	167	0,214	60	221,9	0,342
220	168,7	0,218	200	236,2	0,31
240	173,5	0,225	240	227,2	0,354

The size difference observed between FW and MQ might be related to differences in pH, as higher pH can lead to a larger degree of agglomeration of Ag NPs [2]. Overall, larger particle sizes were observed in both solutions, compared to the information provided by the supplier.

3. Figures complementing the experimental section

Spiking of sediment

Figure S3: Rotating device developed internally at USGS used to mix spiked sediment.

Tubes containing sediment were rotated continuously over the course of 24h, to obtain a fully homogenously spiked sediment.

Dietborne exposure treatments

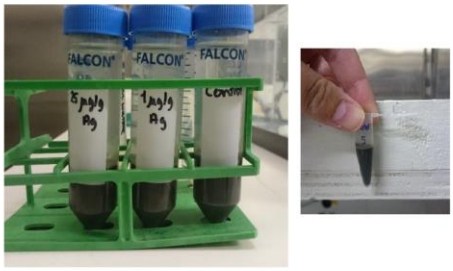

Figure S4: Exposure tubes with contaminated sediment (left) and centrifuge-tube with clean sediment for elimination (right)

50 mL Falcon tubes were used as exposure chambers for the dietborne exposures. Approximately 20 g of wet weight sediment was added to each tube, followed by 30 mL FW. 10-15 worms were exposed in each tube.
1.5 mL acid washed centrifuge-tubes were used as elimination containers. Each tube contained clean sediment and FW, and one worm. All sediment and water were kept for metal analysis after 24h of elimination.

Elimination chambers

Figure S5: Elimination cup with meshed holes (left) and cups in the Elimination tank (right), both with 10 worms added per cup

Elimination chambers used for the up to 20 days' elimination experiment in water. Each cup contained 10 worms, and was submerged into a large tank containing clean FW. Mesh-size was <63 µm to avoid worms from escaping. Mortared Tetramin® was added to each remaining beaker on termination days.

4. Calculation of newly accumulated ^{109}Ag

Briefly, the signal intensity of ^{109}Ag and ^{107}Ag from the ICP-MS analysis were used to determine the relative abundance of [^{109}Ag] (i.e. p^{109}):

$$p^{109} = Intensity\left(\frac{^{109}Ag}{^{109}Ag+^{107}Ag}\right) \qquad \text{(eq. S3)}$$

P^{109} averaged 0.49±0.003 (SD) for samples analyzed on different days (over a period of 1 month), which is consistent with Croteau *et al.* [3]. The product of the relative abundance (p^{109}) and the total Ag concentrations determined from the tracer intensity ([T^{109}Ag]) yielded the total concentration of tracer in worms ($T[^{109}Ag]_{org}$):

$$T[^{109}Ag]_{org} = p^{109} \cdot [T^{109}Ag] \qquad \text{(eq. S4)}$$

The background concentration of Ag occurring in each worm ($[Ag]^0_w$) was calculated as the product of p^{109} and the concentration of Ag inferred from the intensity of the most abundant Ag isotope ($T[^{107}Ag]_{org}$):

$$[Ag]^0{}_{org} = p^{109} \cdot T[^{107}Ag]_{org} \qquad \text{(eq. S5)}$$

Finally, the total amount of newly accumulated ^{109}Ag ($\Delta[^{109}Ag]_{prg}$) was estimated by subtracting $T[^{109}Ag]_{org}$ from $[Ag]^0_{org}$.

5. Calculating Ag-concentration in feces

The amount of Ag in feces was determined by subtracting the concentration of Ag measured in depuration-chamber sediment from the background concentration of Ag in natural sediment:

$$[Ag]_{feces} = [Ag]_{dep.sed} - [Ag]_{BG.sed} \qquad \text{(eq. S6)}$$

6. Figures complementing the result section

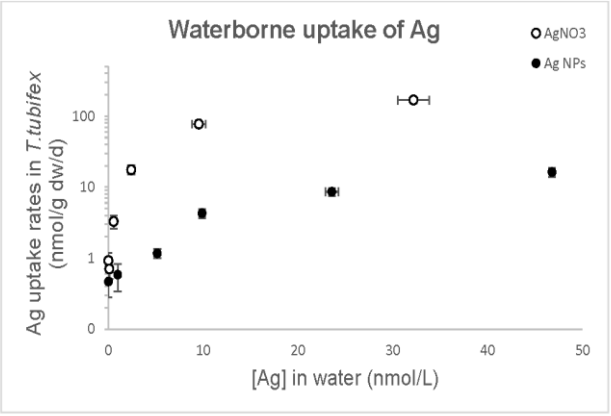

Figure S6: Waterborne uptake rates for Ag after $AgNO_3$ (open circles, ±SD) and Ag NP (closed circles, ±SD) exposure. Ag uptake rates (y-axis) are presented on logarithmic scale. Measured Ag concentrations in water are shown on the x-axis.

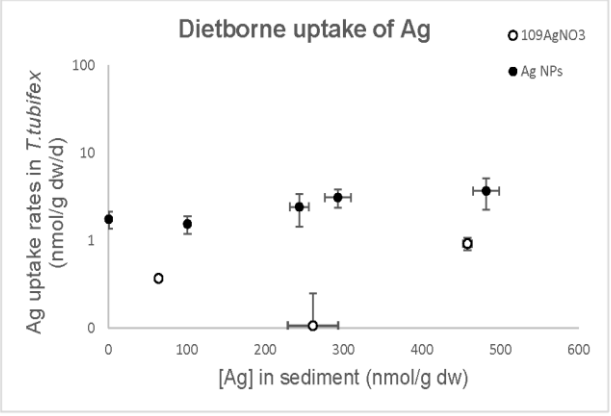

Figure S7: Dietborne uptake rates for ^{109}Ag (open circles, ±SD) and Ag NPs (closed circles, ±SD). Ag uptake rates (y-axis) are presented on logarithmic scale. Measured Ag concentrations in sediment are shown on the x-axis.

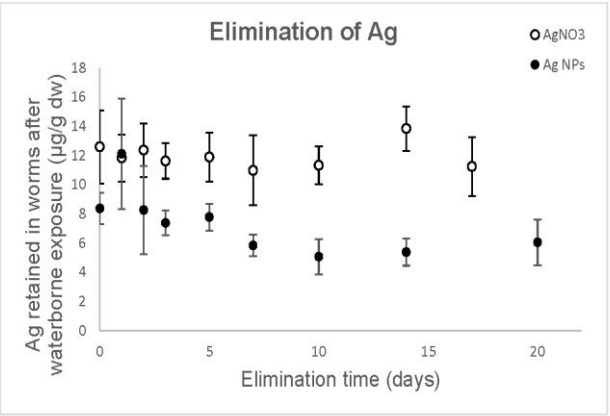

Figure S8: Elimination of Ag after 48h waterborne exposure to 3±1nmol/L $AgNO_3$ (open circles, ±SD) or 58±1nmol/L Ag NPs (closed circles, ±SD) expressed as worm body burden over time

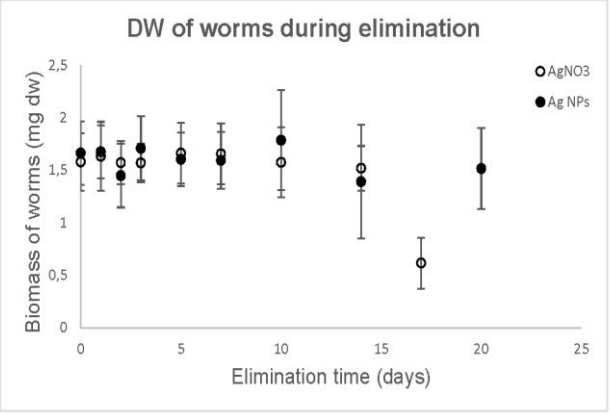

Figure S9: Biomass of worms during elimination after waterborne exposure to $AgNO_3$ (open circles, ±SD) and Ag NPs (closed circles, ±SD)

Figure S9 shows dry weight of worms over the course of the elimination period in clean media. The low biomass seen for worms pre-exposed to $AgNO_3$ after 17 days of elimination, was due to all remaining worms being broken (split into two halves) or partly decomposed.

7. Supplementary information for the discussion

The waterborne uptake of Ag from $AgNO_3$ was conducted at room temperature in the presence of light, whereas the uptake of Ag from Ag NPs was conducted at 15°C and in dark. Generally, higher temperature results in faster reactions (i.e. uptake) and higher toxicity [4], which might explain in part the higher Ag uptake from $AgNO_3$ than from Ag NPs. To test the hypothesis of faster Ag uptake at higher temperatures, we repeated the waterborne $AgNO_3$ exposure (1h) at 15°C in the dark. Results show that worms exposed to $AgNO_3$ at 15°C accumulated Ag at a faster rate than at room temperature:

Table S3: Exposure conditions and Ag uptake in worms after 1h exposure to Ag from $AgNO_3$ in water.

Treatment	Temp (°C)	[Ag]water (nmol/L)	[Ag]worm (nmol/g)*	Uptake rate (nmol Ag/g/d)
Lab *(light)*	23±2	4.5±0.4	1.7±0.4	40.2±10
Cold room *(dark)*	15±1	5.7±0.4	6.6±0.9	154±20

**normalized to account for the different exposure concentrations*

The Ag uptake rate, above, for worms exposed in the supplementary investigation, are similar to those observed for the worms exposed to Ag from $AgNO_3$ in the waterborne exposure setup, which was done under similar conditions. This supports that worms are taking up Ag from $AgNO_3$ faster under lower temperature and darkness, compared to during light and at room temperature. In addition, regardless of the experimental conditions, worms are accumulating Ag from $AgNO_3$ faster than from Ag NPs, confirming that $AgNO_3$ is more bioavailable than Ag NPs for these worms.

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Paper IV

Trophic transfer of Ag ENPs from sediment to fish in a simple freshwater food web

Stine Rosendal Tangaa, Margrethe Winther-Nielsen, Henriette Selck

Manuscript draft

My contributions:

- Developing the experimental idea and plan
- Conducting the experimental work and data-analysis
- Writing the manuscript

Trophic transfer of Ag ENPs from sediment to fish in a simple freshwater food web

Stine Rosendal Tangaa[ab], Margrethe Winther-Nielsen[b] & Henriette Selck[a]

[a]Roskilde University, Dept. of Science and Environment, Roskilde, Denmark

[b]DHI, Dept. of Environment and Toxicology, Hørsholm, Denmark

Abstract

Trophic transfer of metal-based engineered nanoparticles (Me-ENPs) are poorly understood, with only a few studies investigating how these particles are transported within the aquatic food web. We wanted to detect transfer of silver (Ag) ENPs in a simple freshwater food web, including sediment, sediment dwelling worms (*Tubifex tubifex*) and pelagic fish (*Danio rerio*). Ag ENPs were embedded into worm tissue using two distinct methods: via sediment exposure of living worms (AgS) or direct spiking of worm homogenates. Subsequently, worms were homogenized and transformed into palatable food packages (FPs) for the fish (concentrations: AgS: 1.8 μg/g ww food; AgC1: 10 μg/g ww food; AgC2: 500 μg/g ww food). Fish were exposed for 14 days, followed by 14 days of depuration under uncontaminated conditions. Our study shows that Ag embedded as Ag ENPs are transferred from the abiotic sediment to fish, via bioaccumulation in prey worms. Ag was primarily found in fish gut and intestines, with no or very low detectable transfer to remaining fish tissue. Biomagnification was not observed for fish exposed to FPs from either treatment (AgS, AgC1, AgC2). However, an order of magnitude higher BMF-factor was estimated for fish exposed to AgS FPs (AgS_{BMF} = 0.32; $AgC1_{BMF}$ = 0.03; $AgC2_{BMF}$ = 0.01), indicating that the contamination method of Ag ENPs in prey are affecting the bioavailability in predators. This was also reflected in the estimated assimilation efficiencies (AE) for each treatment. All fish depurated Ag after exposure, limiting the possibility of further transporting Ag ENPs up the food chain in the event of reduced exposure. Overall, we demonstrated that Ag ENPs imbedded in the abiotic sediment are bioavailable for both sediment dwelling organisms and their predators. This demonstrates that trophic transfer of metal originating from Me-ENPs (i.e. as metal-ions or intact particles) are possible from the abiotic to biotic compartments in the freshwater ecosystem. Future studies should place emphasis on the sediment community, as well as include more trophic levels and higher organisms, to get a better understanding of how Me-ENPs are affecting the aquatic ecosystems in the long term.

Key words: *Nanoparticles, trophic transfer, sediment, silver*

1. Introduction

Silver nanoparticles (Ag ENPs) are one of the most frequently used metal-based engineered nanoparticles (Me-ENPs), primarily due to their antibacterial effects (Vance et al., 2015). Although monitoring programs for Ag ENPs in the environment is currently lacking, their predicted environmental concentrations in surface water and sediment range from 0.09 to 320 ng/L and 0.15 μg/kg to 14 mg/kg, respectively (Mueller and Nowack, 2008; Gottschalk et al., 2009). Sediment exposure studies have shown that Ag ENPs are bioavailable to benthic organisms including the sediment dwelling ragworm, *Nereis diversicolor* (Cong et al., 2014), the deposit-feeding clam, *Macoma balthica* (Dai et al., 2013), the benthic gastropod, *Potamopyrgus antipodarum,* and the polychaete, *Capitalla teleta* (Ramskov et al., 2015a). All these species accumulated Ag after exposure to sediment amended with Ag ENPs. This uptake of Me-ENPs creates a link between the abiotic environmental compartments and organisms in the aquatic food chain. Ferry *et al.* showed that Au ENPs introduced into an aquatic mesocosm, would distribute between water, organisms and sediment, with large amounts of Au ENPs found in sediment and biofilm after 12 days (Ferry et al., 2009). Thus, benthic organisms such as sediment dwelling worms and benthic grazers, would be the most likely link for Me-ENPs to pass from the abiotic compartments to the aquatic food chain, i.e. via predation by higher pelagic organisms such as demersal fish.

Trophic transfer of anthropogenic contaminants, defined here as the movement of toxicants through the food web via ingestion of prey organisms by predators, has been widely recognized and remains a highly studied ecotoxicological issue. Whilst the movement of aqueous metals in the aquatic food chain is well-known and relatively well understood (Woodward et al., 1994, 1995; Croteau et al., 2005; Rainbow et al., 2006b; Mathews and Fisher, 2008), studies into the potential trophic transfer of particulate metals in the nano-size range, formulated as Me-ENPs, are scarce. Some studies have investigated how Me-ENPs are transported from the lower food chain levels in the pelagic environment (e.g. algae) to higher organisms such as Daphnia (i.e. (Bouldin et al., 2008; Kalman et al., 2015)). In addition, higher organisms such as bivalves and fish, have been shown to accumulate Me-ENPs from contaminated prey organisms such as algae and zooplankton, respectively (i.e. (Conway et al., 2014; Skjolding et al., 2014a)). However, studies including the benthic environment in trophic transfer of Me-ENPs are limited.

Here we investigated the transport of Ag ENPs from sediment to fish in a range of controlled, laboratory experiments to answer the following research questions: 1) to what degree are Ag ENPs bioaccumulated in benthic worms bioavailable for higher trophic organisms, such as fish? 2) does the food contamination process (i.e. pre-exposed worms vs spiked worms) matter regarding bioavailability and bioaccumulation

in fish, and can we use this information to predict if and to what degree Ag ENPs are trophically transported in freshwater ecosystems?

2. Materials and Methods

The experimental work was divided into two parts: 1) exposing *T. tubifex* to sediment spiked with Ag ENPs, and 2) exposing *D. rerio* to Ag ENPs embedded into worm tissue food packages using two distinct methods: via sediment exposure of living worms or direct spiking of worm homogenates. Subsequently, worms were homogenized and transformed into palatable food packages (FPs) for the fish. A total of four dietary treatments were included for the fish in the second experiment: control (non-spiked food; C FPs); sediment exposure of worms (AgS FPs); spiked worm homogenate in two concentrations (AgC1 and AgC2 FPs).

2.1 Experimental organisms

2.1.1 Tubifex tubifex

T. tubifex is an omnipresent, sediment-dwelling oligochaete found in freshwater environments worldwide (Lazim and Learner, 1986). They serve as prey for higher organisms such as demersal fish (Chapman, 2001), and is found at high densities in the environment (i.e. from 30-500·10^4 orgs/m^2 in British waters (Palmer, 1968)). In addition, OECD recommends *T. tubifex* as test organism when assessing bioaccumulation from sediment (OECD, 2008). *T. tubifex* were purchased from a local pet shop (Bonnies Dyrecenter, Rødovre, DK) and reared in artificial freshwater (FW) prior to adding them to sediment (see Section 2.3.1). FW was prepared according to OECD guideline 203 (ISO 6341-1892 (294 mg/L $CaCl_2 \cdot 2H_2O$, 123.3 mg/L $MgSO_4 \cdot 7H_2O$, 63 mg/L $NaHCO_3$, 5.5 mg/L KCl; pH 7.8±0.2; hardness 250 mg/L)), as recommended when using *T. tubifex* (OECD, 2008). Worms used for FPs spiked with Ag ENPs were bought from the supplier and homogenized directly, i.e. no culturing. This was done to ensure no sediment within worm guts prior to FP creation.

2.1.2 Danio rerio

D. rerio (or zebrafish) is an omnivorous freshwater fish, primarily found in tropical regions. Its diet is highly diverse and consists of aquatic and larval form of insects, phytoplankton and zooplankton etc. (Spence et al., 2008). It is a well-known model organism, recommended as test organism by OECD with regards to bioaccumulation of different toxicants (OECD, 2012). Adult zebrafish were purchased from Credo Fish Aps (Nørresundby, DK) and cultivated at DHI (Hørsholm, DK). Two weeks prior to experimental start, fish were acclimated to the experimental conditions. Fish were kept in aquaria containing FW, prepared in the same manner as for *T. tubifex* and according to official guidelines (OECD, 2012). Temperature was kept at 23±2°C, with a light:dark

cycle of 14:10h. The fish loading was kept within the recommended range of 0.1 g to 1.0 g wet weight (ww) fish per liter of water (OECD, 2012). During acclimation, water was exchanged twice a week, whereas the exposure was run as a flow-through setup with one water exchange per aquaria per day. Fish were fed Tetramin® during the first week of acclimation and uncontaminated food packages one week prior to exposure start, to get them acquainted with the food type.

2.2 Chemicals

Paraffin coated Ag ENPs were purchased from Amepox Microelectronics (Lodz, Poland). Information provided by the supplier indicated particle sizes ranging from 3 to 8 nm (TEM, Laser Diffraction). The stock solution had a silver concentration of 1500 ppm (provided by the supplier). In a previous study (Tangaa *et al*, 2017 *In prep*) the hydrodynamic size, size distribution and aggregation were monitored using Dynamic Light Scattering (DLS) (Zetasizer Nano ZS90, Malvern) and UV-vis (UV-1800 Shimadzu). In addition, dissolution of the Ag ENPs was assessed in FW by centrifugal ultrafiltration (Millipore, Amicon, 3KDa membrane filters). As all exposures were done via diet, i.e. Ag ENPs embedded in food packages or sediment, no additional characterization was carried out.

2.3 Experimental procedures

2.3.1 Sediment exposure of T. tubifex

Natural sediment was collected at Isefjorden (Munkholm, Denmark; 55°40'27"N, 11°48'53"E). Sediment from this location have been used as control sediment (i.e. uncontaminated) in several studies (i.e. (Dai et al., 2013; Ramskov et al., 2015a)) due to the low levels of metals and other contaminants. The top 5-10 cm of sediment was scraped off and transported to Roskilde University. Sediment was sieved (<125 μm) with deionized (DI) water and left to settle. After 3-5 days, overlaying water was removed and sediment frozen (-20°C) for min. 24h. Sediment was thawed, rinsed twice in FW, homogenized and left to settle (3 days, room temperature). Overlaying water was carefully siphoned off and the sediment ready to use. Percentage dry weight and organic carbon content (OC) of sediment were 39±1 % and 6±0.6 %, respectively. Background silver concentration in sediment was 0.07 μg/g dry weight (dw).

Sediment was spiked with a Ag ENP stock solution (1500 ppm) to obtain a nominal concentration of 20 μg Ag/g dw sed. Approximately 5 mL Ag ENP stock was added to one beaker containing 500 g ww sed., the beaker was covered in foil and left on the shaking table for 24h. A control treatment (uncontaminated sediment only) was prepared with around 5 mL FW and treated similarly. After 24h, each portion of sediment was homogenized (mixed by hand) and divided into 8 smaller beakers, with 45±3 g ww sed. per beaker (~18 g dw). Then, 30 mL FW were added to each beaker,

and left to settle for 2h. Worms were added to petri dishes, in portions of ~1.2 g ww corresponding to about 75 worms. A total of 8 replicates were employed per treatment (i.e., Ag ENP and control). Overlaying water was carefully renewed with aerated freshly prepared FW in all beakers prior to adding worms. All beakers were placed in a climate cabinet and each was aerated gently. Worms were exposed at 15±2°C in complete darkness, to ensure conditions relatable to previous studies (i.e., Tangaa *et al.* 2017 (*In prep*)). Exposure continued for 21 days. Exposure was terminated by sieving worms from the sediment and rinsing them in FW. Worms were allowed to empty their guts of sediment and fecal matter in clean FW overnight. Hereafter, worms were rinsed in DI-water and frozen (-20°C) for min. 24h.

2.3.2 Trophic transfer study with D. rerio

Preparation of food packages

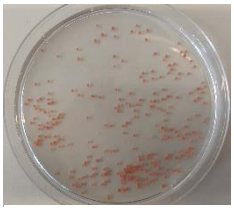

Figure 10: Private photo of palatable food packages created from worm homogenate

Food packages (FPs) were prepared after a method described in Palmqvist *et al.* (Palmqvist et al., 2006), adjusted to fit the used experimental conditions. Frozen worms were thawed, mixed with FW (2 mL per 1.2 g worms) and homogenized using a micro homogenizer (VWR, VDI 12). Sodium alginate was prepared by mixing 159 mg alginic acid sodium salt (Sigma-Aldrich, CAS: 9005-38-3) with 10 mL FW, heating until dissolution and cooled down (<35°C). The alginate-solution was added to the worm homogenate in the ratio of 1:1 and mixed well. A 2 % $CaCl_2$-solution was prepared in DI-water and added to a petri dish (enough to cover the bottom). Small droplets of the worm:alginate mixture was added to the $CaCl_2$-solution, creating gelated, spherical food packages. Food packages were stored cold and dark in FW to keep their form and consistency intact. FPs had a mass of 2.0±0.8 mg ww and a diameter of 1.1±02 mm (n=60) (see Figure 1).

Spiking of T. tubifex

Newly bought worms were rinsed in FW, and divided into smaller portions. Worm-portions were blot dried with tissue paper, transferred to tubes and weighed (~1.2 g ww per tube). Worms were frozen (-20°C) for min 24h before usage. Worms were transformed into food packages as described above, with the only exception of adding Ag ENP to the worm:alginate homogenate before creating the droplets in $CaCl_2$. The Ag ENP was added directly from the stock or as a dilution thereof, and mixed into the worm:alginate homogenate by hand. Thereby FPs with two different Ag-concentrations were created, i.e., AgC1 (10 μg/g) and AgC2 (500 μg/g).

Trophic transfer setup

Before conducting the final trophic transfer experiment, a pilot study was carried out to test whether the experimental conditions were optimal (i.e., exposure time and feeding process). *T. tubifex* were exposed to natural sediment (<125µm) spiked with Ag ENPs for 21 days, at a concentration of 77.9±1.4 µg Ag/g dw sediment. FPs were created as described above. Fish (n=66) were exposed together in four 20 L tanks and fed FPs created from uncontaminated or sediment exposed worms. Results showed that Ag was detectable in fish gut/intestinal tissue after 10 days of exposure (i.e., $[Ag]_{gut}$: 3.47±0.38 µg Ag/g dw). Fish ate the FPs provided, however, the setup prevented a controlled feeding, and hence it was not known if all fish were fed with the same amount of FPs. Therefore, the final experimental setup was altered so fish were exposed individually.

The trophic transfer study was setup according to OECD TG 305 (OECD, 2012). Fish (n=84) were divided into 16 aquaria, each containing 5 or 6 fish and 10 L FW. Fish numbers were kept at a minimum to minimize the quantity of organisms suffering, but still ensuring sufficient replicates for the results to be statistically valid. All aquaria were equipped with a splitting device, creating six individual compartments per aquaria, which ensured that we could control how much feed each fish received (Figure 2). Temperature, pH and O_2 were measured regularly, and kept within the limits of 23±2°C, 7.8±0.2, min. 90 %, respectively (ISO/TC-147, 1996). Fish were fed daily at a feeding rate of 1.5 % of fish body weight per day (OECD, 2012), with FPs created from pre-exposed or spiked *T. tubifex*. Control fish were fed at the same rate, with uncontaminated FPs. Each fish in the four treatments was hand fed 3 FPs per day, and feeding observed to ensure complete ingestion of food. Three fish were sampled from each treatment (including control) on day 2 and 14 during the exposure period. In addition, 5 fish were sampled on day 0 to account for any background concentration of Ag in fish tissue. On day 14, all aquaria were rinsed, new freshly prepared FW was added and the fish were allowed to depurate any assimilated Ag for up to 14 days. During depuration, all fish were fed FPs created from unexposed *T. tubifex* following the same procedures as described for the exposure period. Three fish per treatment were sampled on depuration day 1, 2, 4, 7 and 14. On all sample-days (both during exposure and depuration), a 2 mL water sample was taken from each tank, to measure Ag-level in the water during uptake and depuration.

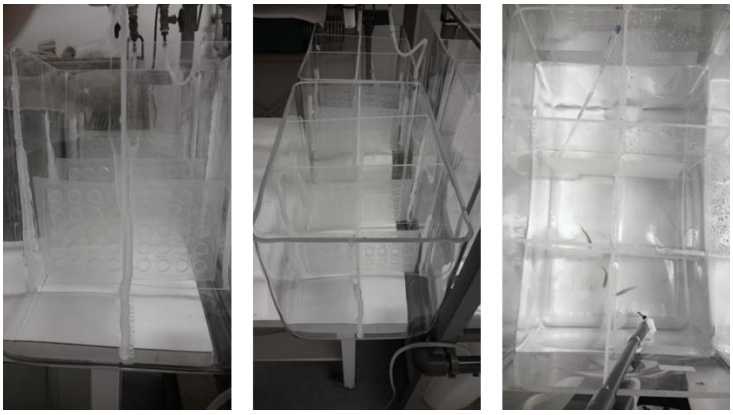

Figure 2: Experimental setup showing aquaria with splitting device

Termination of D. rerio

On termination days, fish were transferred from aquaria with a fish net and into a separate beaker with DI-water and the anesthetic compound MS-222 (ethyl 3-aminobenzoate methanesulfonic acid). MS-222 dosage was chosen to ensure fish euthanasia within 30 seconds (Fouqueray et al., 2013; Skjolding et al., 2014a). Twelve fish (on each sampling day) were weighed and measured (length, cm). Sampled fish were dissected into gut/intestinal regions and "the rest". Fish material were added to Eppendorf-tubes and frozen (-80°C) until further analysis.

2.3.3 Sample preparation and metal analysis

Sample preparation was carried out in accordance with prior studies (Tangaa *et al.* 2017 (*In prep*)) and common practice in the field (i.e. (Croteau et al., 2011c, 2014a)). Frozen samples (sediment, fish) were dried at 40°C for 3-6 days and dry weight determined (Mettler AT250, 5 dec.). FPs and water samples were analyzed wet. Thawed water samples were acidified by addition of 65 % HNO_3 to a final concentration of 5% HNO_3. Sediment, FPs, and fish samples were transferred to acid washed 6 mL Teflon vials and 600 µL 65% HNO_3 were added to each vial. Samples were digested in a Microwave (Milestone Start D) for 40 min at 1380°C. Subsequently, samples were transferred to 5 mL Erlenmeyer flasks, diluted with MilliQ-water (18.2 MΩ·cm) to a final concentration of 5% HNO_3 and shaken by hand. Sediment samples were filtered prior to analysis. Certified reference material LUTS-1 (non defatted lobster hepatopancreas tissue from the National Research Council Canada) were processed similarly, in addition to procedural blanks. An internal standard solution (100 µg/L in 10% HNO_3; CAS No: HNO_3 [7697-37-2]; Agilent) was added to all samples prior to analysis. Samples were analyzed for Ag content by Inductively Coupled Plasma-Mass Spectrometry (ICP-MS; Agilent 7900). Calibration curves were created from external

standards (1000 μg/mL in 5% HNO_3; Agilent) serially diluted (0.1-40 μg/L). Additional quality controls were done by running an independent standard (10 μg/L in 2% HNO_3; Agilent) for every 12 samples. The limit of detection of the ICP-MS for Ag was calculated to be 0.8 μg Ag/L.

2.3.4 Calculations

To investigate the degree of trophic transfer, a biomagnification factor was calculated for each treatment (except control), as the relationship between the Ag assimilation efficiency (AE), food ingestion rate constant (I) and depuration rate constant (k_2). Calculations was based on OECD TG 305 (OECD, 2012). Briefly, AE was estimated as:

$$AE = \frac{C_{0,d} \cdot k_2}{I \cdot C_{food}} \cdot \frac{1}{1 \cdot e^{-k_2 t}}$$

with $C_{0,d}$ being the metal concentration in fish at time 0 of depuration (μg/g), k_2 the depuration rate constant (d^{-1}), I the food ingestion rate constant (g food/g fish/d), C_{food} the metal concentration in food (μg/g) and t the duration of the uptake period (d). $C_{0,d}$ and k_2 was estimated via the intercept and slope of the linear relationship between $\ln([Ag]_{fish})$ and depuration time, where $C_{0,d}$ was calculated as:

$$C_{0,d} = e^{intercept}$$

When these parameters had been determined, BMF was calculated as:

$$BMF = \frac{AE \cdot I}{k_2}$$

2.4 Statistical analysis

All statistical analyses were performed using SYSTAT (version 13.1). Datasets were tested for normal distribution and variance equality using Kolmorogov Smirnoff and Levenes Test, respectively. One-way analysis of variance (ANOVA) was used when conditions were met. Otherwise, the non-parametric Kruskal-Wallis test was used. Tukeys test or Conover-Inman was used for comparison among treatments, if ANOVA or Kruskal-Wallis showed a significant effect, respectively. Statistical significance was obtained if $p \leq 0.05$. All data is presented as actual, measured Ag-concentrations (mean ± standard deviation (SD)) unless stated otherwise.

3. Results and Discussion

3.1 Characterization of Ag ENPs

UV-vis analysis of Ag ENPs dispersed in FW and MilliQ-water showed peaks corresponding to Ag ENPs, i.e. around 410 nm (Bhui et al., 2009). DLS measurements

indicated that particles had an initial average hydrodynamic diameter of 148 nm in FW, with a Polydispersity Index (PdI) of 0.2. Agglomeration/aggregation was observed over a time period of 240 min, with an average particle diameter of 174 nm (PdI = 0.2) after 240 min in FW. Dissolution was less than 2%, regardless of the Ag concentration. Detailed characterization information can be found Tangaa *et al*, 2017 (*In prep*). As methods for characterizing Ag ENPs embedded in sediment or worm-homogenate (FPs) were not available, we assumed that the particles remained in some (nano)particulate form after addition to the complex matrices, which has been suggested in other studies (i.e. (Dale et al., 2013)).

3.2 Exposure concentrations

Table 1: Overview of the actual Ag-concentration detected in sediment and food packages (FP). C: control; AgS: FPs created from sediment-exposed worms; AgC1 and AgC2: FPs created from spiked worms at concentration 1(10μg/g) and 2(500μg/g). All data shown as mean±SD (n=3).

Exposure concentrations			
Treatment	[Ag] (μg/g dw)	*Treatment*	[Ag] (μg/g ww)
Control sediment	0.07±0.0	**C FP**	0.46±0.5
Spiked sediment	21.54±1.8	**AgS FP**	1.77±0.3
		AgC1 FP	10.54±0.3
		AgC2 FP	495.40±140.1

Worms used in the AgS-treatment were exposed to Ag ENP spiked sediment at a concentration of 21.54±1.8 μg/g dw for 21 days, resulting in FPs with an average Ag concentration of 1.77±0.3 μg/g ww. Data shows that the four FP types all differed significantly in Ag concentration ($p<0.05$), creating four different exposure scenarios for the fish (including control).

3.3 General observations

No abnormalities were observed on general fish behavior (i.e. swimming and eating) during the experiment. Fish were eating FPs instantly (i.e. within 5 min.), and swam around with no sign of stress. No difference in behavior was observed between exposed and control organisms. All fish stayed within the weight (0.45±0.07 g ww) and length (3.0±0.1 cm) range of background organisms throughout the experimental time-frame. Some natural variation was observed, but no significant difference was detected for either treatment or timepoint regarding fish weight or length (ANOVA, $p>0.1$).

3.4 Trophic transfer of Ag from worms to fish

Overall, fish accumulated Ag from food packages during the 14 days of exposure (Figure 3). No data is shown for depuration day 7, due to break down of the ICP-MS, resulting in loss of samples. The statistical software (SYSTAT 13) identified some data-points as outliers (i.e., d1 (C and AgS), d2 (C), d4 (AgC1), d14 (AgC2)). These points showed increased Ag-concentrations in fish gut-tissue during depuration, which would only be possible if fish lost weight, which was not observed. These data-points were therefore excluded from the data-set. Ag was primarily found in gut/intestinal tissue of the organisms, with body burdens at day 14 of: 0.36±0.24, 2.51±0.96, 2.57±0.57 and 127±74 μg Ag/g dw for Control, AgS, AgC1 and AgC2 exposed fish, respectively. All exposed fish (AgS, AgC1 and AgC2) had a significantly higher Ag content in gut tissue compared to control after 14 days of exposure (KW, $p<0.05$). Zebrafish are known to accumulate metal from Me-ENPs during dietary exposure (Geffroy et al., 2012; Ladhar et al., 2014). When exposed to Ag ENPs embedded in *Chironomid* larvae, zebrafish showed a higher uptake compared to waterborne exposure (Asztemborska et al., 2014). The authors highlighted that trophic transfer of Ag ENPs from larvae to fish was observed, when larvae were pre-exposed to Ag ENPs in water. Our data shows that Ag uptake was concentration dependent, seen as a higher Ag content found in gut tissues from fish exposed to FPs with the highest Ag-concentration (AgC2; 500 μg/g ww) compared to the remaining treatments after 14 days of exposure (KW, $p<0.05$). However, Ag accumulation in gut was similar in AgS and AgC1 exposed fish, even though the exposure concentration differed with almost a factor of ten (Table 1).

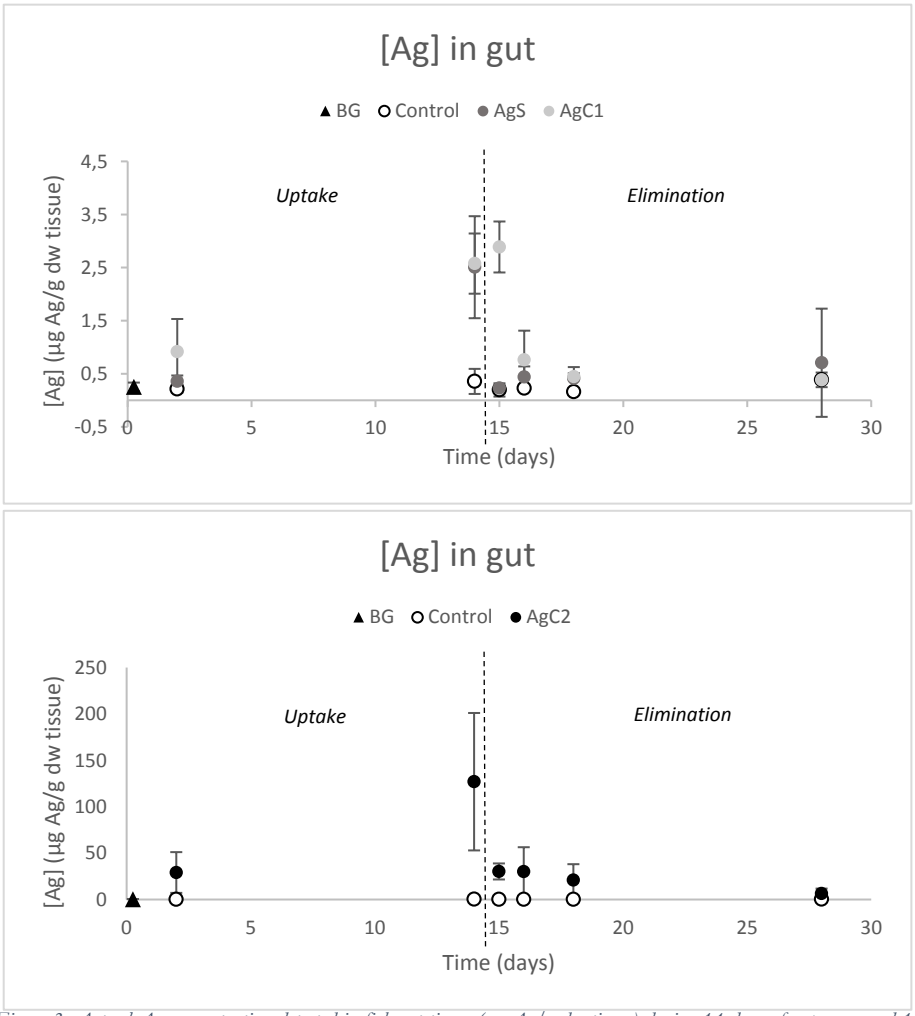

Figure 3: Actual Ag-concentration detected in fish gut tissue (µg Ag/g dw tissue) during 14 days of exposure and 14 days of depuration. No data shown for day 7 of depuration due to loss of samples. BG: background (triangle); C: ccontrol (white circle). ***Top graph****: AgS: FPs created from sediment-exposed worms (dark grey circle); AgC1: FPs created from spiked worm-homogenate (low; 10 µg/g) (grey circle).* ***Bottom graph****: AgC2: FPs created from spiked worm-homogenate (high; 500 µg/g) (black circle). All data shown as mean ± SD. n = 3, except for D1 (C and AgS), D2 (C), D4 (AgC1) were n = 2*

As presented in Table 2, applying the concepts of the biodynamic model (BDM; (Luoma and Rainbow, 2005)) using data from Tangaa *et al.* 2017 (*In prep)* to *T. tubifex*'s accumulation from sediment, theoretical body burdens ($[Ag]_{worm}$) after 21 days of exposure was estimated. $[Ag]_{worm}$ was calculated based on AE & IR or k_{uf} resulting in body burden values of 6.9 and 8.2 µg Ag/g dw tissue, respectively. This is lower than what was actually detected in worms after 21 days of exposure to sediment amended with Ag ENPs in the pilot study (i.e. 27.4±11.3 µg Ag/g dw tissue). The parameters determined in Tangaa *et al.* 2017 (*In prep*) includes assimilation efficiency (AE), ingestion rate (IR) and uptake rate constant from food (k_{uf}), all estimated to be below 1. Using the

measured Ag-concentration found in worms after 21 days of exposure to estimate an uptake rate constant (k_{uf}) results in a k_{uf}-value an order of magnitude higher than what was found in Tangaa *et al.* 2017 (*In prep*) (see Table 2). As a model is only as valid as the input data, this highlights that the parameters from the short-term study are too uncertain to predict long-term results. That is, they are only based upon one study, thereby not representative for all the factors influencing bioaccumulation in *T. tubifex* (e.g., exposure conditions, time and media). Thus, more studies are needed to create model parameters describing the broad range of effects and factors impacting uptake and elimination of Ag ENPs in benthic organisms such as *T. tubifex*.

Table 2: BDM-parameters determined for Ag ENP exposed worms in Tangaa et al. *2017 (In prep) (left column); Long-term exposure data from pilot study (right column); Predicted Ag-concentrations in worms using BDM-parameters (bottom). IR: Ingestion rate; AE: Assimilation efficiency; k_{uf}: uptake rate constant from food; k_e: elimination rate constant*

Data from BDM-study (Tangaa *et al.* 2017 (*In prep*))			**Data from long-term exposure** (Pilot study)		
Parameter		*unit*	Compartment	[Ag]	*unit*
IR	0.6	g/g/d	Sediment	77.9±1.4	µg/g dw
AE	0.7	%	Worms	27.4±11.3	µg/g dw
k_{uf}	0.005	g/g/d			
k_e	<0.001	d^{-1}			

Predicted $[Ag]_{worm}$ (21d) using BDM-parameters compared with measured $[Ag]_{worm}$

Calculations based on	$[Ag]_{worm}$	*unit*
AE & IR	6.9	µg/g dw
k_{uf}	8.2	µg/g dw
Measured Ag (Paper IV)	27.4	µg/g dw
k_{uf} (predicted)	0.02	g/g/d

As mentioned, biomagnification factors were calculated for the exposed fish. The parameters estimated to calculate BMF included AE, I and k_2 according to OECD TG 305 (OECD, 2012), corresponding to AE, IR and k_e in BDM. In addition, steady-state body burdens ($[Ag]_{ss}$) in fish were estimated using the BDM approach (Croteau et al., 2014b). Results are presented in Table 3.

Table 3: Biomagnification factors (BMF) calculated as described in OECD TG 305; Steady-state Ag-concentration in fish gut/intestinal tissue ($[Ag]_{ss}$) calculated based on the biodynamic model (BDM) and measured Ag-concentration in fish tissue after 14 days of depuration ($[Ag]_{fish}$). AgS: FPs created from sediment-exposed worms; AgC1 and AgC2: FPs created from spiked worms at concentration 1(10µg/g) and 2(500µg/g); AE: assimilation efficiency; I: ingestion rate constant; k_2: depuration rate constant

Estimated parameters according to OECD				Calculated		Measured
Treatment	AE (%)	I (g/g/d)	k_2 (d^{-1})	BMF	$[Ag]_{ss}$ (µg/g)	$[Ag]_{fish}$ (µg/g)
AgS	-0.09	0.015	-0.004	0.32	0.57	0.71
AgC1	-0.24	0.015	-0.124	0.03	0.30	0.39
AgC2	-0.12	0.015	-0.162	0.01	5.63	6.58

Use of the BDM approach indicates that fish had not reached their predicted steady-state body burdens after 14 days in uncontaminated conditions (i.e., $[Ag]_{ss} < [Ag]_{fish}$). The estimated AE-values predict that Ag from AgS FPs are most bioavailable, followed by AgC2 and AgC1. The depuration rate constants (k_2) indicate that Ag are eliminated fastest from fish exposed to AgC2 FPs, followed by AgC1 and AgS FPs. These results suggest, that Ag from AgS FPs will be most harmful to fish, i.e. higher accumulation due to larger bioavailability and slower elimination. However, the estimated steady-state BB's indicates that fish will experience the greatest accumulation when exposed to AgC2 FPs (i.e., the highest exposure concentration), consistent with measured data (Figure 2). This highlights that no model parameter can stand alone, and must be considered in a broader perspective, taking both the remaining parameters and actual data into account. The BMF-factors predict that none of the FPs resulted in Ag being biomagnified in fish. A few studies have detected biomagnification (BMF>1) of Me-ENPs in lower organisms, e.g. protozoans fed Cd QDs contaminated bacteria (Werlin et al., 2011), and daphnids fed TiO_2 ENP exposed algae (Chen et al., 2015). Yet when introducing the next food chain level biomagnification is rarely observed. For example, zebrafish fed QD or TiO2 ENP contaminated daphnids showed BMF-values below 1 (Zhu et al., 2010; Lewinski et al., 2011). In fact, Zhu *et al.* showed a similar trend in the estimated BMF's as seen in this study, with higher prey-concentration resulting in lower BMF. The compartmentalization of metal in *T. tubifex* can affect how much of the metal that is assimilated by a predator (common carp, *C. carpio*) (Redeker et al., 2007). Thus, the bioavailability of Ag might be affected by the addition method of Ag ENPs to FPs (i.e., via sediment ingestion or direct spiking), as reflected in the estimated AE-values.

We expected the spiked treatments to result in higher uptake in fish, both due to the higher Ag concentration and the assumption that Ag ENPs spiked into worm-homogenate would be bound "less" than Ag ENPs taken up into worm tissue (Khan et al., 2010). As mentioned, higher BB's was seen for fish exposed to FPs with the highest

Ag-concentration (AgC2), as well as when applying BDM. However, the BMF-values indicate that exposure conditions for prey should be considered when assessing trophic transfer, as fish exposed to AgS FPs showed the highest BMF. This could be due to the concentration difference in exposure concentrations (i.e. AgS being a factor of ten lower than AgC1), however this trend is not consistent when taking AgC2 into account (a factor of 100 higher than AgS). We therefore speculate, that Ag ENPs embedded into sediment and turned into palatable FPs are more bioavailable than Ag ENPs spiked directly into worm homogenate. This is highly important, as dietborne exposure studies today are conducted using the spiking method we applied for the treatments AgC1 and AgC2 (OECD, 2012), which resulted in the lowest BMF-factors. The standard method could therefore, in worst-case, underestimate the bioaccumulation potential of dietary Me-ENPs, similar to what was shown for Cd embedded into natural (*Lumbriculus variegatus*) and commercial feed and fed to rainbow trout (*Oncorhynchus mykiss*) (Ng and Wood, 2008). However, more studies are needed before such conclusions can be made.

Besides detecting Ag concentration in fish gut tissue, the remaining fish tissue ("the rest") was analyzed. Data shows that no detectable amount of Ag was found in fish tissue (i.e. data for all experimental days was below the detection limit of the ICP-MS) for fish exposed to AgS(0.01±0.00 μg Ag/g dw), AgC1(0.01±0.01μg Ag/g dw) and control (0.03±0.05 μg Ag/g dw) FPs. For fish exposed to AgC2 FPs, Ag was measurable in the remaining fish tissue, however no clear trend was observed with regards to exposure and depuration time (see Table 3). This indicates that Ag are not transported into fish tissue, or at least not in amounts high enough to be detected by the analysis method, during the experimental time-frame. This is consistent with other findings, where Me-ENPs are primarily or only detected in gut, intestines or the like after dietary exposure (Lewinski et al., 2011; Ates et al., 2015). This indicates that Me-ENPs are not able to cross the cell-barriers in the gut and transfer into the fish tissue. However, studies including intracellular location of ENPs (i.e., subcellular fractionation and several microscopy techniques, as described in (Jensen et al., 2017)) are needed in order to elucidate this. Zebrafish have been shown to eliminate accumulated Ag during 2 days in uncontaminated conditions. The authors concluded that Ag was not accumulated into tissue, but only present in the gut regions of fish (Asztemborska et al., 2014). In addition, fish are known to create mucus layers within their gut/intestinal systems, used to eliminate contaminants by excreting the mucus-cells (Handy et al., 2000; Khan and McGeer, 2013). Hence, Me-ENPs from contaminated prey or food, might not cause huge effects in fish, as they can be depurated without transport into tissues.

Table 3: Ag detected in remaining fish tissue ("rest") in fish exposed to AgC2 FPs. U: uptake; D: depuration; BG: Background fish

$[Ag]_{rest}$ for AgC2-treated fish	
Day	[Ag] μg/g dw
U2	0.77±0.91
U14	0.47±0.10
D1	0.21±0.09
D2	0.97±0.67
D4	0.21±0.08
D14	0.23±0.05
BG	0.01±0.05

As seen in Figure 3, all fish were able to eliminate any accumulated Ag over the 14 days of feeding on clean food packages. This was visible after only one day of depuration for fish exposed to AgS FPs, and after two days for AgC1 FP exposed fish, where Ag content in the gut/intestinal tissue was no longer different from the control group (KW, $p>0.05$). Fish exposed to AgC2 FPs did not reach their original Ag concentration (i.e. same amount as found in control fish; KW, $p>0.05$) until day 14. This is consistent with other studies, showing that zebrafish are able to eliminate Me-ENPs after exposure (Zhu et al., 2010; Skjolding et al., 2014a). However, this is only true when fish are changing their diet from contaminated to uncontaminated prey. As fish did not show any distress during feeding on contaminated prey in our study, it is likely that they would continue to eat, which may result in even higher body burdens than we observed. This could lead to transfer further up the food chain and have consequences for higher organisms predating on fish, such as humans.

4. Conclusion

Overall, our results demonstrate that Ag ENPs added to sediment, are available for uptake in benthic organisms and thereby Ag can be re-introduced into the pelagic food web, reinforcing the idea of the sediment not just as a sink, but also a source of ENPs in aquatic environments. In addition, we show that Ag bioaccumulation in fish is dependent on the concentration found in prey. However, BMF-factors and AE-values were higher for FPs originating from the lowest exposure treatment, i.e., from worms exposed to sediment amended with Ag ENPs (AgS FPs). Thus, even at low, environmentally realistic exposure scenarios, Ag originating from Ag ENPs can be transported up the food chain, causing unknown effects to the ecosystem. Using the BDM-approach to estimate body burdens in prey and predator, highlighted that many factors must be accounted for when using modelling data to predict the future (e.g., long-term exposure). We urge future studies to take the sediment and benthic

community into account when assessing the risks of Me-ENPs in aquatic environments, as well as include more trophic levels and higher organisms when testing bioavailability and bioaccumulation potential of these particles.

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